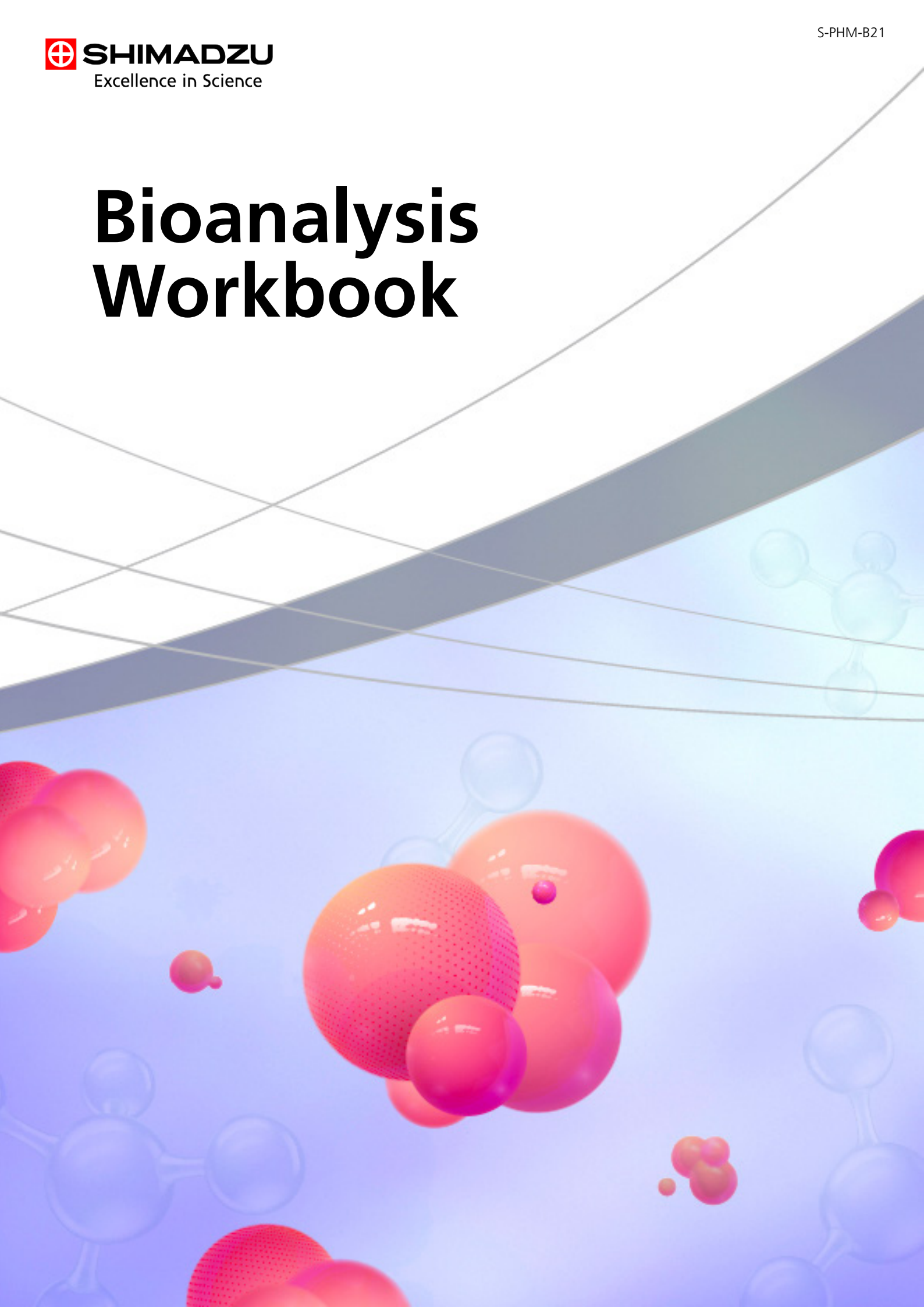
SHIMADZU
Excellence in Science

S-PHM-B21

Bioanalysis Workbook



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Overview

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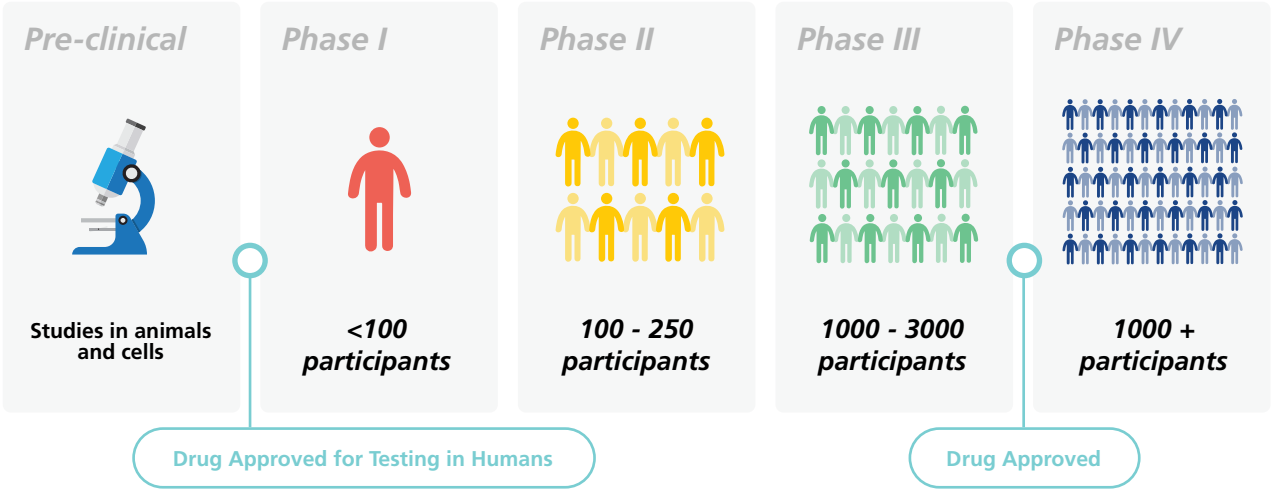
Focus Area

Clinical Trials In Drug Development

The development of a new drug must proceed through several stages of clinical trials to ensure the safety and efficacy for its indicated use in humans. This stringent process is mandatory for drug approval as regulatory authorities, such as the U.S. FDA and Germany BfArM, require successful human clinical trials before a new drug is granted marketing authority.

Preclinical drug development involves researching and designing small molecule or biological drugs. Small molecule drugs are organic compounds with low molecular weight, while biological drugs comprise of macromolecules such as proteins and nucleic acids.

Stages of Human Clinical Trials



Phase I : Pharmacokinetics and dose-escalation studies

This small-scale study is designed to determine the effects of the drug on human body, which include understanding the adsorption, distribution, metabolism and excretion pathways (ADME). The safety and optimal dose of the drug for the proposed medical indication is also assessed. A small pool of <100 healthy participants is often involved.

Phase II : Exploratory therapeutic trial

This phase studies the efficacy and determines proof-of-concept of the drug for the targeted medical indication. The number of participants is extended to between 100-250.

Phase III : Confirmatory Therapeutic Trial

This phase is a large-scale testing to determine whether the drug is both safe and effective. Adverse reactions resulting from long-term usage of the drug are monitored. Upon completion of phase III, pharmaceutical companies can request for marketing approval. This large-scale testing usually encompasses hundreds to thousands of participants.

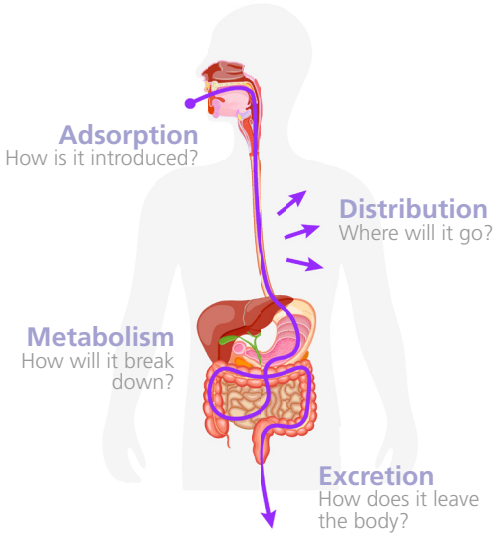
Phase IV : Post-marketing Observational Studies

This phase extends the monitoring of effectiveness and safety of the drug in real-life situations.

Bioanalysis Challenges In Drug Development

Pharmacokinetics and ADME

Pharmacokinetics and ADME are crucial studies to successfully get through the initial stages of clinical trials. These studies systematically track the fate of the drug from the moment of administration up to the point of elimination from the body. Concentration levels of the drug and its corresponding metabolites in blood, plasma, and excreta are closely monitored during this period. Hence, the usage of an accurate, rapid, and reliable analytical method to achieve quantitative bioanalysis is paramount for success in drug development.

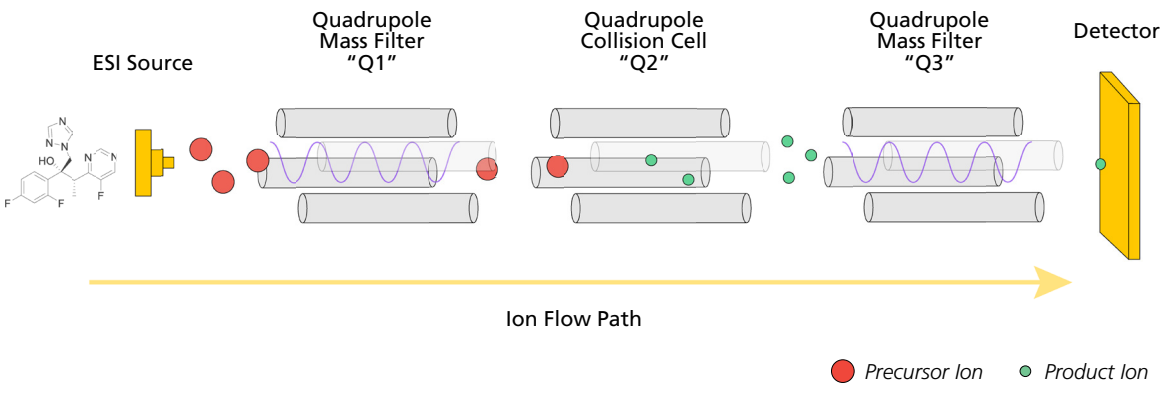


Role of Mass Spectrometry in Drug Development

Mass spectrometry (MS) has emerged as a powerful analytical tool that can deliver accurate, rapid and reliable quantitation of drugs or their metabolites in complex biological matrices. The analysis of complex mixtures is usually assisted by the implementation of a chromatographic separation prior to mass spectrometry. This has spurred the wide adoption of liquid chromatography-mass spectrometry (LC-MS) in the pharmaceutical industry.

The introduction of tandem mass spectrometry (MS/MS) further improves the specificity and sensitivity levels. LC-MS/MS has hence emerged as the primary analytical technique for quantitative bioanalysis. The ability to specifically induce fragmentation on a drug target and exclusively detect its fragment ions delivers unparalleled accuracy for qualitative and quantitative analysis. Coupled with its high dynamic range, multiplexing capability and up to picogram(pg) level of detection in biological matrices, LC-MS/MS utility in all phases of clinical trials is indisputable.

Tandem Mass Spectrometry



Make the Giant Leap with LCMS-8060NX

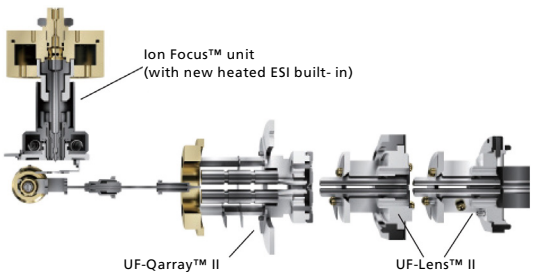
Higher Sensitivity, Speed and Robustness

• Higher Speed and Sensitivity

LCMS-8060NX inherits the excellent speed and sensitivity of LCMS-8060, and further improves the desolvation efficiency through increasing the ESI heat transfer efficiency and the maximum gas flow rate.

• Increased Robustness

The newly-developed Ion Focus™ unit introduces ions into the mass spectrometer with greater efficiency while expelling neutral particles, thus reducing matrix effects and contamination in the instrument. The new ion guide UF-Qarray™II and UF-Lens™II increase the robustness of the instrument while maintaining a high ion transmission rate.



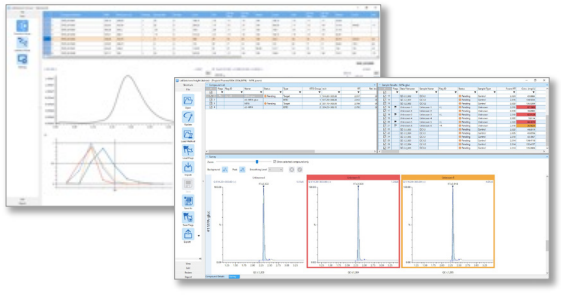
Enhanced Workflow Efficiency

• LabSolutions Connect Software

An all-in-one software that performs everything from optimization of MRM transitions and interface parameters to review of optimization results, creation of method and batch files.

• LabSolutions Insight

A revolutionary software that delivers high data-processing throughput for quantitative analysis. The newly designed interface is highly intuitive and driven by numerous cutting-edge algorithms that simplify the data analysis workflow so that you can focus on the science.



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01

Drugs for Respiratory Diseases

Chronic respiratory diseases, in particular asthma and chronic obstructive pulmonary disease (COPD), are the leading causes of mortality and morbidity worldwide. COPD affects approximately 65 million people in the world and is the third leading cause of mortality while more than 300 million people suffer from asthma.

The main treatment strategies for these diseases involve corticosteroids and bronchodilators, which are usually administered through inhalation. Such administration route poses a challenge to plasma pharmacokinetic studies since the systemic levels of these drugs are usually very low (pg/mL levels).

1 A Rapid, Sensitive and Direct Quantification of Salmeterol in Human Plasma

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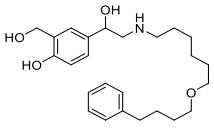
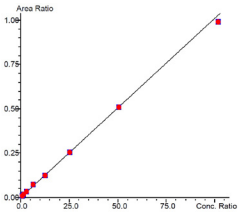
Salmeterol xinafoate is a long-acting beta-agonist bronchodilator.

LCMS-8060 achieves a LLOQ of 0.795 pg/mL in 0.2 mL of plasma and a calibration range of 0.795 – 101.742 pg/mL. Analysis is achieved with a single-step solid phase extraction. The analyte peak eluted within 2.5 min.

Ref: LC-21-ADI-043



Salmeterol xinafoate



2 A Highly Sensitive and Rapid Method for Quantification of Fluticasone Propionate

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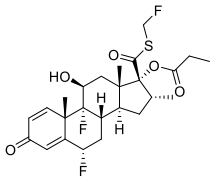
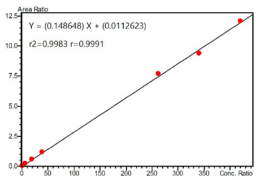


Fluticasone propionate



Fluticasone propionate is a synthetic corticosteroid. LCMS-8060 achieves a LLOQ of 0.2 pg/mL in 0.8 mL of plasma and a calibration range of 0.2 – 120.888 pg/mL. Analysis is achieved with a single-step solid phase extraction and the analyte peak eluted within 6 min.

Ref: ADC/SAIP/B-003

3 Highly Sensitive Quantitative Analysis of Mometasone from Plasma

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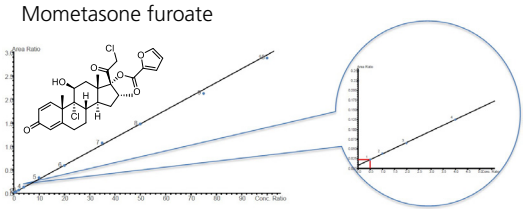
Mometasone furoate is a synthetic corticosteroid. LCMS-8060 achieves a LLOQ of 0.5 pg/mL in plasma and a calibration range of 0.5 – 100 pg/mL. Analysis is achieved upon a simple sample preparation protocol involving solid phase extraction. The analyte peak eluted within 4.5 min.

Ref: TP-774

Mometasone furoate



4 Low Level Quantitation of Fluticasone and Salmeterol from Plasma

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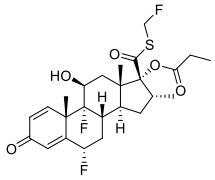
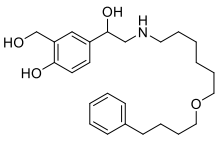
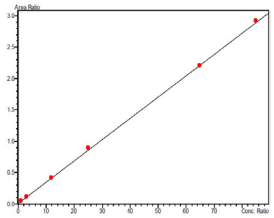


Fluticasone propionate



Salmeterol



Fluticasone propionate is a synthetic corticosteroid while salmeterol is a long-acting beta-agonist bronchodilator. LCMS-8060 achieves a LLOQ of 1 pg/mL for fluticasone propionate and 2 pg/mL for salmeterol. A calibration range of 1 – 100 pg/mL is attained for both. Both analyte peaks eluted within 1.5 min.

Ref: LC-13-ADI-034

5 Highly Sensitive Quantitative Analysis of Budesonide from Plasma

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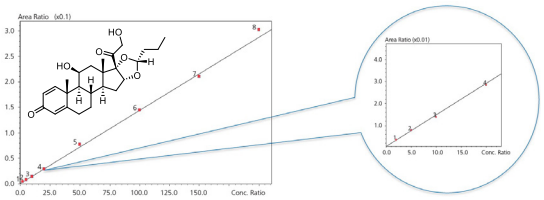
Budesonide is a synthetic corticosteroid. LCMS-8060 achieves a LLOQ of 2.0 pg/mL in plasma and a calibration range of 2 – 200 pg/mL. Analysis is achieved upon a simple sample preparation protocol involving solid phase extraction. The analyte peak eluted within 4 min.

Ref: PO-CON1744E



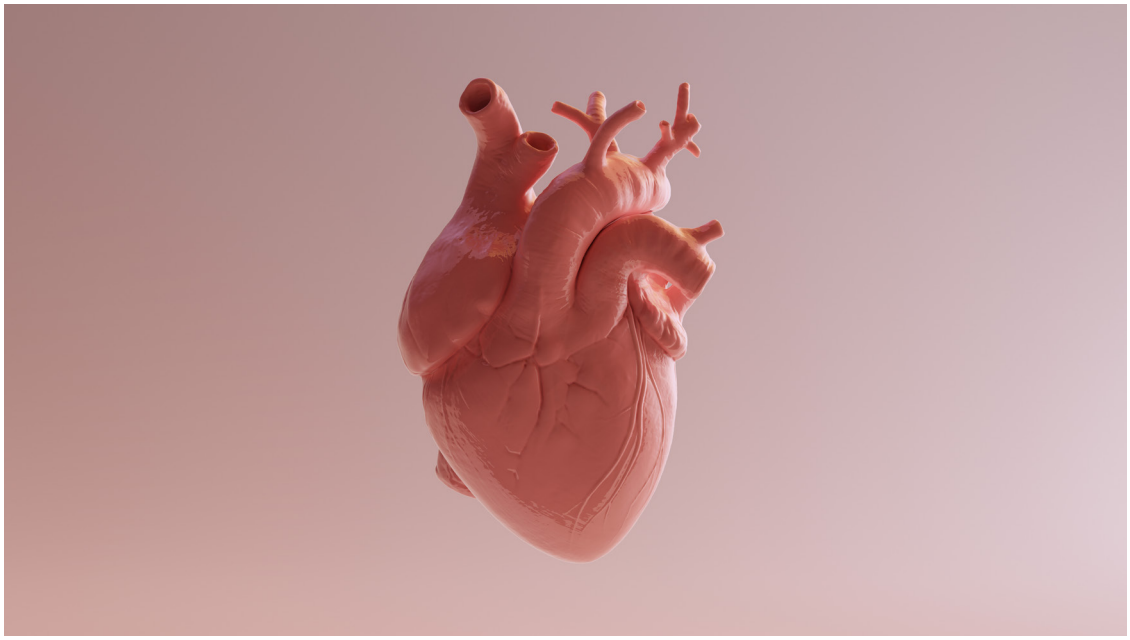
Budesonide

02

Drugs for Cardiovascular and Associated Diseases

Cardiovascular diseases comprise of heart and blood vessels disorders. They are the top causes of death globally, accounting for approximately 30% of all deaths in 2016. Significant risk factors associated with cardiovascular diseases are hypertension and hyperlipidemia which can be effectively managed using a range of drugs.

These drugs usually involve chronic usage and the oral route is the typical method of administration due to its simplicity. The commonly used classes of cardiac drugs include angiotensin receptor blockers and HMG CoA reductase inhibitors.



1 A Fast LC-MS/MS Method for Determination of Telmisartan in Human Plasma

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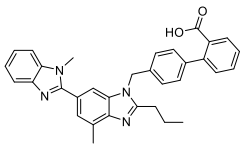
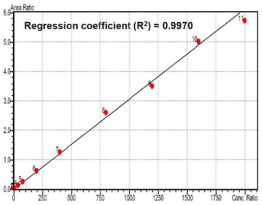
Telmisartan is an angiotensin II receptor antagonist. LCMS-8040 achieves a LLOQ of 4 ng/mL in 0.18 mL of plasma and a calibration range of 4 – 2000 ng/mL. A simple protein precipitation method was employed and the analyte peak eluted within 2 min.

Ref: AD-0072



Telmisartan



2 A High Sensitivity LC-MS/MS Method for Quantitative Determination of Telmisartan in Human Plasma

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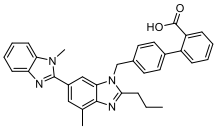
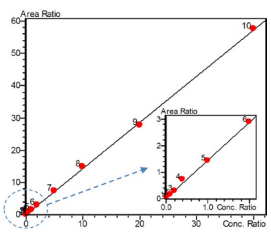


Telmisartan



Telmisartan is an angiotensin II receptor antagonist. LCMS-8050 achieves a LLOQ of 1 pg/mL in 0.15 mL of plasma and a calibration range of 1 – 2000 pg/mL. A simple protein precipitation method was employed and the analyte peak eluted within 2 min.

Ref: AD-0077

3 Fast LC-MS/MS Method for Quantitative Determination of Valsartan in Human Plasma

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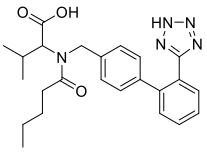
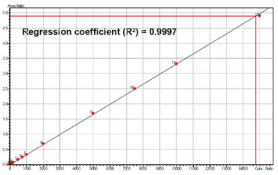
Valsartan is an angiotensin II receptor antagonist. LCMS-8040 achieves a LLOQ of 20 ng/mL in 0.18 µL of plasma and a calibration range of 20 – 15000 ng/mL. A simple protein precipitation method was employed and the analyte peak eluted within 3.5 min.

Ref: AD-0065



Valsartan



4 Highly Sensitive Quantitative Analysis of Felodipine and Hydrochlorothiazide from Plasma

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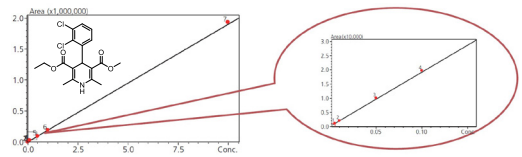
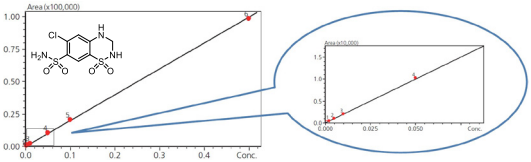


Felodipine



Hydrochlorothiazide

Felodipine is a calcium channel blocker and hydrochlorothiazide is a thiazide diuretic. LCMS-8050 achieves a LLOQ of 5 pg/mL and 2 pg/mL for felodipine and hydrochlorothiazide, respectively, in 0.5 mL of plasma. The respective calibration ranges were 5 – 10000 pg/mL and 2 – 500 pg/mL. A simple sample preparation technique including protein precipitation and liquid-liquid extraction was employed and the analyte peak eluted within 3 min.

Ref: PO-CON1468E

5 Highly Sensitive and Simple Method for Estimation of Tiotropium in Human Plasma

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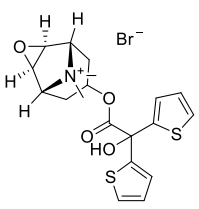
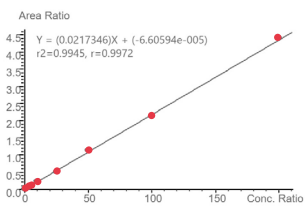
Tiotropium is a bronchodilator. Analysis with LCMS-8060 achieves a LLOQ of 0.2 pg/mL in 450 µL of plasma and with a calibration range of 0.2 – 200 ng/mL. The analyte peak eluted within 6 min. Further validation on selectivity, linearity, recovery, matrix effect, carry-over effect, stability studies as well as inter-day and intra-day precision and accuracy were performed as per FDA guidelines.

Ref: SAIP/ADC/B-009



Tiotropium

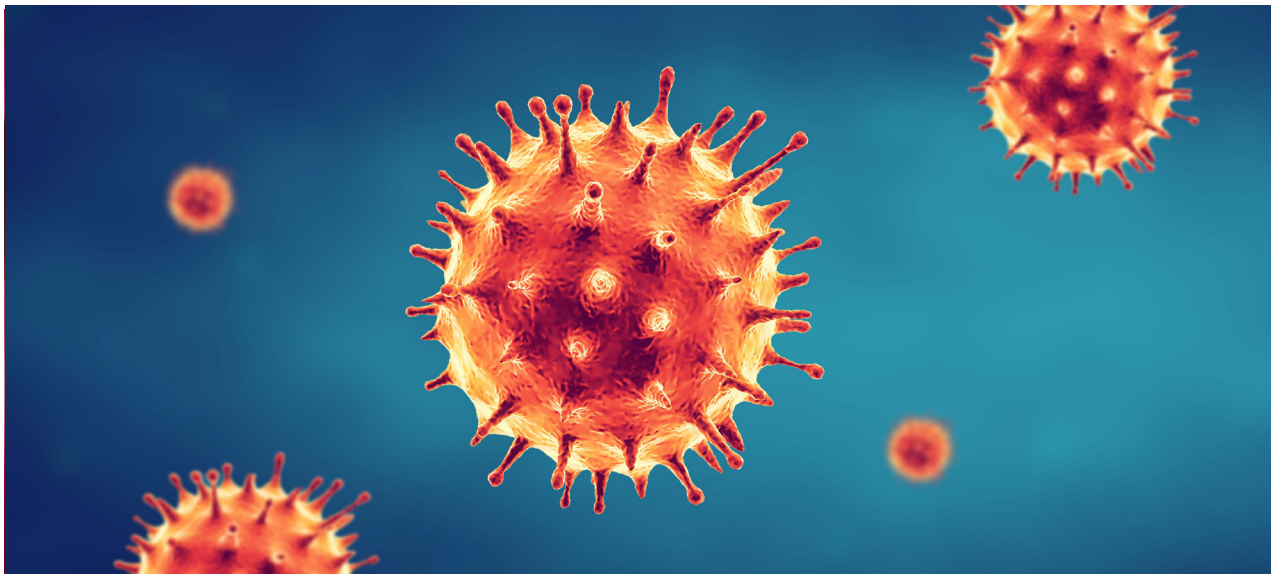


03

Drugs for Infections

Infectious diseases are disorders caused by organisms such as viruses, bacteria, fungi or parasites. Some infectious diseases can be contagious and transmittable by humans, insects, animals or organisms in the environment. In fact, infectious diseases are currently spreading geographically much faster than any time in the human history. Most infectious diseases can be fatal if left untreated.

The prevalence and high economic burdens of infectious diseases have driven worldwide research for new and novel solutions, hoping to prevent epidemic or pandemic events.



1 A Fast and Sensitive LC-MS/MS Method for Quantitation of Fosfomycin in Human Plasma with HILIC Chromatography

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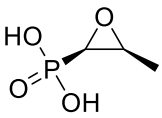
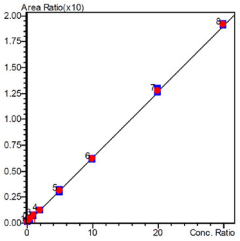
Fosfomycin is a broad-spectrum antibiotic. LCMS-8060 achieves a LLOQ of 20 ng/mL of plasma and a calibration range of 20 – 6000 ng/mL. A simple protein precipitation method was employed and the analyte peak eluted within 2 min.

Ref: THP757



Fosfomycin



2 Quantitative Analysis of β-Lactam Antibiotics in Human Plasma

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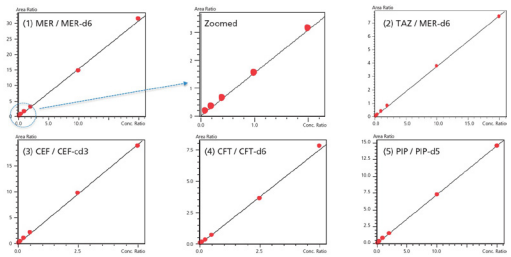

β-lactams are a group of antibiotics with a β-lactam ring in their molecular structures. LCMS-8060 achieves a simultaneous analysis of five β-lactam antibiotics, namely cefepime (CEF), ceftazidime (CFT), meropenem (MER), piperacillin (PIP) and tazobactam (TAZ) with LLOQ of 20 ng/mL with calibration range of 20 – 4000 ng/mL. A simple protein precipitation method was employed and the analyte peak eluted within 3 min.

Ref: PO-CON1738E

3 Bioanalytical Method for Quantification of Polymyxin B1, B2, B3 and Isoleucine-Polymyxin B1 in Human Plasma

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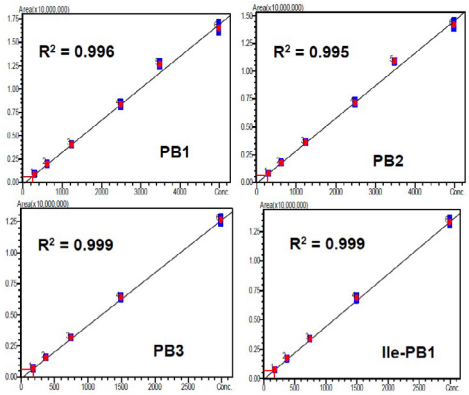
Polymyxins B are a group of lipopeptide antibiotics, consisting mainly of polymyxin B1 (PB1), polymyxin B2 (PB2), polymyxin B3 (PB3) and polymyxin B1-isoleucine (Ile-PB1). LCMS-8060 achieves a LLOQ of 10 ng/mL for PB1, PB2 and PB3, and 30 ng/mL for Ile-B1 in 50 µL of plasma. Calibration range of 0.1 – 5 µg/mL was achieved for PB1 and PB2 while PB3 and Ile-PB1 were 0.05 – 3.75 µg/mL. A simple protein precipitation method was employed and the analyte peak eluted within 9 min.

Ref: TP118



4 A High Sensitivity LC-MS/MS Method for Quantitative Analysis of Eight Antifungal Drugs in Human Serum

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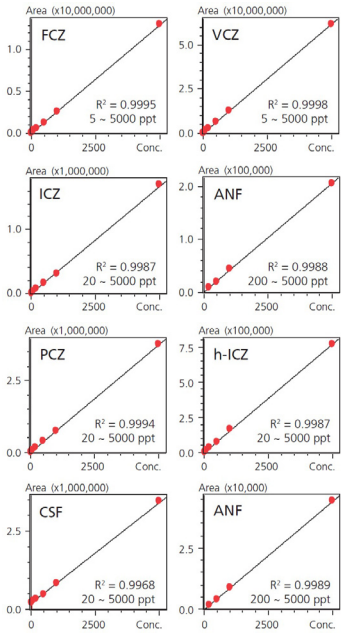

Triazoles and echinocandins are two classes of drugs commonly used for fungal infections. LCMS-8060 achieves a simultaneous analysis of five triazoles, namely fluconazole (FCZ), posaconazole (PCZ), voriconazole (VCZ), hydroxy itraconazole (h-ICZ) and itraconazole (ICZ) and three echinocandins, mainly anidulafungin (ANF), caspofungin (CSF) and micafungin (MCF). The LLOQ achieved with LCMS-8060 for FCZ, VCZ, PCZ, h-ICZ, ICZ, ANF, CSF, MCF were 4.9 pg/mL, 1.3 pg/mL, 9.5 pg/mL, 18.3 pg/mL, 18.9 pg/mL, 126.2 pg/mL, 9.3 pg/mL and 130.8 pg/mL, respectively, in 100 µL of human serum. Calibration range of FCZ and VCZ are from 5 – 5000 pg/mL, PCZ, h-ICZ , ICZ and CSF from 20 – 5000 pg/mL and ANF and MCF from 200 – 5000 pg/mL. A simple protein precipitation method was employed and the analyte peak eluted within 3 min.

Ref: PO-CON1740E

5 High Throughput Quantitation for Therapeutic Drug Monitoring with Open Access LC-MS/MS

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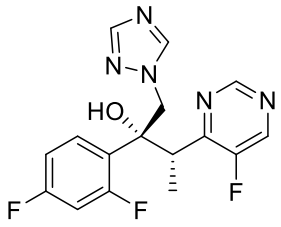
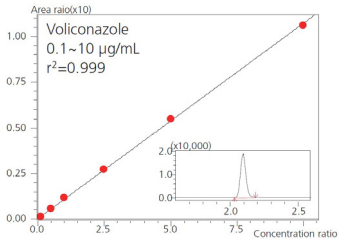
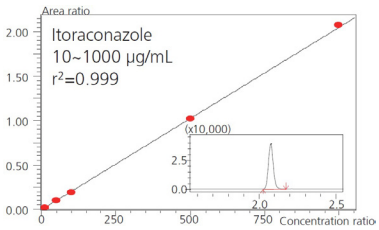
Voriconazole and itraconazole are triazole antifungal agents. Two methods were developed for the analysis of voriconazole as well as for itraconazole and its hydroxyl metabolite. LCMS-8050 achieves a LLOQ of 0.1 µg/mL for voriconazole and 10 µg/mL for itraconazole and hydroxy itraconazole. Calibration range of 0.1 – 10 µg/mL and 10 – 10000 µg/mL were attained for itraconazole and hydroxy itraconazole. A simple protein precipitation method was employed and the analyte peaks eluted within 2.5 min.

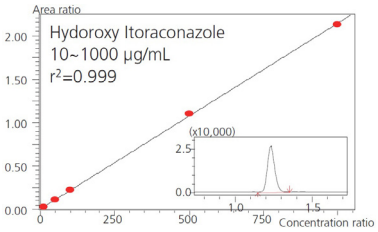
Ref: PO-CON1643E



Voriconazole





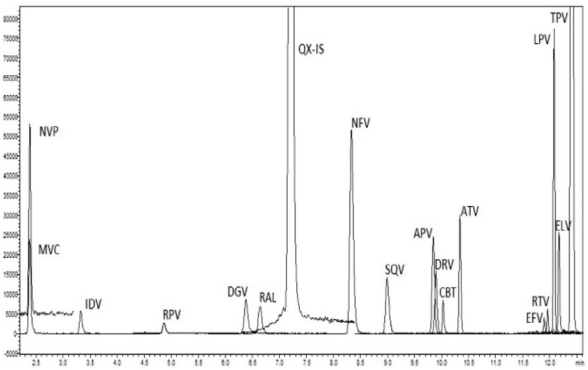


6 Quantification of 16 Anti-HIV Drugs from Human Plasma

Go to Page

Sixteen anti-HIV drugs, namely maraviroc, nevirapine, indinavir, rilpivirine, dolutegravir, raltegravir, nelfinavir, saquinavir, darunavir, cobicistat, amprenavir, atazanavir, ritonavir, elvitegravir, lopinavir, efavirenz and tipranavir were analyzed with LCMS-8050. A simple protein precipitation method was employed and all analyte peaks eluted within 13 min.

Ref: SCA_210_019

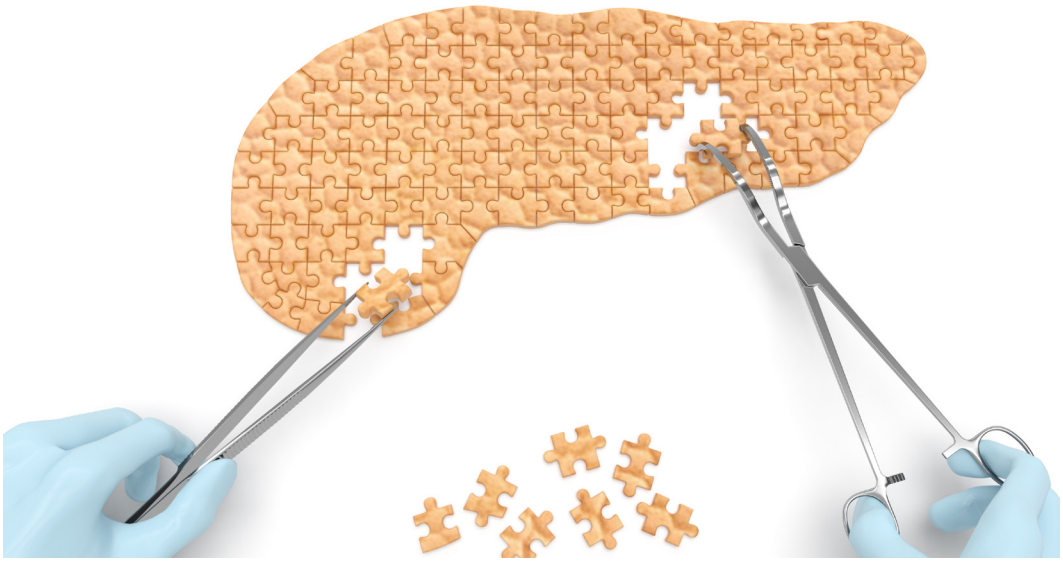


04

Drugs for Hormonal or Metabolic Disorders

Peptides represent an exclusive class of drug due to its molecular size, biochemical and therapeutic distinctiveness. Peptide drugs are essentially applied as replacement therapy to supplement the lack or absence of endogenous peptide hormones. One of the earliest known peptide therapy was the usage of insulin for the regulation of blood glucose level.

The limitations of endogenous peptides have spurred the rapid development of analogs with improved pharmaceutical properties. Further advancement in peptide screening and computational biology are expected to increase the discovery of new peptide drugs in the future.

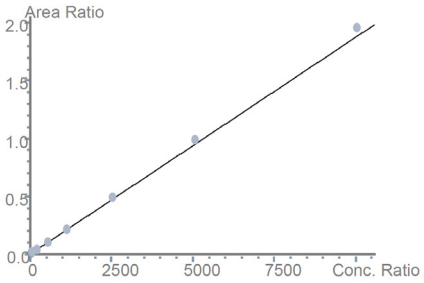


1 Development and Validation of a Sensitive Bioanalytical Method for Estimation of Intact Insulin in Human Plasma

Go to Page

Insulin is a hormone made up of 51 amino acids which regulates the blood glucose level. LCMS-8060 achieves a LLOQ of 50 pg/mL in 500 µL of plasma with a calibration range of 51.5 – 10058.6 pg/mL. The analyte peak eluted within 13 min.

Ref: SAIP/ADC/B-005



2 Multidimensional LC-MS/MS Method for the Quantification of Intact Human Insulin

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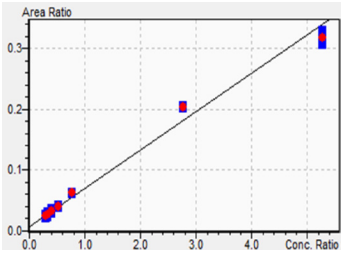


Insulin is a hormone made up of 51 amino acids which regulates the blood glucose level. Analysis with 2D-LC-MS/MS set up on LCMS-8060 achieves a LLOQ of 0.6 ng/mL with a calibration range of 49.9 – 9989.8 pg/mL. A simple protein precipitation method was employed and the analyte peak eluted within 6 min.

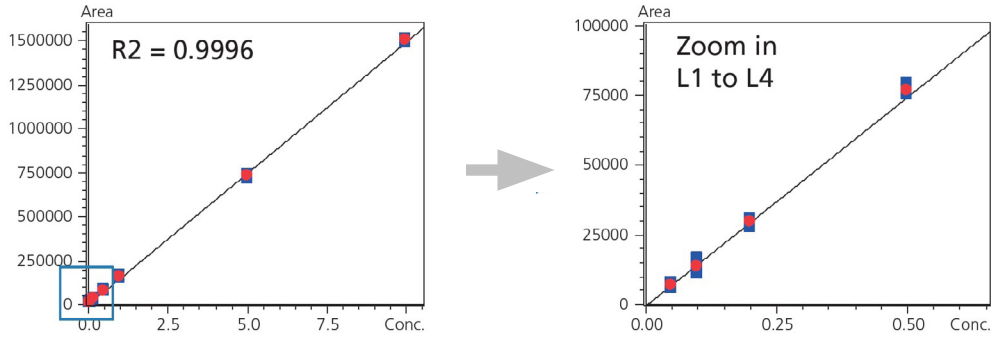
Ref: AD-0182

3 Development of 2D-LC-MS/MS Bioanalytical Method for Quantitative Determination of Insulin Glargine in Human Plasma

Go to Page

Insulin glargine is a recombinant insulin analogue. 2D-LC-MS/MS set up on LCMS-8060 achieved a LLOQ of 70 pg/mL in 500 µL plasma with a calibration range of 50 – 10000 pg/mL. A simple protein precipitation method was employed and the analyte peak eluted within 5 min.

Ref: PO-CON1645E

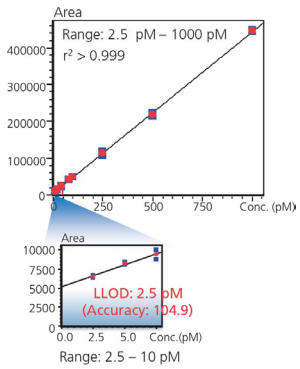


4 Selective and Sensitive Quantification of Glucagon and Glucagon-Related Peptide Hormones in Human Plasma

Go to Page

Glucagon is a hormone made up of 29 amino acids which regulates the blood glucose level. Analysis with LCMS-8060 achieves a LLOQ of 8.7 pg/mL in 500 µL of plasma with a calibration range of 8.7 – 3482 pg/mL. A simple solid phase extraction method was employed and the analyte peak eluted within 7 min.

Ref: PO-CON1631E



5 Chromatographically Resolved Sensitive Method for Estimation of Octreotide in Human Plasma

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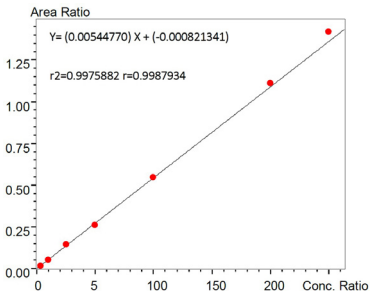
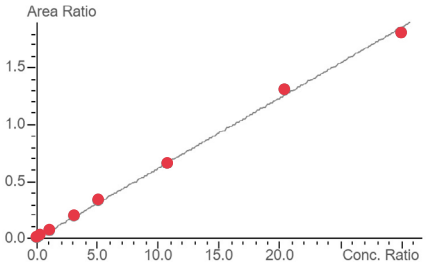
Octreotide is a synthetic hormone made up of 8 amino acids which mimics the effects of somatostatin. Analysis with LCMS-8060 achieves a LLOQ of 3.0 pg/mL in 500 µL of plasma with a calibration range of 3 – 250 pg/mL. A simple sample preparation method including solid phase extraction was employed and the analyte peak eluted within 5 min.

Ref: SAIP/ADC/B-004



6 Selective and Sensitive Method for Estimation of Liraglultide in Human Plasma

Go to Page



Liraglutide is an incretin mimetics hormone used to treat diabetes mellitus type 2 and obesity. Analysis with LCMS-8060 achieves a LLOQ of 0.5 ng/mL in 200 µL of plasma and with a calibration range of 0.5 – 200 ng/mL. The analyte peak eluted within 5 min. Further validation on selectivity, linearity, recovery, carry-over effect as well as inter-day and intra-day precision and accuracy were performed as per FDA guidelines.

Ref: SAIP/ADC/B-007

7 Simple and Sensitive Method for Estimation of Leuprolide in Human Plasma

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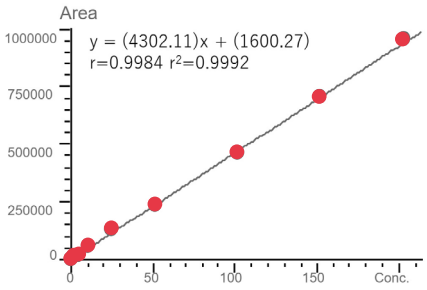
Leuprolide is a gonadotropin-releasing hormone agonist that is used to treat hormone-responsive cancers. Analysis with LCMS-8045 achieves a LLOQ of 0.025 ng/mL in 200 µL of plasma and with a calibration range of 0.025 – 30 ng/mL. The analyte peak eluted within 4 min. Further validation on selectivity, linearity, recovery, matrix effect, carry-over effect as well as inter-day and intra-day precision and accuracy were performed as per FDA guidelines.

Ref: SAIP/ADC/B-008



05

Endogenous or Synthetic Hormonal Steroids

Steroid hormones help to regulate metabolism, immune functions, inflammation and development of sexual characteristics. Elevation or reduction of endogenous steroid hormone levels are usually associated with metabolic disorder in clinical research and diagnosis. Steroid hormones are also commonly administered to regulate their endogenous levels or used for cancer pharmacotherapy.

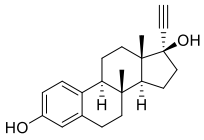
However, accurate quantification of trace-level hormonal steroids with mass spectrometry is challenging due to their intrinsic difficulty to be ionised and matrix interferences.

1 LC-MS/MS Method for Quantitative Determination of Ethinyl Estradiol in Human Plasma

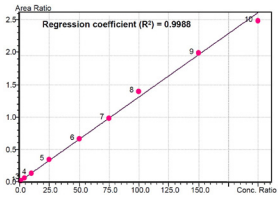
Go to Page

Ethinyl estradiol is a synthetic estrogen. Analysis with LCMS-8040 achieves a LLOQ of 1.0 pg/mL in 713 µL of plasma with a linear calibration range of 1.0 – 200.0 pg/mL. The sampling protocol includes liquid-liquid extraction, derivatisation with dansyl chloride and solid phase extraction. The analyte peak eluted within 6 min.

Ref: AD-0078

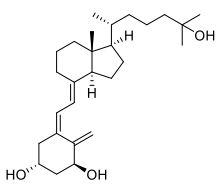


Ethinyl estradiol

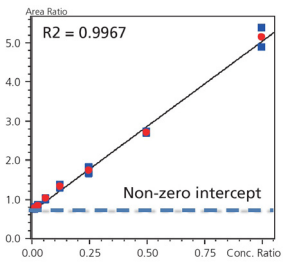


2 Development of 2D-LC/MS/MS Method for Quantitative Analysis of 1α,25-Dihydroxylvitamin D3 in Human Serum

Go to Page



1α,25-Dihydroxylvitamin D3



1α,25-Dihydroxylvitamin D3 is an active form for Vitamin D in the body. Analysis with LCMS-8050 achieves a LLOQ of 10 pg/mL in 150 µL of plasma with a calibration range of 3.1 – 200.0 pg/mL. A simple protein precipitation method was applied and the peak eluted within 23 min.

Ref: PO-CON1470E

3 A Direct LC-MS/MS Method for Quantitative Determination of 25-Hydroxyvitamin D2 and D3 in Human Plasma

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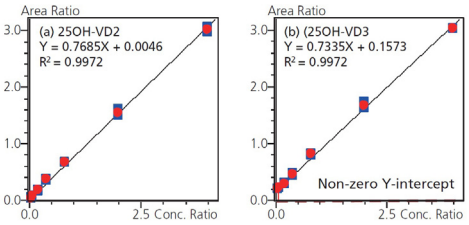
25-Dihydroxylvitamin D2 and D3 are active forms for vitamin D in the body. Analysis with LCMS-8050 achieves a LLOQ of 3 ng/mL in 125 µL of plasma with a calibration range of 1.0 – 100.0 ng/mL. A simple protein precipitation method was employed and the analyte peak eluted within 8.6 min.

Ref: PO-CON1537E

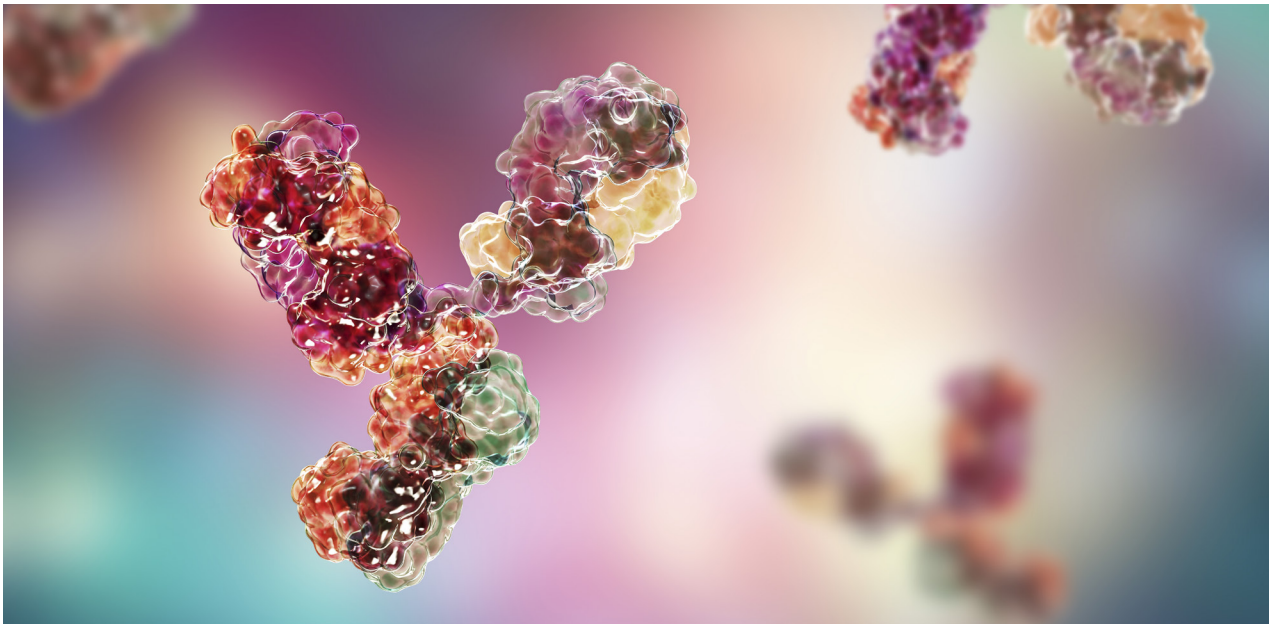


06

Antibody Drugs

Human monoclonal antibodies are highly specific and can act through multiple mechanisms to deliver targeted therapeutic effects. Monoclonal antibodies are currently used for cancer treatment and autoimmune diseases.

The current bioanalysis technique of monoclonal antibodies, which is based on ligand binding assay, tends to show large variability and requires long assay development time. The application of nSMOL™ reagent kits achieves a paradigm shift in the bioanalysis of antibody drugs as it shortens analytical time, improves sensitivity and increases robustness of LC-MS analysis.

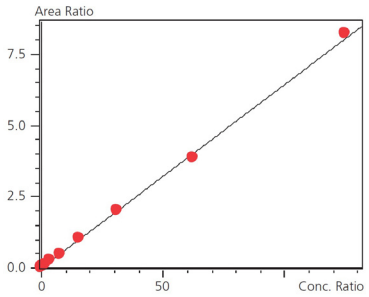


1 LC-MS/MS Quantitative Method Development of Herceptin Based on Selective Hydrolysis (nSMOL™) Technology and Skyline Software

Go to Page

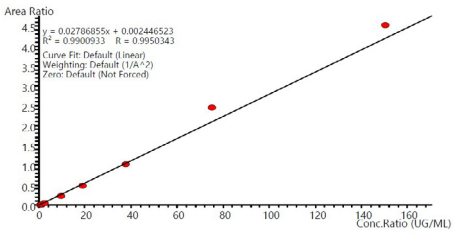
Herceptin (Trastuzumab) is a monoclonal antibody targeting HER2 receptors. Analysis with LCMS-8060 achieves a LLOQ of 0.122 µg/mL with a calibration range of 0.122 – 125 µg/mL. The sample protocol required includes nSMOL™ pretreatment for selective enzymolysis of Fab region of monoclonal antibody. The analyte peak eluted within 10 min.

Ref: PO-CON1779E



2 Bioanalytical Assessment of Cetuximab in Wistar Rat Plasma Using nSMOL™

Go to Page



Cetuximab is an epidermal growth factor receptor inhibitor. Analysis with LCMS-8060 achieves a LLOQ of 0.295 µg/mL with a calibration range of 0.295 – 150 µg/mL. The sample preparation includes nSMOL™ pretreatment for selective enzymolysis of Fab region of monoclonal antibody. A simple protein precipitation method was applied and the peak eluted within 5.5 min.

Ref: LC-31-ADI-54

3 *Bioanalysis of Denosumab in Human Plasma Using nSMOL™*

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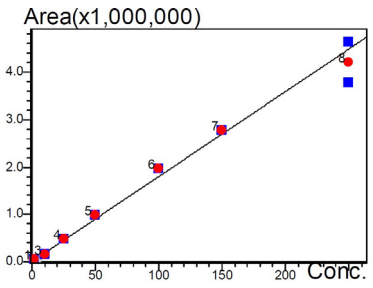
Denosumab is a RANK ligand inhibitor. Analysis with LCMS-8060 achieves a LLOQ of 2 µg/mL with a calibration range of 2 – 250 µg/mL. The sample protocol required includes nSMOL™ pre-treatment for selective enzymolysis of Fab region of monoclonal antibody and the analyte peak eluted within 9.5 min.

Ref: LAAN-A-LC-E291



07

Drugs for Other Therapeutic Areas

There exists a broad range of other drug classes which do not fall into the categories listed in this booklet. Several other drug types encompass immunosuppressant, tumor suppressor, anti-histamine and gastric acid suppressor. These drugs are important and crucial in tackling the medical conditions that they are designed for.

For example, immunosuppressants play a central role in the success of tissue or organ transplants. However, due to their potent immunosuppressant effects and relatively narrow therapeutic index, the monitoring of drug level in patients is required to ensure efficacy and minimize toxic side effects.

1 Quantification of Immunosuppressant Drugs in Whole Blood by LC-MS/MS

Go to Page

Cyclosporin A, tacrolimus, sirolimus and everolimus are immunosupressants. LCMS-8040 achieves a LLOQ of 0.2 ng/mL for everolimus, tacrolimus and sirolimus, and 6.2 ng/mL for cyclosporin A in 300 µL of whole blood. The calibration range for everolimus, tacrolimus and sirolimus is 0.2 – 25 ng/mL while cyclosporin A is 6.2 – 800 ng/mL. The sample preparation includes protein precipitation. The analyte peak eluted within 4 min.

Ref: LC-08-ADI-029

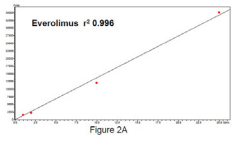


Figure 2A

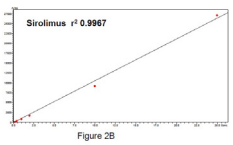


Figure 2B

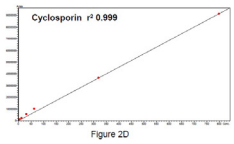


Figure 2D

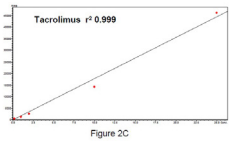
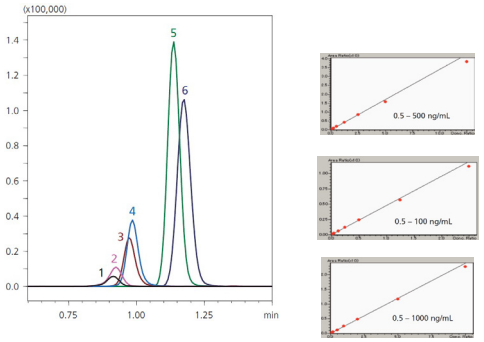


Figure 2C

2 Accelerated and Robust Monitoring for Immunosuppressants Using Triple Quadrupole Mass Spectrometry

Go to Page



Cyclosporin A, tacrolimus, sirolimus and everolimus are immunosuppressants. LCMS-8050 achieves a LLOQ of 0.5 ng/mL for all analytes in 2.7 mL of whole blood. The calibration ranges are 0.5 – 1000 ng/mL for cyclosporin A and tacrolimus, 0.5 – 1000 ng/mL, 0.5 – 500 ng/mL for sirolimus and 0.5 – 100 ng/mL for everolimus. A liquid-liquid extraction step was applied and the analytes eluted within 1.8 min.

Ref: PO-CON1449E

3 High Throughput Quantitation for Therapeutic Drug Monitoring with Open Access LC-MS/MS System

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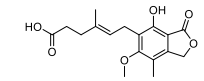
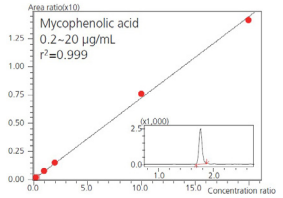
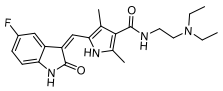
Mycophenolic acid is an immunosuppressant while sunitib and axitinib are anti-cancer drugs. Analysis with LCMS-8050 achieves a LLOQ of 0.2 µg/mL for mycophenolic acid, 3 µg/mL for sunitinib and 0.3 µg/mL for axitinib. The calibration ranges are 0.2 – 20 µg/mL for mycophenolic acid, 3 – 300 µg/mL for sunitinib and 0.3 – 30 µg/mL for axitinib. A simple protein precipitation method was employed and the analyte peak eluted within 5 min.

Ref: PO-CON1643E

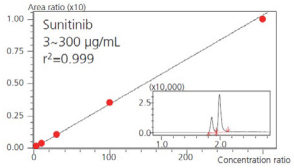


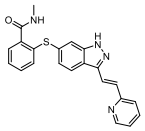
Mycophenolic acid



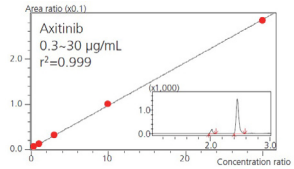


Sunitib



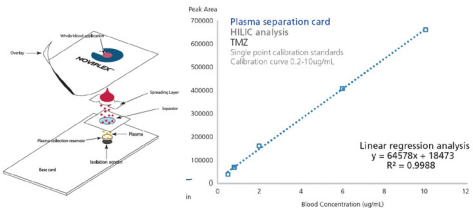


Axitinib



4 Next Generation Plasma Collection Technology for the Clinical Analysis of Temozolomide by HILIC/MS/MS

Go to Page



Temozolomide is an imidazotetrazine alkylating agent. LCMS-8060 achieved a LLOQ of 0.2 µg/mL in 50 µL of blood collected on plasma separation card with a calibration range of 0.2 – 10 µg/mL. The sample preparation protocol includes the collection of blood on plasma separation card and extraction using acetonitrile. The analyte peak eluted within 2 min.

Ref: PO-CON1482E

5 Fast LC-MS/MS Method for Quantitative Determination of Omeprazole in Human Plasma

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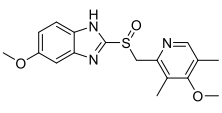
Omeprazole is a proton pump inhibitor. Analysis with LCMS-8040 achieves a LLOQ of 1.0 ng/mL in 180 µL of plasma with a calibration range of 1.0 – 2000 ng/mL. A simple liquid-liquid phase extraction was employed and the analyte peak eluted within 2.5 min.

Ref: LAAN-A-LC-E291



Omeprazole

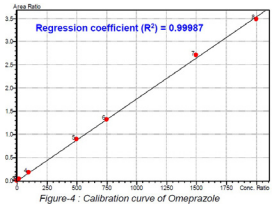


Figure-4 : Calibration curve of Omeprazole

SHIMADZU
Excellence in Science

Application News

Liquid Chromatography Mass Spectrometry

A rapid, sensitive and direct quantification of Salmeterol in human plasma using LCMS-8060

No.LC-21-ADI-043

Introduction

Salmeterol xinafoate combined with fluticasone propionate is used in the management of asthma and Chronic Obstructive Pulmonary Disease (COPD). The combination of the long-acting beta-agonist salmeterol and the inhaled corticosteroid fluticasone propionate may reduce mortality among patients with COPD. Dosing of salmeterol and fluticasone through inhalation leads to low systemic concentrations. Hence, it is imperative to develop a highly sensitive LC/MS/MS assay for bioanalytical quantification.

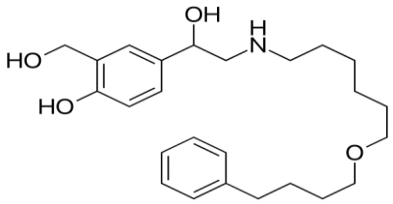


Fig. 1 Chemical structures of salmeterol

We have developed and validated most sensitive LC-MS/MS method capable of quantifying 0.8 pg/mL of salmeterol in 0.2 mL human plasma. The given method describes calibration curve for salmeterol ranging from 0.8 pg/mL to 100 pg/mL. The method was validated as per USFDA guidelines. To the best of our knowledge, this is the lowest LLOQ achieved with low sample volume, single step extraction, short run time and direct injection method for quantification of salmeterol. These factors enhance productivity of the pharmacokinetic investigation involving large number of study samples.

Calibration Standards & Quality Control

One of the biggest challenge of salmeterol measurement apart from sensitivity is contamination during sample preparation. Suitable precautions were taken during stock and sample preparation.

Stable isotope labelled internal standard of salmeterol-D3 (ISTD) was used .
Calibration levels were prepared for 9 concentration levels ranging from 0.795 to 101.742 pg/mL. A blank and a blank containing ISTD were injected before the calibration standards.
QC levels covering the entire calibration range were prepared in six replicates for every series

Sample Preparation

Standards or QC samples (200 µL) were spiked with ISTD solution and diluted with aqueous solution. The diluted sample was then submitted to solid phase extraction (SPE). The eluate was evaporated to dryness and reconstituted.

Results - Sensitivity

Calibration curve was analyzed between 0.795- 101.742 pg/mL, with regression coefficient (r) > 0.995 as shown in table 1 and figure 2. Signal to noise ratio (s/n) for 0.795 pg/mL was >15, across 7 PA batches.

Table 1 Calibration levels of salmeterol

Level	Area Ratio
CC1	0.00727
CC2	0.01704
CC3	0.03173
CC4	0.07146
CC5	0.12243
CC6	0.25081
CC7	0.50884
CC8	0.98692
CC9	0.00727

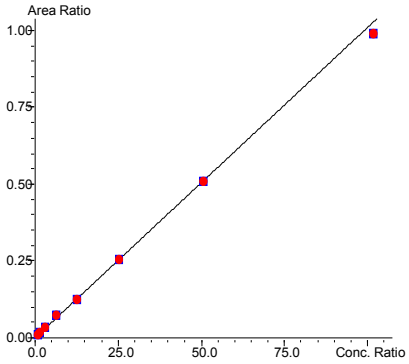


Fig. 2 Calibration curve for salmeterol

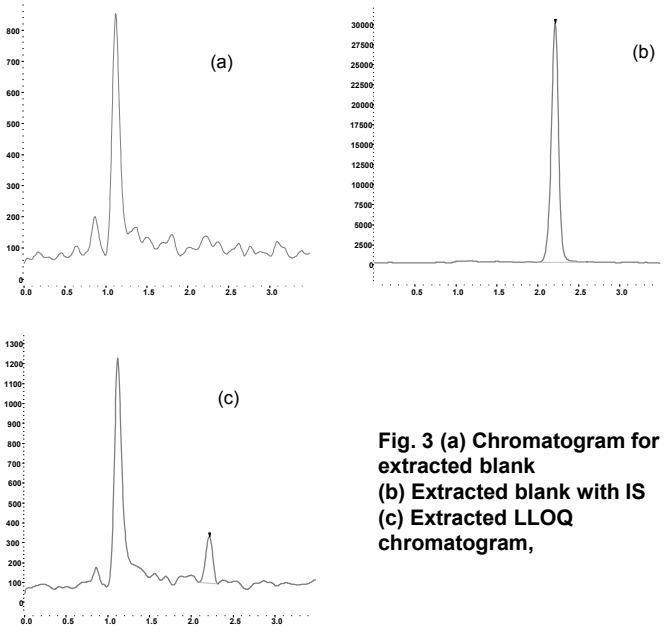


Fig. 3 (a) Chromatogram for extracted blank
(b) Extracted blank with IS
(c) Extracted LLOQ chromatogram,

The lower limit of quantification (LLOQ) was set to 0.795 pg/mL. Figure 3 shows representative chromatograms of the extracted blank, extracted blank with IS and extracted LLOQ.
Method was assessed for various validation parameters, like precision and accuracy, recovery, matrix effect, stability etc. These values were found to be well under acceptable levels as shown in table 2.

Table 2 Method validation summary

Calibration curve range			0.795 pg/mL to 101.742 pg/mL	
Intraday precision and accuracy	Accuracy (% Nominal)		88.00 to 112.43	
	Precision (% CV)		5.09 to 9.89	
Inter day precision and accuracy	Accuracy (% Nominal)		92.24 to 108.94	
	Precision (% CV)		4.26 to 14.96	
Recovery	% Recovery		66.51	
	Precision (% CV)		4.95	
Matrix effect	Matrix Factor		0.91	
	IS normalized Matrix Factor		0.97	
Bench top stability in matrix (6.0 hrs.)	% Change		LQC=-3.58	HQC=0.64
	Precision (% CV)	Stability Sample	LQC=4.98	HQC=0.56
		Comparison Sample	LQC=3.81	HQC=3.36
	Accuracy (% Nominal)	Stability Sample	LQC=109.03	HQC=105.36
		Comparison Sample	LQC=113.08	HQC=104.70
Auto sampler stability in matrix (30.0 hrs.)	% Change		LQC= 0.63	HQC=-0.10
	Precision (% CV)	Stability Sample	LQC=7.08	HQC=1.49
		Comparison Sample	LQC=6.49	HQC=1.49
	Accuracy (% Nominal)	Stability Sample	LQC=108.90	HQC=99.36
		Comparison Sample	LQC=108.22	HQC=99.46
Freeze thaw stability in matrix (Third Cycle)	% Change		LQC=-1.47	HQC=1.51
	Precision (% CV)	Stability Sample	LQC=7.95	HQC=1.26
		Comparison Sample	LQC= 3.81	HQC= 3.36
	Accuracy (% Nominal)	Stability Sample	LQC=111.42	HQC=106.28
		Comparison Sample	LQC=113.08	HQC=104.70
Long term stability in matrix at -80°C (15 Days)	% Change		LQC=-0.26	HQC=2.14
	Precision (% CV)	Stability Sample	LQC=9.10	HQC=0.89
		Comparison Sample	LQC=3.81	HQC=3.36
	Accuracy (% Nominal)	Stability Sample	LQC=112.78	HQC=106.93
		Comparison Sample	LQC=113.08	HQC=104.70

Conclusion

LCMS-8060, along with special sample preparation provides best in class sensitivity for demanding bioanalytical assay such as that of salmeterol.

The criticality of these assays is huge both w.r.t technical challenges and commercial impact for pharmaceutical organizations. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavours.

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First Edition: May 2018

A HIGHLY SENSITIVE AND RAPID METHOD FOR QUANTIFICATION OF FLUTICASONE PROPIONATE USING SHIMADZU LC-MS/MS 8060

Medha Wagh, Chaitanya Atmakuri, Dr. Avinash Gaikwad- Shimadzu Application Development Centre (SAIP)

INTRODUCTION

Fluticasone propionate, a medium-potency synthetic corticosteroid is administered as nasal spray or drops for the treatment of allergic and non-allergic rhinitis; or by oral inhalation for the treatment of asthma. Therapeutic dose of fluticasone results in very low plasma concentrations and requires a sensitive bioanalytical method for accurate quantification of the drug in plasma.

Shimadzu Application Development Centre (ADC), Mumbai has developed and validated the most sensitive method with lowest limit of quantification (LLOQ) of 0.2 pg/mL. The method has used a single step sample extraction technique and direction injection approach to eliminate environmental contamination. These factors enhance productivity of the pharmacokinetic investigation involving high-throughput analysis.

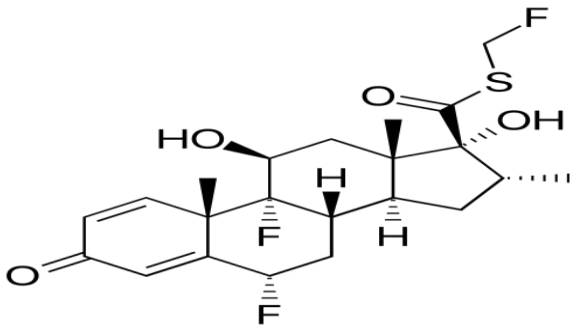

Figure 1. Structure of fluticasone

SALIENT FEATURES

1- **Quantitative method** for estimation of **Fluticasone** in human plasma was developed. Method was **validated** as per USFDA guidelines (results are presented in table 1).

2- Effective **high throughput** for quantitative assessment is increased by use of a quick **single step extraction** procedure along with short analyte retention time of 5.8 min contributed by **Ultra-fast technologies** of **LCMS-8060.**

3- **New UF Qarray ion guide technology** with increased ion production and consistent signal intensity enables sensitive quantification of Fluticasone using a **single MRM transition**.

4- **Optimum plasma volume** (800µL) avoided unnecessary wastage of plasma samples and at the same time increased the life of the mass spectrometer.

5- **Steady gradient method**, helped to eliminate endogenous interference and to reduce background noise for both analyte and its deuterated internal standard.

6- Method was validated for the following criteria:

   a. Selectivity
   b. Linearity
   c. Inter-day and Intra-day precision and accuracy (PA)
   d. Matrix effect
   e. Recovery
   f. Stability

Table 1. Method validation summary

Calibration curve range			0.200 pg/mL to 120.888 pg/mL	
Intra Day precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)		100.00	
	Precision (% CV)		16.33	
Intra Day precision and accuracy (For LQC, MQC, HQC)	Accuracy (% Nominal)		100.24 to 107.89	
	Precision (% CV)		4.98 to 7.41	
Global precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)		102.50	
	Precision (% CV)		18.33	
Global precision and accuracy (For LQC, MQC, HQC)	Accuracy (% Nominal)		102.17 to 108.34	
	Precision (% CV)		7.73 to 11.14	
Global % recovery	% Recovery		64.29	
	Precision (% CV)		7.26	
Matrix effect	Matrix Factor		1.00	
	IS normalized Matrix Factor		1.02	
Bench top stability in matrix (6.0 hrs.)	% Change		LQC=-4.92	HQC=0.81
	Precision (% CV)	Stability	LQC=6.13	HQC=3.05
		Comparison	LQC=7.63	HQC=5.90
	Accuracy (% Nominal)	Stability	LQC=96.85	HQC=106.11
		Comparison	LQC=101.87	HQC=105.26
Auto sampler stability in matrix (50.0 hrs.)	% Change		LQC= -3.12	HQC=-1.63
	Precision (% CV)	Stability	LQC=12.83	HQC=5.07
		Comparison	LQC=8.67	HQC=11.84
	Accuracy (% Nominal)	Stability	LQC=100.32	HQC=110.57
		Comparison	LQC=103.55	HQC=112.40
Long term matrix stability at -80 °C (24 days)	% Change		LQC=-4.80	HQC=6.12
	Precision (% CV)	Stability	LQC=8.34	HQC=7.33
		Comparison	LQC= 7.63	HQC= 5.90
	Accuracy (% Nominal)	Stability	LQC=96.98	HQC=111.70
		Comparison	LQC=101.87	HQC=105.26

METHOD VALIDATION RESULTS:

LINEARITY

Linearity experiments were conducted for fluticasone quantitation in human plasma. Calibration curve was plotted from 0.200-120.888 pg/mL (refer table 2) with r^2> 0.995 as shown in figure 2. Signal to noise ratio (s/n) for 0.200 pg/mL was more than 10, across 5 PA batches. Representative chromatograms are shown in figure 3.

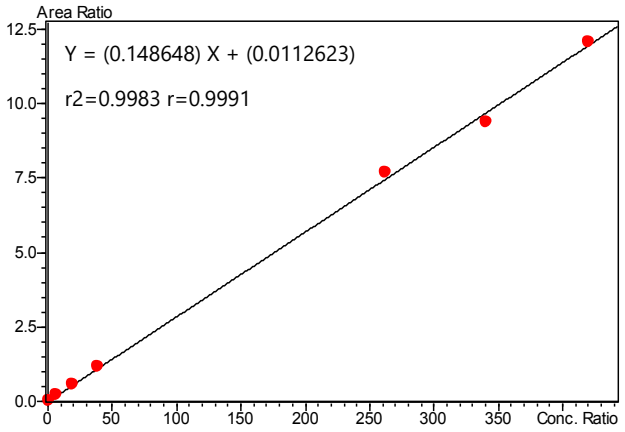

Figure 2. Calibration curve

Table 2.

Calibration curve range

Level	Nominal Conc
CC1	0.200
CC2	0.505
CC3	1.009
CC4	3.154
CC5	9.461
CC6	21.024
CC7	42.048
CC8	84.096
CC9	120.888

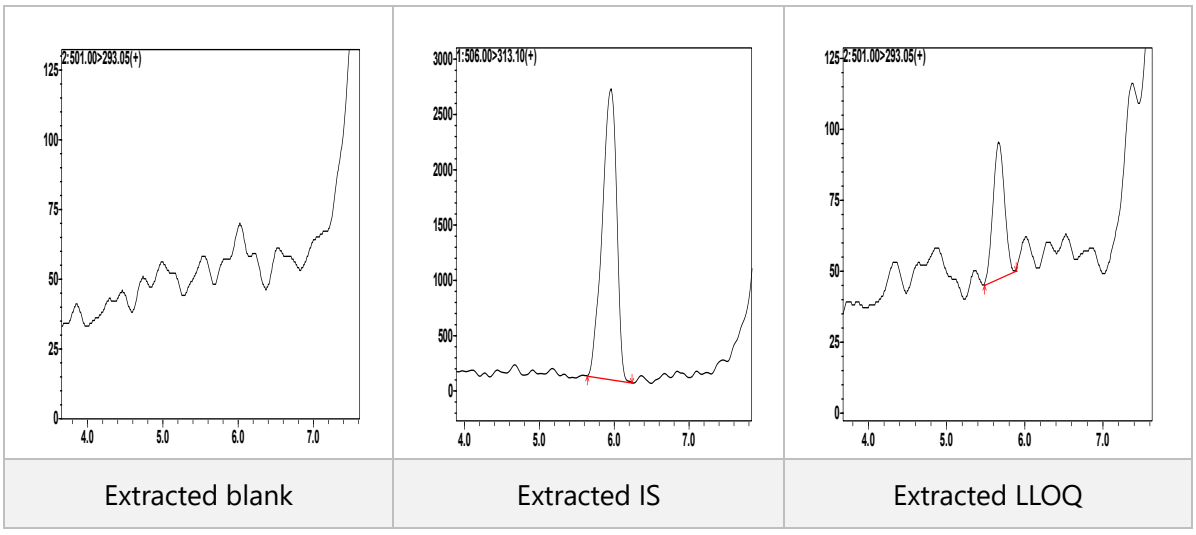

Figure 3. Chromatograms of fluticasone (extracted blank, extracted IS, extracted LLOQ)

SELECTIVITY

Selectivity of this method was assessed in different lots of plasma. Interference from blank matrix was assessed for both fluticasone and fluticasone-D5 (refer figure 3). Percentage interference was found to be less than 10.00 % of LLOQ area response for fluticasone, as shown in table 3 below.

Table 3. Percentage interference in blank with respect to LLOQ

	Fluticasone			Fluticasone-D5		
Lot no	Area in blank matrix at RT of fluticasone	LLOQ area	% Interference at RT of fluticasone	Area in blank matrix at RT of fluticasone-D5	IS area	% Interference at RT of fluticasone-D5
P4458	46	493	9.33	122	66,851	0.18
P4791	0	689	0.00	220	70,195	0.31
P5515	0	627	0.00	153	51,592	0.30
P5517	68	798	8.52	143	53,529	0.27
P5518	0	571	0.00	374	59,237	0.63
P5519	0	890	0.00	89	62,717	0.14

INTRA DAY PRECISION AND ACCURACY

Intraday precision and accuracy was conducted using 6 replicates of LLOQ-QC, LQC, MQC and HQC in a P&A batch. Figure 4 represents the stability of QC samples (X-axis represents number of QC injection and Y-axis represents calculated concentration in log scale). Quantitative data is summarised in table 4.

Table 4. Quantitative tabulation of intra-day QC assessment

QC	LLOQQC	LQC	MQC	HQC
NOMINAL CONC	0.220	1.682	26.280	105.120
PA batch Observed concentration (pg/mL)	0.174	1.516	30.625	112.791
	0.228	1.705	27.378	110.937
	0.181	1.604	27.395	106.078
	0.256	1.822	28.953	100.496
	0.258	1.697	30.559	113.291
	0.223	1.772	25.218	103.049
Mean	0.2200	1.6860	28.3547	107.7737
STDEV	0.0359	0.1114	2.1017	5.3625
%CV	16.33	6.60	7.41	4.98
% Nominal	100.00	100.24	107.89	102.52

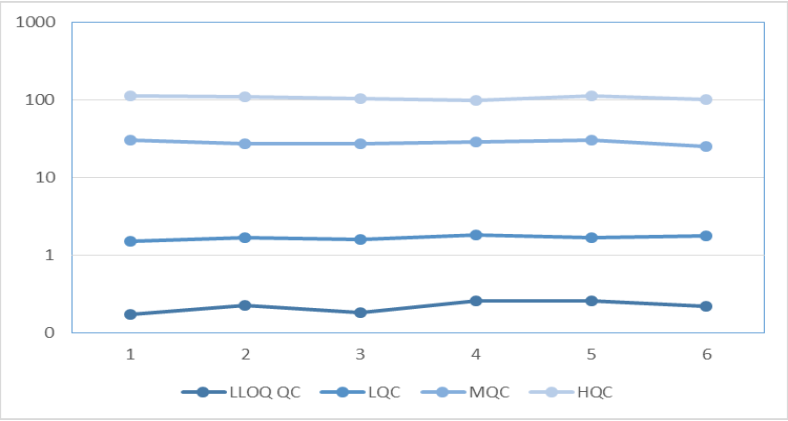

Figure 4. Trend plot of intra-day QC assessment
X-axis represents the number of injections and Y-axis represents calculated concentration (log scale)

GLOBAL PRECISION AND ACCURACY

Precision and accuracy experiments were conducted on 5 batches. Excellent accuracy and repeatability was observed with % CV < 12.00 % and % accuracy > 100.00 % for LQC, MQC and HQC. At LLOQQC the % CV was found to be 18.33 % and % accuracy 112.64 %. The results are presented in table 5. Figure 5 shows trend plots of 30 QC at each level, analysed over 5 batches.

Table 5. Quantitative tabulation of inter-day QC assessment

QC level (n=30)	Mean Conc. (pg/mL)	% CV	% Accuracy
LLOQ QC (0.220 pg/mL)	0.2255	18.33	102.50
LQC (1.682 pg/mL)	1.7184	7.73	102.17
MQC (26.280 pg/mL)	28.4715	10.51	108.34
HQC (105.120 pg/mL)	112.3098	11.14	106.84

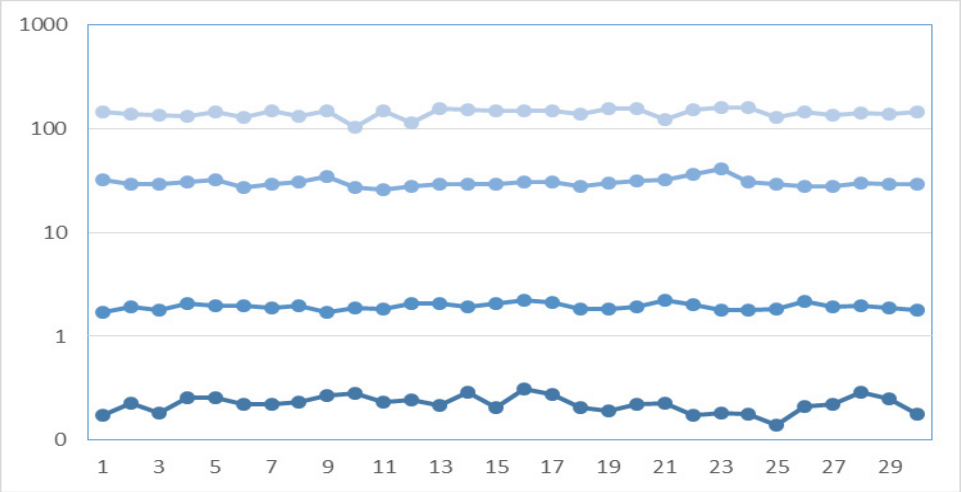

Figure 5. Trend plot of inter-day QC assessment
X-axis represents the number of injections and Y-axis represents calculated concentration (log scale)

MATRIX EFFECT

Matrix effect was studied for both fluticasone and fluticasone-D5 using LQC and HQC samples. Mean matrix factor and IS normalised matrix factor was found to be 1.00 and 1.02 respectively. Representative data of matrix effect is shown in table 6. The results confirm the suitability of method for quantitative estimation of fluticasone in human plasma.

Table 6. Quantitative tabulation of matrix effect

	Response of aqueous standard	Response of post extracted spike sample	Matrix factor
LQC	4792	5550	1.16
	5320	5709	1.07
	4789	5331	1.11
	5450	5389	0.99
	5333	5522	1.04
	5292	5157	0.97
HQC	365280	348228	0.95
	352894	337526	0.96
	348430	350845	1.01
	362809	342762	0.94
	364014	326980	0.90
	357803	336132	0.94
Mean Matrix Factor of fluticasone			1.00
Standard Deviation			0.08
% CV			7.73

RECOVERY

Recovery experiments were conducted to judge combined effect of sample extraction efficiency and instrument method. Average recovery was found to be 64.29 % as shown in table 7.

Table 7. Quantitative tabulation of % recovery

LQC	69.54
MQC	60.61
HQC	62.70
Mean	64.29
SD	4.67
% CV	7.26

CARRY OVER EFFECT

Carry over was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carry over was present/observed at the retention time and MRM transition of fluticasone in the extracted blank sample following the highest standard calibrator.

OTHER EXPERIMENTATION

Based on validation guidelines, method was assessed for following stability studies.

1- Bench top stability

2- Auto sampler stability

3- Long term stability at -80 °C

CONCLUSION

LCMS-8060, along with special sample preparation provides best in class sensitivity for demanding bioanalytical assay of fluticasone. The criticality of these assays is huge, both w.r.t technical challenges and commercial impact for pharmaceutical organizations. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavours.

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Highly sensitive quantitative analysis of mometasone from plasma using LC-MS/MS

TP-774

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Highly sensitive quantitative analysis of mometasone from plasma using LC-MS/MS

1. Introduction

Mometasone furoate is a synthetic corticosteroid used to reduce inflammation of the skin and in airways. It is a prodrug of the free form of mometasone. Administration of mometasone is usually in form of topical creams or low concentration inhalers. Hence the amount of drug present in the blood is very low (due to decreased absorption in topical forms and initial low concentrations in inhalers).

These factors dictate need of establishing highly sensitive bioanalytical assays for quantitation of mometasone furoate in blood. In this study, a highly sensitive method is established for analysis of mometasone in human plasma at low levels required for clinical studies. Developed method demonstrated required amount of sensitivity and specificity. Stability and reproducibility of instrument as well as method were assessed over multiple precision and accuracy batches, results of which are discussed further.

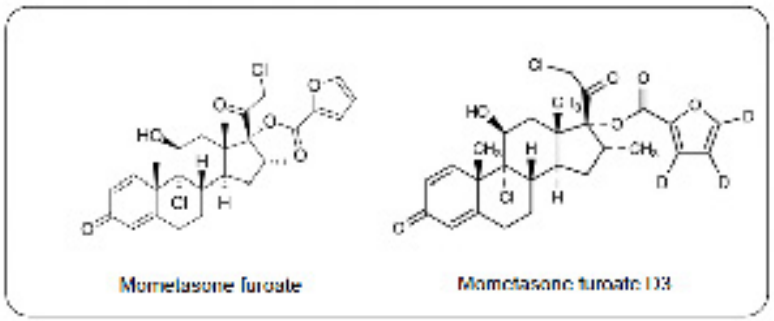

Figure 1. Structure of mometasone furoate and mometasone furoate D3

2. Materials and methods

2.1 Sample Preparation

- **Preparation of spiked calibration standards and quality control (QC) samples**

Mometasone furoate calibration standards at concentration levels of 0.5 pg/mL, 1 pg/mL, 2 pg/mL, 4 pg/mL, 10 pg/mL, 20 pg/mL, 35 pg/mL, 50 pg/mL, 75 pg/mL, 100 pg/mL and quality control samples at concentration levels of LLQC (0.5 pg/mL), LQC (4.5 pg/mL), MQC (40 pg/mL) and HQC (80 pg/mL) were prepared in plasma.

- **Sample Extraction**

Spiked calibration standards and quality control samples in plasma were taken in 4 mL RIA vials to which internal standard solution (mometasone D3) was added except in blank and vortexed. Added precipitant, vortexed and contents werecentrifuged at 4500 rpm for 5 mins.

The samples were extracted by solid phase extraction technique based on :
1. Conditioning (methanol followed by water)
2. Loading (entire plasma sample)
3. Washing (by washing solutions)
4. Elution

SPE eluent was evaporated in low pressure nitrogen evaporator. The residue was reconstituted in 200 µL mobile phase, vortexed and filled in HPLC vials for injection.

2.2 LC-MS/MS analysis

Figure 2. Nexera X2 with LCMS-8060

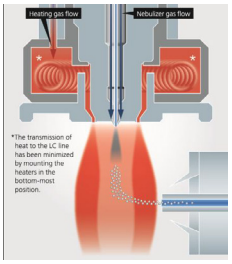

Figure 3. Heated ESI probe

LCMS-8060 triple quadrupole mass spectrometer by Shimadzu (shown in Figure 2), sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UFsensitivity), ultra fast scanning speed of 30,000 u/sec (UFscanning) and polarity switching speed of 5 msec (UFswitching). This system ensures highest quality of data, with very high degree of reliability.

In order to improve ionization efficiency, the newly developed heated ESI probe (shown in Figure 3) combines high-temperature gas with the nebulizer spray, assisting in the desolvation of large droplets and enhancing ionization. This development allows highsensitivity analysis of a wide range of target compounds with considerable reduction in background.

The details of analytical conditions are given in Table 1.

Table 1. Instrument parameters for analysis of mometasone

UHPLC condition (Nexera X2)	
Column	C18 column (100 mm × 3 mm, 2.1 um)
Mobile phase	A: buffer
	B: acetonitrile
Flow rate	0.5 mL/min
Elution mode	Flow gradient mode
Column temperature	50 ^{0}C
MS parameters (LCMS-8060)	
MS interface	Electro Spray Ionization (ESI)
Nitrogen gas flow	Nebulizing gas- 3 L/min; Drying gas- 10 L/min
Zero air flow	Heating gas- 15 L/min
MS temperatures	Desolvation line- 150 ^{0}C; Heating block- 350 ^{0}C; Interface- 250 ^{0}C
MRM transitions (positive)	Mometasone-TIC of (521 > 355) + (521 > 373) Mometasone D3- (524 > 355)

3. Results

LLOQ of 0.5 pg/mL was achieved for mometasone in plasma. Overlay of MRM chromatograms of blank and LLOQ spiked standards are shown in Figure 4A. Similarly, overlay of the MRM chromatograms of blank and zero standard (Blank+IS) for mometasone D3 are shown in the Figure 4B. No interfering peaks were seen in blank plasma at the retention time of these compounds which confirms the absence of any interference.

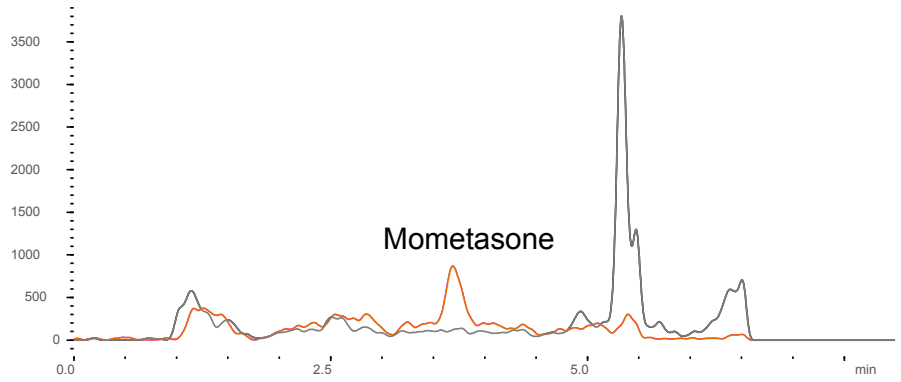

Figure 4A. Overlay of MRM chromatograms of blank and 0.5 pg/mL spiked standard

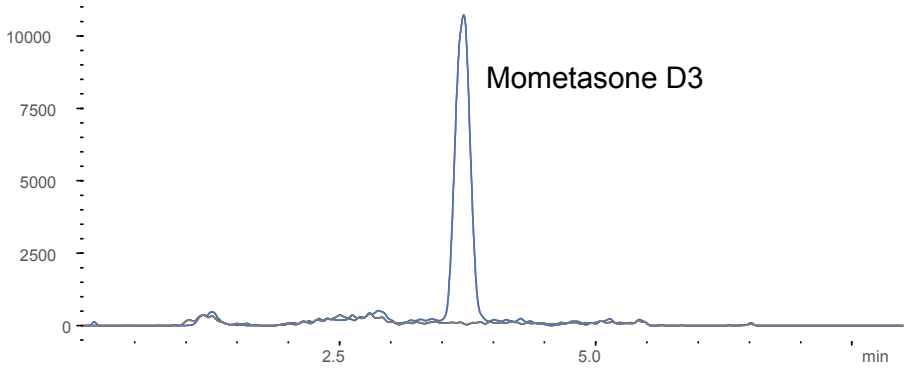

Figure 4B. Overlay of MRM chromatograms of blank and zero standard for mometasone D3

Linearity test was carried out using internal standard calibration method with correlation coefficient of 0.9987 for mometasone as shown in Figure 5.

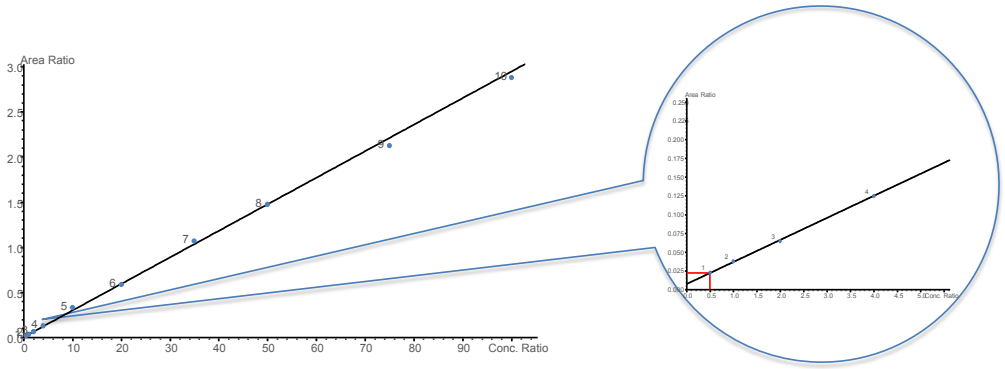

Figure 5. Calibration curve for mometasone spiked standards

Developed method was assessed for precision and accuracy across many PA batches. Results are summarized in Tables 2A and 2B, which are found to be under acceptable criteria for both calibration standards and QC samples [1],[2],[3].

Table 2A. Results of accuracy for mometasone spiked calibration standards

Name of compound	Standard concentration (pg/mL)	Calculated concentration from calibration graph (pg/mL)	% accuracy
Mometasone	0.50	0.47	93.41
	1.00	1.09	108.89
	2.00	2.05	102.62
	4.00	3.93	98.37
	10.00	10.81	108.14
	20.00	19.62	98.09
	35.00	35.77	102.20
	50.00	49.63	99.25
	75.00	71.52	95.36
	100.00	96.98	96.98

Table 2B. Results of accuracy and repeatability for quality control samples

Name of compound	Standard concentration (pg/mL)	Calculated average concentration from calibration graph (pg/mL)	Average % accuracy (n=6)	Average % RSD for area counts (n=6)
Mometasone	0.50 (LLQC)	0.51	101.02	8.32
	4.50 (LQC)	4.64	103.18	5.05
	40.00 (MQC)	37.55	93.88	4.17
	80.00 (HQC)	77.84	97.30	4.64

4. Conclusion

LCMS-8060, along with special sample preparation provides best in class sensitivity for demanding bio analytical assay such as that of mometasone. The criticality of these assays is huge both w.r.t technical challenges and commercial impact for pharmaceutical organizations. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavours.

5. References

[1]EMA Guideline on Bioanalytical Method Validation. EMEA/CHMP/EWP/192217/2009 Rev.1 Corr. 2.
[2]US FDA Guidance for Industry. Bioanalytical Method Validation. Draft September 2013 Rev. 1
[3] Japan MHLW. Guideline on Bioanalytical Method Validation in Pharmaceutical Development (25 July 2013).

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Application Note

LCMS-8060

Low level quantitation of Fluticasone and Salmeterol from plasma using LC/MS/MS

No. - LC-13-ADI-034

❑ Introduction

Fluticasone propionate belongs to a class of drugs known as corticosteroids, specifically glucocorticoids, which are hormones that predominantly affect the metabolism of carbohydrates and, to a lesser extent, fat and protein. It is used to treat asthma, allergic rhinitis, nasal polyps, various skin disorders and Crohn's disease and ulcerative colitis. It is also used to treat eosinophilic esophagitis. Salmeterol is a long-acting beta$_2$-adrenergic receptor agonist drug used in the maintenance and prevention of asthma symptoms and maintenance of chronic obstructive pulmonary disease (COPD) symptoms[1][2].

LC/MS/MS has been increasingly employed in pharmacokinetic studies due to its specificity and sensitivity. This also allows the development of assays with minimal sample preparation.

LC/MS/MS method has been developed for ultra-trace level quantitation of these molecules from plasma using LCMS-8060, a triple quadrupole mass spectrometer from Shimadzu Corporation, Japan. Ultra high sensitivity of LCMS-8060 with heated ESI source enabled development of a low level quantitation method for both these molecules with good repeatability even in presence of complex matrix like plasma.

❑ Experimental

Preparation of standards : All the standards were prepared in organic solvents

Plasma extraction procedure

Analytical conditions:

Analysis was performed on Ultra High Performance Liquid Chromatography (UHPLC) Nexera coupled with LCMS-8060 triple quadrupole system (Shimadzu Corporation, Japan). MRM transitions were optimized for Fluticasone using Automatic MRM optimization function of Shimadzu LAB Solutions software. Two MRM events were optimized (501.1.>292.15, 501.1.>313.25) and TIC were used for quantitation. The details of analytical conditions are given below:

Nexera parameters	
Column	Chromolith RP-18e, (100 mm L x 2 mm I.D.)
Mobile phase	A:10mM ammonium formate B: acetonitrile (A:B, 35:65) v/v
Flow rate	1.0 mL / min
Oven temp.	40 °C
Injection volume	50 µl

LCMS-8060 parameters	
Interface	ESI
Polarity	positive
Nebulizing gas flow	3 L / min
Heating Gas	18 L/min
Drying gas flow	10 L / min
Interface temp	300 °C
Desolvation line temp.	200 °C
Heat block temp.	500 °C

❑ Results and discussion

LC/MS/MS method was developed for analysis Fluticasone in plasma matrix .LOQ was checked as per following criteria – **A**. % RSD for area < 15% , **B**. % accuracy between 80-120 % and **C**. Signal to noise ratio (S/N) > 1 and D. Linearity <0.99.

For L1 of Fluticasone, A. % RSD for area <4%, B. % accuracy-103%, C. Signal to noise ratio (S/N) – 50 and D. Linearity-0.9993 (refer table 1 and figure1)

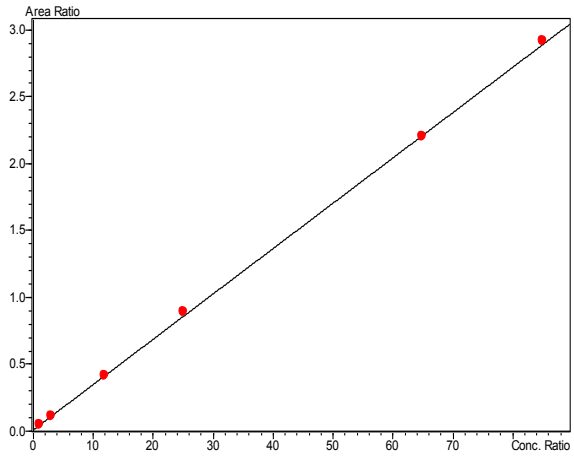

Figure 1: Fluticasone Linearity in plasma (1pg/mL to 85pg/mL)

R^2= 0.99937

Table 1: Linearity results of Fluticasone

Sr.No.	Levels	Concentration (pg/mL)	Area	Height	% accuracy
1	L1	1.0	4340	1520	103.0
			4139	1871	100.5
2	L2	3.0	10910	4754	98.3
			9993	4172	94.9
3	L3	12.0	36257	15220	98.3
			38030	15827	98.3
4	L4	25.0	74913	29847	106.0
			69812	27932	100.8
5	L5	40.0	NA	NA	NA
			NA	NA	NA
6	L6	65.0	197159	80398	97.3
			201204	83351	102.0
7	L7	85.0	264214	109429	103.5
			268824	112074	98.4
8	L8	100	NA	NA	NA
			NA	NA	NA

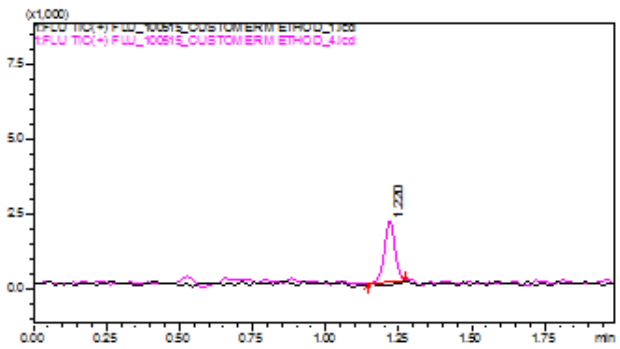

Figure 2: Overlay TIC chromatograms of blank and 1pg/mL of Fluticasone in solvent

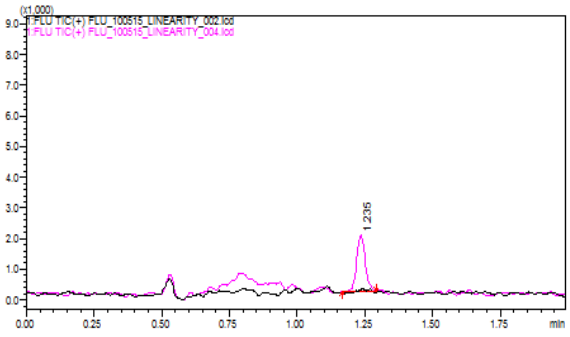

Figure 3: Overlay TIC chromatogram of blank and 1pg/mL of Fluticasone in plasma matrix

Overlay TIC chromatograms of blank and 1pg/mL of Fluticasone in solvent and in plasma matrix are given in figure 2 and 3 respectively. Signal to noise ratio (S/N) of Fluticasone in solvent and plasma matrix is mentioned in below table 2.

Table 2: Fluticasone sensitivity table

Sr. No.	Molecule	Medium	Concentration	S/N (RMS)
1	Fluticasone	Solvent	1pg/mL	50.26
		Plasma	1pg/mL	47.38

Application Note **No. LC-13-ADI-034**

Experimental conditions for Salmeterol

Preparation of standards All the standards were prepared in organic solvents

Plasma extraction procedure

Analytical conditions

Analysis was performed on Ultra High Performance Liquid Chromatography (UHPLC) Nexera coupled with LCMS-8060 triple quadrupole system (Shimadzu Corporation, Japan). MRM transitions were optimized for salmeterol using Automatic MRM optimization function of Shimadzu LAB Solutions software. MRM transition 416.10.>232.20 was used for quantitation.
The details of analytical conditions are given below:

Analytical conditions for Salmeterol

Nexera parameters	
Column	Chromolith RP-18e, (100 mm L x 2 mm I.D.)
Mobile phase	A:10mM ammonium formate B: acetonitrile (A:B, 30:70) v/v
Flow rate	0.8 mL / min
Oven temp.	40 ˚C
Injection volume	20 µl

LCMS-8060 parameters	
Interface	ESI
Polarity	positive
Nebulizing gas flow	3 L / min
Heating Gas	18 L/min
Drying gas flow	15 L / min
Interface temp	400 ˚C
Desolvation line temp.	300 ˚C
Heat block temp.	500 ˚C

❑ **Results and discussion of Fluticasone**

LC/MS/MS method was developed for analysis Salmeterol in plasma matrix.

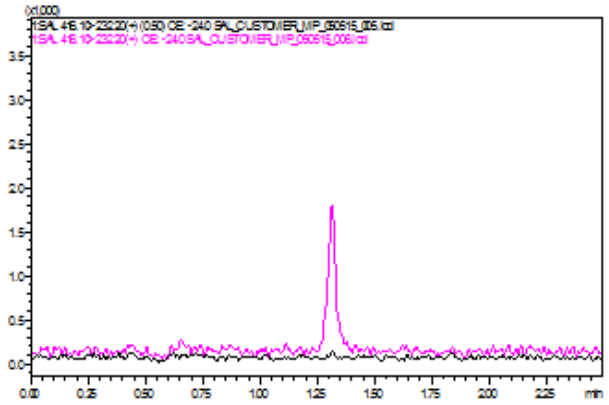

Figure 4: Overlay MRM chromatogram of blank and 2 pg/mL of Salmeterol in solvent

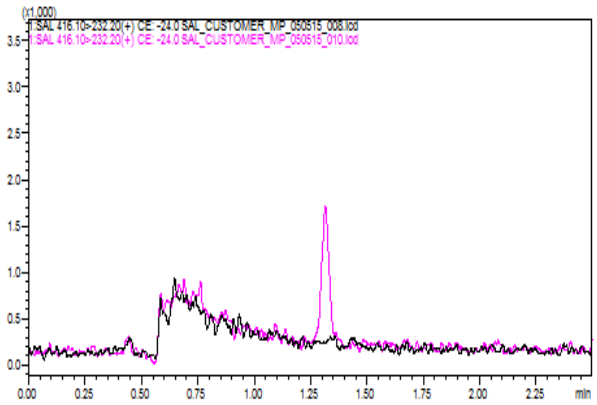

Figure 5: Overlay MRM chromatogram of blank and 2 pg/mL of Salmeterol in plasma

Overlay TIC chromatograms of blank and 2 pg/mL of Salmeterol in solvent and in plasma matrix are given in figure 4 and 5 respectively. Signal to noise ratio (S/N) of Salmeterol in solvent and plasma matrix is mentioned in below table 3.

Table 3: Salmeterol sensitivity table

Sr. No.	Molecule	Medium	Concentration	S/N (RMS)
1	Salmeterol	Solvent	2 pg/mL	76.79
		Plasma	2 pg/mL	46.23

❑ Conclusion

High sensitive method was developed for low level quantitation of fluticasone and salmeterol in plasma.

For L1 level, Fluticasone (1pg/mL) got 47 s/n ratio and for Slameterol (2pg/mL) got 46 s/n ratio in plasma by RMS method. Still there is lot of scope to achieve 3-4 times less concentration than current L1 value as LOQ.

Heated ESI probe of LCMS-8060 system enables drastic augment in sensitivity with considerable reduction in background. Hence, LCMS-8060 system from Shimadzu gives a complete solution for bioanalysis.

❑ References

1.R. R. Shah et al., International Journal of PharmTech Research, Volume 3, Number 3, (2011), 1801-1806.
2.Sriram Krishnaswami et al., Journal of Pharmaceutical and Biomedical Analysis, Volume 22, (2000), 123-129.

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Highly sensitive quantitative analysis of budesonide from plasma using LC/MS/MS

ASMS 2017 ThP 710

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Highly sensitive quantitative analysis of budesonide from plasma using LC/MS/MS

Introduction

Budesonide is a glucocorticoid used in the management of asthma, treatment of various skin disorders, and allergic rhinitis. Budesonide is provided as a mixture of two epimers (22R and 22S). Interestingly, the 22R form is two times more active than the 22S epimer. The two forms do not interconvert. [1]

Inflammation is an important component in the pathogenesis of asthma. Corticosteroids like budesonide have been shown to have a wide range of inhibitory activities against multiple cell types (e.g., mast cells, eosinophils, neutrophils, macrophages, and lymphocytes) and mediators (e.g., histamine, eicosanoids, leukotrienes, and cytokines) involved in allergic and non-allergic-mediated inflammation. These anti-inflammatory actions of budesonide contribute to their efficacy in the aforementioned diseases. Budesonide undergoes significant first-pass elimination and its bioavailability is 10 %, which demands its low level quantitation in plasma for bioanalysis. Budesonide is formulated as an extended release tablet and inhalers. [2] Here, an LC/MS/MS method has been developed for highly sensitive quantitation of budesonide (as shown in Figure 1) from plasma using LCMS-8060, a triple quadrupole mass spectrometer from Shimadzu Corporation, Japan.

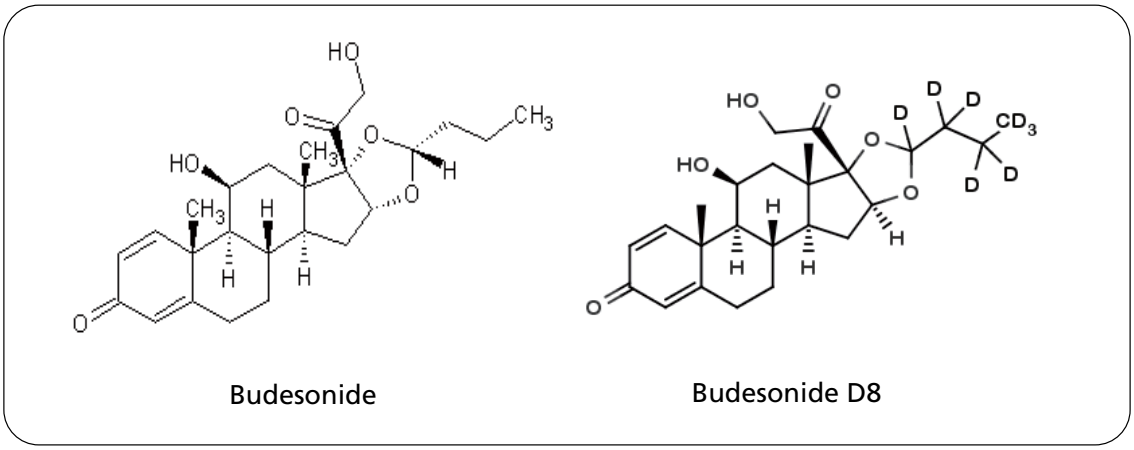

Figure 1. Structure of budesonide and budesonide D8

Materials and method

Sample preparation

- **Preparation of spiked calibration standards and quality control (QC) samples**

Budesonide (procured from TRC) calibration standards at concentration levels of 2 pg/mL, 5 pg/mL, 10 pg/mL, 20 pg/mL, 50 pg/mL, 100 pg/mL, 150 pg/mL, 200 pg/mL and quality control samples at concentration levels of LQC (7.5 pg/mL), MQC (75 pg/mL) and HQC (175 pg/mL) were prepared in plasma.

Highly sensitive quantitative analysis of budesonide from plasma using LC/MS/MS

• **Sample extraction**

Spiked calibration standards and quality control samples in plasma were taken in 4 mL RIA vials to internal standard solution (Budesonide D8 procured from TRC) was added except in blank. Then above plasma was centrifuged at 4500 rpm for 5 mins.

• **The samples were extracted by solid phase extraction (SPE) technique as followed:**

1. Conditioning (1mL methanol followed by 1 mL water)
2. Loading (entire plasma sample)
3. Washing (1 mL water followed by 1 mL 5 % methanol)
4. Elution (1 mL methanol)

SPE eluent was evaporated at 50 °C for 25 minutes in low pressure nitrogen evaporator. The residue was reconstituted in 200 µL mobile phase, vortexed and filled in HPLC vials for injection.

LC/MS/MS analysis

Figure 2. Nexera with LCMS-8060

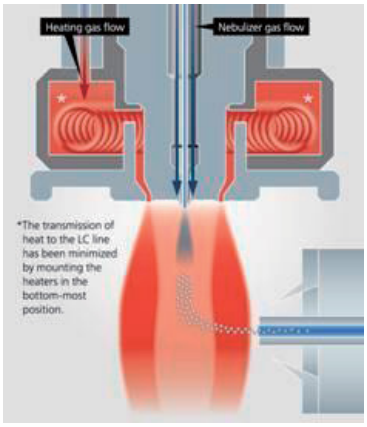

Figure 3. Heated ESI probe

LCMS-8060 triple quadrupole mass spectrometer by Shimadzu (shown in Figure 2), sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UFsensitivity), ultra fast scanning speed of 30,000 u/sec (UFscanning) and polarity switching speed of 5 msec (UFswitching). This system ensures highest quality of data, with very high degree of reliability.
In order to improve ionization efficiency, the newly developed heated ESI probe (shown in Figure 3) combines high-temperature gas with the nebulizer spray, assisting in the desolvation of large droplets and enhancing ionization. This development allows high-sensitivity analysis of a wide range of target compounds with considerable reduction in background.

The details of analytical conditions are given in Table 1.

Highly sensitive quantitative analysis of budesonide from plasma using LC/MS/MS

UHPLC conditions (Nexera X2 system)	
Column	: C18 column100 mm×3 mm, 2.1 um
Mobile phase A	: Buffer
B	: Acetonitrile
Flow rate	: 0.4 mL/min
Time program	: B conc. 40% (0.01 min) - 90% (0.01-1min) - 90% (1.01-2.50min) - 40% (2.51-4.00min)
Injection vol.	: 20 uL
Column temperature	: 40 °C

MS conditions (LCMS-8060)	

Table 1. MRM transitions

Compound	Polarity	MRM transition
Budesonide	positive	431.10 > 323.20
Budesonide D8	positive	439.10 > 323.20

MS interface	: Electro Spray Ionization (ESI)
Nitrogen gas flow	: Nebulizing gas- 3 L/min; Drying gas- 10 L/min
Zero air flow	: Heating gas- 10 L/min
MS temperatures	: Desolvation line- 250 °C; Heating block- 400 °C Interface- 300 °C

Results

LLOQ of 2 pg/mL was achieved for budesonide in plasma. Overlay of MRM chromatograms of blank and LLOQ for budesonide spiked standards are shown in Figure 4A. Similarly, overlay of the MRM chromatograms of blank and zero standard (Blank+IS) for budesonide D8 are shown in the Figure 4B. No interfering peaks were seen in blank plasma at the retention time of these compounds which confirms the absence of any interference.

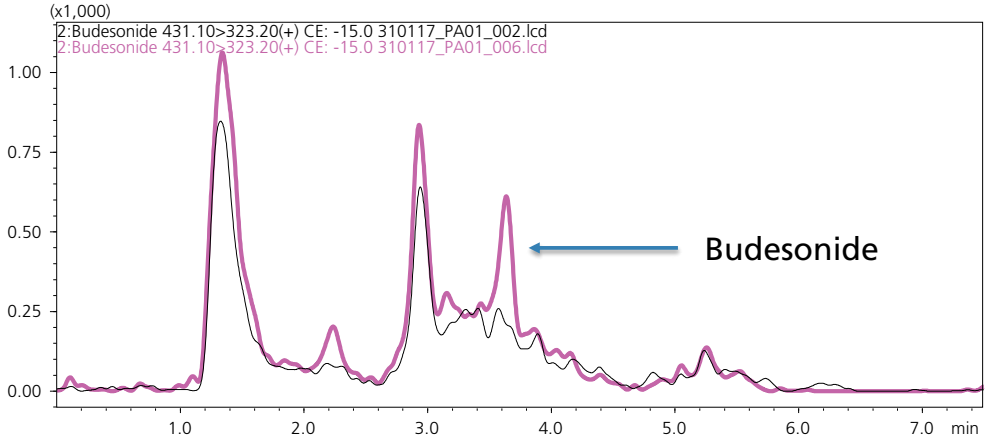

Figure 4A. Overlay of MRM chromatograms of blank and 2 pg/mL for budesonide spiked standard

Highly sensitive quantitative analysis of budesonide from plasma using LC/MS/MS

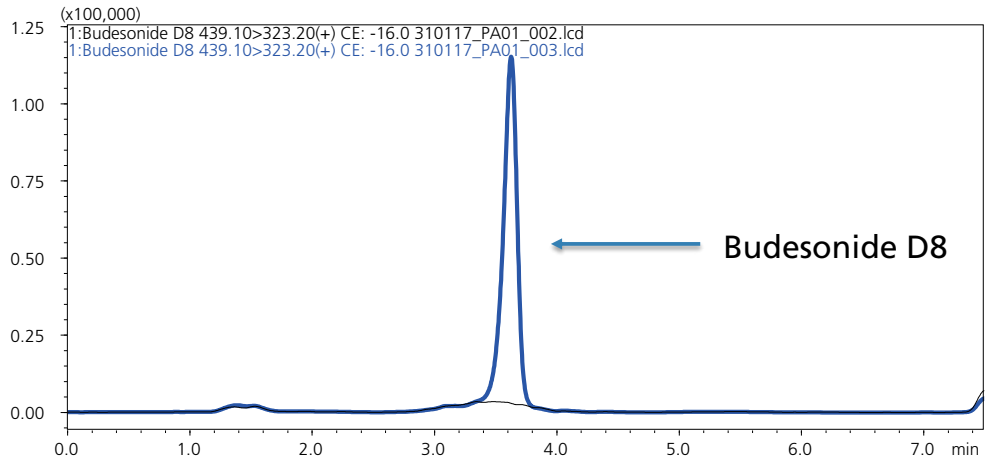

Figure 4B. Overlay of MRM chromatograms of blank and zero standard for budesonide D8

Linearity test was carried out using internal standard calibration method with correlation coefficient of 0.9992 for budesonide as shown in Figure 5.

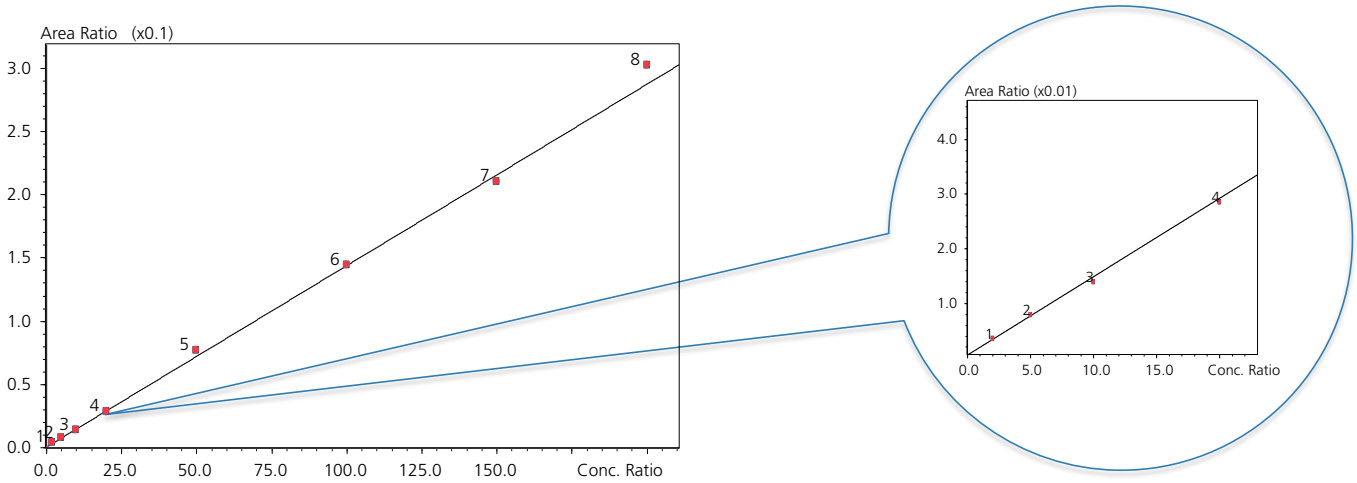

Figure 5. Calibration curve for budesonide spiked standards

Highly sensitive quantitative analysis of budesonide from plasma using LC/MS/MS

Precision and accuracy batch of budesonide meets the bioanalytical acceptance criteria. Results are shown in table 2A and 2B.

Table 2A. Results of accuracy for budesonide spiked calibration standards

Name of compound	Standard concentration (pg/mL)	Calculated concentration from calibration graph (pg/mL)	% accuracy
Budesonide	2	1.997	99.9
	5	5.164	103.3
	10	9.448	94.5
	20	20.088	100.4
	50	51.995	104.0
	100	98.700	98.7
	150	148.574	99.0
	200	202.831	101.4

Table 2B. Results of accuracy and repeatability for budesonide quality control samples

Name of compound	Standard concentration (pg/mL)	Calculated average concentration from calibration graph (pg/mL)	Average % accuracy (n=6)	Average % RSD for area counts (n=6)
Budesonide	7.5 (LQC)	7.264	96.9	5.74
	75 (MQC)	75.285	100.4	6.39
	175 (HQC)	176.429	101.0	4.36

Conclusions

- Ultra-fast and highly sensitive quantitative analysis of budesonide from plasma was developed on LCMS-8060 system.
- Heated ESI probe of LCMS-8060 helped in achieving LLOQ of 2 pg/mL for budesonide with considerable reduction in background. Hence, LCMS-8060 system from Shimadzu gives a complete solution for bioanalysis.

Highly sensitive quantitative analysis of budesonide from plasma using LC/MS/MS

References

[1] Deventer K., Mikulcikova P., Van Hoecke H., Van Eenoo P., Delbeke F.T. Detection of budesonide in human urine after inhalation by liquid chromatography–mass spectrometry. J. Pharm. Biomed. Anal. 2006;42:474–479.
[2] Streel B., Cahay B., Klinkenberg R. Using total error concept for the validation of liquid chromatography-tandem mass spectrometry method for the determination of budesonide epimers in human plasma. J. Chromatogr. B.

First Edition: June, 2017

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Application News

No. AD-0072

LCMS-8040 UFMS

A Fast LC/MS/MS method for Determination of Telmisartan in Human Plasma by LC-MS-MS

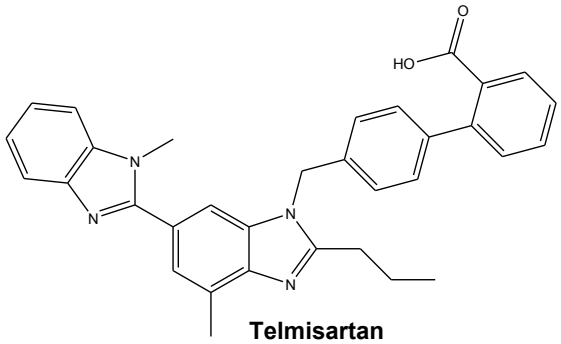
Manikandan Venkateswaran, Vinod Pillai. S.L, Venkat Manohar and Arvind Thyagarajan
*Indian Institute of Chromatography & Mass Spectrometry (IICMS)**

Abstract

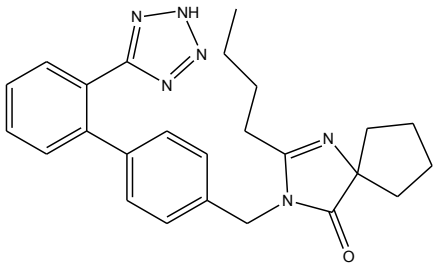
A fast LC/MS/MS method for quantitative determination of Telmisartan in human plasma using UHPLC Nexera coupled to LCMS-8040 Triple Quadrupole Mass Spectrometer was described. A simple protein precipitation method for extraction of Telmisartan with internal standard (Irbesartan) from the biological matrix was employed. The new advanced LCMS-8040 enabled the quantification of Telmisartan from human plasma samples over a concentration range of 4.0 (LLOQ) to 2000 ng/mL. The constructed calibration curve was linear with a regression of coefficient more than 0.99.

❑ Introduction

Telmisartan (2-(4-{[4-methyl-6-(1-methyl-1*H*-1,3-benzodiazol-2-yl)-2-propyl-1*H*-1,3-benzodiazol-1-yl]methyl}phenyl)benzoic acid) is an angiotensin II receptor antagonist widely used in treatment of hypertension. It undergoes minimal bio-transformation in the liver to form the inactive Telmisartan-1-o-acylglucoronide as its principal metabolite. The long half-life and selectivity of Telmisartan for angiotensin II receptors allows once daily dosing with minimal side effects. Liquid chromatography with tandem mass spectrometry (LC/MS/MS) has been widely employed for bioassays of drugs. In this study, a simple and fast method for quantitative determination of Telmisartan in human plasma with Irbesartan as internal standard is described using the UHPLC Nexera coupled to LCMS-8040 instrument.



Telmisartan



Irbesartan (IS)

Figure 1 : Chemical structure of Telmisartan and Irbesartan (IS)

❑ Experimental

Preparation of Aqueous Standards: Stock solution at concentration of 1 mg/mL of Telmisartan was prepared in acetonitrile:water (80:20 v/v) mixture. This solution was further serially diluted using acetonitrile:water (80:20 v/v) mixture to obtain aqueous standards containing Telmisartan at the concentration of 40, 80, 200, 400, 800, 2000, 4000, 8000, 12000, 16000 and 20000 ng/mL respectively. Similarly, a stock solution of Irbesartan, which is used as the internal standard (IS) was prepared at the concentration of 1 mg/mL in acetonitrile:water (80:20 v/v) mixture. This solution was further diluted to obtain internal standard working solution (ISTD) at a concentration of 3000 ng/mL.

Preparation of Plasma Calibration Standards (CC): 180 µL of human plasma was spiked with 20 µL of each aqueous Telmisartan standard solution and 20 µL of ISTD solution, vortexed for 30 seconds to obtain plasma calibration standard whose concentration ranged from 4.0 – 2000 ng/mL. Each of these samples was then extracted according to the procedure as described under sample preparation.

Preparation of Plasma Quality Control Standards (QC): The quality control standard solutions were prepared at three intermediate concentrations of CC standards namely 12.0, 900.0 and 1800.0 ng/mL (LQC, MQC and HQC respectively). Six individual preparations of each of the QC standards were prepared to evaluate precision and recovery. Each of these sample preparation was then extracted according to the procedure as described under sample preparation.

** Address: No. 7, Chakrapani Road, Guindy, Chennai - 600 032, Tamil Nadu, India*

Sample Preparation: A simple protein precipitation method for extraction of Telmisartan and the IS from the plasma matrix was employed. All plasma samples were treated with 1000 µL of ice-cold acetonitrile and vortexed for 5 minutes. The samples were then centrifuged at 10,000 rpm for 10 minutes. The supernatant layer was directly taken for injection into the LC/MS/MS system.

Table 1 : Analytical conditions

Column	: Zorbax Eclipse Plus C18, 100 x 2.1mm, 1.8 µm		
Mobile phase-A	: 0.02M Ammonium acetate in water		
Mobile phase-B	: Acetonitrile		
Isocratic	: A:B (60:40 v/v)		
Flow rate	: 600 µL/min	DL temp	: 250 ^{0}C
Column temp	: 50 ^{0}C	Heat block	: 300 ^{0}C
Drying gas	: 15 L/min	Interface	: ESI
Nebulizing gas	: 3.0 L/min	Interface volt	: 4.5 kV
For Telmisartan			
MRM	: 515.00 → 276.10	Polarity	: Positive
Dwell time	: 100 ms	CE	: -49.0 V
Q1 pre-bias	: -40.0 V	Q3 pre-bias	: -26.0 V
For Irbesartan (IS)			
MRM	: 429.00 → 207.00	Polarity	: Positive
Dwell time	: 100 ms	CE	: -24.0 V
Q1 pre-bias	: -32.0 V	Q3 pre-bias	: -40.0 V

The LC-MS conditions are summarized in Table 1. Precursor ions of Telmisartan and Irbesartan (IS) were determined by injecting a solution containing these compounds in the Q1 scan mode. Under these conditions, the analyte and the IS yielded predominantly the quasi molecular ions of m/z 515 and m/z 429 respectively. Each of these precursor ions was subjected to collision induced dissociation (CID) in order to generate product ions. This operation was done automatically by the use of SSS (Synchronized Survey Scan) function in the software to obtain optimized parameters. Based on this, the ion transitions of m/z 515.00 → 276.00 and m/z 429.00 → 207.00 (Figure 4) were used in MRM mode for Telmisartan and Irbesartan (IS) respectively.

❑ Results and Discussion

LLOQ

The concentration of Telmisartan at lower limit of quantitation (LLOQ) was determined to be 4.0 ng/mL. This was confirmed from the coefficient of variance (CV) being less than 20% for the six replicate injections of Telmisartan at this concentration. The overlay mass chromatograms corresponding to Telmisartan at its LLOQ and the blank is presented in Figure 3.

Linearity

A linear dynamic range of 4.0 to 2000.0 ng/mL was achieved for Telmisartan with R^2 value of 0.9970. The CC standards were used to construct a calibration curve by plotting the area ratio of Telmisartan with respect to IS versus the concentration of CC standards. Linear curve fit type was used and weighted ($1/x^2$). Figure 2 shows a representative calibration curve of Telmisartan in plasma using Irbesartan as internal standard.

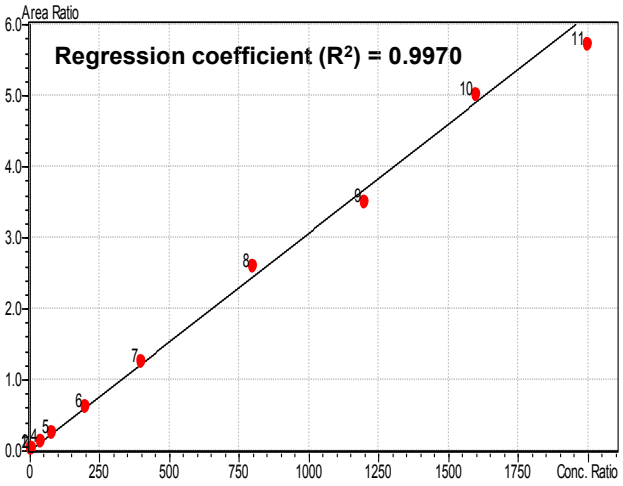


Figure 2 : Calibration curve of Telmisartan

The calculated concentrations for all the CC standards were within ±10% of the nominal value as determined by bias calculation (Table 2).

Table 2: Accuracy of Telmisartan in CC samples

Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Accuracy*
4.0	3.9	98.6
8.0	8.4	105.5
20.0	18.1	90.5
40.0	41.9	104.8
80.0	79.7	99.6
200.0	203.0	101.5
400.0	404.8	101.2
800.0	848.5	106.1
1200.0	1149.1	95.8
1600.0	1643.7	102.7
2000.0	1873.4	93.7

* Expressed as Bias = (mean concentration / nominal concentration) x 100

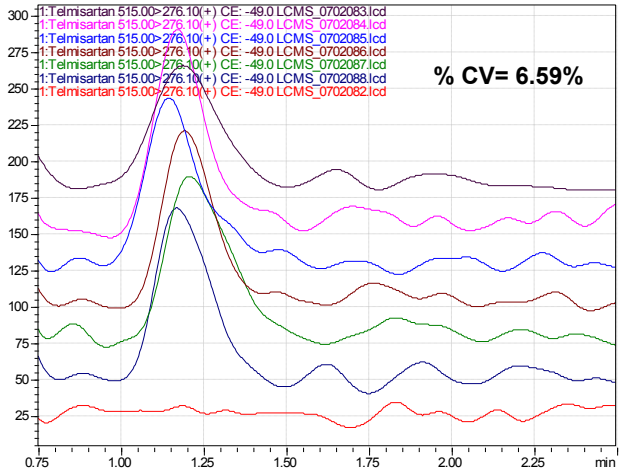


Figure 3 : Overlay chromatograms of Telmisartan at LLOQ conc.with blank (bottom)

Precision & Accuracy of QC Samples

Low, middle and high QC samples containing Telmisartan were prepared at concentrations of 12, 900 and 1800 ng/mL in plasma. The precision (%CV, n=6) of the QCs for Telmisartan varied from 3.6 to 5.3% and accuracy from 85.0 to 103.0% of the nominal value (Table 3).

Table 3: Precision and accuracy of Telmisartan in QC samples

Nominal Conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy*	Precision (n=6)
12.0	10.8	90.0	5.3
	10.5	87.5	
	11.5	95.8	
	11.9	99.2	
	11.0	91.7	
	10.2	85.0	
900.0	848.3	94.3	3.6
	868.0	96.4	
	855.4	95.0	
	923.8	102.6	
	860.4	95.6	
	854.0	94.9	
1800.0	1840.3	102.2	4.7
	1817.1	101.0	
	1819.4	101.1	
	1630.0	90.6	
	1853.2	103.0	
	1816.3	100.9	

* Expressed as Bias = (mean concentration/nominal concentration) x 100

Recovery of QC Samples

The recovery of Telmisartan was calculated by comparing the peak area obtained for QC samples that were subjected to extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations. Good recoveries were obtained (Table 4) for Telmisartan demonstrating the efficiency of analyte extraction in the presence of biological matrix.

Table 4: Recovery of Telmisartan in QC samples

QC sample	Number of Preparations	% Recovery
		Telmisartan
LQC	1	85.94
	2	91.21
	3	99.37
	4	101.37
	5	98.20
	6	89.94
MQC	1	86.95
	2	93.72
	3	96.67
	4	100.16
	5	95.41
	6	95.37
HQC	1	87.95
	2	94.26
	3	94.45
	4	96.16
	5	97.51
	6	98.66

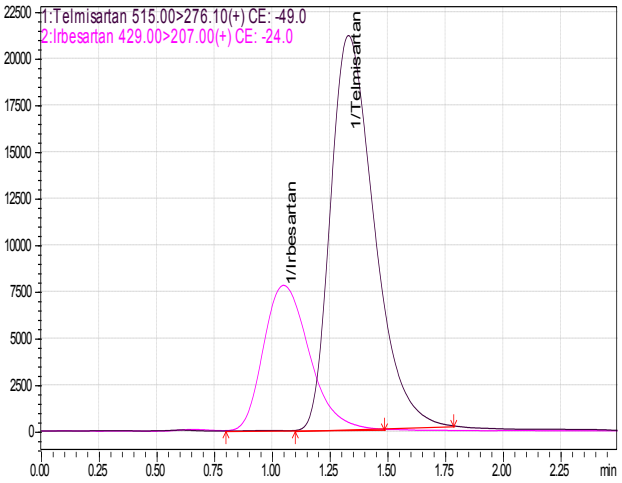


Figure 4 : Mass chromatogram of Telmisartan at MQC

❑ Conclusions

A simple, high throughput LC/MS/MS method for quantitative determination of Telmisartan in human plasma was developed. The LLOQ of the method was determined at 4.0 ng/mL. The linear dynamic range of the calibration curve was 4.0 – 2000 ng/mL with a regression of R^2 = 0.9970. Good recoveries (between 85.9 to 101.4 %) were obtained for all the three levels of QC samples repeatedly.

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Application News

No. AD-0077

Quantitative Bioanalysis / LCMS-8050

A High Sensitivity LC/MS/MS Method for Quantitative Determination of Telmisartan in Human Plasma

❑ Introduction

Telmisartan is an angiotensin II receptor antagonist commonly used in the treatment of hypertension and heart failure. Bioanalysis of telmisartan has been reported for human pharmacokinetics and bioequivalence studies, which is carried out mostly on LC/MS/MS with solid phase extraction (SPE) method for high sensitivity and selectivity. The reliability of a bioassay depends on the performance of LC/MS/MS system employed and the method of sample pre-treatment. With rapid progress of interface and triple quadrupole MS techniques, simpler sample pre-treatment without SPE may be adopted to achieve not only reliable quantitative results, but also higher throughput and lower running cost. In this issue of Application News, a high sensitivity LC/MS/MS method for quantitative determination of telmisartan in human plasma is described. The method was developed on LCMS-8050, a tandem mass spectrometer with a heated ESI interface. The high sensitivity and robust interface design of the system allow the use of protein-precipitation only in plasma pre-treatment and achieve a LLOQ of 4 pg/mL in plasma.

❑ Experimental

Preparation of calibrants and Samples

Pooled human plasma obtained from i-DNA Biotechnology was used as bio-matrix in this work. Stock solutions of telmisartan and irbesartan, the latter as internal standard (IS), were prepared from high purity solid chemicals in the diluent (acetonitrile and 0.1% formic acid - water, 60/40, vol/vol). The sample pre-treatment without SPE or other cleanup method is shown in Figure 1. Pure acetonitrile was added to the plasma, with or without spiked telmisartan and IS, in a ratio of 3:1 for protein precipitation.

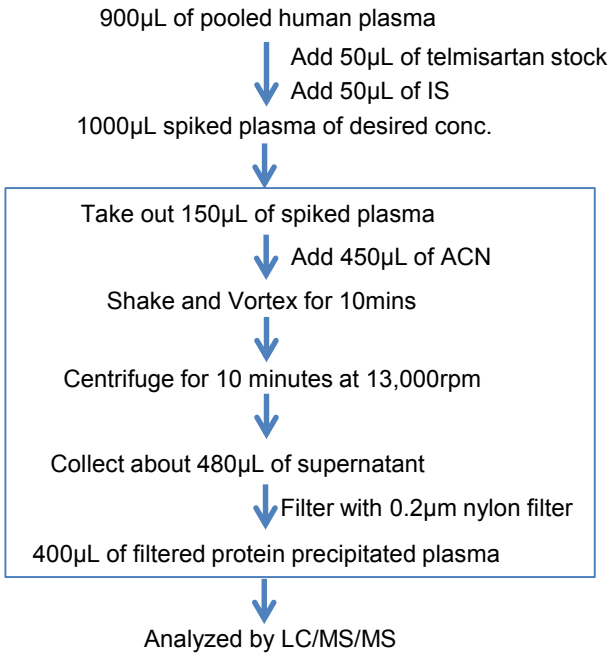

Figure 1: Flow chart of protein-precipitation (steps in the box) of a pre-spiked plasma sample.

Analytical system and conditions

Shimadzu's LCMS-8050 with a heated ESI interface coupled with a Nexera UHPLC system was employed. A ZORBAX Eclipse Plus C18 column (3.0mmID, 100mmL, 1.8 µm) was used. An isocratic elution method was adopted with a mobile phase of 40% water - 60% acetonitrile with 0.04% formic acid, a flow rate of 0.4 mL/min and oven temperature of 50°C. The MS interface conditions were: ESI in positive mode, block Temp at 400°C, DL Temp. at 200°C, nebulizing gas (N_2) at 2 L/min, drying gas (N_2) at 6 L/min and heating gas (purified air) at 14 L/min. The injection volume of sample was 5 µL.

❑ Results and Discussion

MRM method with internal standard (IS)

A MRM method was established on the LCMS-8050 with the optimized MRM parameters as shown in Table 1. Telmisartan and IS were eluted as sharp peaks under the isocratic LC conditions as shown in Figure 2. The first MRMs of both compounds were used to establish internal standard (IS) calibration method. The second MRMs of them were used as reference ions for identification.

Table 1: Retention times, MRMs and optimized CID voltages for analysis of telmisartan with IS on LCMS-8050

Name	RT (min)	Transition (m/z)	Voltage (V)		
			Q1 Pre Bias	CE	Q3 Pre Bias
Telmisar-tan	1.127	515.2 > 276.1	-26	-49	-30
		515.2 > 497.2	-26	-35	-25
Irbesartan (IS)	1.448	429.2 > 207.0	-22	-25	-22
		429.2 > 195.1	-22	-23	-21

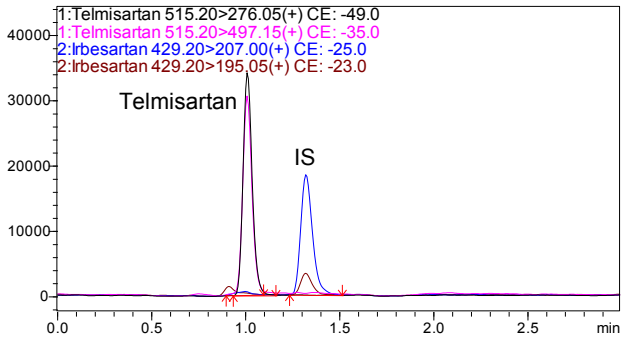

Figure 2: MRM chromatograms of telmisartan (50 pg/mL) and IS (50 pg/mL) post-spiked in plasma.

Calibration curve and linearity

A quantification method for telmisartan in plasma was set up based on the first MRM transitions of telmisartan and IS in Table 1. Linear calibration curves were established by IS method for plasma samples prepared by pre-spiked and post-spiked procedures, the latter is displayed in Figure 3. Excellent linearity was obtained with R^2 coefficient greater than 0.999 across the range from 1.0 pg/mL to 2000 pg/mL. The details of the calibrant series, accuracy and recovery in duplicate measurements are shown in Table 2.

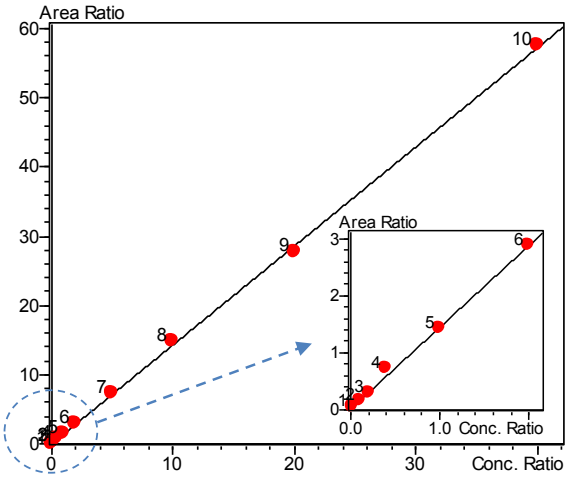

Figure 3: IS calibration curve of telmisartan post-spiked in plasma, ranging from 1 pg/mL ~ 2000 pg/mL.

Evaluation of method performance

The LLOQ of the method was determined with post-spiked plasma sample at 1.0 pg/mL. The average S/N ratio of the telmisartan peak was 11, which resulted in LOQ of 0.8 pg/mL. The repeatability of this level obtained was RSD = 13.3% (see Table 3). With QC1 sample (5.0 pg/mL post-spiked in plasma), the average S/N obtained was 40 and the LOQ calculated from the LabSolutions was 1.1 pg/mL (RSD = 3.2%, see Table 3). Based on the above results, it can be confirmed that the LLOQ of the method is at 1 pg/mL in plasma solution and 4 pg/mL in plasma before adding three volumes of acetonitrile for protein precipitation (dilution factor of sample preparation: 4.0).

Table 2: Calibrant series and performance of IS calibration method for quantitation of telmisartan in plasma

Level[1]	Conc. Calc. (pg/mL)	Tel/IS. Ratio[2]	Conc. Mea. (pg/mL)	Accuracy (%)	Recovery (%)
1	1	0.02	0.85	85.2	132.1
2	5	0.1	4.69	93.8	107.4
3	10	0.2	9.67	96.7	95.4
4	20	0.4	24.7	123.5	91.7
5	50	1	49.6	99.3	90.2
6	100	2	99.8	99.8	89.4
7	250	5	252.0	100.8	93.7
8	500	10	518.2	103.7	95.2
9	1000	20	970.9	97.1	94.1
10	2000	40	2008.2	100.4	96.4

[1] Duplicate injections of each level; [2] IS: 50 pg/mL

Table 3: Performance evaluation of quantitation method for telmisartan in plasma with LLOQ and low QC samples

Sample	Conc. Cal. (pg/mL)[1]	Ave. Conc. (pg/mL)	Accuracy (%)	RSD (%)
LLOQ	1	0.83	82.8	13.3
QC1	5	4.65	93.8	3.2
QC2	20	23.91	123.5	2.3

[1] IS: 50 pg/mL

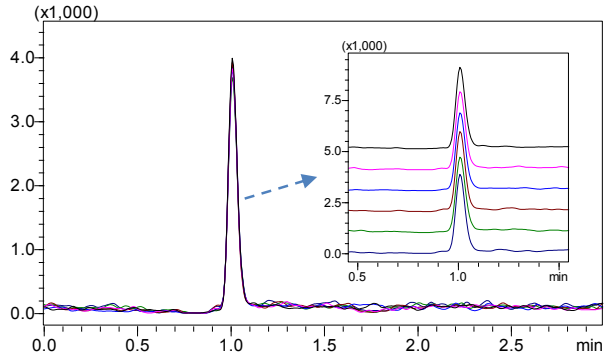

Figure 4: Overlay of six chromatograms of QC1 (5.0 pg/mL telmisartan post-spiked in plasma)

The accuracy and precision of the method were evaluated with low QC samples. The results are compiled into Table 3. The six MRM chromatograms of consecutive injections of QC1 are plotted into Figure 4, which shows impressively the excellent reproducibility of the analysis on LCMS-8050.

It is worth to note that the method exhibited a certain level of ion enhancement at all concentrations. In addition, a small interference peak was found from the plasma due to the simple sample pretreatment and fast elution of telmisartan peak. Validation of the method is needed for applying it in bioanalysis of actual samples.

❑ Conclusions

A high sensitivity bioanalytical method for determination of telmisartan in human plasma was established on LCMS-8050 with a heated ESI. The method allows the use of simple sample pre-treatment, achieving high sensitivity and reliable performance required for bioanalysis.

Note: The data shown in this Application News are for Research Use Only. Not used for clinical diagnostic purposes.

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Application News

No. AD-0065

LCMS-8040 UFMS

Fast LC/MS/MS Method for Quantitative Determination of Valsartan in Human Plasma

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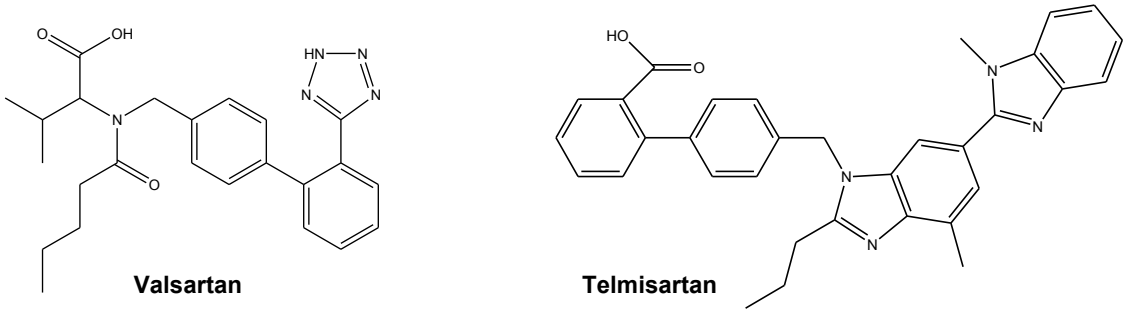
*Indian Institute of Chromatography & Mass Spectrometry (IICMS)**

Abstract

A sensitive and fast LC/MS/MS method for the quantitative determination of valsartan in human plasma using UHPLC NEXERA coupled to LCMS-8040 triple quadrupole mass spectrometer was described. A simple protein precipitation method was employed to extract valsartan and the internal standard from the plasma samples.

❑ Introduction

Valsartan (N-Valeryl-N[[2'-(1H-tetrazol-5-yl)biphenyl-4-yl]methyl valine) is an orally active, potent and specific competitive angiotensin II antagonist acting at the ATI receptor, which mediates all known effects of angiotensin II on the cardio vascular system. Valsartan is widely used in treatment of hypertension. Several analytical methods for determination of Valsartan have been reported in the literature. For routine analysis of bio-fluids, simple sample preparation protocols that are sensitive and specific are preferred. LC/MS/Ms is then the method of choice for analytes extracted from biological matrices. In this note, a simple, fast and a sensitive method for quantitative determination of Valsartan in human plasma with Telmisartan as internal standard is described using the Nexera UHPLC coupled to a LCMS-8040 instrument.



Valsartan **Telmisartan**

Figure1 Chemical structures of valsartan and Telmisartan (IS)

❑ Experimental

Preparation of aqueous standards: Stock solutions of Valsartan was prepared separately at 1mg/mL concentration in Water : Acetonitrile (20:80) mixture. The Internal standard (IS) stock solution of Telmisartan was prepared at 1mg/mL concentration in Methanol. The stock solution of internal standard was further diluted with Water : Acetonitrile (20:80) mixture to obtain an internal standard working solution (ISTD) at a concentration of 50µg/mL. The stock solution of Valsartan was then serially diluted with Water : Acetonitrile (20:80) mixture to obtain aqueous Valsartan standards at concentration 0.2, 0.5, 1, 2, 5, 7.5, 10, 20, 50, 75, 100, and 150 µg/mL respectively.

Preparation of plasma calibration standards (CC): 180µL of human plasma was spiked with 20 µL of each aqueous valsartan standard solution and vortexed for 30 seconds to obtain plasma calibration standard whose concentration ranged from 20 ng/mL to 15000 ng/mL. Each of these samples were then extracted according to the procedure as described under sample preparation.

Preparation of plasma quality control standards (QC): Three QC samples (LQC, MQC and HQC) were prepared in plasma at concentrations of 60, 6000 and 12000ng/mL respectively. Six individual preparations for every level was performed to evaluate precision and accuracy. Each of these samples were then extracted according to the procedure as described under sample preparation.

Sample preparation: A simple protein precipitation method was used to extract valsartan and internal standard from plasma matrix. All plasma samples were treated with 1000 µL of methanol and vortexed for 5 minutes. The samples were then centrifuged at 13,000 rpm for 10 minutes. The supernatant layer was directly taken for injection into the LC/MS/MS system.

The LC/MS/MS conditions are as summarized in Table 1. Precursor ions of valsartan and Telmisartan (IS) were determined by injecting a solution containing these

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Table 1: Analytical conditions

Column	: Zorbax Eclipse Plus C18, 100 x 2.1mm, 1.8µm		
Mobile phase-A	: 0.2% v/v Formic acid in water		
Mobile phase-B	: Acetonitrile		
Isocratic : A:B::60:40 v/v			
Flow rate	: 600 µL/min	DL temp	: 200 ^{0}C
Column temp	: 50 ^{0}C	Heat block	: 200 ^{0}C
Drying gas	: 15 L/min	Interface	: ESI
Nebulizing gas	: 3.0 L/min	Interface volt	: 4.5 kV
For Valsartan			
MRM	: 436.00 → 291.00	Polarity	: Positive
Dwell time	: 100 ms	CE	: - 17.0V
Q1 pre-bias	: -32.0V	Q3 pre-bias	: -30.0V
For Telmisartan			
MRM	: 515.00 → 276.10	Polarity	: Positive
Dwell time	: 100 ms	CE	: - 49.0V
Q1 pre-bias	: -40.0V	Q3 pre-bias	: -26.0V

compounds in the Q1 scan mode. Under these conditions, the analyte and the IS yielded predominantly the protonated molecular ions of m/z 436 and m/z 515 respectively. Each of these precursor ions was subjected to collision induced dissociation (CID) in order to generate product ions. This operation was done automatically by the use of SSS (Synchronized Survey Scan) function in the software and all parameters were optimized. Based on this, the ion transitions of m/z 436.00 → 291.00 and m/z 515.00 → 276.10 were used as MRM for Valsartan and Telmisartan (IS) respectively.

❑ Results and Discussion

The concentration of valsartan at lower limit of quantification (LLOQ) was determined as 20.0 ng/mL. This was confirmed from the coefficient of variance (CV) being less than 20% for the six replicate injections of valsartan at this concentration. The overlay chromatograms at LLOQ concentration for the six replicate analysis are shown in Figure 2.

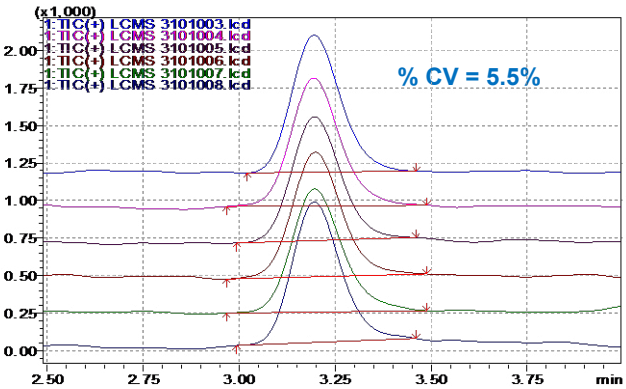

Figure 2: Overlay chromatograms of valsartan at LLOQ

The CC standards were used to construct a calibration curve by plotting the area ratio of Valsartan with respect to IS versus the concentration of CC standards. Linear curve fit type was used and weighted ($1/x^2$). A linear dynamic range of 20.0 to 15000.0 ng/mL was achieved for Valsartan with a R^2 value of 0.9997.

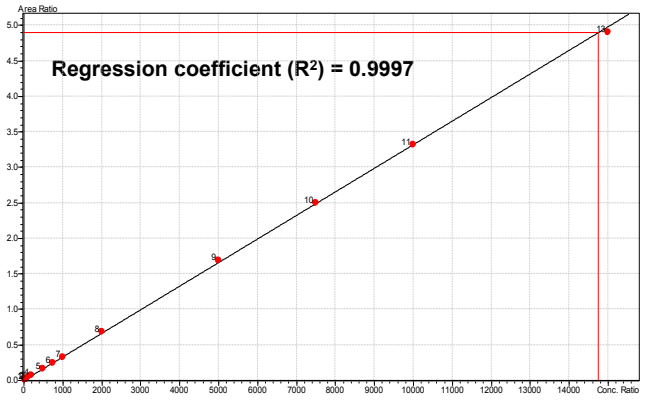

Figure-3 : Calibration curve of valsartan

Figure-3 shows a representative calibration curve of valsartan in plasma using irbesartan as internal standard. The calculated concentrations for all the CC standards were with in the ±10% of the nominal value as determined by bias calculation (refer Table 2).

Table 2 : Calculated concentrations of CC standards

Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Accuracy*
20.0	19.9	99.7
50.0	50.1	100.2
100.0	101.6	101.6
200.0	199.6	99.8
500.0	495.2	99.0
750.0	731.5	97.5
1000.0	985.9	98.6
2000.0	2062.5	103.1
5000.0	5094.1	101.9
7500.0	7521.9	100.3
10000.0	9987.9	99.9
15000.0	14744.8	98.3

* Expressed as Bias = (mean concentration / nominal concentration) x 100

Low, middle and high QC samples containing valsartan was prepared at concentrations of 60, 6000 and 12000 ng/mL in plasma. The precision (%CV, n=6) for the QCs for Valsartan varied from 1.6 to 6.5% and accuracy from 81.7 to 101.5% of the nominal value (Table-3).

The recovery of Valsartan was calculated by comparing the peak area ratio obtained for QC samples that were subjected to extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations. Good recoveries were obtained (Table-4) for Valsartan demonstrating the efficiency of analyte extraction in the presence of biological matrix. The mass chromatogram of Valsartan at LQC and HQC levels are as shown in figure 4 and 5 respectively.

Table 3: Precision and accuracy of Valsartan in QC samples

Nominal Conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy*	Precision (n=6)
60.0	58.2	97.0	3.9
	58.6	97.7	
	54.0	90.0	
	57.6	96.0	
	60.9	101.5	
	59.4	99.0	
6000.0	4902.6	81.7	6.5
	5389.5	89.8	
	5622.3	93.7	
	5043.7	84.1	
	5810.6	96.8	
	5550.7	92.5	
12000.0	10636.5	88.6	1.6
	10960.0	91.3	
	10761.8	89.7	
	10902.7	90.9	
	11146.9	92.9	
	10853.8	90.4	

* expressed as Bias = (mean concentration/nominal concentration) x 100

Table 4: Recovery of Valsartan in QC samples

QC sample	Number of Preparations	% Recovery
		Valsartan
LQC	1	98.5
	2	99.2
	3	91.6
	4	97.6
	5	103.1
	6	100.6
MQC	1	96.9
	2	106.5
	3	111.1
	4	99.9
	5	114.8
	6	109.7
HQC	1	95.1
	2	98.0
	3	96.2
	4	97.5
	5	99.7
	6	97.0

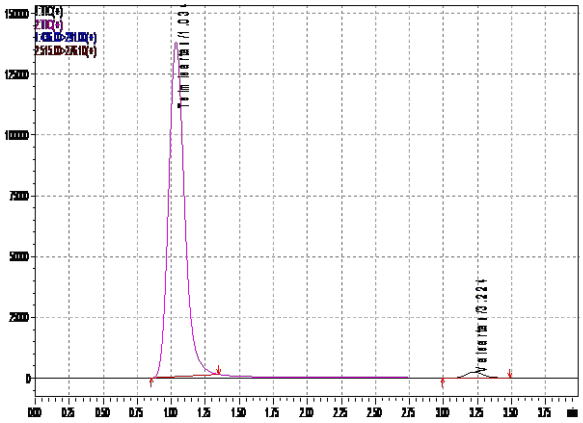

Figure 4: Mass chromatogram of valsartan at LQC

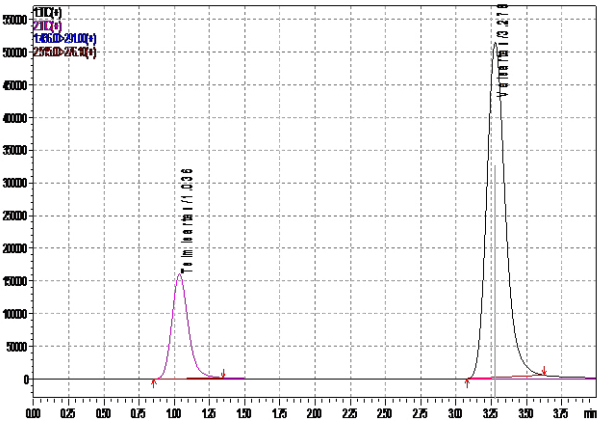

Figure 5: Mass chromatogram of valsartan at HQC

❑ Conclusions

A simple, high throughput LC/MS/MS method for quantitative determination of valsartan in human plasma was developed. The LLOQ of the method was determined as 20.0 ng/mL. The linear dynamic range of the calibration curve was 20.0 – 15000 ng/mL with a regression of R^2 = 0.9997. Good recoveries (between 91.6 to 114.8 %) were obtained at all the three levels of QC samples with repeatability.

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Highly sensitive quantitative analysis of Felodipine and Hydrochlorothiazide from plasma using LC/MS/MS

ASMS 2014 TP497

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Highly sensitive quantitative analysis of Felodipine and Hydrochlorothiazide from plasma using LC/MS/MS

Introduction

Felodipine is a calcium antagonist (calcium channel blocker), used as a drug to control hypertension[1]. Hydrochlorothiazide is a diuretic drug of the thiazide class that acts by inhibiting the kidney's ability to retain water. It is, therefore, frequently used for the treatment of hypertension, congestive heart failure, symptomatic edema, diabetes insipidus, renal tubular acidosis and the prevention of kidney stones[2].
Efforts have been made here to develop high sensitive methods of quantitation for these two drugs using LCMS-8050 system from Shimadzu Corporation, Japan. Presence of heated Electro Spray Ionization (ESI) probe in LCMS-8050 ensured good quantitation and repeatability even in the presence of a complex matrix like plasma. Ultra high sensitivity of LCMS-8050 enabled development quantitation method at low ppt level for both Felodipine and Hydrochlorthiazide.

Felodipine

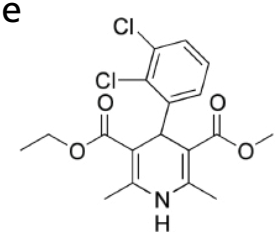

Figure 1. Structure of Felodipine

Felodipine is a calcium antagonist (calcium channel blocker). Felodipine is a dihydropyridine derivative that is chemically described as ± ethyl methyl 4-(2,3-dichlorophenyl)1,4-dihydro-2,6-dimethyl-3,5-pyridin edicarboxylate. Its empirical formula is $C_{18}H_{19}Cl_2NO_4$ and its structure is shown in Figure 1.

Hydrochlorothiazide

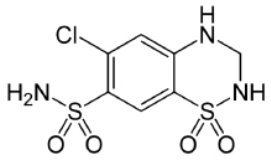

Figure 2. Structure of Hydrochlorothiazide

Hydrochlorothiazide, abbreviated HCTZ (or HCT, HZT), is a diuretic drug of the thiazide class that acts by inhibiting the kidney's ability to retain water.
Hydrochlorothiazide is 6-chloro-1,1-dioxo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide.Its empirical formula is $C_7H_8ClN_3O_4S_2$ and its structure is shown in Figure 2.

Method of Analysis

Preparation of matrix matched plasma by protein precipitation method using cold acetonitrile

To 100 µL of plasma, 500 µL of cold acetonitrile was added for protein precipitation then put in rotary shaker at 20 rpm for 15 minutes for uniform mixing. It was centrifuged at 12000 rpm for 15 minutes. Supernatant was collected and evaporated to dryness at 70 °C and finally reconstituted in 200 µL Methanol.

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Preparation of matrix matched plasma by liquid-liquid extraction method using diethyl ether and hexane mixture (1:1 v/v)

To 500 µL plasma, 100 µL sodium carbonate (1.00 mol/L) and 5 mL of diethyl ether : hexane (1:1 v/v) was added. It was placed in rotary shaker at 20 rpm for 15 minutes for uniform mixing and centrifuged at 12000 rpm for 15 minutes. Supernatant was collected and evaporated to dryness at 60 °C. It was finally reconstitute in 1000 µL Methanol.

Preparation of calibration standards in matrix matched plasma

Response of Felodipine and Hydrochlorothiazide were checked in both above mentioned matrices. It was found that cold acetonitrile treated plasma and diethyl ether: hexane (1:1 v/v) treated plasma were suitable for Felodipine and Hydrochlorothiazide molecules respectively. Calibration standards were thus prepared in respective matrix matched plasma.

- Felodipine Calibration Std : 5 ppt, 10 ppt, 50 ppt, 100 ppt, 500 ppt, 1 ppb and 10 ppb
- HCTZ Calibration Std : 2 ppt, 5 ppt, 10 ppt, 50 ppt, 100 ppt, and 500 ppt

Figure 3. LCMS-8050 triple quadrupole mass spectrometer by Shimadzu

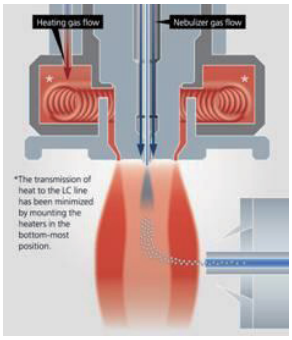

Figure 4. Heated ESI probe

LCMS-8050 triple quadrupole mass spectrometer by Shimadzu (shown in Figure 3), sets a new benchmark in triple quadrupole technology with an unsurpassed sensitivity (UFsensitivity), Ultra fast scanning speed of 30,000 u/sec (UFscanning) and polarity switching speed of 5 msecs (UFswitching). This system ensures highest quality of data, with very high degree of reliability.

In order to improve ionization efficiency, the newly developed heated ESI probe (shown in Figure 4) combines high-temperature gas with the nebulizer spray, assisting in the desolvation of large droplets and enhancing ionization. This development allows high-sensitivity analysis of a wide range of target compounds with considerable reduction in background.

LC/MS/MS analysis

Compounds were analyzed using Ultra High Performance Liquid Chromatography (UHPLC) Nexera coupled with LCMS-8050 triple quadrupole system (Shimadzu Corporation, Japan), The details of analytical conditions are given in Table 1 and Table 2.

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Table 1. LC/MS/MS conditions for Felodipine

• Column	: Shim-pack XR-ODS (75 mm L x 3 mm I.D.; 2.2 µm)
• Flow rate	: 0.3 mL/min
• Oven temperature	: 40 °C
• Mobile phase	: A: 10 mM ammonium acetate in water B: methanol
• Gradient program (%B)	: 0.0 – 3.0 min → 90 (%); 3.0 – 3.1 min → 90 – 100 (%); 3.1 – 4.0 min → 100 (%); 4.0– 4.1 min → 100 – 90 (%) 4.1 – 6.5 min → 90 (%)
• Injection volume	: 10 µL
• MS interface	: ESI
• Nitrogen gas flow	: Nebulizing gas 1.5 L/min; Drying gas 10 L/min;
• Zero air flow	: Heating gas 10 L/min
• MS temperature	: Desolvation line 200 °C; Heating block 400 °C Interface 200 °C

Table 2. LC/MS/MS conditions for Hydrochlorothiazide

• Column	: Shim-pack XR-ODS (100 mm L x 3 mm I.D.; 2.2 µm)
• Flow rate	: 0.2 mL/min
• Oven temperature	: 40 °C
• Mobile phase	: A: 0.1% formic acid in water B: acetonitrile
• Gradient program (%B)	: 0.0 – 1.0 min → 80 (%); 1.0 – 3.5 min → 40 – 100 (%); 3.5 – 4.5 min → 100 (%); 4.5– 4.51min → 100 – 80 (%) 4.51 – 8.0 min → 90 (%)
• Injection volume	: 25 µL
• MS interface	: ESI
• Nitrogen gas flow	: Nebulizing gas 2.0 L/min; Drying gas 10 L/min;
• Zero air flow	: Heating gas 15 L/min
• MS temperature	: Desolvation line 250 °C; Heating block 500 °C Interface 300 °C

Results

LC/MS/MS analysis results of Felodipine

LC/MS/MS method for Felodipine was developed on ESI positive ionization mode and 383.90>338.25 MRM transition was optimized for it. Checked matrix matched plasma standards for highest (10 ppb) as well as lowest concentrations (5 ppt) as seen in Figure 5 and Figure 6 respectively. Calibration curves as mentioned with R^2 = 0.998 were plotted (shown in Figure 7). Also as seen in Table 3, % Accuracy was studied to confirm the reliability of method. Also, LOD as 2 ppt and LOQ as 5 ppt was obtained.

Highly sensitive quantitative analysis of Felodipine and Hydrochlorothiazide from plasma using LC/MS/MS

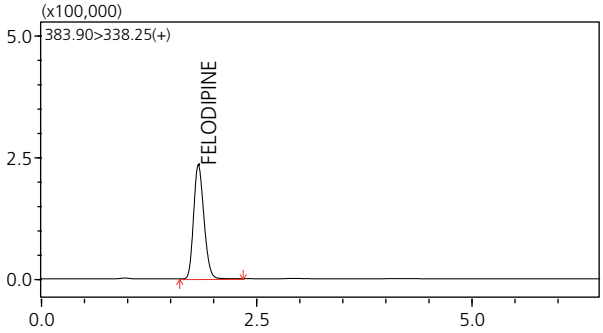

Figure 5. Felodipine at 10 ppb in matrix matched plasma

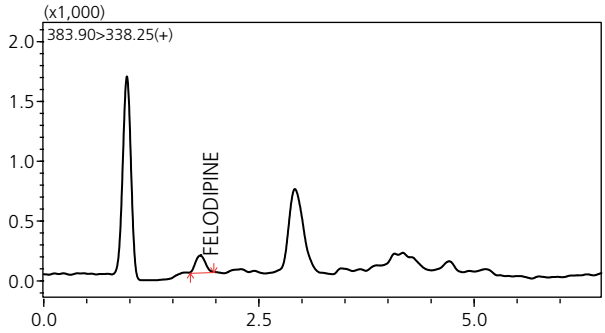

Figure 6. Felodipine at 5 ppt in matrix matched plasma

Table 3: Results of Felodipine calibration curve

Sr. No.	Standard	Nominal Concentration (ppb)	Measured Concentration (ppb)	% Accuracy (n=3)	% RSD for area counts (n=3)
1	STD-FEL-01	0.005	0.005	97.43	9.87
2	STD-FEL-02	0.01	0.010	103.80	8.76
3	STD-FEL-03	0.05	0.053	104.47	2.24
4	STD-FEL-04	0.1	0.103	103.13	1.23
5	STD-FEL-05	0.5	0.469	94.88	1.33
6	STD-FEL-06	1	0.977	97.33	0.95
7	STD-FEL-07	10	10.023	100.90	0.60

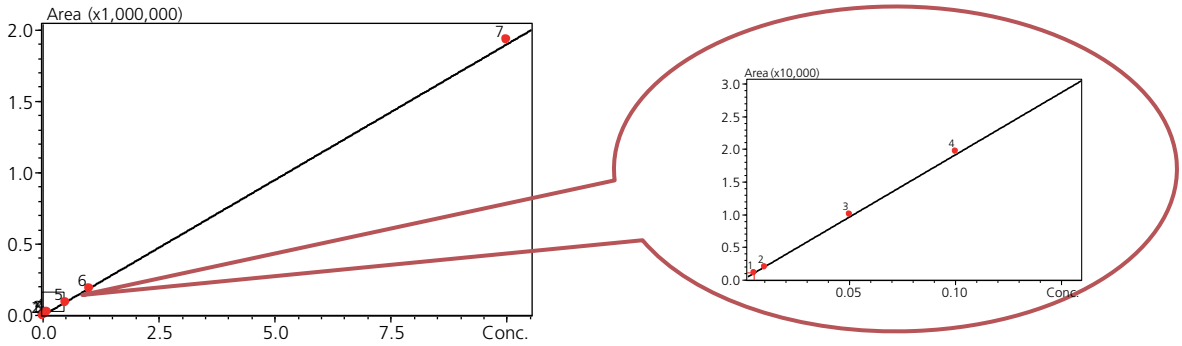

Figure 7. Calibration curve of Felodipine

LC/MS/MS analysis results of Hydrochlorothiazide

LC/MS/MS method for Hydrochlorothiazide was developed on ESI negative ionization mode and 296.10>204.90 MRM transition was optimized for it. Checked matrix matched plasma standards for highest (500 ppt) as well as lowest (2 ppt) concentrations as seen in Figures 8 and 9 respectively.

Calibration curves as mentioned with R^2=0.998 were plotted (shown in Figure 10). Also as seen in Table 4, % Accuracy was studied to confirm the reliability of method. Also, LOD as 1 ppt and LOQ as 2 ppt were obtained.

Highly sensitive quantitative analysis of Felodipine and Hydrochlorothiazide from plasma using LC/MS/MS

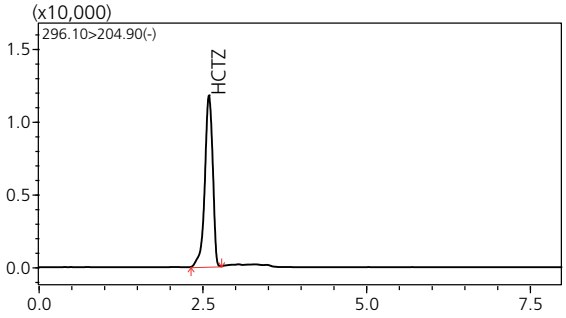

Figure 8. Hydrochlorothiazide at 500 ppt in matrix matched plasma

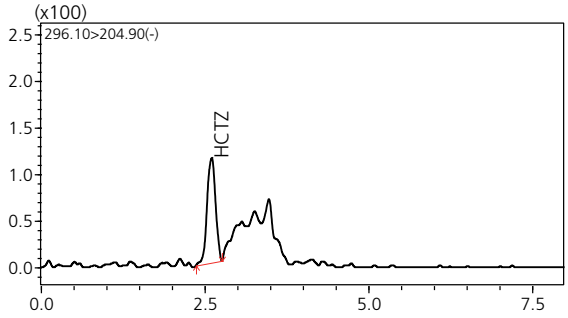

Figure 9. Hydrochlorothiazide at 2 ppt in matrix matched plasma

Table 4. Results of Hydrochlorothiazide calibration curve

Sr. No.	Standard	Nominal Concentration (ppb)	Measured Concentration (ppb)	% Accuracy (n=3)	% RSD for area counts (n=3)
1	STD-HCTZ-01	0.002	0.0020	102.03	6.65
2	STD-HCTZ-02	0.005	0.0048	95.50	3.53
3	STD-HCTZ-03	0.01	0.0099	100.07	3.80
4	STD-HCTZ-04	0.05	0.0512	102.67	1.60
5	STD-HCTZ-05	0.1	0.1019	102.11	3.58
6	STD-HCTZ-06	0.5	0.4944	102.13	1.68

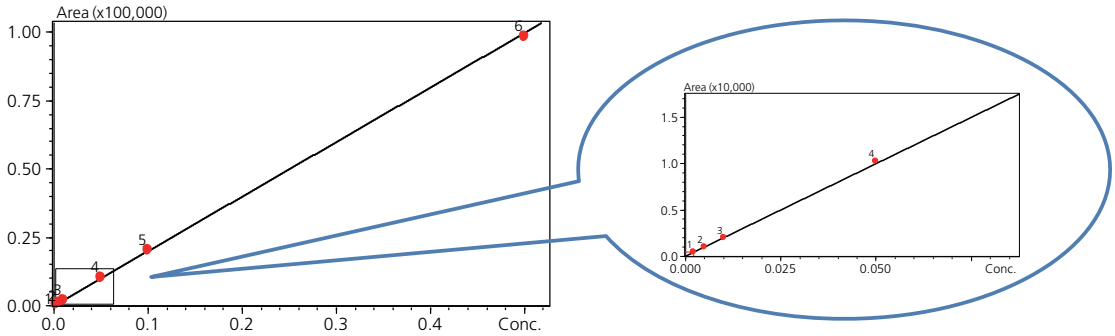

Figure 10. Calibration curve of Hydrochlorothiazide

Conclusion

- Highly sensitive LC/MS/MS method for Felodipine and Hydrochlorothiazide was developed on LCMS-8050 system.
- LOD of 2 ppt and LOQ of 5 ppt was achieved for Felodipine and LOD of 1 ppt and LOQ of 2 ppt was achieved for Hydrochlorothiazide by matrix matched methods.
- Heated ESI probe of LCMS-8050 system enables drastic augment in sensitivity with considerable reduction in background. Hence, LCMS-8050 system from Shimadzu is an all rounder solution for bioanalysis.

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Application News

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Pharma CRO / LCMS-8060

HIGHLY SENSITIVE AND SIMPLE METHOD FOR ESTIMATION OF TIOTROPIUM IN HUMAN PLASMA USING SHIMADZU LCMS-8060

Yogesh Arote, Chaitanya Atmakuri, Dr. Avinash Gaikwad

❑ Introduction

Tiotropium is an inhaled long-acting anti-cholinergic for the maintenance treatment of COPD (chronic obstructive pulmonary disease) [1,2]. It is chemically described as (1α, 2β, 4β, 5$^{\alpha}$, 7β) -7- [(Hydroxydi-2-thienylacetyl)oxy] – 9.9 – dimethyl - 3 - oxa - 9 – azoniatricyclo [3.3.1.0] nonane bromide monohydrate. It is a synthetic, non chiral, quarternary ammonium compound [3]. Structure of tiotropium is provided in figure 1 [4]

The long duration of action with tiotropium is owing to prolonged, competitive binding to M(3) muscarinic receptors. Tiotropium is poorly absorbed following inhalation, which largely limits side effects. Metabolism of absorbed drug is minimal and excretion is largely through the kidneys. Tiotropium is efficacious and well tolerated by patients with COPD[2]

Therapeutic doses of tiotropium results in very low concentrations and requires a sensitive bio-analytical method for accurate quantification of the drug in plasma.

Shimadzu Application Development Centre (ADC-SAIP), Mumbai has developed and validated the most sensitive method with the lowest limit of quantification (LLOQ) of 0.2 pg/mL. The method has used a single step extraction technique and direct injection approach to eliminate environmental contamination. These factors enhance the productivity of the pharmacokinetic investigation involving high-throughput analysis.

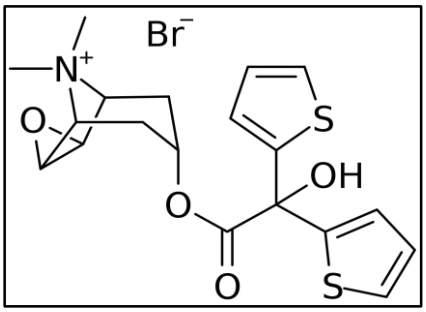

Fig.1 Structure of tiotropium

❑ Salient features

- Quantitative method for estimation of tiotropium in human plasma was developed and validated as per USFDA Guidelines (results are presented in Table 1).
- Effective throughput for quantitative assessment is increased by use of a quick and simple extraction method.
- Heated ESI along with New UF-Qarray ion guide technology contributes by increasing ion production and enhancing transmission respectively. This ensures sensitive and selective quantification of tiotropium at 0.2 pg/mL.
- Optimum plasma volume (450 µL) avoided unnecessary wastage of plasma samples and at the same time increased the life of the mass spectrometer.
- Customised gradient method enhances the chromatographic resolution of tiotropium with consistent and reproducible peak area and retention time.
- Method was validated for the following criteria:
  - ✓ Selectivity
  - ✓ Linearity
  - ✓ Inter-day and intra-day precision and accuracy (PA)
  - ✓ Recovery
  - ✓ Matrix effect
  - ✓ Carry over effect
  - ✓ Stability studies

Table 1. Method validation summary

Calibration curve range			0.20 pg/mL to 200.00 pg/mL	
Intraday precision and accuracy (at LLOQ QC)	Accuracy (% Nominal)		112.96	
	Precision (% CV)		10.03	
Intraday precision and accuracy (at LQC, MQC and HQC)	Accuracy (% Nominal)		102.88 to 109.00	
	Precision (% CV)		1.35 to 4.34	
Global precision and accuracy (at LLOQ QC)	Accuracy (% Nominal)		106.92	
	Precision (% CV)		18.63	
Global precision and accuracy (at LQC, MQC and HQC)	Accuracy (% Nominal)		102.84 to 105.35	
	Precision (% CV)		6.35 to 10.40	
Global % recovery	Recovery (%)		57.61	
	Precision (% CV)		8.66	
Matrix effect	IS normalized Matrix Factor		1.17	
Bench top stability in matrix (6.0 hrs.)	% Change		LQC=-1.33	HQC=-12.57
	Precision (% CV)	Stability Sample	LQC=3.25	HQC=-12.14
	Accuracy (% Nominal)	Stability Sample	LQC=103.25	HQC=87.86
Auto sampler stability in matrix (30.0 hrs.)	% Change		LQC=-8.15	HQC=-12.69
	Precision (% CV)	Stability Sample	LQC=-3.89	HQC=-12.26
	Accuracy (% Nominal)	Stability Sample	LQC=96.11	HQC=87.74
Freeze thaw stability in matrix (Three Cycles)	% Change		LQC=-0.88	HQC=-1.06
	Precision (% CV)	Stability Sample	LQC=3.72	HQC=-0.58
	Accuracy (% Nominal)	Stability Sample	LQC=103.72	HQC=99.42

❑ Results and Discussion

A. Linearity

Linearity was assessed by 6 calibration curves in human plasma on 4 consecutive days. Calibration curve for tiotropium was found linear from 0.20-200.00 pg/mL (refer table 2) The correlation coefficient was greater than 0.98. Signal to noise ratio (s/n) at LLOQ level was found to be more than 20:1, across 6 PA batches. Representative chromatogram is shown in figure 3.

Table 2. Calibration curve range

Level	Back Cal. Conc. (pg/mL)
CC1	0.20
CC2	0.38
CC3	0.68
CC4	1.85
CC5	5.05
CC6	10.92
CC7	23.23
CC8	52.22
CC9	98.01
CC10	203.57

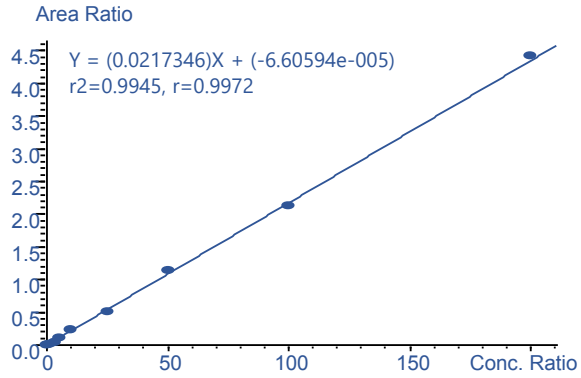

Fig.2 Calibration curve

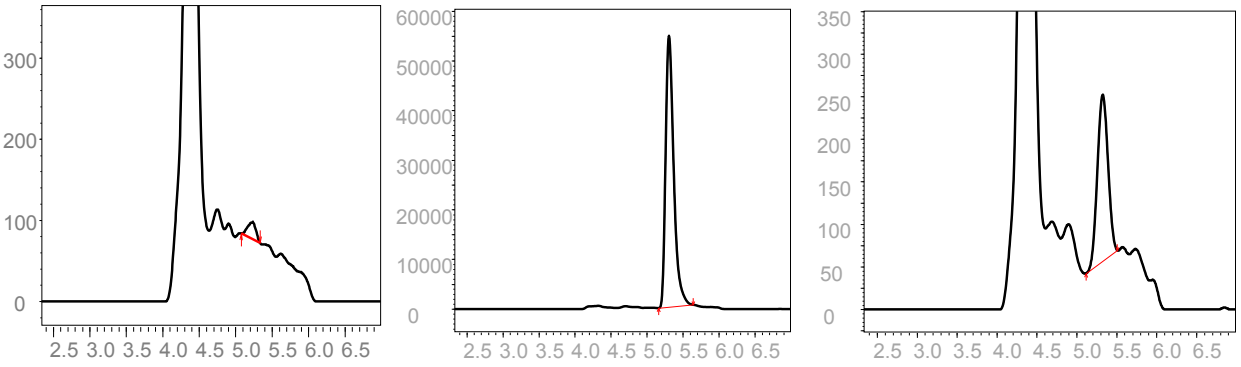

Fig.3 Chromatograms of Tiotropium (extracted blank, extracted IS, extracted LLOQ)

B. Selectivity

Selectivity was evaluated by analyzing 7 different lots of blank human plasma. No significant interference was observed at the retention time and MRM transition of analyte and IS (refer figure 3).

Table 3. Percentage interference in blank with respect to LLOQ

	Tiotropium		
Plasma lot no.	Area in blank matrix	LLOQ area	% Interference
V 5548	133	774	17.18
V 4547	160	1,652	9.69
V 0984	250	5,704	4.38
V 4556	128	1,303	9.82
V 4555	218	1,303	16.73
V 4554	113	1,515	7.46
V 4560	319	1,709	18.67

C. Intra-day precision and accuracy

Intraday precision and accuracy was evaluated by processing 6 replicates of LLOQQC, LQC, MQC and HQC in one P&A batch. Figure 4 represents the consistency of QC samples (X-axis represents number of QC injections and Y-axis represents calculated concentration in log scale). Statistical data is summarized in table 4.

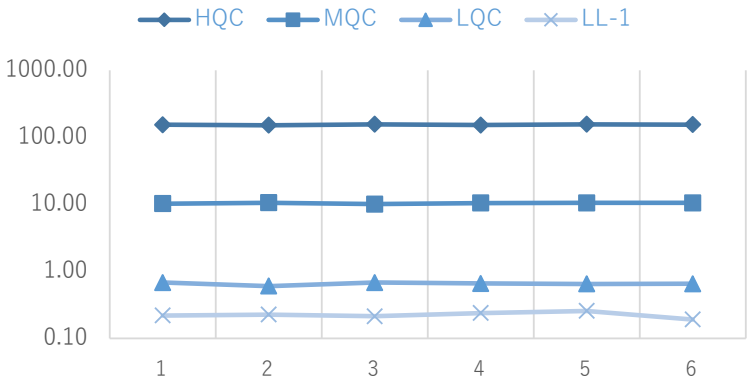

Fig 4. Trend plot of intra-day QC assessment

Table 4. Intra-day precision and accuracy batch

QC (n=6)	LLOQQC	LQC	MQC	HQC
Nominal concentration	0.20	0.60	10.00	150.00
PA batch observed concentration (pg/mL)	0.22	0.68	10.22	153.93
	0.23	0.60	10.56	151.71
	0.21	0.68	10.10	156.62
	0.24	0.66	10.47	152.21
	0.26	0.65	10.50	156.48
	0.19	0.65	10.49	154.98
Mean	0.22	0.65	10.39	154.32
Standard deviation	0.02	0.03	0.18	2.09
% CV	10.03	4.34	1.78	1.35
% Nominal	112.96	109.00	103.89	102.88

D. Global precision and accuracy

Precision and accuracy experiments were evaluated at LLOQ, LQC, MQC and HQC level in 6 batches. Excellent accuracy and repeatability was observed with % CV < 10.40 % and % accuracy between 102.84 to 105.35% at LQC, MQC and HQC level. At LLOQQC level, the % CV was found to be 18.63 % and % accuracy as 106.92 %. The results are presented in table 5. Figure 5 shows trend plots of 36 QC at LLOQ, LQC, MQC and HQC level, analyzed over 6 batches.

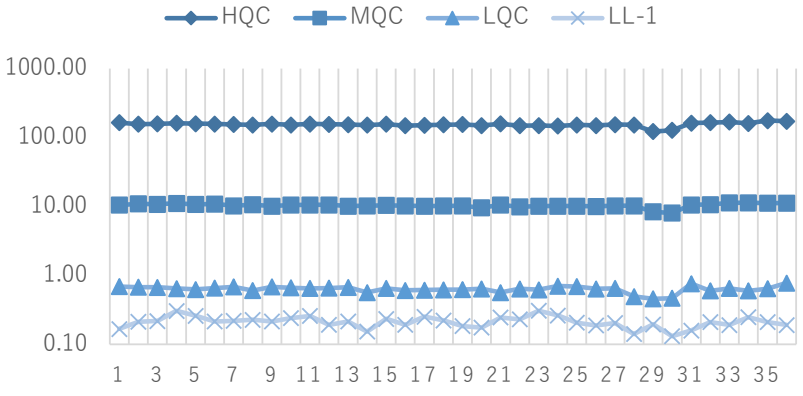

Fig 5. Trend plot of inter-day QC assessment. X-axis represents the number of injections and Y-axis represents calculated concentration (log scale)

Table 5. Global precision and accuracy batch

QC level (n=36)	Mean Conc. (pg/mL)	% CV	% Accuracy
LLOQ QC (0.20 pg/mL)	0.21	18.63	106.92
LQC (0.60 pg/mL)	0.63	10.40	105.35
MQC (10.00 pg/mL)	10.31	6.48	103.13
HQC (150.00 pg/mL)	154.26	6.35	102.84

E. Matrix effect

Matrix effect was experiment was performed at LQC and HQC levels for tiotropium. IS normalised matrix factor was found to be 1.17 and thus meets the acceptance criteria.

F. Recovery

Recovery experiment was evaluate at extracted LQC, MQC and HQC level. Mean recovery for tiotropium is found to be 57.61 %. Recovery of tiotropium is found to be precise, consistent and reproducible at all QC levels.

H. Stability

Analyte stability in human plasma was studied for bench top stability (6.0 hrs), autosampler stability (30.0 hrs) and freeze thaw stability (three cycles). Stability samples were processed and analyzed along with freshly analyte spiked calibration standards and comparison QC samples. Analyte stability in human plasma was found to meet the acceptance criteria.

G. Carry-over effect

Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was present/observed at the retention time and MRM transition of the analyte in the extracted blank sample following the highest standard calibrator.

❑ Conclusion

LCMS-8060, along with special sample preparation, optimized chromatography provides a very selective and sensitive method for bioanalytical assay of Tiotropium. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple mobile phase at a minimal gradient flow rate of 0.2 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavours.

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A Fast and Sensitive LC/MS/MS Method for Quantitation of Fosfomycin in Human Plasma with HILIC Chromatography

ThP 757

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A Fast and Sensitive LC/MS/MS Method for Quantitation of Fosfomycin in Human Plasma with HILIC Chromatography

1. Overview

Fosfomycin (FOM) was analysed in human plasma using a HILIC-LC/MS/MS method in MRM mode on LCMS-8060. Racemic fosfomycin-13C3 benzylamine salt was added in the samples as the internal standard. The MRM transitions for quantitation are 137.05>79.0 for fosfomycin and 140.0>79.0 for the internal standard. The developed method is fast and sensitive for quantitation of fosfomycin in biological samples.

2. Introduction

Fosfomycin is an old and broad-spectrum antibiotic drug manufactured since 1970s. It is mainly used for treatment of urinary tract infections (UTIs). However, the development of bacterial resistance occurs frequently, making fosfomycin unsuitable for treatment of severe infections in the past. Recently, use of fosfomycin formulations was approved in several countries, because it was found to be active against many multidrug-resistant (MDR) pathogens. Quantification of fosfomycin in human plasma may provide insight into its pharmacokinetics characteristics, which is crucial for current therapy modification.

Therefore, a reliable analytical method is needed for determination of fosfomycin in biological samples. In this study, a LC/MS/MS method with HILIC chromatography was developed and used for quantification

3. Experimental

The standard of fosfomycin (in powder form) was obtained and used in this study. Two pooled human plasma samples were obtained from a commercial supplier. A stock solution of 1000 mg/L fosfomycin in Milli-Q water was used to prepare calibration standards in blank plasma samples. The stock solution was stored at -20oC before use. Racemic fosfomycin-13C3 benzylamine as the internal standard, was used in this experiment. A stock solution of 100 mg/L racemic fosfomycin-13C3 benzylamine was prepared in ammonium acetate solution (5 mM). Quality control samples (QC) were prepared in the same manner as the calibration standards. Sample pre-treatment was carried out by a protein crashing procedure, adding mixed organic solvent (ACN/MeOH, 1:1). The ratio of plasma and solvent mixture was 1:4 (v/v). The crashed plasma was centrifuged for 10min and then filtered using a 0.22-micron nylon filter. The filtered solution was diluted with 5mM

Table 1. Analytical conditions of fosfomycin analysis on LCMS-8060

Column	Shimadzu Shim-pack GIS HILIC Column (150 x 3.0 mm, 3µm)	Interface & temp.	Heated ESI, 300°C
Flow rate	0.3 mL/min	MS mode	MRM (-)
Mobile phase	A: 5 mM ammonium acetate in water B: Acetonitrile	Block temp.	350°C
		DL temp.	250°C
		CID gas	Ar (230 kPa)
Elution mode	Isocratic, 10% B	Nebulizing gas flow	N_2, 3 L/min
Oven temp.	40°C	Drying gas flow	N_2, 10 L/min
Injection vol.	5.0 µL	Heating gas flow	Zero air, 10 L/min

4. Results and Discussion

4.1 Development of MRM method for fosfomycin in human plasma

A MRM method in negative mode was developed for quantitative analysis of fosfomycin in human plasma samples on a triple quadrupole LCMS-8060. The MRM chromatogram of pre-spiked plasma standard and the internal standard are shown in Figure 1. Racemic fosfomycin-13C3 benzylamine was used as internal standard added to the plasma sample. MRM transition of 137.05>79.0 was selected as the quantifier ion for fosfomycin, and transitions of 137.05>63.0 and 137.05>81.05 were used as reference ions. For the internal standard fosfomycin-13C3, the MRM transition of 140.0>79.0 was used as the quantifier. A calibrant series of eight concentration levels were prepared by pre-spiked fosfomycin standards in the blank human plasma. The concentrations of the calibration curve were at 0.02, 0.1, 0.2, 0.4, 1, 2, 4 to 6 ppm, which correspond to concentrations of fosfomycin of 1, 5, 10, 20, 50,100, 200 and 300 ppm pre-spiked in the plasma (Table 2). The established calibration curve (Figure 2) was applied for determination of fosfomycin in post-spiked human plasma and the neat fosfomycin standards. QC samples were prepared in the same way (pre-spiked) for evaluation of method performance.

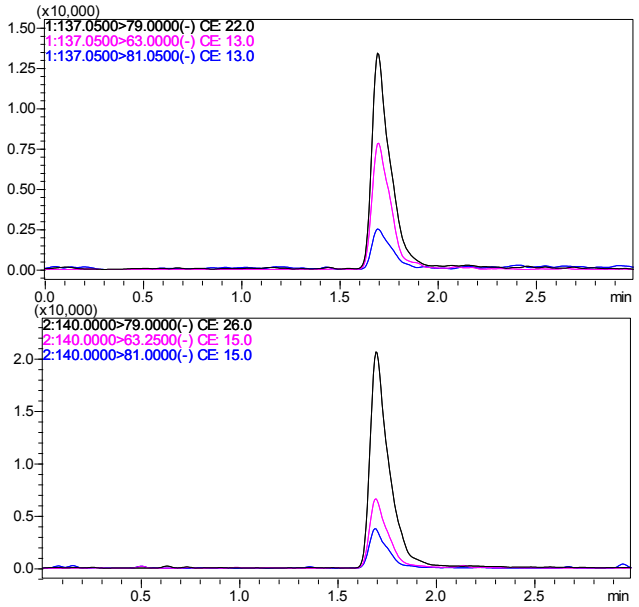

Figure 1. MRM chromatograms of pre-spiked standard of fosfomycin in human plasma, fosfomycin of 0.2 ppm (top) and fosfomycin-$^{13}C_3$ of 0.2 ppm (bottom).

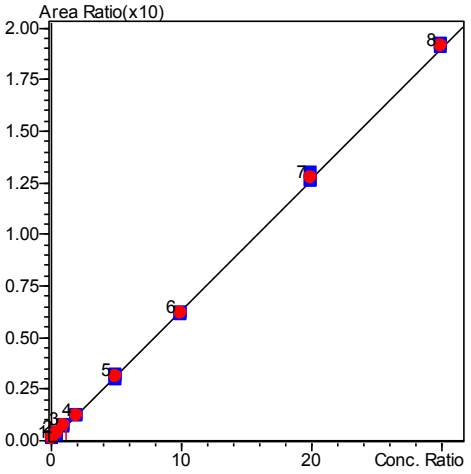

Figure 2. IS calibration curve of fosfomycin pre-spiked in blank human plasma, eight concentration levels ranging from 0.02 ppm to 6ppm on LCMS-8060 (IS: 0.2 ppm).

Table 2. Summary of MRM quantification method for analysis of fosfomycin in plasma on LCMS-8060: calibration range, linearity, accuracy and repeatability (%RSD, area) in two pooled plasmas

Name	RT (Min)	MRM (negative)	Calib. range (ppm)	R^2	Accuracy (%)	%RSD (n=6)		
						Low conc. (0.1ppm)	Medium conc. (0.4 ppm)	High conc. (2 ppm)
FOM (Plasma 1)	1.70	137.05>79.0	0.02 ~ 6	0.999	93.4	4.2	4.7	2.1
FOM (Plasma 2)	1.72	137.05>79.0	0.02 ~ 6	0.999	97.0	4.7	2.0	0.9

4.2 Performance evaluation for quantitation method of fosfomycin

Linearity and LLOQ of MRM quantitation: The linearity of the plotted calibration curve (R2) with IS method was 0.999 for the range from 0.02 ppm to 6.0 ppm. The LLOQ was determined with 0.02 ppm pre-spiked sample, obtaining S/N>/=10 and RSD% (n=6) < 8% (Figure 3). A blank plasma prepared following the similar sample preparation procedure without addition of fosfomycin showed no interference peaks for fosfomycin and the internal standard (IS). The repeatability of the method was checked with low, medium and high conc. standards. The %RSD for the peak area (n=6) were calculated to be at 0.9~4.7% (Table 2). In order to investigate the accuracy of the method, QC samples of varying concentrations (0.5 ppm and 3 ppm) were prepared in the same manner of the calibration standards for both sets of plasma samples. The accuracy, deviation and also precision were calculated. The results are summarized in Table 3. All calculated values were within the acceptance criteria of ±15% of the mean concentrations.

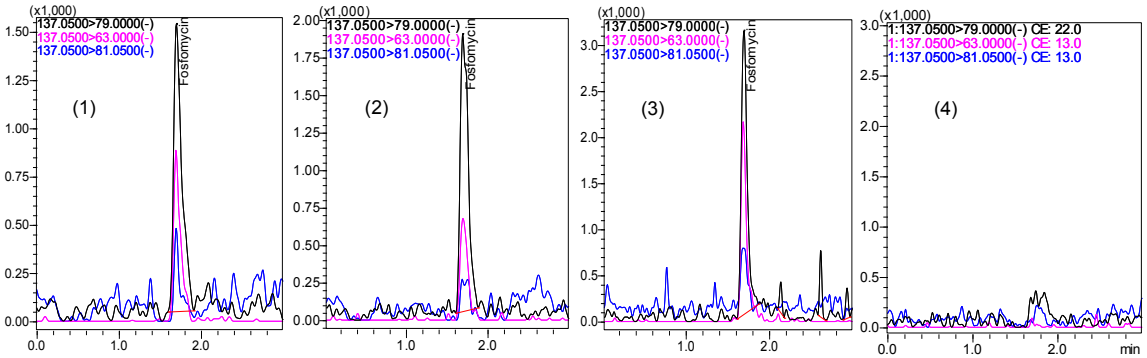

Figure 3. MRM chromatograms of fosfomycin in human plasma of 0.02 ppm in (1) pre-spiked, (2) post-spiked and (3) in neat solution. (4) MRM chromatogram of blank plasma sample.

Table 3. Accuracy, deviation and precision results of QC samples obtained from the two human plasma

Sample	Prepared QC conc. (ppm)	Measured QC conc. (ppm)	Accuracy (%)	Deviation (%)	%RSD (n=6) (peak area)
QC plasma 1	0.5	0.49	98.4	-1.6	1.5
	3.0	2.98	99.3	-0.7	2.5
QC plasma 2	0.5	0.50	100.8	0.8	2.4
	3.0	3.07	102.3	2.3	3.3

Recovery and matrix effect: Three sets of standard samples of fosfomycin, i.e., prespiked, post-spiked and neat solution were prepared for investigation of recovery and matrix effect. Two plasma matrixes were used to prepared these samples. For calculation of the recovery (%), the response of the post-spiked standard was compared to that of the pre-spiked standard to obtain the percentage. The results were summarized in Table 4. It can be seen that the recovery for plasma 1 was in the range of 60.7~81.2%, and for plasma 2, it was in the range of 63.8~77.5%. Matrix effect (%) was calculated by comparing the response of the post-spiked standards and that of the neat standards. The results of the matrix effect were shown in the same table. For plasma sample 1, matrix effect was calculated to be 52.2~74.8%, and for plasma 2, the matrix effect was in the range of 64.4~76.5%.

Table 4. Evaluation of recovery (%) and matrix effect (%) using various fosfomycin standards spiked in the plasma and neat solution

Sample	Conc. of FOM standard (ppm)	Recovery (%)	Matrix effect (%)
Plasma 1	0.02	81.2	74.8
	0.1	71.0	67.5
	0.2	68.8	65.0
	0.4	60.7	56.2
	1.0	64.9	52.2
	4.0	68.3	59.6
	6.0	68.8	60.7
Plasma 2	0.1	67.1	76.5
	0.4	77.5	70.6
	1.0	64.5	64.4
	6.0	63.8	73.7

5. Conclusion

A fast and sensitive LC/MS/MS method was developed for determination of fosfomycin in human plasma samples. The calibration range used in the method is 0.02 ppm ~ 6 ppm, which correspond to its concentrations of 1 ppm ~ 300 ppm (dilution factor = 50) in plasma. The LLOQ of the method is determined to be 0.02 ppm in solution, which corresponds to the concentration of 1.0 ppm in plasma. Recovery and matrix effect were investigated with pre-spiked, post-spiked and neat standard solution.

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Quantitative Analysis of β-Lactam Antibiotics in Human Plasma by High Sensitivity LC/MS/MS Method

ASMS 2017 ThP 079

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Quantitative Analysis of β-Lactam Antibiotics in Human Plasma by High Sensitivity LC/MS/MS Method

Introduction

The β-lactam type antibiotics are used in the treatment of various bacterial infections in human over decades. One of the consequences of continuous uses of antibiotics is the progressive development of drug resistance of bacteria in human [1]. Therapeutic Drug Monitoring (TDM) aims at obtaining pharmacokinetic pattern of an antibiotic in patient to develop personalized medicine treatment. Conventional TDM methods such as immunoassays are well-established. However, one of the drawbacks of immunoassays is lack of specificity due to cross-reactivity with metabolites, which may give false positives [2,3]. Recently, LC/MS/MS has been used for fast and direct measurement of β-lactam antibiotics such as amoxicillin [4] and piperacillin, etc. [5,6] in human plasma. In this application news, a fast LC/MS/MS method with a simple sample pre-treatment procedure for quantitative analysis of five β-lactam antibiotics, meropenem (MER), tazobactam (TAZ), piperacillin (PIP), cefepime (CEF) and ceftazidime (CFT) is described. A small injection volume of sample of this MRM-based method is required only, which minimizes the contamination of sample matrix, as such, reducing the cleaning and maintenance time of the interface of LC/MS/MS in clinical research work

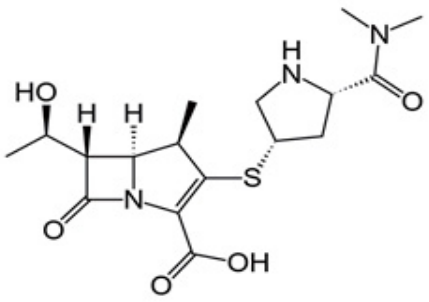

Figure 1: Structure of meropenem (MER) with a β-lactam ring.

Experimental

Sample preparation and analytical conditions

Five antibiotics used in this study are meropenem (MER), tazobactam (TAZ), piperacillin (PIP), cefepime (CEF) and ceftazidime (CFT). The compounds and four stable isotope-labelled meropenem-d6, piperacillin-d5, cefepime-cd3 and ceftazidime-d6 as internal standards were purchased from certified suppliers. Pool human plasma was obtained from i-DNA Biotechnology Pte Ltd and used as matrix. The sample pre-treatment and spiked sample preparation procedure are illustrated in Figure 1. A simple protein crash method was applied by adding ACN:MeOH (1:1) to plasma in a ratio of 3:1, followed by vortex and centrifuge. A calibration series of spiked standard samples were prepared: 20, 40, 80, 200, 400, 2000 and 4000 ng/mL in plasma. The concentrations of internal standards were 200 ng/mL or 800 ng/mL in these calibrants. A LCMS-8060, a triple quadrupole LC/MS/MS system with heated ESI was employed in this work. The analytical conditions and instrumental parameters are compiled into Table 1.

Quantitative Analysis of β-Lactam Antibiotics in Human Plasma by High Sensitivity LC/MS/MS Method

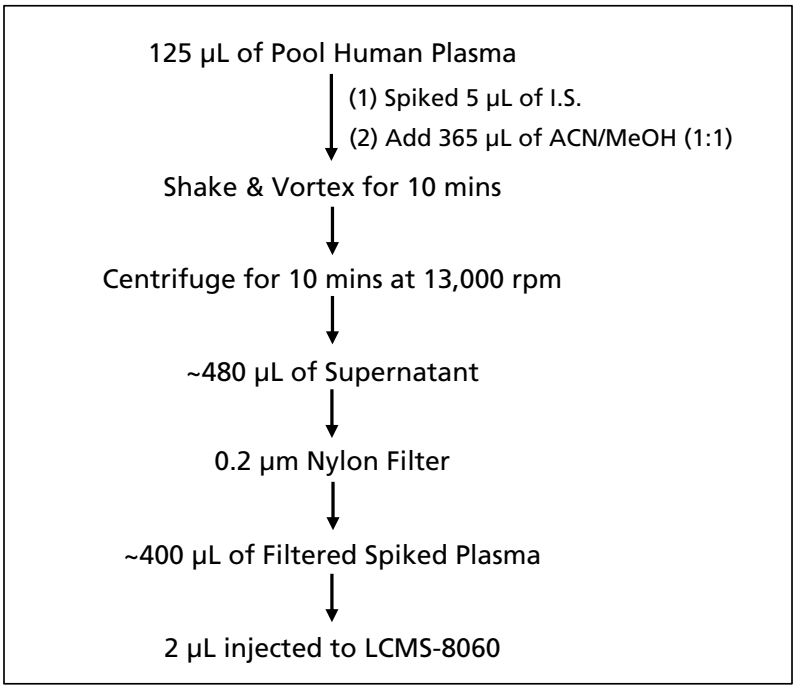

Figure 2: Procedure of protein crash and spiked-sample preparation

Table 1: Analytical conditions and parameters on LCMS-8060

Column	: Kinetex 1.7µ C18 100A (100 mmL x 2.10mm I.D.)
Mobile Phase	: A: Water with 0.1% FA
	B: Acetonitrile with 0.1% FA
Elution Program	: Gradient elution (5.5 minutes)
	B: 5% (0 to 0.2 min) → 90% (3.5 to 4.0 min) → 5% (4.1 to 5.5 min)
Flow Rate	: 0.5 mL/min
Oven Temp.	: 40°C
Injection	: 2 µL
Interface	: ESI (heated)
MS Mode	: MRM, Positive
Block Temp.	: 400°C
DL Temp.	: 250°C
Interface Temp.	: 300°C
CID Gas	: Ar, 270 kPa
Nebulizing Gas	: N_2, 3.0 L/min
Drying Gas	: N_2, 5.0 L/min
Heating Gas	: Zero Air, 15L/min

Results and Discussion

Fast MRM-based method for five β-lactam antibiotics

Table 2 shows the summarized results of optimized MRM transitions and parameters of the five β-lactam antibiotics studied and four stable isotope-labelled internal standards. Two MRM transitions were selected for each compound, with one as the quantitation ion and the other for confirmation.

Furthermore, a fast gradient elution MRM method was established with a total run time of 5 minutes. The MRM chromatograms of a mixed standard sample in plasma are shown in Figure 3. Due to lack of stable isotope-labelled tazobactam, MER-d6 was also used as the internal standard for tazobactam (TAZ) in this work.

Table 2: MRM transitions and parameters of five β-lactam antibiotics and internal standards on LCMS-8060

Compd.	Formula	E. Mass	MRM (m/z)	CE (V)	Int (%)
Tazobactam (TAZ)	$C_{10}H_{12}N_4O_5S$	300.05	301.1>168.2	-15	100
			301.1>122.1	-22	92
Cefepime (CEF)	$C_{19}H_{24}N_6O_5S_2$	480.13	481.1>86.2	-15	100
			481.1>396.0	-13	63
Meropenem (MER)	$C_{17}H_{25}N_3O_5S$	383.15	384.1>68.1	-41	100
			384.1>141.1	-16	64
Ceftazidime (CFT)	$C_{22}H_{22}N_6O_7S_2$	546.10	547.1>468.0	-13	100
			547.1>396.1	-19	42
Piperacillin (PIP)	$C_{23}H_{27}N_5O_7S$	517.16	518.2>143.1	-21	100
			518.2>160.1	-15	25
CEF-cd3	$C_{18}{}^{13}CH_{21}D_3N_6O_5S_2$	484.13	485.2>86.1	-16	100
			485.2>400.1	-13	66
MER-d6	$C_{17}H_{19}D_6N_3O_5S$	389.15	390.2>147.2	-18	100
			390.2>114.1	-27	74
CFT-d6	$C_{22}H_{16}D_6N_3O_7S_2$	552.10	553.1>474.0	-16	100
			553.1>319.1	-20	59
PIP-d5	$C_{23}H_{22}D_5N_5O_7S$	522.16	523.1>148.1	-21	100
			523.1>160.1	-14	23

Quantitative Analysis of β-Lactam Antibiotics in Human Plasma by High Sensitivity LC/MS/MS Method

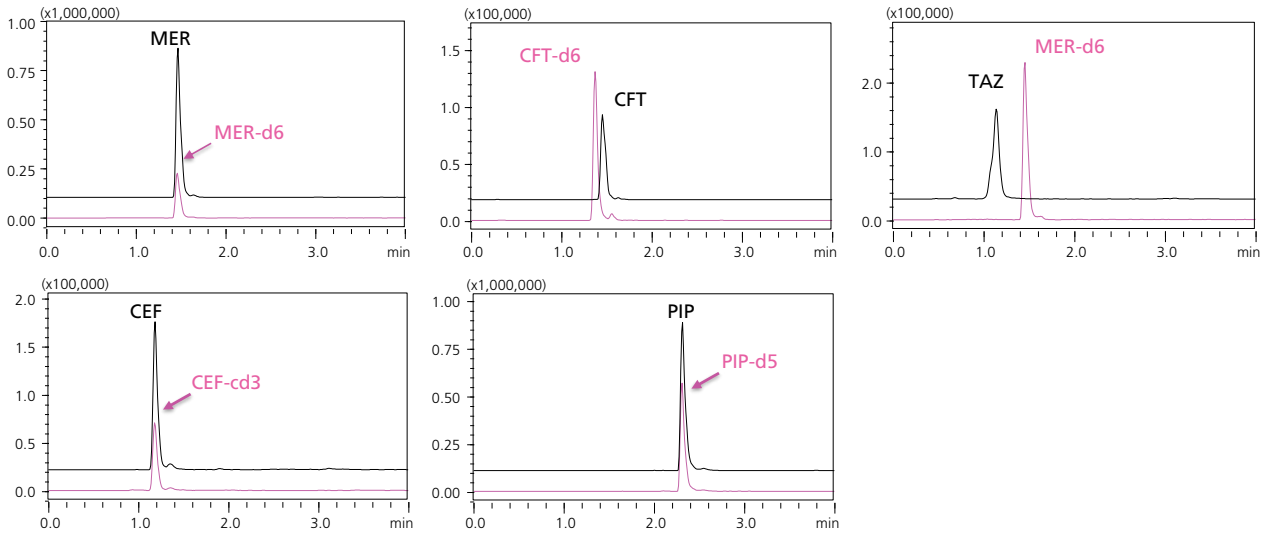

Figure 3: MRM chromatograms of five β-lactam antibiotics each (400 ng/mL) with internal standards in plasma on LCMS-8060

Calibration curves with IS

As shown in Figure 4, linear calibration curves with IS method were constructed using the standard samples prepared by pre-spiked in plasma matrix. The method parameters are summarized in Table 3. It can be seen that good linearity with R2 greater than 0.997 was obtained for the five compounds in the range from 20 ng/mL to 4000 ng/mL in plasma.

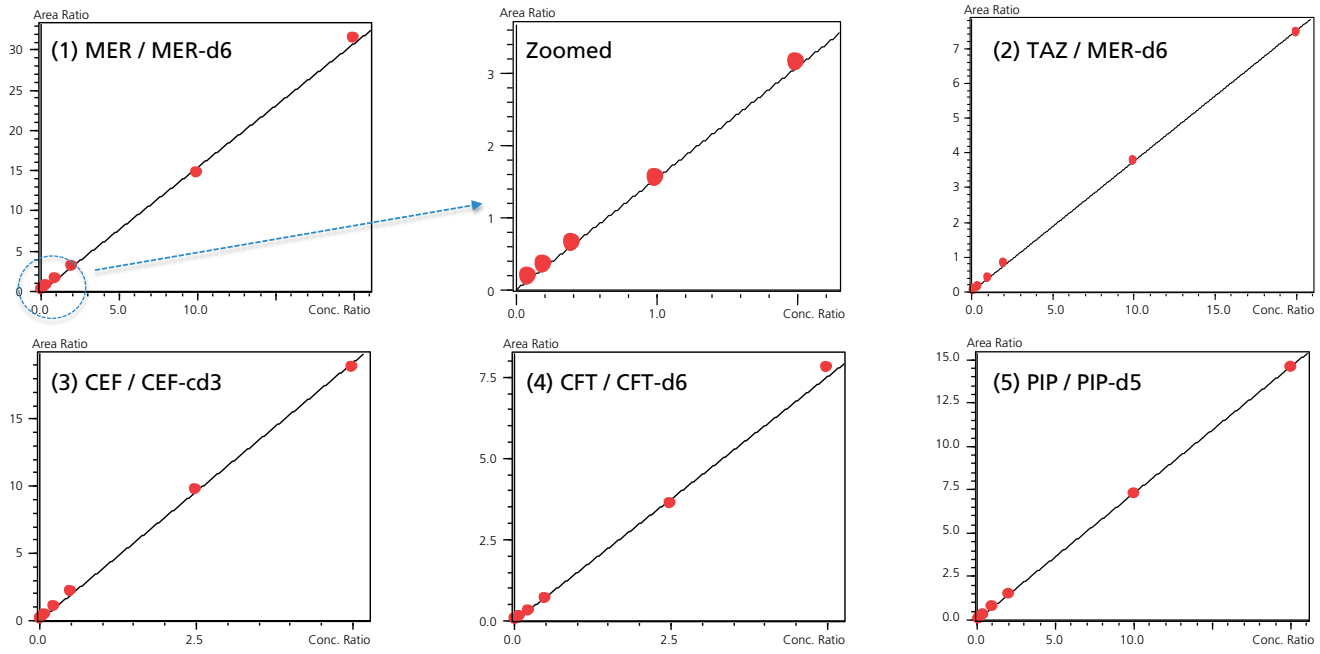

Figure 4: Calibration curves of five β-lactam antibiotics with stable isotope labelled internal standards in human plasma on LCMS-8060. Details of the calibration information are shown in Table 3.

Quantitative Analysis of β-Lactam Antibiotics in Human Plasma by High Sensitivity LC/MS/MS Method

Table 3: MRM quantitation method of five β-lactam antibiotics with internal standards on LCMS-8060

Compd.	RT (min)	qMRM (m/z)	IS	IS (ng/mL)	Range (ng/mL)	R^2
TAZ	1.18	301.1 > 168.1	MER-d6	200	20~4000	0.9993
CEF	1.21	481.1 > 86.1	CEF-cd3	800	20~4000	0.9991
MER	1.47	384.1 > 68.1	MER-d6	200	20~4000	0.9989
CFT	1.47	547.1 > 468.0	CFT-d6	800	20~4000	0.9971
PIP	2.33	518.2 > 143.1	PIP-d5	200	20~4000	0.9999

Evaluation of method performance

Accuracy of the quantitation method was evaluated with pre-spiked standard samples at all concentration levels with duplicate injections. The results are shown in Table 4, which indicate that reliable quantitation accuracy was obtained, except CFT at 20 ng/mL, due to employing IS method.

Repeatability of the method on LCMS-8060 was evaluated with pre-spiked samples, post-spiked samples and mixed standards in solvent at low, middle and high concentration levels. The %RSD results of pre- and post-spiked sample are shown in Table 5. The results indicate excellent repeatability achieved, which is believed to be due to employing IS method and the excellent operation stability of the LCMS-8060 system.

Table 4: Results of accuracy (%, n=5) of five β-lactam antibiotics with IS in plasma samples on LCMS-8060

Compd.	Accuracy (%)						
	20 ng/mL	40 ng/mL	80 ng/mL	200 ng/mL	400 ng/mL	2000 ng/mL	4000 ng/mL
TAZ	91	97	100	106	107	101	99
CEF	87	97	102	105	109	101	98
MER	97	102	102	99	102	95	102
CFT	126	103	93	87	91	96	104
PIP	99	100	97	103	102	100	100

Table 5: Repeatability (RSD %, n=5) of five β-lactam antibiotics with IS in plasma samples on LCMS-8060

Compd.	At 40 ug/mL		At 200 ng/mL		At 2000 ng/mL	
	Post-spiked	Pre-spiked	Post-spiked	Pre-spiked	Post-spiked	Pre-spiked
TAZ	4.1	1.8	4.0	4.2	4.9	3.9
CEF	5.1	5.0	4.2	2.0	1.9	3.8
MER	4.2	4.5	1.2	1.1	1.0	2.0
CFT	6.4	5.4	7.2	7.2	4.9	5.1
PIP	5.1	3.8	5.3	3.6	3.6	1.9

Recovery of the sample pre-treatment method was evaluated based on the peak area ratios of pre-spiked samples and post-spiked samples at all concentration levels. The results shown in Table 6 indicate excellent recovery were obtained.

Quantitative Analysis of β-Lactam Antibiotics in Human Plasma by High Sensitivity LC/MS/MS Method

Matrix effect of the method was determined by the peak area ratios of spiked samples and mixed standards in pure solvent at all concentration levels. The results are shown in Table 7. It can be seen that strong matrix effect occurred for CFT (33%~40%) and TAZ (128%~149%). This could be due to interference from plasma, which causes ion suppression and ion amplification. By further dilution of 2.5 times of the plasma samples with pure water before injection into LCMSMS, the matrix effects of CFT and TAZ were improved significantly to 62%~85% and 95%~109%, respectively.

Table 6: Recovery (%) of five β-lactam antibiotics in plasma samples by protein crash pre-treatment

Compd.	Recovery (%)						
	20 ng/mL	40 ng/mL	80 ng/mL	200 ng/mL	400 ng/mL	2000 ng/mL	4000 ng/mL
TAZ	86	89	90	94	97	92	97
CEF	94	87	87	88	90	88	89
MER	103	104	98	102	102	97	103
CFT	113	94	93	97	103	100	101
PIP	104	106	98	105	103	98	102

Table 7: Results of matrix effect (%) of five β-lactam antibiotics in plasma samples on LCMS-8060

Compd.	Matrix effect (%)						
	20 ng/mL	40 ng/mL	80 ng/mL	200 ng/mL	400 ng/mL	2000 ng/mL	4000 ng/mL
TAZ*	149	140	145	139	137	134	128
CEF	118	115	115	120	117	123	117
MER	93	93	101	98	99	99	103
CFT*	33	38	37	39	40	36	34
PIP	96	94	102	99	99	101	97

*Note: the matrix effect was improved significantly by diluting the plasma sample with pure water before injection

Specificity of the method for detection and confirmation of the five β-lactam antibiotics is demonstrated in Figure 5. In addition, the confirmation criteria include the MRM transitions, the ratios with reference MRM transitions (variation < 30%) as well as retention time (shift < 5%).

Limit of quantitation (LOQ) of the method was estimated from the chromatograms of the lowest level spiked sample (20 ng/mL). Based on S/N = 10, the estimated LOQ of the method are 5.8, 6.0, 1.9, 2.9 and 0.7 ng/mL for TAZ, CEF, MER, CFT and PIP, respectively.

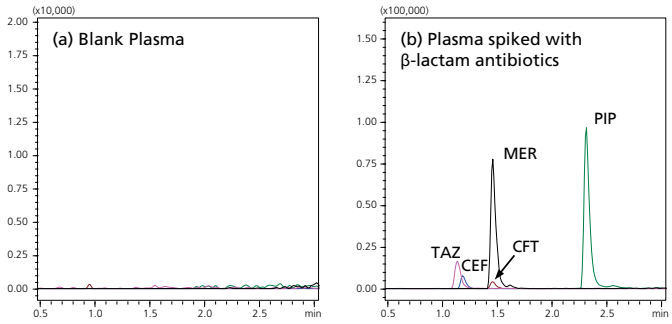

Figure 5: MRM chromatograms of blank plasma and plasma spiked with five β-lactam antibiotics (40 ng/mL)

Quantitative Analysis of β-Lactam Antibiotics in Human Plasma by High Sensitivity LC/MS/MS Method

Conclusions

A fast MRM-based method for quantitation of five β-lactam antibiotics tazobactam, cefepime, meropenem, ceftazidime and piperacillin in human plasma was developed on LCMS-8060. A simple sample pre-treatment with protein crash by organic solvent was applied and a small injection volume of 2 µL was required due to the high sensitivity of the LCMS-8060 employed. The method performance was evaluated on the linearity, accuracy, repeatability, recovery, matrix effect, specificity and limit of quantitation (LOQ). The estimated LOQs of the method for the five antibiotics are in the range from 0.7 ng/mL to 6.0 ng/mL with an injection volume of 2 uL.

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Bioanalytical Method for Quantification of Polymyxin B1, B2, B3 and Isoleucine-polymyxin B1 in Human Plasma

TP 118

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Bioanalytical Method for Quantification of Polymyxin B1, B2, B3 and Isoleucine-polymyxin B1 in Human Plasma

1. Overview

- A simple bioanalytical MRM based method developed for quantitative analysis of Polymyxin B1, B2, B3 and Ile-B1 in human plasma sample on Shimadzu LCMS-8060
- Trichloroacetic acid is successfully used as an protein precipitation and ion-pairing reagent for the bioanalytical method development

2. Introduction

Polymyxin B (PB) is widely used as a last selection of infection therapy due to the emergence of multi-drug resistant bacteria. The commercial formulation of PB is a chemical mixture containing over 30 polymyxin B polypeptides. It was reported that there were variations in the composition of PB components in different products [1]. The different components may not exhibit equivalent pharmacological activity and toxic propensity [2].

Therefore, monitoring of all the main forms of polymyxin B is needed for accurate assessment of their pharmacokinetic properties and toxicity. Thus, we develop a simple, sensitive and selective LC/MS/MS method for the quantitation of four main forms of polymyxin B in human plasma including PB1, PB2, PB3 and Ile-PB1, which account for more than 95% of the polypeptides in commercial formulation.

3. Experimental

Polymyxin B1, B2, B3 and Ile-B1 standards were dissolved in the Milli-Q water (primary stock solution, 1000 µg/mL) and diluted to different working solutions to create the calibration curves and quality control standards, ranging from 0.5 to 100 µg/mL. Polymyxin Bs pre-spiked in human plasma were extracted by protein precipitation with 40% trichloroacetic acid solution. The procedure is shown in Figure 1. Briefly, 10 µL of 40% (w/v) trichloroacetic acid solution was added to 50 µL of plasma samples and mixed vigorously for 1 min. After precipitation for 10 min, 190 µL of MQ water added to the sample for dilution. After centrifugation at 15,000×g for 10 mins, the supernatant was transferred into a HPLC glass vial. The obtained sample was injected to LCMS-8060 for analysis. The analytical conditions on LC-MS /MS 8060 are shown in Table 1.

Table 1. Analytical conditions of polymyxin Bs on LCMS-8060

Column	Shim-pack GISS C18 (100 mm. x 2.1mm I.D., 1.9µm)
Flow Rate	0.4 mL/min
Mobile Phase	A : 0.01% Trifluoroacetic acid (TFA) in milli-Q water with 0.5% Formic acid B : 0.01% Trifluoroacetic acid (TFA) in Acetonitrile (ACN) with 0.5% Formic acid
Elution Mode	Gradient elution, LC program 10 minutes 14%B (0.01 min to 1.00 min)→25%B (2.00 min) →28% B (7.00 min to 8.00 min) →22%B (9.00min)
Oven Temp.	40ºC
Injection Vol.	20 µL

Interface	ESI
MS Mode	MRM, Positive
Heat Block temp.	350ºC
DL temp.	250ºC
Interface temp.	350 ºC
Nebulizing gas flow	Nitrogen, 3.0 L/min
Drying gas flow	Nitrogen, 10.0 L/min
Heating gas flow	Zero air, 10 L/min
CID gas	270 kPa (Ar)

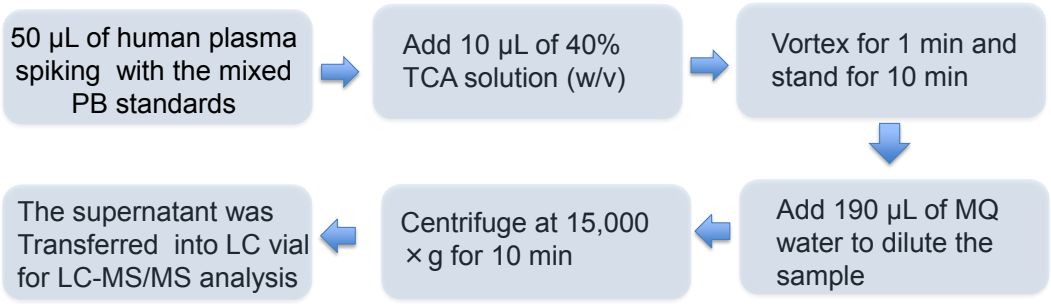

Figure 1. The procedure for human plasma sample preparation

MRM method of polymyxin Bs

Polymyxin B1 and Ile-PB are structural isomers and polymyxin B2 and B3 are also isomers. Therefore, it is expected to set up chromatographic conditions to be able to separate the isomers. As shown in Table 2 and Figure, the LC method established can separate the isomer pairs completely under the conditions. The main precursors of Polymyxin Bs are doubly-charged. MRM optimization was performed using an Automated MRM optimization program in LabSolutions. The details of the MRM parameters obtained are shown in Table 2.

Table 2. MRM transitions and compound-dependent MS parameters for Polymyxin Bs on LCMS-8060

Compound	RT (min)	MRM Transition		Pause time (msec)	Dwell time (msec)	Q1 Pre Bias (V)	C.E. (V)	Q3 Pre Bias (V)
		Precursor	Product					
Polymyxin B1	8.59±0.02	602.65 (2+)	241.20	3	20	-24	-24	-27
			101.10	3	20	-24	-35	-20
Ile-PB1	7.64±0.04	602.65 (2+)	241.15	3	20	-22	-24	-17
			101.15	3	20	-26	-35	-19
Polymyxin B2	6.14±0.03	595.75 (2+)	227.20	3	20	-24	-20	-16
			101.10	3	20	-26	-40	-21
Polymyxin B3	6.56±0.03	595.70 (2+)	227.15	3	20	-24	-24	-11
			101.05	3	20	-24	-33	-19

4. Results and Discussion

4.1 Effects of TCA as ion-pairing reagent added in samples to separation andpeak shape of polymyxin Bs

Ion-pairing chromatography has been applied normally with addition of an ionparing reagent in the mobile phase. An alternative way is used recently, where the ion-paring reagent is only deposited on the column brought with sample injection. This was preferred especially for LC-MS/MS analysis. Successful analytical methods of this kind of ion-pairing chromatography were reported to achieve desirable retention and resolution on a reversed phase column with so-called LC-MS friendly mobile phase without addition of ion-pairing reagent [3]. TCA is selected as the ionpairing regent in this work. It is added to only the samples, but not in the mobile phase. It was observed that with TCA added to sample only, the LC separation of the polymyxins Bs was improved significantly in peak shape and tailing effect.

Using this simple sample preparation with TCA added only in protein crashing of plasma sample, desirable retention and good resolution of the four polymyxin Bs were achieved on a Shim-pack GISS C18 (100 mm. x 2.1mm I.D., 1.9µm) column with mobile phases without TCA. The effect of the different concentration of trichloroacetic acid on the protein precipitation and the chromatographic behavior were investigated. The results showed that 40% TCA solution works best for the good efficiency in protein precipitation and achieved the baseline separation and symmetry peaks for all four forms of polymyxin Bs.

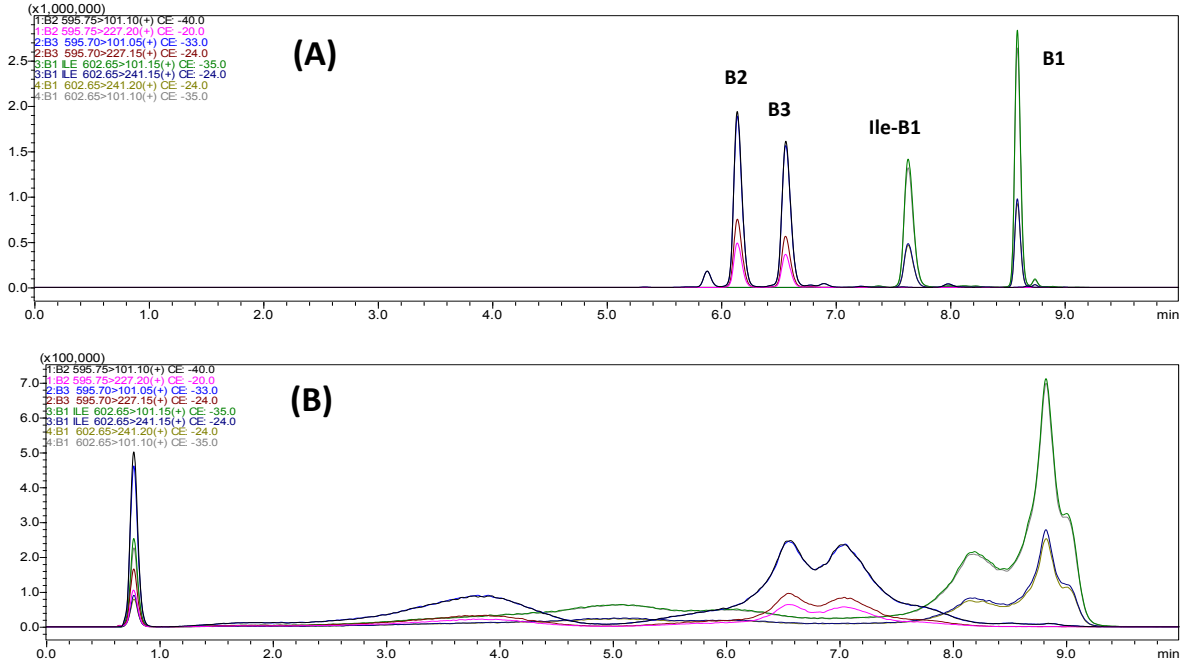

Figure 2. Chromatograms obtained using MRM to detect polymyxin B1, B2, B3 and Ile-B1 with (A) 40%TCA spiked in or (B) without TCA in standard solutions.

4.2 Performance evaluation for quantitation of polymyxin Bs in human plasma

Linearity, LOD and LOQ: Respectable linearities (R2 >0.995) were achieved for polymyxin B1 and B2 in the range of 0.1 ~ 5 µg/mL, PB1 and Ile PB1 in the range of 0.05 ~ 3.75 µg/mL. Calibration linear ranges were different for the four forms of polymyxin B because of their different concentration level in commercial formulation of polymyxin B.

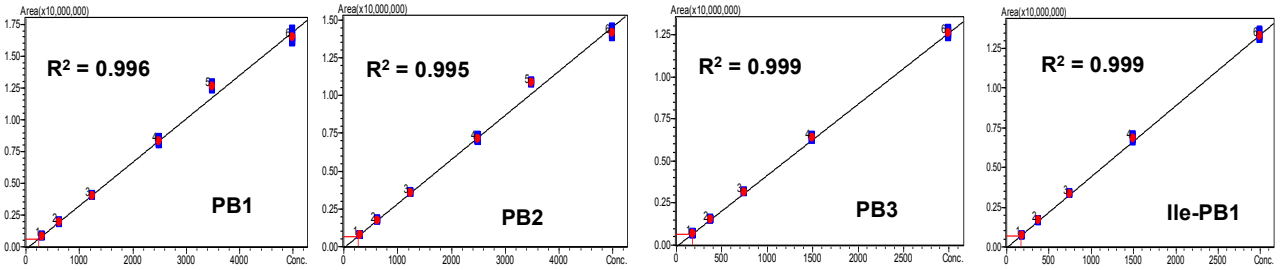

Figure 2. Representative calibration curves of PB1, PB2, PB3 and ile-PB1 on LC/MS/MS

The LOD and LOQ for the four forms of polymyxin B standards ranged from 0.32 to 5.40 µg/mL and 1.13 to 17.99 µg/mL, respectively as shown in Table 3.

Accuracy tests were performed by testing polymyxin Bs standards at low, medium and high concentration levels within their respective calibration ranges. The errors in accuracy were less than 20%, and prevision is demonstrated was less than 15% RSD. Extraction recovery and matrix effect were evaluated and the results were shown in Table 4.

Table 3. Linearity, LOD and LOQ of polymyxin Bs in human plasma

Compound	Range (µg/mL)	R^2	LOD (ng/mL)	LOQ (µg/mL)
PB1	0.1 – 5	0.996	5	0.01
PB2	0.1 – 5	0.995	5	0.01
PB3	0.05 – 3.75	0.999	5	0.01
Ile-PB1	0.05 – 3.75	0.999	5	0.03

Table 4. Method performance for the quantification of polymyxin Bs in human plasma

Compound	RT (min)	Conc. Level (µg/mL)	Accuracy (n=6)	RSD (%)	Conc. Level (µg/mL)	Matrix effect	Recovery (%) (n=2)
PB1	8.59 ± 0.02	5	97.18	2.19	5	121.63	91.16
		2.5	99.4	2.53	1	128.48	103.82
		0.625	98.08	2.53	0.1	–	58.93
PB2	6.14 ± 0.03	5	97.23	1.52	5	134.16	95.10
		2.5	98.20	1.86	1	115.13	104.03
		0.625	98.37	2.21	0.1	–	60.04
PB3	6.56 ± 0.03	3	99.70	1.47	5	109.84	93.15
		1.5	100.93	1.61	1	121.61	102.08
		0.375	100.04	2.37	0.1	–	65.34
Ile-B1	7.64 ± 0.04	3	99.43	1.70	5	123.01	90.58
		1.5	102.28	2.20	1	115.27	107.47
		0.375	99.67	2.81	0.1	–	60.46

5. Conclusion

We described the development of an analytical method with trichloroacetic acid (TCA) used as a precipitation reagent and ion-pairing reagent added to plasma samples only. This novel and simple LC-MS/MS method exhibits excellent separation for the isomers of polymyxin B1 and Ile-PB1, as well as polymyxin B2 and B3 and with good peak shape. The quantification performance of polymyxin B1, B2, B3 and ile-B1 in human plasma samples was evaluated including linearity, LOD, LOQ, accuracy, recovery and matrix effect. One additional advantage of the method is avoiding ion-pairing reagent in the mobile phase, allowing the

Acknowledgement

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A High Sensitivity LC/MS/MS Method for Quantitative Analysis of Eight Antifungal Drugs in Human Serum

ASMS 2017 ThP 073

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A High Sensitivity LC/MS/MS Method for Quantitative Analysis of Eight Antifungal Drugs in Human Serum

Introduction

Triazoles and echinocandins are commonly used for the treatment of invasive fungal infections via systemic antifungal chemotherapy. However, these drugs exhibit substantial pharmacokinetic variability in patients such as bioavailability and drug-drug interactions [1,2]. Clinicians often find it challenging to select proper drug doses and evaluate the potential toxicity effects. Therapeutic drug monitoring (TDM) of antifungals is essential to maximise the efficacy and minimise drug overdose risk in patients, hence individualising the treatment [3]. In this study, we aim at developing a fast and reliable LC/MS/MS method with high sensitivity and simple sample pre-treatment. The method is established for simultaneous determination of two classes of antimycotic compounds, five triazoles and three echinocandins in human serum. The method performance is evaluated with spiked serum samples thoroughly before further implementation and validation with clinical samples.

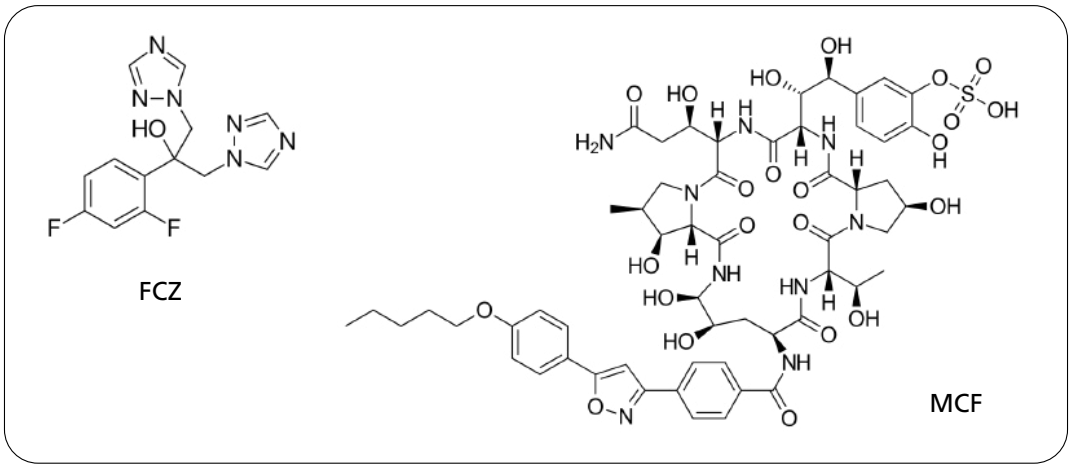

Figure 1 Structure of fluconazole (FCZ) with triazoles and micafungin (MCF) with a cyclic hexapeptoid

Methods and Materials

Human serum was treated with methanol/acetonitrile mixture (1:1, v/v) in the ratio of 1:3 to precipitate the proteins, followed by vortex and centrifugation. A Shimadzu LCMS-8060 triple quadrupole with a heated ESI interface coupled with a Nexera UHPLC was used to develop the method for high sensitivity quantitative analysis of eight antifungal drugs: fluconazole (FCZ), posaconazole (PCZ), voriconazole (VCZ), hydroxyitraconazole (h-ICZ), itraconazole (ICZ), anidulafungin (ANF), caspofungin (CSF) and micafungin (MCF) .

A High Sensitivity LC/MS/MS Method for Quantitative Analysis of Eight Antifungal Drugs in Human Serum

Table 1 Analytical conditions and MS/MS parameters on LCMS-8060

Column	: Kinetex C18 100Å (100 x 2.1 mm, 1.7 µm)
Flow rate	: 0.4 mL/min
Mobile phase	: A: Water with 0.1 % formic acid B: Acetonitrile with 0.1 % formic acid
Elution mode	: 0.00 min (5% B) → 4.00 – 5.50 mins (90% B) → 5.51 – 9.00 min (5% B)
Oven temp.	: 40 °C
Inj. Vol.	: 10 µL
Interface	: ESI, 300 °C
MS mode	: Positive, MRM
Block temp.	: 400 °C
DL temp.	: 250 °C
CID gas	: Ar (270 kPa)
Nebulizing gas flow	: N_2, 2 L/min
Drying gas flow	: N_2, 10 L/min
Heating gas flow	: 0 air, 10 L/min

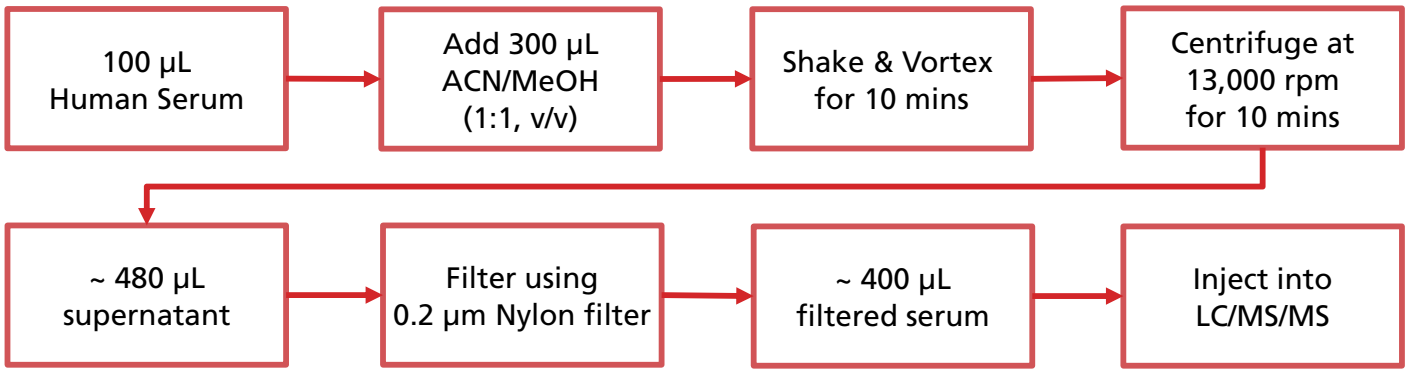

Figure 2 Pre-treatment and Protein Crash procedure for Human Serum

Results and Discussion

Method development for antifungals

Table 1 shows the summary of LC/MS/MS analytical conditions and parameters while Table 2 describes the optimised MRM transitions and parameters for the eight antifungal drugs. With the automated MRM optimisation program, three transitions were chosen for each compound, with one quantifier and two confirmatory transitions. Predominantly, the antifungals form $[M+H]^+$ ions except $[M-H]^-$ ion for ANF.

Table 2 MRM transition and parameters for eight antifungals on LCMS-8060

Antifungals		Precursor ion	Product ion	Q1 Pre-bias (V)	CE (V)	Q3 Pre-bias (V)
Triazole	Fluconazole (FCZ)	307.20	220.10	-27	-20	-22
			238.15	-29	-15	-11
			169.15	-28	-24	-11
	Posaconazole (PCZ)	701.40	683.30	-32	-35	-26
			126.95	-20	-55	-24
			614.35	-20	-38	-22
	Voriconazole (VCZ)	350.20	281.15	-30	-19	-20
			127.10	-30	-37	-13
			224.15	-30	-20	-11
	Hydroxy-itraconazole (h-ICZ)	721.30	408.15	-22	-42	-29
			392.20	-20	-36	-20
			430.30	-36	-37	-13
	Itraconazole (ICZ)	705.30	392.20	-34	-39	-27
			432.20	-36	-36	-30
			256.05	-36	-42	-18
Echinocandin	Anidulafungin (ANF)	1140.40	1122.30	-34	-19	-44
			1104.55	-34	-26	-32
			343.15	-34	-45	-24
	Caspofungin (CSF)	547.50	131.10	-22	-23	-26
			137.05	-20	-26	-14
			86.10	-22	-51	-16
	Micafungin (MCF)	(-)1268.45	(-)247.05	36	55	15
			(-)469.15	36	55	21
			(-)390.00	36	55	12

A High Sensitivity LC/MS/MS Method for Quantitative Analysis of Eight Antifungal Drugs in Human Serum

Quantitative performance with spiked human serum

Figure 3 shows the total MRM chromatograms for the eight antifungal drugs with a total LC/MS/MS runtime of nine minutes through gradient elution. Calibration curves with external standard method were plotted on standard-spiked serum matrix, obtaining linearity of R^2 > 0.996. The linear ranges 5~5000 ppt for FCZ and VCZ, while 20~5000 ppt for PCZ, ICZ, h-ICZ and CSF. Due to lower sensitivity, the dynamic ranges of the method for ANF and MCF are 200~5000 ppt.

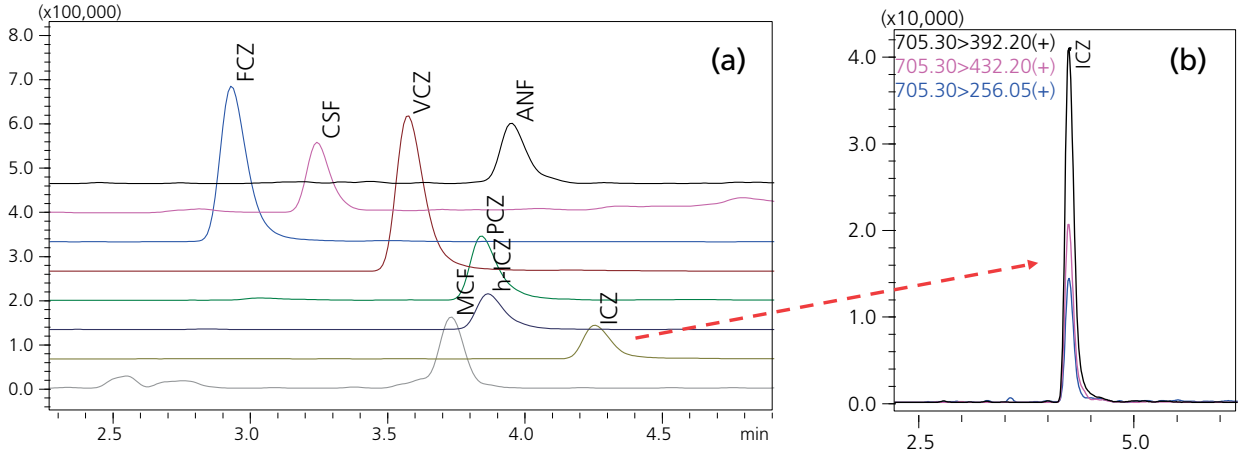

Figure 3 (a) Total MRM chromatograms of antifungal drugs spiked in serum (5ppb each); (b) Individual MRMs of ICZ

Table 3 Calibration results for eight antifungal drugs in spiked human serum

Antifungal	RT (min)	Range (ppt)	R^2	LOD (ppt)	LOQ (ppt)	% RSD (n=3)	
						200 ppt	1000 ppt
FCZ	3.912	5 ~ 5000	0.9995	1.6	4.9	13.5	11.3
VCZ	3.192	5 ~ 5000	0.9998	0.4	1.3	10.8	9.1
PCZ	3.681	20 ~ 5000	0.9994	3.0	9.5	9.6	10.6
h-ICZ	2.860	20 ~ 5000	0.9987	6.0	18.3	13.3	11.3
ICZ	3.503	20 ~ 5000	0.9987	6.2	18.9	18.7	13.2
ANF	3.797	200 ~ 5000	0.9988	41.6	126.2	23.6	14.9
CSF	3.835	20 ~ 5000	0.9968	3.1	9.3	17.3	14.1
MCF	4.230	200 ~ 5000	0.9989	43.2	130.8	32.8	13.2

A High Sensitivity LC/MS/MS Method for Quantitative Analysis of Eight Antifungal Drugs in Human Serum

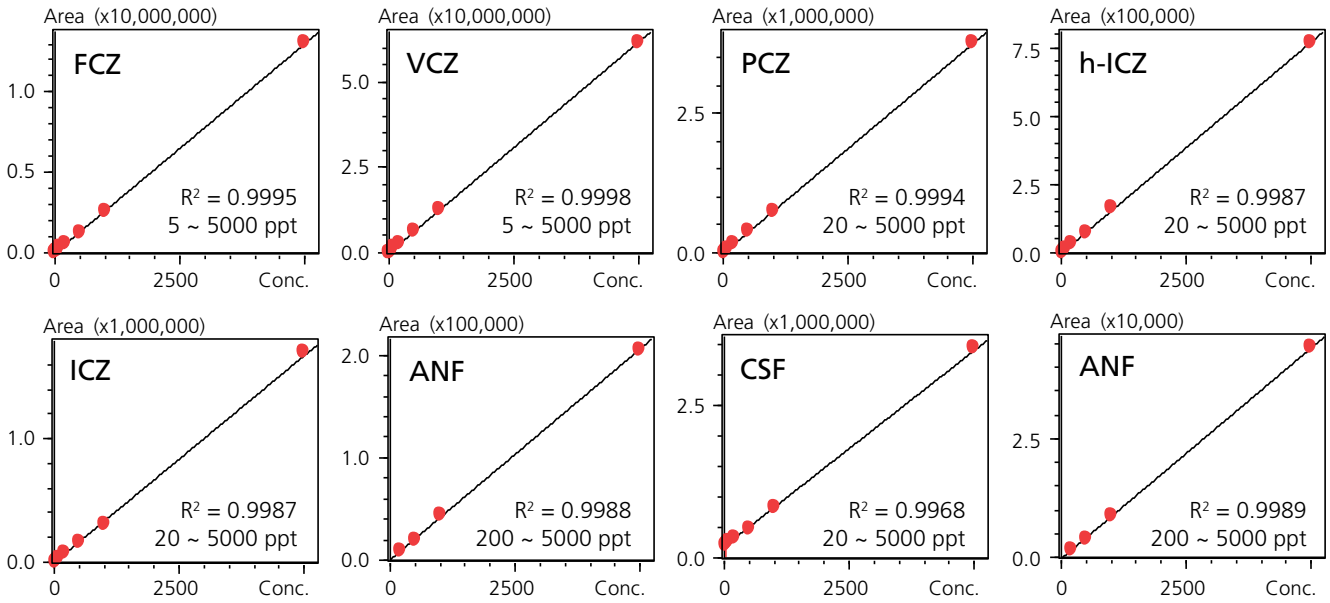

Figure 4 Calibration curve and respective linearity of eight antifungal drugs

The quantitative performance for the eight antifungal drugs are shown in Table 3. It shows good linearity (R^2) of 0.9968 – 0.9998, and their respective limit of detection (LOD) and limit of quantitation (LOQ) are also described. Repeatability study were carried out at two concentrations, 200 ppt (mid level) and 1000 ppt (high level) with n=3. ANF and MCF have larger %RSD values as compared to other antifungals as the low concentration level done was the LOQ.

Table 4 Recovery and matrix effect of antifungal drugs in spiked human serum

Antifungal	Recovery (%)		Matrix effect (%)	
	200 ppt	1000 ppt	200 ppt	1000 ppt
FCZ	124.1	110.3	82.8	80.7
VCZ	117.0	117.9	53.3	50.8
PCZ	108.3	128.9	61.0	55.5
h-ICZ	96.6	125.7	51.4	61.5
ICZ	103.0	119.0	38.9	41.9
ANF	135.0	123.6	86.2	65.0
CSF	101.4	95.4	119.2	103.7
MCF	69.7	88.2	94.2	63.2

A High Sensitivity LC/MS/MS Method for Quantitative Analysis of Eight Antifungal Drugs in Human Serum

Recovery (RE%) of the MRM method was determined by comparing the ratio between the peak area the pre-spiked standards and post-spiked in serum, while matrix effect (ME%) was determined with the ratio between post-spiked standards in serum to those in the diluent. The study was performed in triplicates and the results are shown in Table 4. The recovery for all the antifungals ranges from 69% to 135% while the matrix effect calculated is in between 39% and 119%. The recovery shows that the protein precipitation would cause some variations on the extraction efficiency whereas human serum poses effects on most antifungal analytes.

Conclusions

A fast MRM-based method for high sensitivity quantitative analysis of eight antifungal drugs, fluconazole (FCZ), posaconazole (PCZ), voriconazole (VCZ), hydroxyitraconazole (h-ICZ), itraconazole (ICZ), anidulafungin (ANF), caspofungin (CSF) and micafungin (MCF), has been developed on LCMS-8060. With remarkable sensitivity of the LOQ ranging at 1.3~131 ppt, the LC/MS/MS method could be applied for sensitive quantitation of the antifungal drugs in human serum for therapeutic drug monitoring (TDM) or other clinical research study.

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High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

ASMS 2016 ThP 682

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High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

Introduction

In this paper, we introduce four research use only LC/MS/MS methods for therapeutic drug monitoring (TDM), mycophenolic acid, sunitinib and axitinib, voriconazole, itraconazole. TDM is indispensable for managing drug dosage based on the drug concentration in blood in order to conduct a rational and efficient drug therapy. Liquid chromatography coupled with tandem quadrupole mass spectrometry (LC/MS/MS) is increasingly used in TDM because it can perform selective and sensitive analysis by simple sample pretreatment. In the field of TDM, it is necessary to measure the specimen such as plasma or serum quickly after suitable pretreatment and report the precise result. LC/MS/MS system with a simple and user-friendly interface can provide a streamlined workflow and reduce the load of analysts. We developed high-throughput LC/MS/MS methods for TDM with a new data acquisition and processing software.

Method

Instruments and LC/MS/MS analytical conditions

For LC/MS/MS analysis, a LCMS-8050 triple quadrupole mass spectrometer coupled to a Nexera X2 UHPLC system with mobile phase switching unit (Shimadzu corporation, Japan) was used. In all the methods, the compounds were separated by a reversed phase mode using a common column, Shim-pack GIS (Shimadzu corporation, Japan). All data acquisition and processing were performed by Open Solution QuantAnalytics (Shimadzu corporation, Japan), a software package for acquiring and reviewing quantitative LC/MS/MS data with ease.

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

Table 1 LC/MS/MS consitions

	Method 1	Method 2
Target	Mycophenolic acid (Immuno-suppressant)	Voriconazole (Triazole antifungal agent)
Column	Shim-pack GIS (2.1 mmI.D. x 75mmL., 3um)	
Column Oven	40°C	
Flow rate	0.3 mL/min	0.4 mL/min
Mobile phase A	1% Acetic acid-water	10 mM Ammonium acetate-water
Mobile phase B	Acetonitrile	Methanol
Gradient	A/B = 1/1, isocratic	Bconc. 30% (0 - 0.50min) → 100% (1.50 - 3.00min) → 30% (3.01 - 5.00min)
Injection volum	5 µL	1 µL
Ionization	ESI-positive	
MRM transition	321.40 > 207.30	350.20 > 281.20
Run time	4 min.	5 min.
	Method 3	**Method 4**
Target	Sunitinib and Axitinib (Anti-cancer drug)	Itraconazole (Triazole antifungal agent)
Column	Shim-pack GIS (2.1 mmI.D. x 75mmL., 3um)	
Column Oven	40°C	
Flow rate	0.3 mL/min	0.4 mL/min
Mobile phase A	10 mM Ammonium acetate-water	10 mM Ammonium acetate-water
Mobile phase B	Acetonitrile	Acetonitrile
Gradient	Bconc. 10% (0 - 0.25min) → 80% (2.00 - 3.00min) → 10% (3.01 - 5.00min)	Bconc. 65% (0 - 1.00 min) → 95% (1.50 - 2.50min) → 65% (2.51 - 4.50min)
Injection volum	5 µL	3 µL
Ionization	ESI-positive	
MRM transition	Sunitinib 399.40 > 283.30 Axitinib 387.40 > 356.30 SU12662 (Active metabolite of Sunitinib) 371.40 > 283.30	Itraconazole 705.40 > 392.40 Hydroxy Itraconazole (Active metabolite of Itraconazole) 721.40 > 408.40
Run time	5 min.	4.5 min.

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

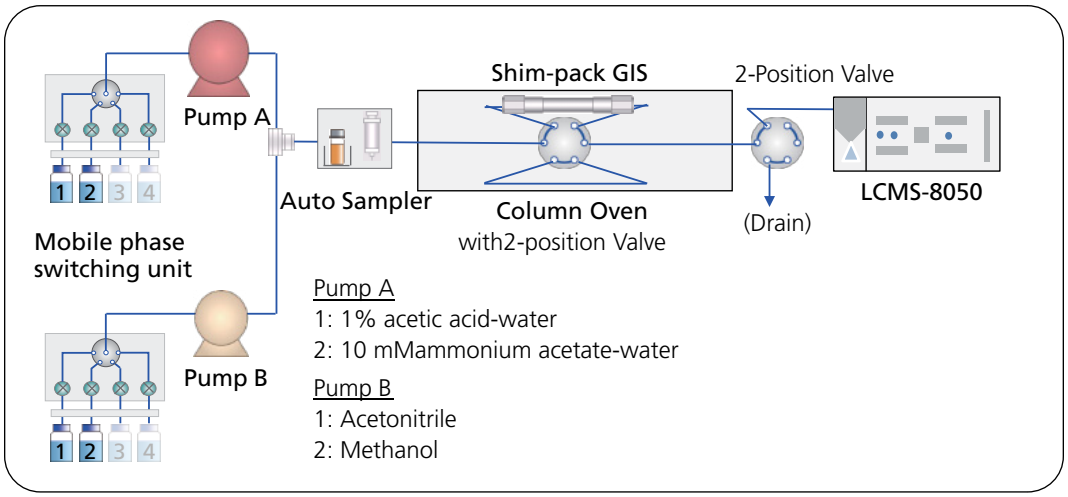


Figure 1 Nexera X2 UHPLC system with mobile phase switching unit

Calibration standards and QC samples

For each compound, more than five calibration standards and three QC samples were prepared. Samples were precipitated in a simple way of deproteination using organic solvent such as methanol or acetonitrile. The resulting supernatant was diluted and injected into LC/MS/MS without filtration.

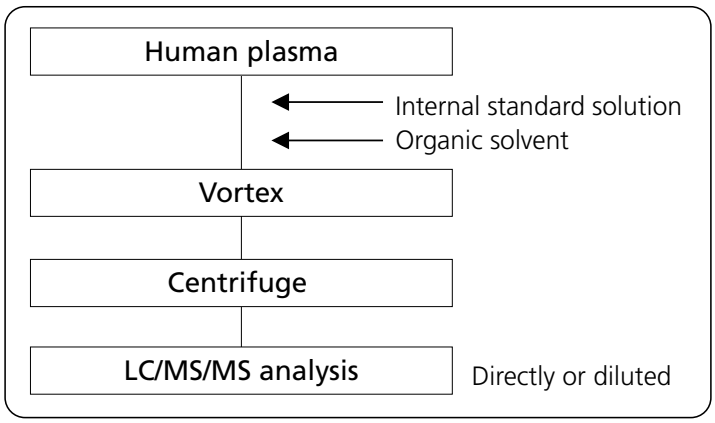


Figure 2 Work flow of the pretreatment

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

Result and discussion

Precision, accuracy and linearity

Table 2 illustrates linearity of all compounds and Table 3 illustrates accuracy and precision of the QC samples. Determination coefficient (r2) of all calibration curves was larger than 0.995, the precision (n=6) was within 15% RSD, and the accuracy (n=6) was within 80-120%.

Excellent linearity, accuracy and precision were obtained within a specific concentration range. Furthermore, All the methods took less than 5 minutes per one LC/MS/MS analysis, including column rinsing.

Table 2 Linear dynamic range for each compound

	Compound	Linearity (µg/mL)			r2
Method 1	Mycophenolic acid	0.2	~	20	0.999
Method 2	Voriconazole	0.1	~	10	0.999
Method 3	Sunitinib	3	~	300	0.999
	Axitinib	0.3	~	30	0.999
	SU12662	3	~	300	0.999
Method 4	Itoraconazole	10	~	1000	0.999
	Hydoroxy Itorazonazole	10	~	1000	0.999

Table 3 Precision and accuracy for analysis of QCs

	Compound	Concentrations of QC samples (µg/mL)			Precision (%)			Accuracy (%)		
		Low	Middle	High	Low	Middle	High	Low	Middle	High
Method 1	Mycophenolic acid	0.5	5	15	1.6	3.66	2.55	108.5	104.9	103.5
Method 2	Voriconazole	0.2	4	8	1.74	1.56	1.7	98.6	103.6	99
Method 3	Sunitinib	5	50	250	4.05	1.36	2.24	100.7	95.5	96
	Axitinib	0.5	5	25	8.26	4.56	4.43	85.3	88.5	91.2
	SU12662	5	50	250	4.07	1.26	3.17	97.2	96.5	97.8
Method 4	Itoraconazole	25	250	750	0.65	0.82	0.38	98.7	98.8	103.3
	Hydoroxy Itorazonazole				3.27	4.36	3.1	81.3	90.4	86.9

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

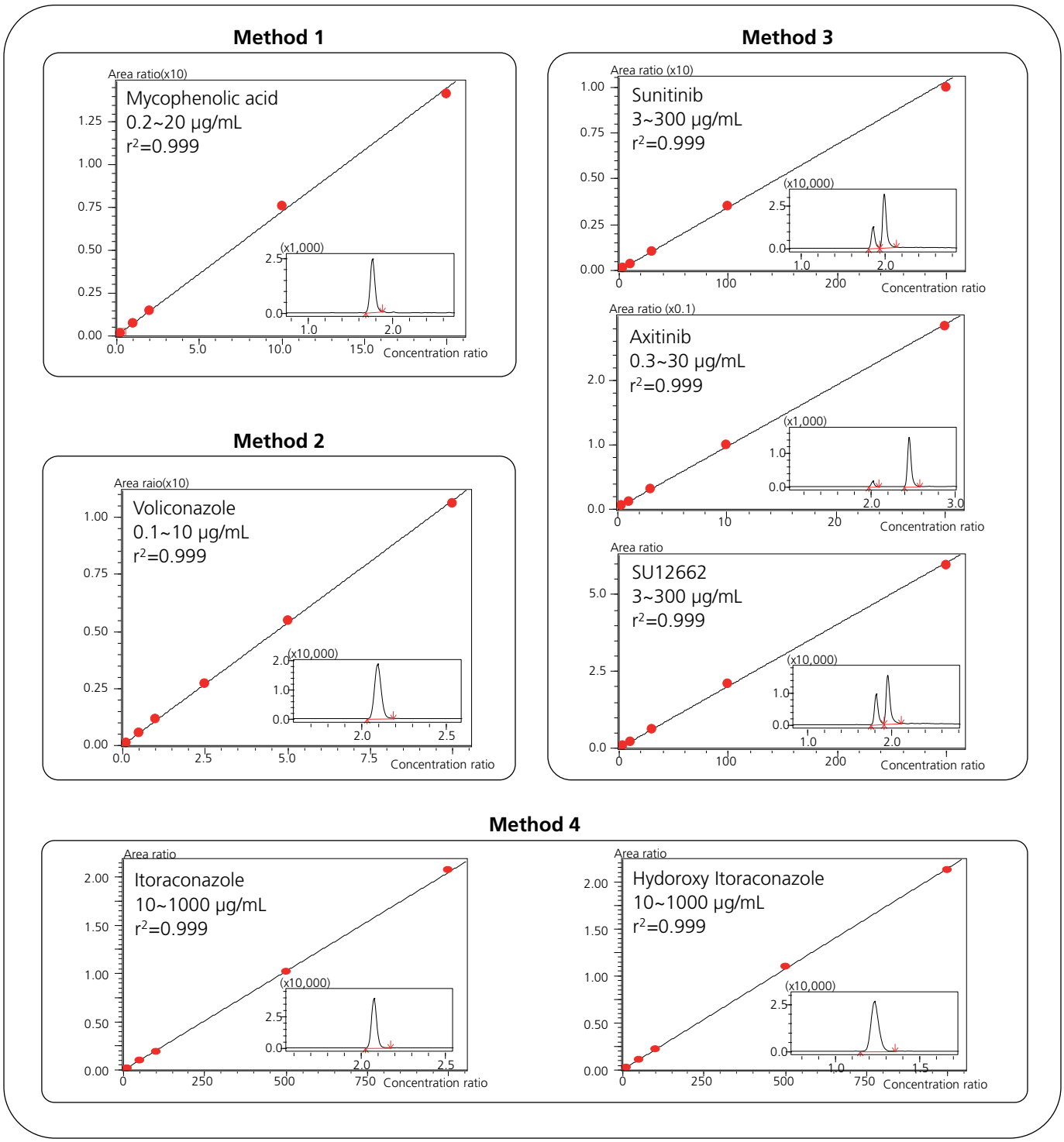
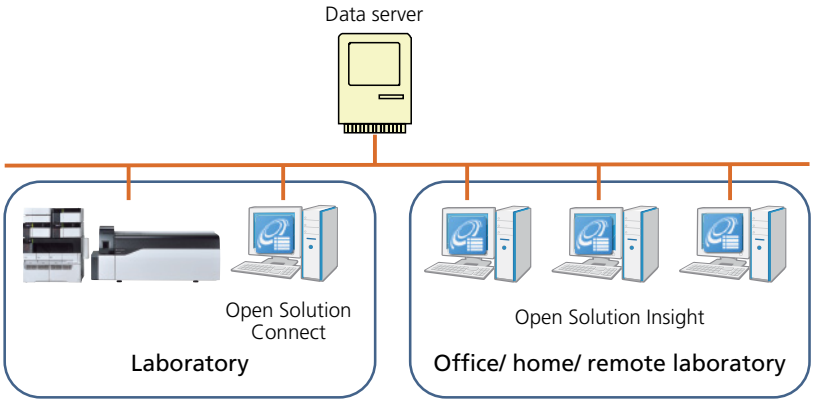


Figure 3 Calibration curves for each compound

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

Easy data acquisition with Open access software

The developed platform was used one common column for all methods and mobile phase changeover could be automatically done by just selecting method file. Open Solution QuantAnalytics software enables users to submit sample queue and set the LC/MS/MS condition easily and quickly. Users can intuitively start LC/MS/MS measurement by just selecting the predefined method and placing sample vials in the specified autosampler plate positions guided by software. The resulting data can be reviewed in office as soon as it becomes available on the designated data server. This system enables easy and quick data acquisition without tedious manual operation such as replacement of a column and mobile phases.



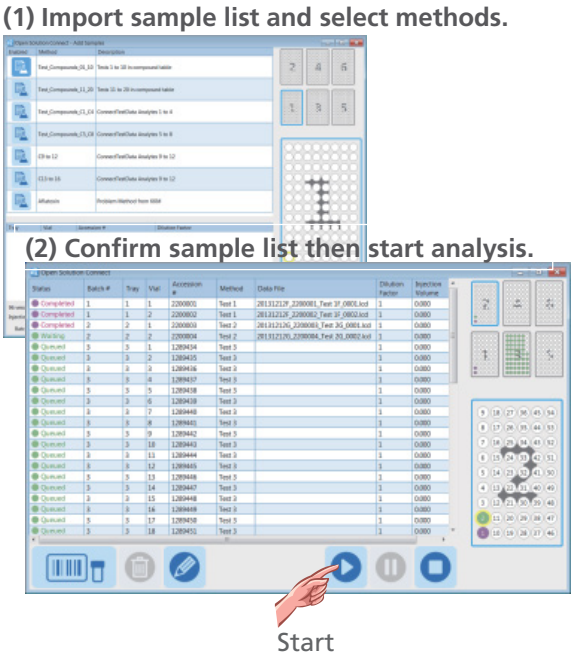


Figure 4 User interface for sample queue submission

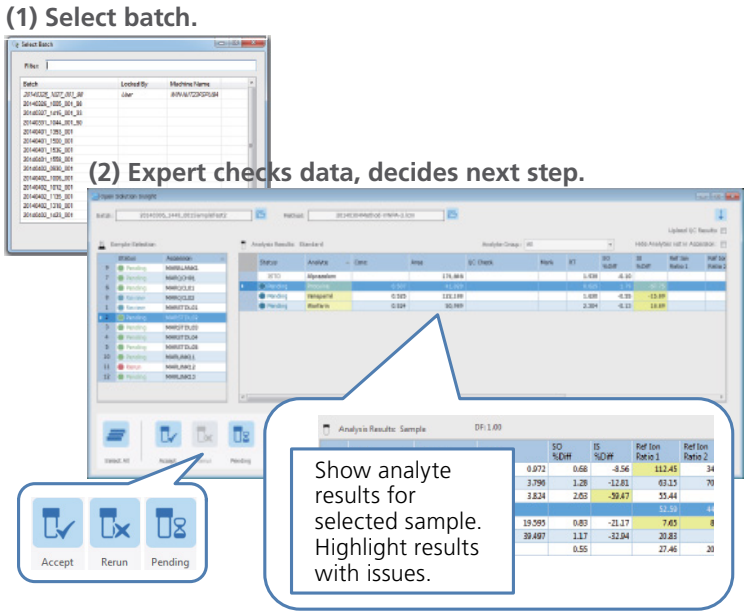


Figure 5 User interface for checking the result

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

Conclusions

- The combination of the Open Solution QuantAnalytics software and LC/MS/MS system with mobile phase switching unit enables easy sample submission and efficient data acquisition. The user only describes the vial position of samples, chooses a predefined method and the resulting data can be reviewed in office as soon as it becomes available on the designated data server.
- Saving time and effort for changing system conditions among each target compounds was achieved with mobile phase switching system and high through-put methods using a common column.

Disclaimer: Shimadzu LCMS-8050 CL and certain Nexera X2 UHPLC components are registered in the U.S. as a Class I device and is not specifically cleared for TDM. Other UHPLC components, Shim-pack GIS, and OpenSolution QuantAnalytics are intended for Research Use Only (RUO). Not for use in diagnostic procedures.

First Edition: June, 2016

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Application News

LCMS-8050

No. SCA_210_019

Quantification of 16 anti-HIV drugs from human plasma

Introduction

This Application News describes a simple LC-MS/MS analytical method for the simultaneous quantification of new agents dolutegravir, elvitegravir, rilpivirine and other thirteen anti-HIV drugs from human plasma by Shimadzu Nexera X2 coupled with Triple Quadrupole Mass Spectrometer LCMS-8050.

Sample preparation

Protein precipitation step

A simple protein precipitation solution of 600 µL (acetonitrile:methanol 50:50 v/v) and 40 µL of IS working solution (quinaxoline 5 µg/ml in H_20:MeOH 50:50) were added to 100 µL of plasma samples in a PTFE microfuge tube. After vortexing for 30 seconds, the mixture was centrifuged at 12000 rpm (7200 g) for 10 minutes at 4 °C. Then the supernatant was diluted 1:1 with water in vial for the injection.

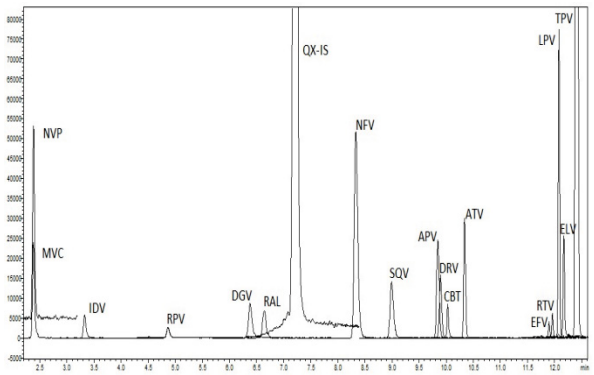

Figure 1. Chromatogram of a CAL1/LOQ

Table 1. Experimental Condition

HPLC conditions (Nexera X2)	
Analytical Column	AQUITY UPLC HSS T3 1.8 µm (2.1 x 150 mm)
Solvent A	H_2O + Formic Acid 0.05%
Solvent B	ACN + Formic Acid 0.05%
Flow rate	0.500 mL/min
Injection Volume	2 µL
Autosampler temp.	15 °C
Column oven temp.	45 °C

GRADIENT	
Time (min)	Solvent B%
0,0	30
8,0	43
9,0	55
10,0	60
11,0	75
11,1	95
13,0	95
13,1	30
15,0	30

MS settings (LCMS-8050)	
Ionization mode	ESI (positive-negative)
Interface voltage	4 kV
Nebulizer gas	3.0 L/min
Drying gas	10 L/min
Heating gas	10 L/min
Interface temp.	300 °C
DL temp.	250 °C
Heat block temp.	400 °C
CID Gas	270 kPa

Table 2. MRM Transitions

Compounds	Tag	Polarity	Precursor (m/z)	Product (m/z)
Maraviroc	MVC	+	514.15	388.95
Nevirapine	NVP	+	267.10	225.90
Indinavir	IDV	+	614.15	421.00
Rilpivirine	RPV	+	367.15	128.15
Dolutegravir	DGV	+	420.15	277.10
Raltegravir	RAL	+	445.00	109.05
Nelfinavir	NFV	+	568.10	135.10
Saquinavir	SQV	+	671.20	570.10
Darunavir	DRV	+	548.05	391.95
Cobicistat	CBT	+	776.15	606.00
Amprenavir	APV	+	506.00	245.00
Atazanavir	ATV	+	705.20	168.05
Ritonavir	RTV	+	721.10	295.90
Elviltegravir	ELV	+	447.80	344.00
Lopinavir	LPV	+	629.15	155.20
Efavirenz	EFV	-	313.85	243.95
Tipranavir	TPV	+	603.05	411.00
Quinoxaline	QX-IS	+	312.85	245.95

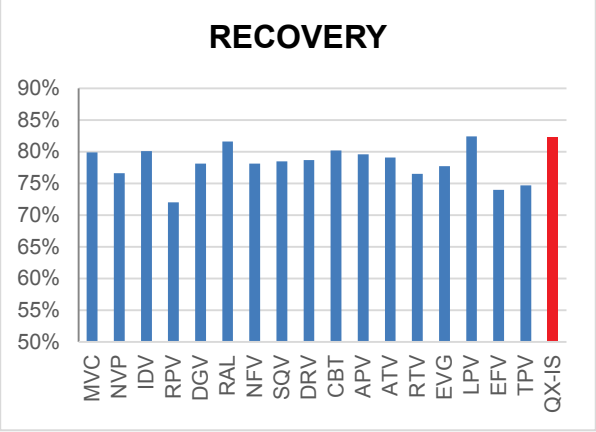

Figure 2. Recovery for all drugs was above 70% with a matrix effect below 10%

The high performance and sensitivity of the instruments allows to utilize only few microliters of plasma ensuring in any case precision and accuracy also for very low concentrations.

Table 3. Accuracy and precision

Drugs	QCs Conc. ng/mL	Accuracy (%)	Intraday Precision RSD% Assay n°=6	Interday Precision RSD%
MVC	2000	101,3	3,7	4,7
	500	103,5	2,5	3,1
	50	99,5	4,0	4,7
NVP	6400	101,5	2,8	4,2
	1600	102,4	3,1	3,4
	160	99,2	2,8	3,4
IDV	6400	101,9	3,7	5,2
	1600	103,8	2,6	3,0
	160	101,0	3,5	6,2
RPV	2000	106,8	5,2	10,5
	500	107,5	7,3	9,5
	50	98,7	4,2	5,4
DGV	4800	101,5	2,6	3,6
	1200	103,2	2,8	3,8
	120	101,5	2,7	4,9
RAL	2400	101,5	2,4	3,4
	600	102,0	2,7	2,8
	60	98,9	3,0	5,2
NFV	6400	102,1	4,1	5,8
	1600	104,5	2,6	3,0
	160	97,5	3,7	7,0
SQV	5600	102,5	4,2	5,4
	1400	107,4	2,7	3,1
	140	101,4	3,8	9,9
DRV	8000	101,4	3,6	4,8
	2000	103,5	2,5	3,1
	200	96,8	2,9	4,6
CBT	2400	102,9	4,3	6,0
	600	106,0	3,1	3,3
	60	101,1	4,8	7,3
APV	8000	101,7	3,3	4,7
	2000	102,8	2,5	3,0
	200	96,3	2,7	4,3
ATV	4800	101,9	4,2	5,8
	1200	105,2	2,7	3,2
	120	101,4	3,7	7,3
RTV	2000	100,6	5,0	7,7
	500	104,5	3,2	3,9
	50	99,8	5,2	5,9
ELV	2400	101,8	2,9	5,6
	600	103,1	2,8	4,1
	60	99,4	5,2	7,0
LPV	12000	104,1	4,5	6,3
	3000	104,1	2,9	4,7
	300	98,8	3,1	6,9
EFV	6400	103,9	3,8	5,4
	1600	104,4	3,1	3,9
	160	97,5	4,1	5,9
TPV	144000	105,2	7,7	9,7
	360000	104,6	3,9	4,4
	3600	100,4	3,8	10,7

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Application News

No. SAIP/ADC/B-005

Pharma CRO / LCMS-8060

Development and validation of a sensitive bioanalytical method for estimation of intact Insulin in human plasma using Shimadzu LCMS-8060

Chaitanya Atmakuri, Dr.Avinash Gaikwad)

❑ Introduction

Insulin is a hormone made by the pancreas that controls blood sugar levels, hence plays a very critical role in maintaining human health and metabolism. Figure 1 shows the amino acid sequence of insulin. It is one of the most popular protein based drug used by clinicians globally.

Insulin has been classically measured using various approaches of ligand binding assays. However, these methods present some inherent challenges like cross reactivity, poor specificity and selectivity.

As a natural choice, LC-MS/MS is considered as an alternate technique for measurement of large amount of samples generated from different formats of clinical studies.

Earlier approaches for insulin measurement using LCMS involved long and laborious sample preparation techniques which still were not able to deliver required performance and sensitivity.

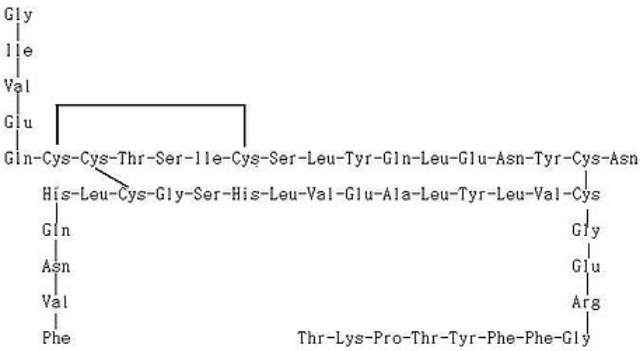

Fig.1 Amino acid sequence of human insulin

The analytical method used to evaluate insulin should address many challenges such as poor ionization, non-specific adsorption, carry-over and low extraction recovery. Subcutaneous insulin release therapy results in very low plasma levels which adds to complexity of and hence requires a sensitive method for estimation.

We have therefore developed a method with high chromatographic resolution and sensitivity giving lowest limit of quantification (LLOQ) of 50 pg/mL for insulin in human plasma using LCMS-8060. Method was developed keeping some key criteria in focus- namely simpler extraction process, highly optimized chromatography, enhanced sensitivity and repatibility. These factors enable high selectivity and increased throughput for analysis of insulin for the pharmacokinetic investigation.

❑ Salient features

- Quantitative method for estimation of insulin in human plasma was developed. Method was validated as per USFDA Guidelines (results are presented in Table 1)
- The sample preparation method used effectively managed blank interference and non-specific binding
- Instrument parameters were completely optimized to eliminate carryover throughout the calibration range
- To make method more patient friendly, method utilizes only 500 uL of plasma
- Customised gradient method enhances the chromatographic resolution of Insulin with consistent and reproducible peak area and retention time
- Method was validated for the following criteria:
  - ✓ Selectivity
  - ✓ Linearity
  - ✓ Inter-day and intra-day precision and accuracy (PA)
  - ✓ Recovery
  - ✓ Stability

Table 1 Method validation summary

Calibration curve range			51.517 pg/mL to 10058.59 pg/mL	
Intraday precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)		91.11	
	Precision (% CV)		11.25	
Intraday precision and accuracy (For LQC, MQC, HQC)	Accuracy (% Nominal)		93.25 to 106.85	
	Precision (% CV)		4.24 to 8.30	
Global precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)		99.89	
	Precision (% CV)		15.19	
Global precision and accuracy (For LQC, MQC, HQC)	Accuracy (% Nominal)		96.68 to 109.01	
	Precision (% CV)		6.56 to 8.41	
Global % recovery	% Recovery		53.38	
	Precision (% CV)		1.67	
Matrix effect	Matrix Factor		1.05	
	IS normalized Matrix Factor		1.06	
Bench top stability in matrix (6.0 hrs.)	% Change		LQC=5.41	HQC=0.18
	Precision (% CV)	Stability Sample	LQC=6.74	HQC=4.19
		Comparison Sample	LQC=8.30	HQC=4.24
	Accuracy (% Nominal)	Stability Sample	LQC=98.29	HQC=104.94
		Comparison Sample	LQC=93.25	HQC=104.75
Auto sampler stability in matrix (39.0 hrs.)	% Change		LQC= -5.35	HQC=4.02
	Precision (% CV)	Stability Sample	LQC=6.31	HQC=6.79
		Comparison Sample	LQC=12.25	HQC=12.15
	Accuracy (% Nominal)	Stability Sample	LQC=96.10	HQC=110.47
		Comparison Sample	LQC=101.54	HQC=106.20
Freeze thaw stability in matrix (Cycle-4)	% Change		LQC= 0.67	HQC=2.94
	Precision (% CV)	Stability Sample	LQC=4.33	HQC=5.76
		Comparison Sample	LQC=8.30	HQC=4.24
	Accuracy (% Nominal)	Stability Sample	LQC=93.87	HQC=107.83
		Comparison Sample	LQC=93.25	HQC=104.75
Dry extract stability in matrix (4 days 19 hours)	% Change		LQC= 0.04	HQC=-0.23
	Precision (% CV)	Stability Sample	LQC=9.30	HQC=3.16
		Comparison Sample	LQC=8.30	HQC=4.24
	Accuracy (% Nominal)	Stability Sample	LQC=93.28	HQC=104.51
		Comparison Sample	LQC=93.25	HQC=104.75
Extended batch (113 samples)	Precision (%)	LQC=6.79	MQC=4.90	HQC=4.17
	Accuracy (%)	LQC=94.41	MQC=100.48	HQC=107.74

❑ Results and Discussion

A. Linearity

Linearity experiments were conducted for insulin quantitation in human plasma. Calibration curve was plotted from 51.517-10058.586 pg/mL (refer Table 2) with r2> 0.980 as shown in Figure 2. Signal to noise ratio (s/n) for 51.517 pg/mL was more than 10, across 4 PA batches. Representative chromatograms are shown in Figure 3.

Table 2 Calibration curve range

Level	Cal. Conc
CC1	51.52
CC2	77.27
CC3	105.37
CC4	225.28
CC5	560.81
CC6	1147.12
CC7	2532.60
CC8	5065.20
CC9	10058.59

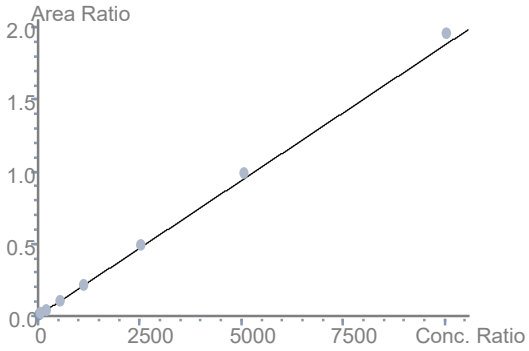

Fig.2 Calibration curve

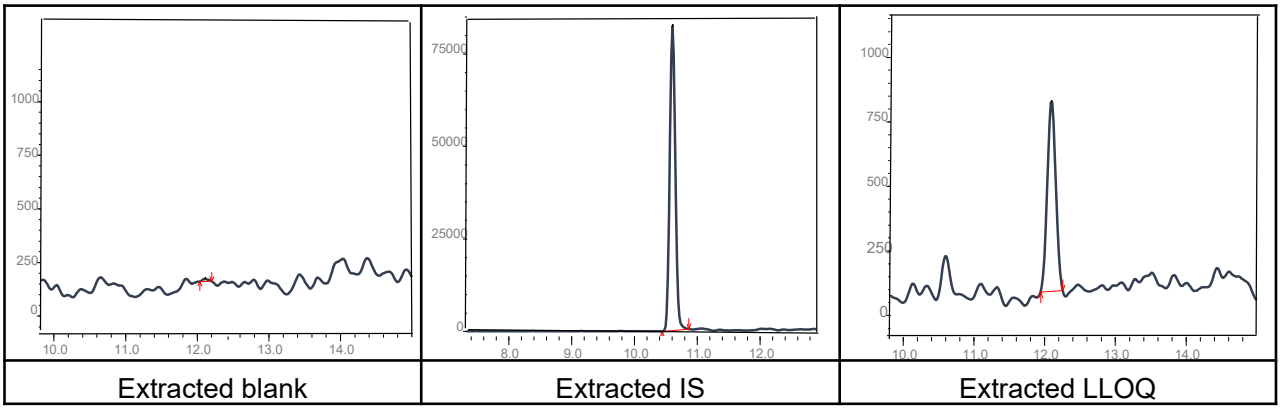

Fig.3 Chromatograms of insulin (extracted blank, extracted IS, extracted LLOQ)

B. Selectivity

Selectivity of this method, with respect to insulin, was assessed in different lots of plasma. Insulin analogue was used as IS. Interference from blank matrix was assessed for both insulin and insulin analogue (refer Figure 3) and no significant interference was detected as shown in Table 3 below.

Table 3. Percentage interference in blank with respect to LLOQ

Plasma lot no.	Insulin			Insulin analogue		
	Area in blank matrix	LLOQ area	% Interference	Area in blank matrix	IS area	% Interference
P4458	215	6,913	3.11	0	582,880	0.00
P4791	0	5,629	0.00	275	484,932	0.06
P5515	92	5,489	1.68	382	476,040	0.08
P5516	421	6,681	6.3	0	478,279	0.00
P5517	0	6,301	0.00	73	466,710	0.02

C. Intra-day precision and accuracy

Intraday precision and accuracy was conducted using 6 replicates of LLOQ-QC, LQC, MQC and HQC over one P&A batch. Figure 4 represents the stability of QC samples (X-axis represents number of QC injections and Y-axis represents calculated concentration in log scale). Quantitative data is summarised in Table 4.

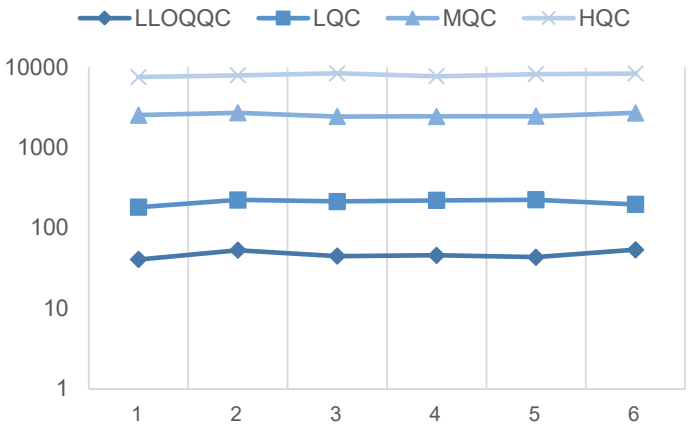

Fig 4. Trend plot of intra-day QC assessment

Table 4. Quantitative tabulation of intra-day QC assessment

QC (n=6)	LLOQQC	LQC	MQC	HQC
Nominal concentration	51.52	225.28	2532.60	7496.50
PA batch observed concentration (pg/mL)	40.76	181.33	2531.36	7542.13
	52.95	223.72	2706.89	7919.60
	44.78	213.70	2435.52	8370.28
	45.75	220.54	2448.08	7711.84
	43.55	224.45	2454.92	8193.10
	53.82	196.69	2692.50	8320.95
Mean	46.94	210.07	2544.88	8009.65
Standard deviation	5.279	17.441	124.615	339.407
% CV	11.25	8.30	4.90	4.24
% Nominal	91.11	93.25	100.48	106.85

D. Global precision and accuracy

Precision and accuracy experiments were conducted for 4 batches. Excellent accuracy and repeatability was observed with % CV < 8.41 % and % accuracy between 96.68 to 109.01 % for LQC, MQC and HQC. At LLOQQC, the % CV was found to be 15.19 % and % accuracy as 99.89 %. The results are presented in Table 5. Figure 5 shows trend plots of 24 QC at each level, analysed over 4 batches.

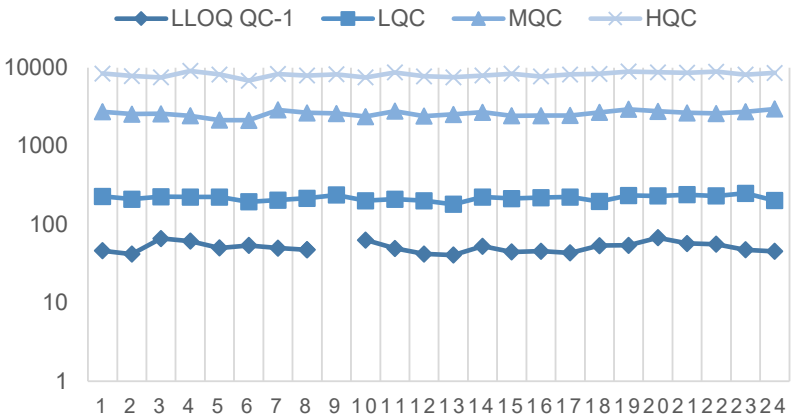

Fig 5. Trend plot of inter-day QC assessment.
X-axis represents the number of injections and Y-axis represents calculated concentration (log scale)

Table 5. Quantitative tabulation of inter-day QC assessment

QC level (n=24)	Mean Conc. (pg/mL)	% CV	% Accuracy
LLOQ QC (51.52 pg/mL)	51.46	15.19	99.89
LQC (225.28 pg/mL)	217.81	7.65	96.68
MQC (2532.60 pg/mL)	2595.22	8.41	102.47
HQC (7496.50 pg/mL)	8172.06	6.56	109.01

Note: LOQQC-3 of PA-2 was not reported due to sample loss

E. Matrix effect

Matrix effect was studied for both insulin and insulin analogue using LQC and HQC samples. Mean matrix factor and IS normalised matrix factor was found to be 1.05 and 1.06 respectively.

Representative data of matrix effect is shown in Table 6. The results confirm the suitability of method for quantitative estimation in human plasma.

Table 6. Quantitative tabulation for checking of matrix effect

	Response ratio of aqueous standard	Response ratio of post extracted spike sample	Matrix factor
LQC	0.031	0.029	0.96
	0.030	0.032	1.05
	0.030	0.031	1.04
	0.031	0.033	1.06
	0.030	0.029	0.99
	0.030	0.031	1.02
HQC	1.643	1.745	1.06
	1.588	1.655	1.04
	1.347	1.608	1.19
	1.675	1.659	0.99
	1.525	1.733	1.14
	1.567	1.677	1.07
	Mean matrix factor of insulin		1.05
	Standard deviation		0.06
	% CV		6.16

F. Treated vs untreated plasma analysis

In compliance with the regulations, since the initial calibration curves and QC were made using charcoal treated plasma, method's applicability was tested against untreated plasma QC as well.

6 replicates of untreated LQC, MQC and HQC were checked against a calibration curve made by spiking treated plasma sample, as shown in Table 7. This data was analyzed over 5 such batches with precision and accuracy both under acceptable criteria. % accuracy for both treated and untreated QC are shown in Table 8 and 9

Table 7. Quantitative tabulation results for PA batch (calibration levels)

Linearity pg/ mL	STD 9	STD 8	STD 7	STD 6	STD 5	STD 4	STD 3	STD 2	STD 1
	10058.59	5065.2	2532.6	1147.119	560.814	225.284	105.375	77.275	51.517
1	10305.072	5439.095	2564.963	1095.912	509.596	209.452	101.954	71.973	55.6353
2	10956.935	5221.227	2622.465	1136.956	541.012	221.065	119.291	71.296	54.9353
AVG	10631.003	5330.161	2593.714	1116.434	525.304	215.259	110.622	71.635	55.285
%STDEV	460.937	154.056	40.66	29.022	22.215	8.212	12.259	0.479	0.495
%CV	4.34	2.89	1.57	2.6	4.23	3.81	11.08	0.67	0.9
% NOM	105.69	105.23	102.41	97.33	93.67	95.55	104.98	92.7	107.32

Table 8. Results for quality control samples prepared in treated plasma

CONC	225.28	2532.6	7496.5
Sr.No.	LQC	MQC	HQC
1	223.12	2665.48	8008.33
2	207.04	2537.79	7654.98
AVG	215.079	2601.633	7831.657
STDEV	11.372	90.29	249.858
% CV	5.29	3.47	3.19
% NOM	95.47	102.73	104.47

Table 9. Results for quality control samples prepared in untreated plasma

CONC	590.49	2898.45	7860.18
Sr.No.	LQC	MQC	HQC
1	596.33	3336.59	8927.13
2	609.95	3201.97	9082.49
3	660.66	3300.09	8388.07
4	612.16	3367.19	8301.57
5	618.87	2990.87	8407.03
6	562.56	3165.37	8484.31
AVG	610.086	3227.012	8598.432
STDEV	31.9	139.42	323.847
% CV	5.23	4.32	3.77
% NOM	103.32	111.34	109.39

G. Recovery

Recovery experiments were conducted to judge combined effect of sample extraction efficiency and instrument method. Average recovery was found to be 53.38 % as shown in Table 10.

Table 10. Quantitative tabulation for % recovery

LQC	53.67
MQC	52.38
HQC	54.09
Mean	53.38
SD	0.89
% CV	1.67

H. Carry-over effect

Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was present/observed at the retention time and MRM transition of the analyte in the extracted blank sample following the highest standard calibrator.

I. Other experiments

Based on validation guidelines, method was assessed for following criteria:
Bench top stability
Auto sampler stability
Freeze thaw stability
Dry extract stability
Extended batch verification

❑ Conclusion

LCMS-8060, along with special sample preparation, optimized chromatography provides a very selective and sensitive method for bioanalytical assay of insulin. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple mobile phase at a minimal gradient flow rate of 0.3 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavours.

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Application News

No. AD-0182

Clinical Research / LCMS-8060

Multidimensional LC-MS/MS Method for the Quantification of Intact Human Insulin

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❑ Overview

In this study, we developed and validated a novel multidimensional LC-MS/MS method for the analysis of human insulin in serum samples. Insulin was extracted from serum samples by protein precipitation using ice-cold methanol. Stable isotope-labelled human insulin (insulin-d40) was added in the extraction step as internal standard. The method was validated based on FDA guidance for industry on bioanalytical on method validation: selectivity, accuracy, precision, recovery, calibration curve, sensitivity, reproducibility and stability.

❑ Introduction

Insulin is a peptide hormone produced by pancreatic islets that regulated the metabolism of carbohydrates, fats and proteins by promoting the absorption of glucose. Historically, insulin has been analyzed by radioimmunoassay or enzyme-linked immunosorbent assay. These assays could suffer from the lack of standardization or cross reactivity, and this has driven the development of alternative assays using LC-MS/MS which provide greater specificity. Hence, method development was done using Shimadzu NexaraX2 LCxLC coupled to LCMS-8060 for the quantification of intact human insulin in human serum. In this study, we developed and validated a novel multidimensional LC-MS/MS method for the analysis of human insulin in serum samples. The validation results have been evaluated with the FDA guidance for industry on bioanalytical on method validation.

❑ Experimental

Sample preparation and analytical conditions

Insulin was extracted from serum samples by protein precipitation using ice-cold methanol; (1:1; vol/vol, serum:methanol). Stable isotope-labelled human insulin (insulin-d40) was added in the extraction step as internal standard. Chromatographic separation was performed on LC system comprising of a loading column (Kinetex C18, 50 × 2.1 mm, 2.6 µm) on which insulin peak was heart-cut and transferred to the analytical column (Kinetex C18, 50 × 2.1 mm, 1.3 µm) shown in figure 1. A Gradient elution of 7.5mins was used for both the loading and analytical columns. Detection and quantification of intact insulin was performed based on multiple reaction monitoring (MRM) under positive electrospray ionization mode in the Shimadzu LCMS-8060 triple quadrupole mass spectrometer.

Table 1. Analytical conditions and parameters on LCMS-8060

Column 1	Kinetex 2.6µ C18 100A (100 mmL x 2.10mm I.D.)
Column 2	Kinetex 1.3um C18 100A (50 mmL x 2.10mm I.D.)
Mobile Phase A & C	Water with 0.1% FA
Mobile Phase B & D	Acetonitrile:water (9:1) with 0.1% FA
Elution Program 1	B: 25% (0 to 0.5 min) ➔ 50% (3.0 min) ➔ 90% (3.1 to 4.5 min) ➔ 25% (4.6 to 7.5 min)
Elution Program 2	D: 25% (0 to 4.0 min) ➔ 100% (6.5 to 7.5 min)
Flow Rate for column 1	0.25 mL/min
Flow Rate for column 2	0.35 mL/min
Oven Temp.	40ºC
Injection	50 µL

Interface	ESI
Interface temp. & Voltage	350 ^{O}C & 2 kV
MS Mode	MRM, Positive
Heat Block Temp.	500 ^{O}C
DL Temp.	250 ^{O}C
CID Gas	Ar, 270 kPa
Nebulizing Gas	N_2, 2.5 L/min
Drying Gas	N_2, 5.0 L/min
Heating Gas	Dry Air, 10L/min

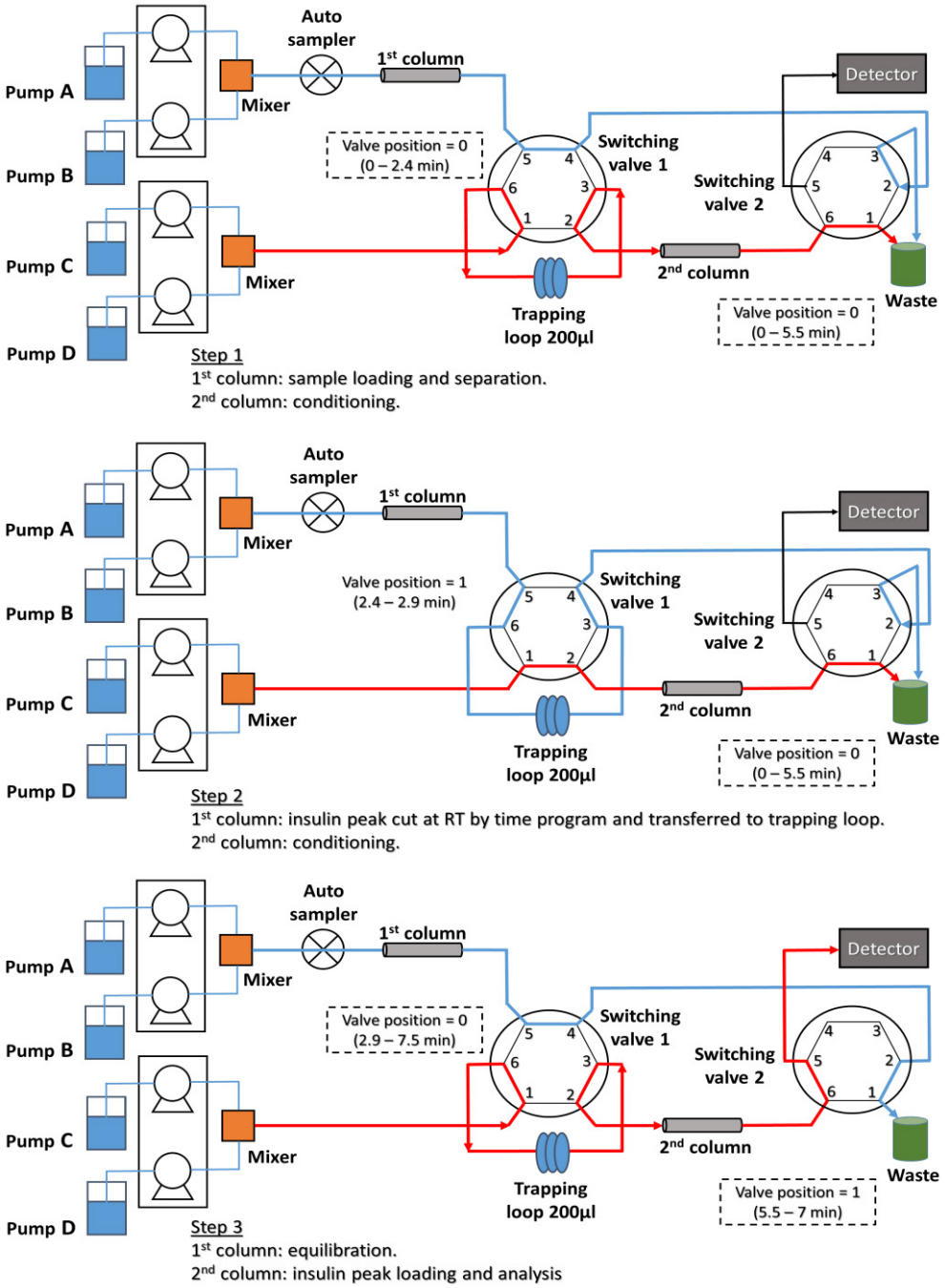

Figure 1. Mutli-dimensional LC-MS/MS schematic diagram and operation scheme

❑ Results and Discussion

Method Development

Insulin and insulin-d40 (internal standard) were used for setting up the MRM quantitation method. Table 2 shows the summarized results of optimized MRM transitions and parameters of the insulin standards and isotope-labelled internal standards. Two MRM transitions were selected for each compound, with one as the quantitation ion and the other for confirmation. Furthermore, a gradient elution MRM method was established with a total run time of 7.5 minutes. The MRM chromatogram of insulin in human serum is shown in Figure 3. The retention time of insulin peak is at 5.8mins

Table 2. MRM transitions and parameters of Insulin on LCMS-8060

Standard				Internal Standard			
Compd.	**R.T (min)**	**MRM (m/z)**	**CE (V)**	**Compd.**	**R.T (min)**	**MRM (m/z)**	**CE (V)**
Insulin	5.8	1162.1>1158.4	-33	Insulin-d40	5.8	1170.3>1166.7	-34
		1162.1>1410.1	-29			1170.3>1419.9	-37

Method validation

Selectivity of insulin in serum was studied. In figure 2 (a) and (b), it shows endogenous compound was found in the blanks. Therefore, insulin standard + endogenous compound shown in figure 2(c) was used to construct the calibration. In addition, the confirmation criteria include the MRM transitions as well as retention time.

Accuracy, Precision and Recovery of the quantitation method was validated within and between-run. The results are shown in Table 3 and 4, which indicate that reliable quantitation accuracy, precision and recovery were obtained. Accuracy and precision was being measured at five determinations per concentration level with four different concentration levels (LLOQ, Low, Med and High). Recovery and matrix effect is studied at three differently concentration levels (Low, Med and High). The accuracy result obtained for intra and inter day are within 15.0% of the nominal value. The precision CV for intra and inter day are <6.8%. The recovery obtained for insulin ranged from 87.5 to 100.5% with CV% of <3.7% for inter day. Furthermore, matrix effect was evaluated with results ranging from 92.6% to 111.4%.

Calibration curve was established using the internal standard method prepared by pre-spiked in serum matrix (see figure 3). It can be seen that good linearity with R^2 greater than 0.99 was obtained for the insulin in the range from 8.6 pmol/L to 1720 pmol/L in serum.

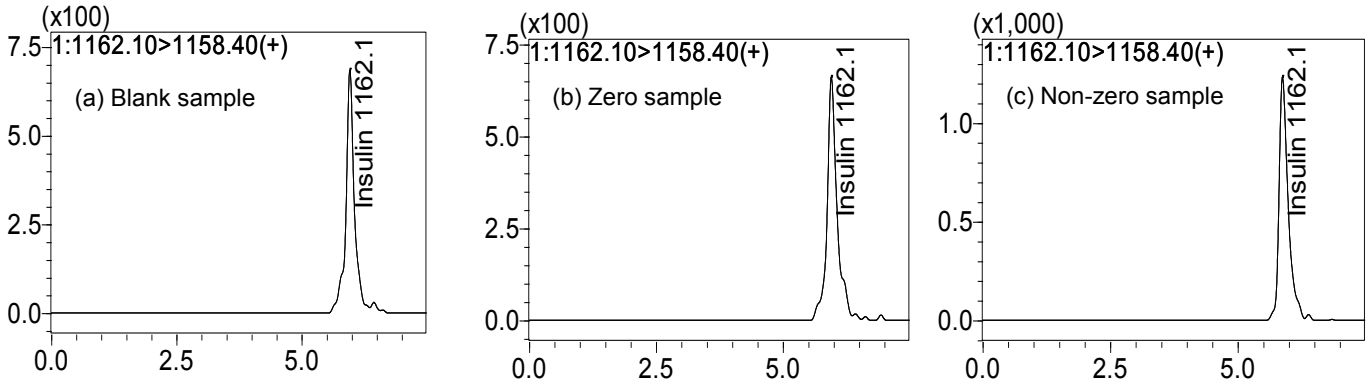

Figure 2. Chromatogram of (a) Blank, (b) Zero and (c) Non-Zero samples (172.2ppt).

Table 3. Intra- and inter-day accuracy and precision of insulin

Nominal Conc. (pmol/L)	Intra-day (n=5)			Inter-day (n=5)		
	Found conc. (pmol/L)	Accuracy (%)	Precision (% CV)	Found conc. (pmol/L)	Accuracy (%)	Precision (% CV)
LLOQ: 103.3 (Endogenous +8.6)	92.9 ± 4.8	89.93	5.21	100.8 ± 6.1	97.62	6.07
Low: 120.5 (Endogenous +25.8)	126.7 ± 8.5	105.13	6.71	126.2 ± 6.7	104.67	5.32
Med: 783.4 (Endogenous +688.7)	825.1 ± 53.5	105.32	6.49	842.3 ± 33.6	107.52	3.99
High: 1472.1 (Endogenous +1377.4)	1306.9 ± 69.1	88.78	5.29	1368.4 ± 90.1	92.95	6.58

Table 4. Matrix effect and recovery of insulin

Nominal Conc. (pmol/L)	Mean peak area (x10^3) (n=5)			Inter-day (n=5)		Matrix Effect (%)
	Set A	Set B	Set C	Mean (%)	CV (%)	
25.8	2.64	2.94	2.17	87.46	3.67	111.36
688.7	24.73	22.89	23.00	100.48	1.24	92.57
1377.4	41.50	46.04	41.93	91.07	1.55	110.93

Set A was neat solution standards. Set B and Set C were standards spiked in extracted serum and serum respectively.

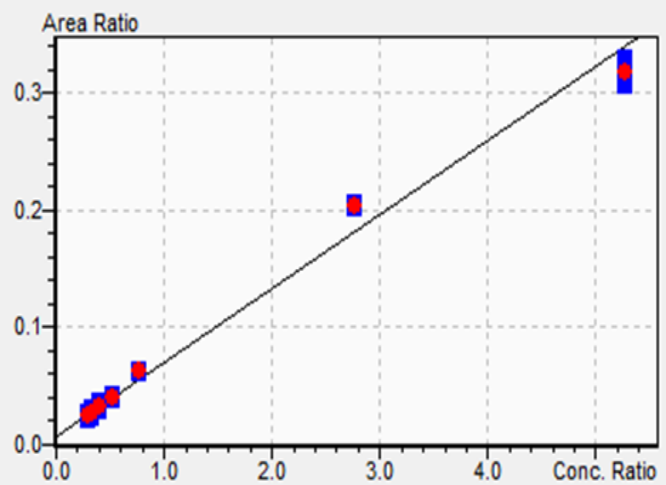

Figure 3. Insulin calibration curve from 8.6 to 1720 pmol/L with r^2>0.99.

Sensitivity of the method for LLOQ of 103.3 pmol/L nominal concentration was evaluated and giving an accuracy of 89.9% for intra day and 97.6% for inter day. The precision for both intra and inter day is < 6.1%. Both accuracy and precision for LLOQ being measured is acceptable.

Reproducibility of the method was assessed. This includes the QCs and incurred samples. Reinjection reproductivity was also evaluated for instrument interruption

Stability of the analyte insulin was assessed after freeze and thaw, at room temperature, cold room and long term stability (see table 5). The mean recovered concentration (%) was within ±15.0% of the nominal concentration except for the low concentration of 25.8 pmol/L which is -17.6%.

Table 5. Stability assessment of insulin

Nominal Conc. (pmol/L)	Room Temp. (25°C, 4h)		Cold-room (4°C, 24h)		Freeze-thaw (-80°C, 3 cycles)		Long term stability (-80°C, 14 weeks)	
	Mean recovered conc. (%)	CV (%)	Mean recovered conc. (%)	CV (%)	Mean recovered conc. (%)	CV (%)	Mean recovered conc. (%)	CV (%)
25.8	105.85	6.96	92.19	3.99	82.42	0.40	96.39	3.58
688.7	97.58	6.63	96.32	2.91	98.74	6.63	99.87	2.90
1377.4	98.08	5.64	97.51	5.64	97.41	1.37	99.79	2.81

Method Application

Table 6. Comparison of insulin sample concentrations assayed by 2D-LC-MS/MS and immunoassay methods.

Sample	2D-LC-MS/MS conc. (pmol/L)	Immunoassay conc. (pmol/L)	% Difference
1	671	656	2.33
2	636	646	-1.49
3	562	590	-4.75
4	647	615	5.10
5	806	786	2.57
6	1261	772	48.15
7	414	610	-38.32
8	404	654	-47.31
9	817	767	6.30
10	932	909	2.45

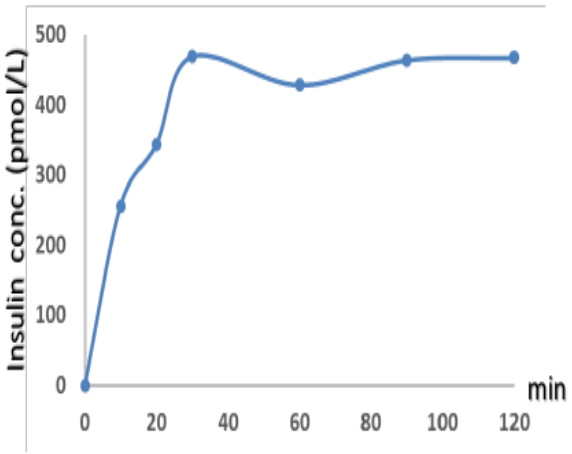

Figure 4: Pharmacokinetic curve of insulin derived from a human subject*.

*Samples were derived from a study of insulin sensitivity via euglycaemic, hyperinsulinaemic clamp technique. After an overnight fast of ten hours, two polythene cannulae are inserted, one into an antecubital vein for infusion of 20% dextrose solution and insulin, and the second into the contralateral antecubital vein for regular blood sampling.

The 2D-LC-MS/MS validated method was assayed and it was compared against immunoassay method. The results are shown in table 6, the difference % is <±6.5% except sample 6, 7, and 8 which is >±38.3%. In figure 4, a pharmacokinetic studies using this method was done for 2 hrs with intervals of 20mins.

❑ Conclusions

A 2D-LC-MS/MS method for quantitation of insulin in human serum was developed and validated. The results data have been evaluated with the FDA guidance for industry on bioanalytical on method validation; based on the selectivity, accuracy, precision, recovery, calibration curve, sensitivity, reproducibility and stability.

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Application News

No. AD-0118

Quantitative Bioanalysis / LCMS-8060

Development of 2D-LC/MS/MS Bioanalytical Method for Quantitative Determination of Insulin Glargine in Human Plasma

ASMS 2016 MP 311

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Development of 2D-LC/MS/MS Bioanalytical Method for Quantitative Determination of Insulin Glargine in Human Plasma

Introduction

Human insulin is an essential factor for regulating the metabolism of carbohydrates. Insulin glargine (Lantus) is one of the recombinant insulin analogues widely-used in diabetic patients to regulate glucose levels. Various bioassays have been used for quantitation of insulin and the analogues in serum/plasma for research and diagnosis. Recently, LC/MS/MS has been applied for quantitative analysis of glargine and other insulin analogues, because it enables to distinguish human insulin and recombinant analogues as well as their metabolites. The American Diabetes Association has recommended the sensitivity of bioassay to achieve an LLOQ of 70 pg/mL [1], which is challenging to the current LC-MS systems. The bioassay reported always require tedious solid phase extraction (SPE) for clean up before injecting into the LC/MS/MS [2, 3]. This motivated this study, aiming at developing a high sensitivity method on the latest LCMS-8060 to determine quantitatively insulin glargine in human plasma with lesser sample preparation steps. We describe a newly developed 2D-LC/MS/MS method for high sensitivity quantitation of insulin glargine spiked in human plasma without SPE clean up.

Methods and Materials

Insulin glargine (MW 6063) was obtained from Sigma-Aldrich. Commercially available pooled human plasma supplied by Innovative Research (USA) was used in this study. A 2D-LC/MS/MS system (see Figure 2) was set up on Shimadzu LCMS-8060 triple quadrupole system coupled to Nexera UHPLC with a column switching valve installed in column oven controlled by LabSolutions workstation. A stock solution of insulin Glargine was prepared with a mixed solvent of water-methanol (80/20) with 0.1% acetic acid. The procedure of sample preparation of spiked human plasma samples is shown in Figure 1. It includes protein precipitation step by adding ACN-MeOH solvent into the human plasma in 3 to 1 ratio followed by vortex and centrifuge at high speed. The supernatant was filtered and blown dry with nitrogen gas. The dried supernatant was reconstituted with mixed solvent of water-methanol (80/20) with 0.1% acetic acid before analysis on 2D-LC/MS/MS. Both pre-spiked and post-spiked plasma samples of various concentrations were prepared freshly from high concentration stock in method development. The details of columns, mobile phases and gradient programs of 1stD and 2nd D UHPLC separations and MS/MS conditions are compiled into Tables 1(a) and 1(b).

Table 1 (a): 2D-UHPLC conditions (on Nexera X2 system)

Column	: 1st D: Kinetex, 2.6um C18 100A (50 x 2.1mm)
	2nd D: Kinetex, 1.3um C18 100A (50 x 2.1mm)
Mobile Phase of 1st D	: A: 0.1% formic acid in Water
	B: 0.1% Formic acid in Acetonitrile: Isopropanol (50:50)
Mobile Phase of 2nd D	: C: 0.1% formic acid in Water
	D: 0.1% formic acid in Acetonitrile
1st D gradient program	: B: 15% (0 to 0.1min) → 90% (2.5 to 4.0min) → 15% (5.0 to 7.0min)
& flow rate	Flow rate: 0.3mL/min
2nd D gradient program	: D: 15% (0 to 2.25min) → 90% (4.0 to 5.0min) → 15% (5.5 to 7.0 min)
& flow rate	Peak cutting: from 1.50 to 1.73
	Flow rate: 0.35mL/min
Oven Temp.	: 40°C
Injection Vol.	: 50 µL

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Development of 2D-LC/MS/MS Bioanalytical Method for Quantitative Determination of Insulin Glargine in Human Plasma

Table 1 (b): LCMS-8060 MS conditions

Interface	ESI
MS mode	Positive
Block Temperature	300°C
Interface Temp.	400°C
DL Temperature	300°C
CID Gas	Ar (350kPa)
Nebulizing Gas Flow	N2, 2.0L/min
Drying Gas Flow	N2, 10.0L/min
Heating Gas Flow	Zero Air, 10.0L/min

1500µL of ACN/MeOH (1:1; vol/vol)

↓

500µL of Human Plasma

↓

Shake and Vortex 10mins

↓

0.2µm nylon filter

↓

Centrifuge for 10 minutes at 13000rpm

↓

1800µL of filtered protein precipitated Human Plasma

↓ Blow Dry by N2

Reconstitute with 360uL of pre-mixed solvent water/methanol (80/20) with 0.1% acetic acid

Figure 1: Flow chart of human plasma sample pre-treatment method (dilution factor: 4)

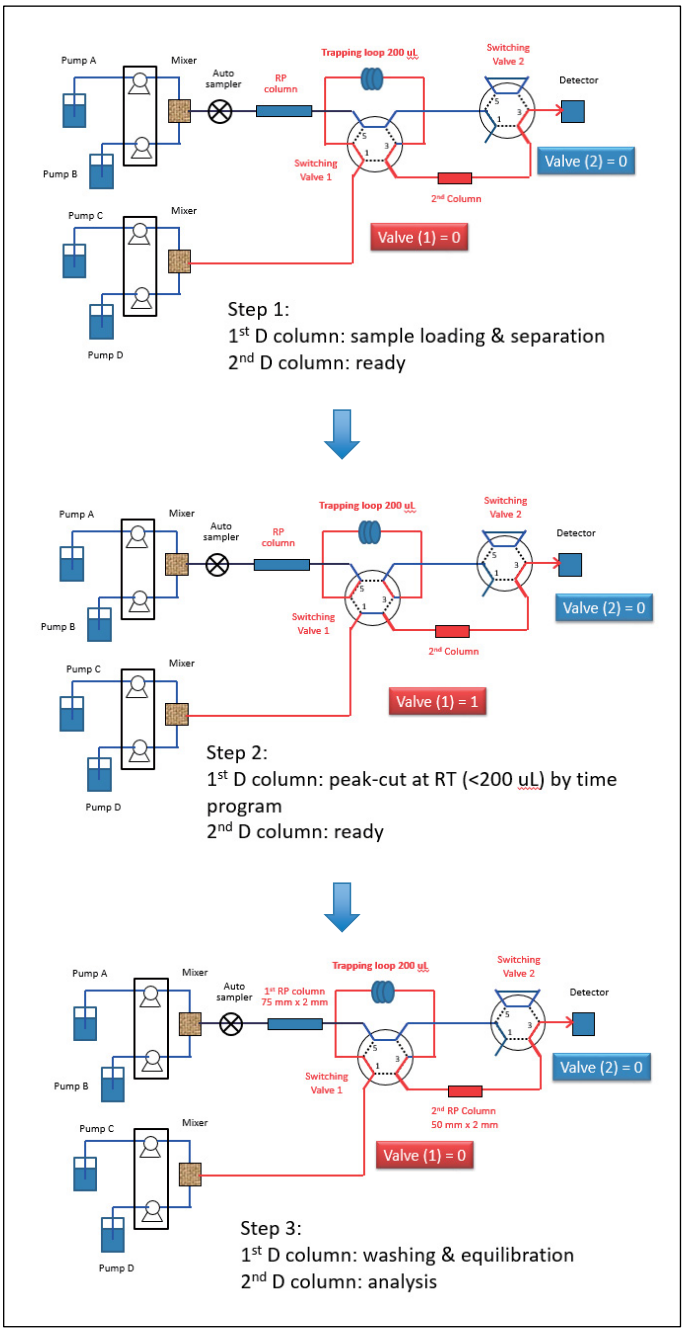

Figure 2: Schematic diagram of 2D-LC/MS/MS and operation scheme

Development of 2D-LC/MS/MS Bioanalytical Method for Quantitative Determination of Insulin Glargine in Human Plasma

Results and Discussion

Development of 2D-LC/MS/MS method

An ESI interference was employed for effective ionization of insulin glargine ($C_{267}H_{404}N_{72}O_{78}S_6$, MW 6063). A MRM quantitation method for insulin glargine was developed. MRM optimization was performed using an automated MRM optimization program with LabSolutions workstation.

The insulin glargine molecules form multiply charged ions in ESI ionization and the precursors selected for MRM optimisation were charges +6 and +7. Two MRM transitions were used (Table 2), the first transition for quantitation and the second transition for confirmation.

Table 2: MRM transitions and CID parameters of insulin glargine on LCMS-8060

Name	RT (min)	Transition (m/z)	Voltage (V)		
			Q1 Pre Bias	CE	Q3 Pre Bias
Glargine	3.926	867.00 > 984.00	-20	-20	-22
		1011.50 > 1179.00	-34	-32	-42

The 2D-LC/MS/MS method developed in this study involves "cutting the targeted peak" in the 1st-D elution precisely (1.50~1.73 min) and the trapped elute in a stainless steel sample loop (200 uL) being transferred into the 2nd-D column for separation and MS/MS detection (see Figure 2). The operation was accomplished automatically by a control program with the LabSolutions switching the 6-way valves in and out during an analysis. Both 1st-D and 2nd-D separations were carried out in gradient elution mode (Table 1(a)). The advantages of this 2D-UHPLC separation are firstly to remove effectively the plasma matrix and, secondly, to minimize interferences which occurred in 1D-LC/MS/MS analysis. In addition, the MS interface and lens system are kept clean from contamination, which is very critical when a large amount of plasma sample was injected for obtaining higher detection sensitivity. As such, with 2D-LC/MS/MS, SPE clean-up is not needed to achieve the desired sensitivity (70 pg/mL).

Calibration curve, linearity and accuracy

Seven levels of post-spiked calibrants of insulin glargine from 0.05 ng/mL to 10 ng/mL were prepared freshly for establishment of calibration curve (See Table 3). A linear calibration curve (R2 > 0.9996) with weighting method 1/C was established as shown Figure 3. The accuracy of the method is between 98 % and 105 %. The chromatograms of the 1ng/mL post-spiked glargine standard sample is shown in Figure 4.

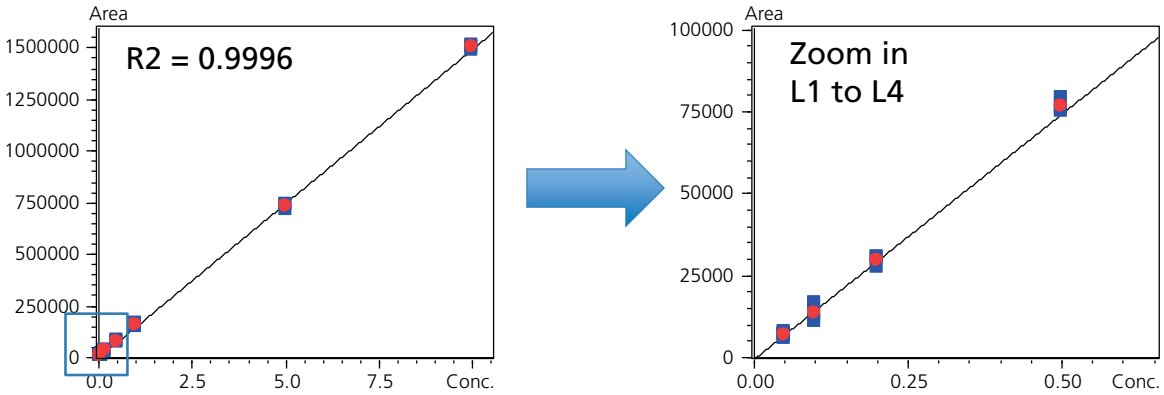

Figure 3: Calibration curve of insulin glargine in human plasma, full range and zoomed

Development of 2D-LC/MS/MS Bioanalytical Method for Quantitative Determination of Insulin Glargine in Human Plasma

Table 3: Calibration curve standard samples in plasma

Cali. level [1]	Conc. (ng/mL)	Avg. Area	Avg. Accuracy %
L1	0.05	6827	100.2
L2	0.1	13604	104.5
L3	0.2	29667	100.7
L4	0.5	76704	101.1
L5	1.0	156677	98.9
L6	5.0	732260	98.6
L7	10.0	1501911	100.6

[1] Calibration levels were injected in triplicates (n=3) except for level 1 and level 2 (n=6)

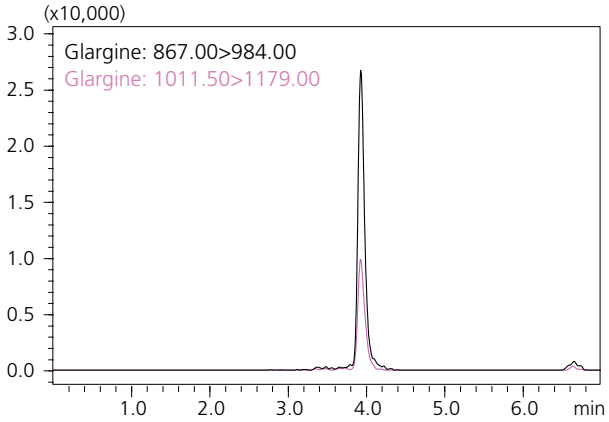

Figure 4: MRM chromatograms of insulin glargine spiked in human plasma (1 ng/mL)

Recovery, repeatability, LOQ and specificity

Recovery of the 2D-LC/MS/MS method was determined by comparison of the peak areas of pre-spiked and post-spiked samples at L1, L2, L4 and L6. As shown in Table 4, the recovery of the sample preparation method are between 63 % and 87 %. The repeatability (RSD%, n=7) of the method obtained is 11.1% at L1 and between 7.8 % and 2.3 % for L2, L4 and L6. It is worth to note that matrix effect was not evaluated due to the non-specific absorption nature of insulins, which may cause significant lost of the molecules in neat solution at low concentrations. The LOQ of the method is estimated to be 0.018 ng/mL from the L1 chromatograms of pre-spiked and post-spiked samples (see Figure 5). This corresponds to a LOQ of ~70 pg/mL of insulin Glargine in plasma before pre-treatment. The specificity of the method relies on several criteria: two MRMs (867.0>984.0 and 1011.5>1179), their intensity ratio and RT in 2nd-D MRM chromatogram as shown in Figures 4 and 5.

Development of 2D-LC/MS/MS Bioanalytical Method for Quantitative Determination of Insulin Glargine in Human Plasma

Table 4: Summary of method performance of insulin Glargine spiked in human plasma (n = 3~6)

Sample	Conc. (ng/mL)	Area (Pre-spiked)	Area (post-spiked)	%RSD	Recovery%
L1	0.05	5,944	6,827	11.1	87.1
L2	0.1	10,216	13,604	7.8	75.1
L4	0.5	58,905	76,704	5.1	76.8
L6	5	465,387	732,260	2.3	63.6

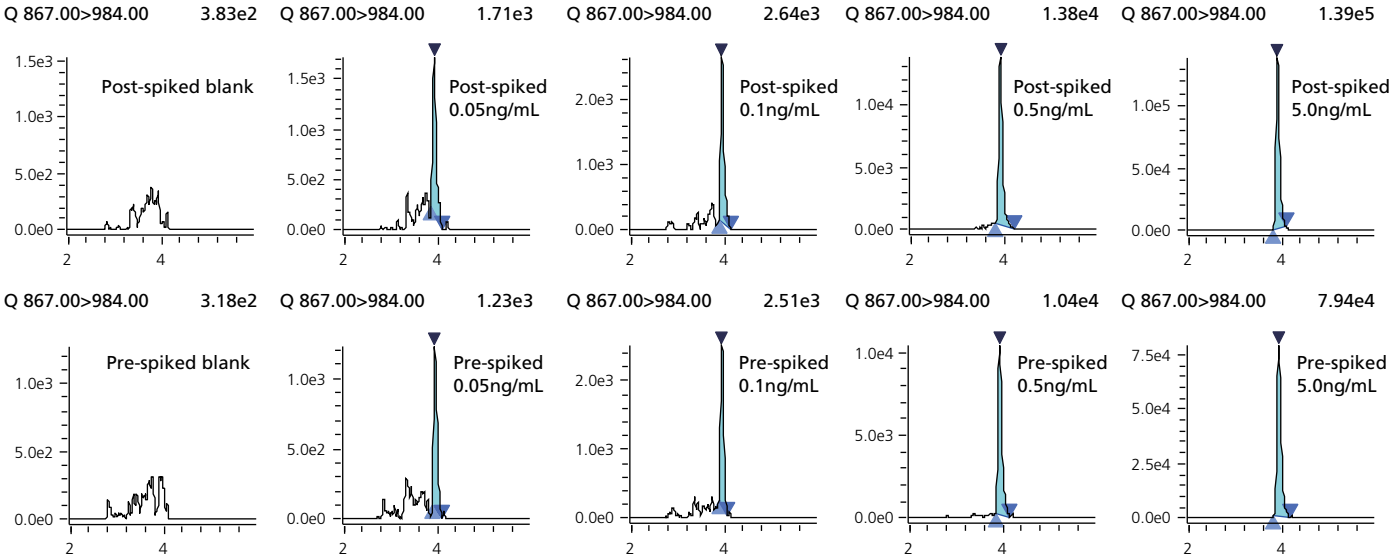

Figure 5: MRM peak of insulin glargine post-spiked (top) and pre-spiked (bottom) in human plasma

Conclusions

A 2D-LC/MS/MS method has been developed on LCMS-8060 for quantitative analysis of insulin Glargine in human plasma without use of SPE extraction and clean-up. The performance of the method was evaluated thoroughly, including linearity, accuracy, repeatability, recovery, sensitivity and specificity. The LOQ of the method is estimated to be 70 pg/mL in human plasma. **The advantages of the 2D-LC/MS/MS method are not only simplifying the sample pre-treatment and enhancing the sensitivity, but also reducing the potential contamination of plasma samples to the interface and lens of MS/MS system.**

Development of 2D-LC/MS/MS Bioanalytical Method for Quantitative Determination of Insulin Glargine in Human Plasma

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Selective and sensitive quantification of glucagon and glucagon-related peptide hormones in human plasma using conventional LC/MS/MS

ASMS 2016 ThP-494

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Selective and sensitive quantification of glucagon and glucagon-related peptide hormones in human plasma using conventional LC/MS/MS

Introduction

Impaired secretion of endogenous bioactive peptides such as peptide hormones and cytokines is associated with the development and pathophysiology of various diseases. Glucagon is a peptide hormone known to increase blood glucose levels. Glucagon-like peptide 1 (GLP-1), a peptide hormone generated from the same precursor as glucagon, regulates glucose metabolism by enhancing insulin secretion from pancreatic β-cells.
Because of similarities between amino acid sequences of glucagon and glucagon-related peptides derived from proglucagon (Figure 1), the quantification of glucagon in blood by conventional immunoassay methods have been hampered by cross-reactivity of anti-glucagon antibodies with glucagon-related peptides.
In the present study, to selectively quantify these peptide hormones in human plasma, we developed a sensitive method using a LC/MS/MS.

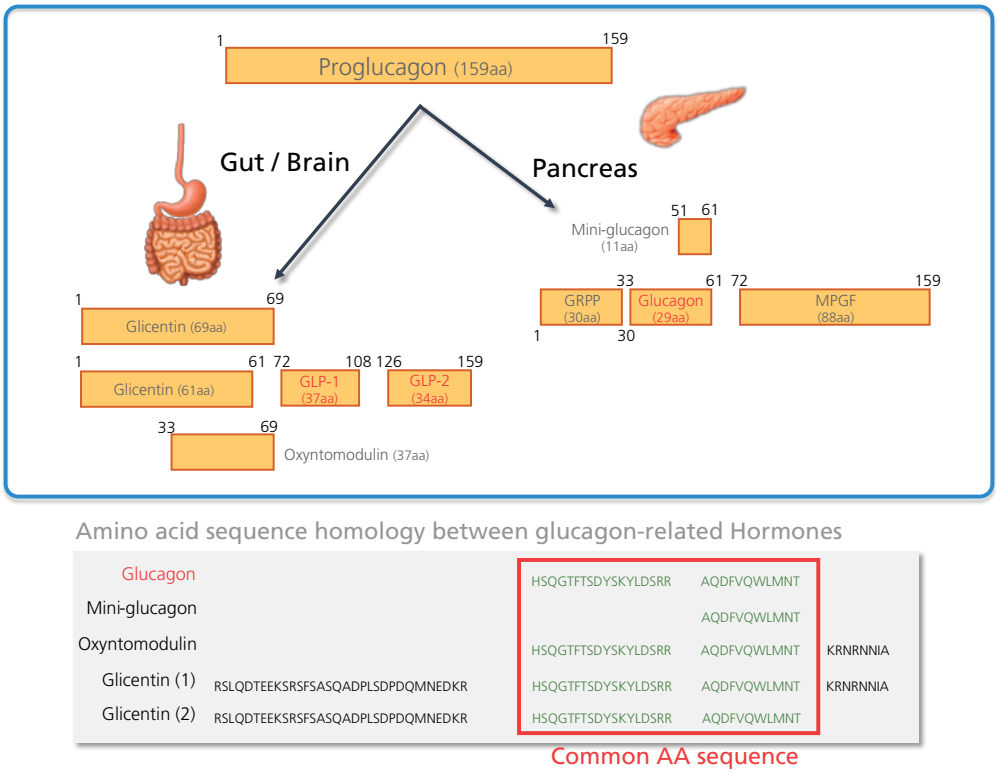


Figure 1. Processing of proglucagon in pancreas and gut / brain.

Methods

Intact peptides (insulin, glucagon, GLP-1 (7-36) amide, GLP-1 (7-37), exenatide, and liraglutide) were analyzed using a triple quadrupole mass spectrometer (LCMS-8060; Shimadzu, Japan) coupled with conventional flow liquid chromatography (Nexera *X2*; Shimadzu). The LC separation was performed using Shim-pack ODS II column (1.6 µm, 2.0 mm × 150 mm, Shimadzu) or Kinetex 2.6u XB-C18 100A (2.1 mm × 100 mm, Phenomenex) with binary gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The absolute concentration of each peptide was calculated from the calibration curve using the peak area of external standard. Plasma samples were collected using a blood collection tube containing protease inhibitors cocktail to prevent degradation of glucagon and glucagon-related peptides, and pretreated by solid phase extraction using EVOLUTE EXPRESS AX 30 mg (Biotage, Sweden).

Selective and sensitive quantification of glucagon and glucagon-related peptide hormones in human plasma using conventional LC/MS/MS

Result

Development of analytical method for intact peptide hormones by a LCMS-8060

Peptide hormones are detected as multiple charged ions. Mainly observed charge distribution of glucagon and insulin are 3+ to 5+ and 4+ to 6+, respectively (Figure 2). Sensitivity of measurement was evaluated using standard peptides (Figure 3). HPLC and SRM conditions for simultaneous analysis of insulin, glucagon, GLP-1, and GLP-1 analogues were optimized by measuring each intact peptide (Figure 4).

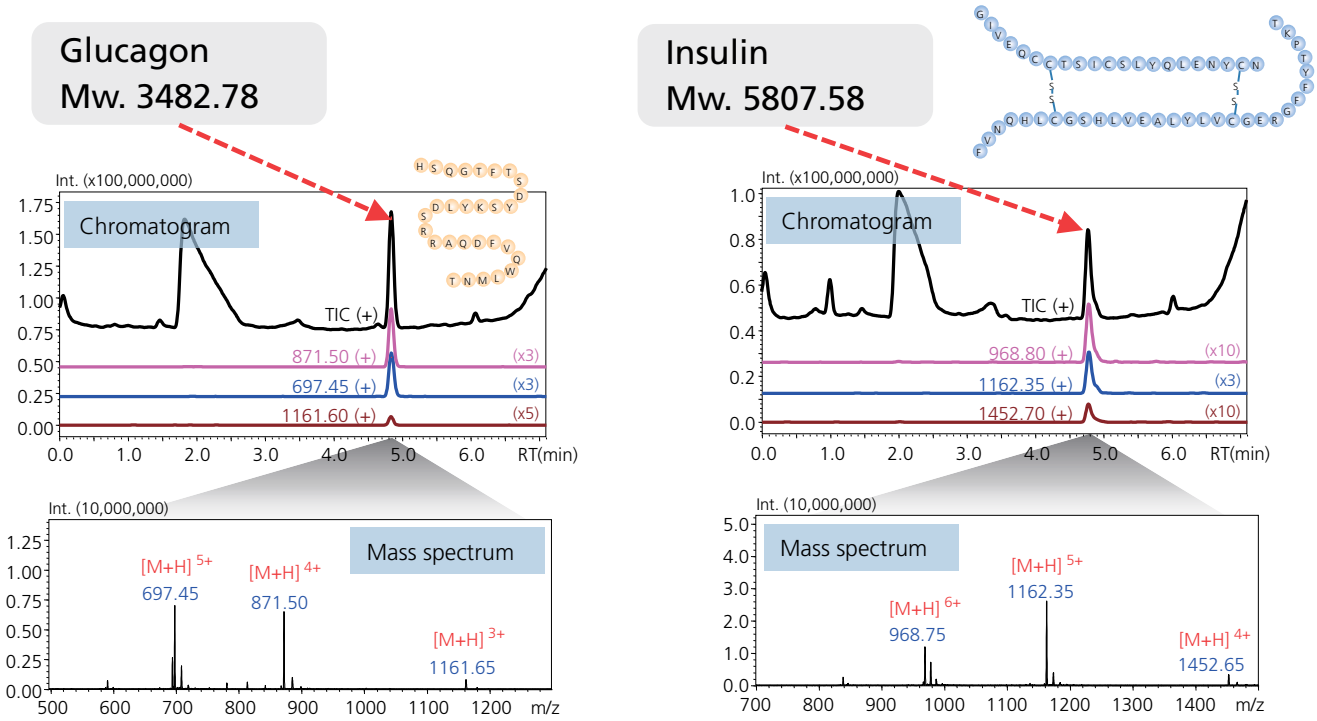


Figure 2. Precursor scan analysis of intact glucagon and insulin.

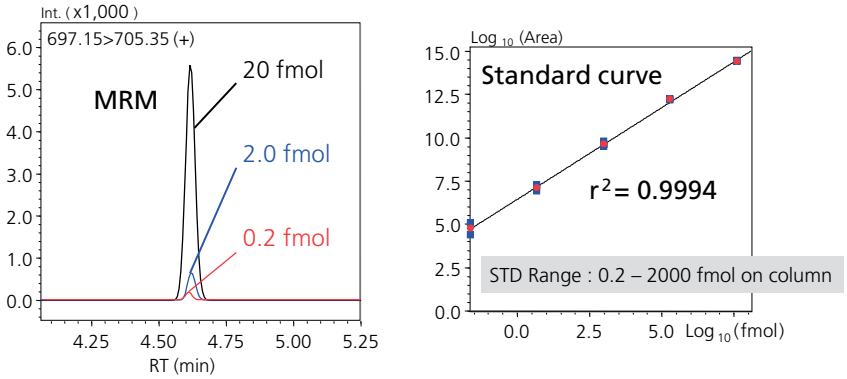
HPLC conditions (Nexera X2)	
Column	: Shim-Pack ODS II (2.0mmI.d x 150 mm)
Column temperature	: 40 deg. C
Mobile phase A	: 0.1 % formic acid / water
Mobile phase B	: 0.1 % formic acid / acetonitrile
Solvent for sample loading	: 0.1% formic acid / water
Flow rate	: 0.3 mL/min
Total cycle time	: 10 min
MS conditions (LCMS-8060)	
Ionization	: ESI, Positive
Gas flow	: 2.5/ 10/ 5.0 L/min (Neb./ Heating/ Drying)
Temp.	: 350/ 250/ 500 deg. C (IF/ DL/ Heat block)
CID gas	: 350 kPa

Selective and sensitive quantification of glucagon and glucagon-related peptide hormones in human plasma using conventional LC/MS/MS

MRM parameter

Protein	Charge	Transition	CE	Type
Glucagon	5+	697.15>705.35	-23	target
	4+	871.15>225.10	-40	ref.
	4+	871.15>940.10	-30	ref.
	4+	871.15>1002.15	-29	ref.
	5+	697.15>1002.15	-22	ref.
	5+	697.15>751.85	-19	ref.
Insulin	5+	1162.50>1410.10	-34	target
	5+	1162.50>1129.40	-34	ref.
	5+	1162.50>1158.40	-25	ref.
	6+	968.95>651.85	-24	ref.

Glucagon



Insulin

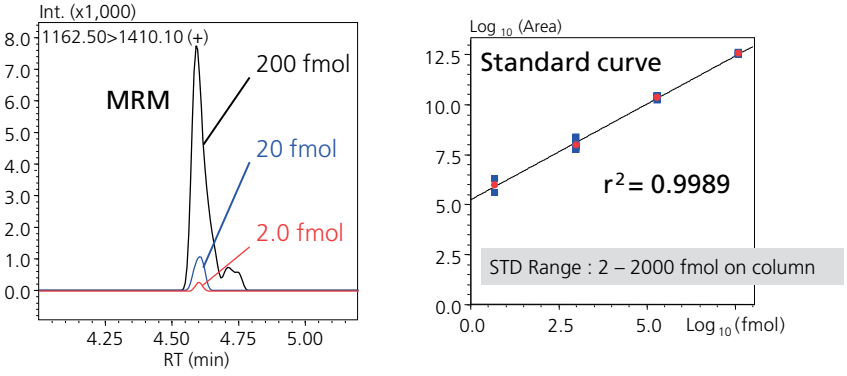


Figure 3. Evaluation of analytical method using standard sample.

Selective and sensitive quantification of glucagon and glucagon-related peptide hormones in human plasma using conventional LC/MS/MS

HPLC conditions (Nexera X2)	
Column: Kinetex 2.6u XB-C18 100A (2.1 mm × 100 mm, Phenomenex)	
Column temperature	: 40 deg. C
Mobile phase A	: 0.1 % formic acid / water
Mobile phase B	: 0.1 % formic acid / acetonitrile
Solvent for sample loading	: 0.1% formic acid / water
Flow rate	: 0.15mL/min
Total cycle time	: 13 min

MS conditions (LCMS-8060)	
Ionization	: ESI, Positive
Gas flow	: 2.5/ 10/ 5.0 L/min (Neb./ Heating/ Drying)
Temp.	: 350/ 250/ 500 deg. C (IF/ DL/ Heat block)
CID gas	: 350 kPa

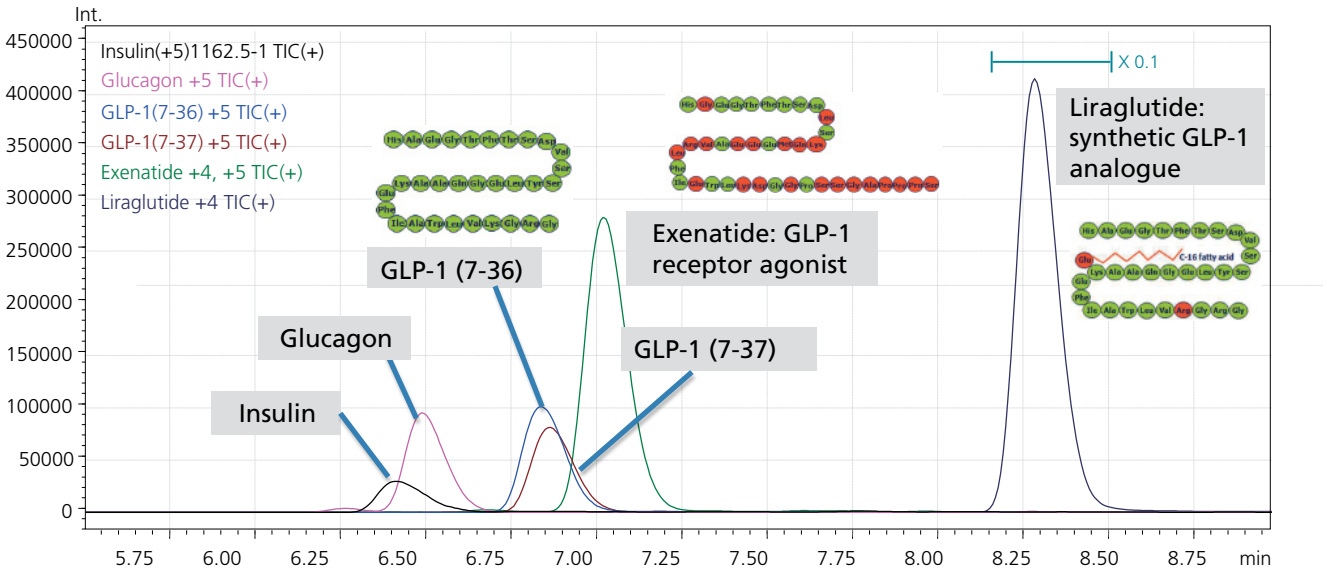


Figure 4. Analytical condition and MRM chromatogram of insulin, glucagon, GLP-1, and GLP-1 analogues (10 fmol of each intact peptides standards).

Selective and sensitive quantification of glucagon and glucagon-related peptide hormones in human plasma using conventional LC/MS/MS

Sample preparation for glucagon in plasma sample (Figure 5)

To release a protein-protein interaction of glucagon in blood, 500 µl of plasma collected using blood collection tube containing protease inhibitor cocktail was acidified with 10 µl of 40% acetic acid at once. Then, to alkalize the solutions, 500 µl of 5% ammonium hydrate are added to samples. Total 1000 µl of sample is applied to solid phase extraction. Glucagon is eluted with 200 µl of elution buffer and diluted with 100 µl of 60% acetic acid. Of the 300 µl of eluate, 45 µl is subjected to LC-MS/MS analysis.

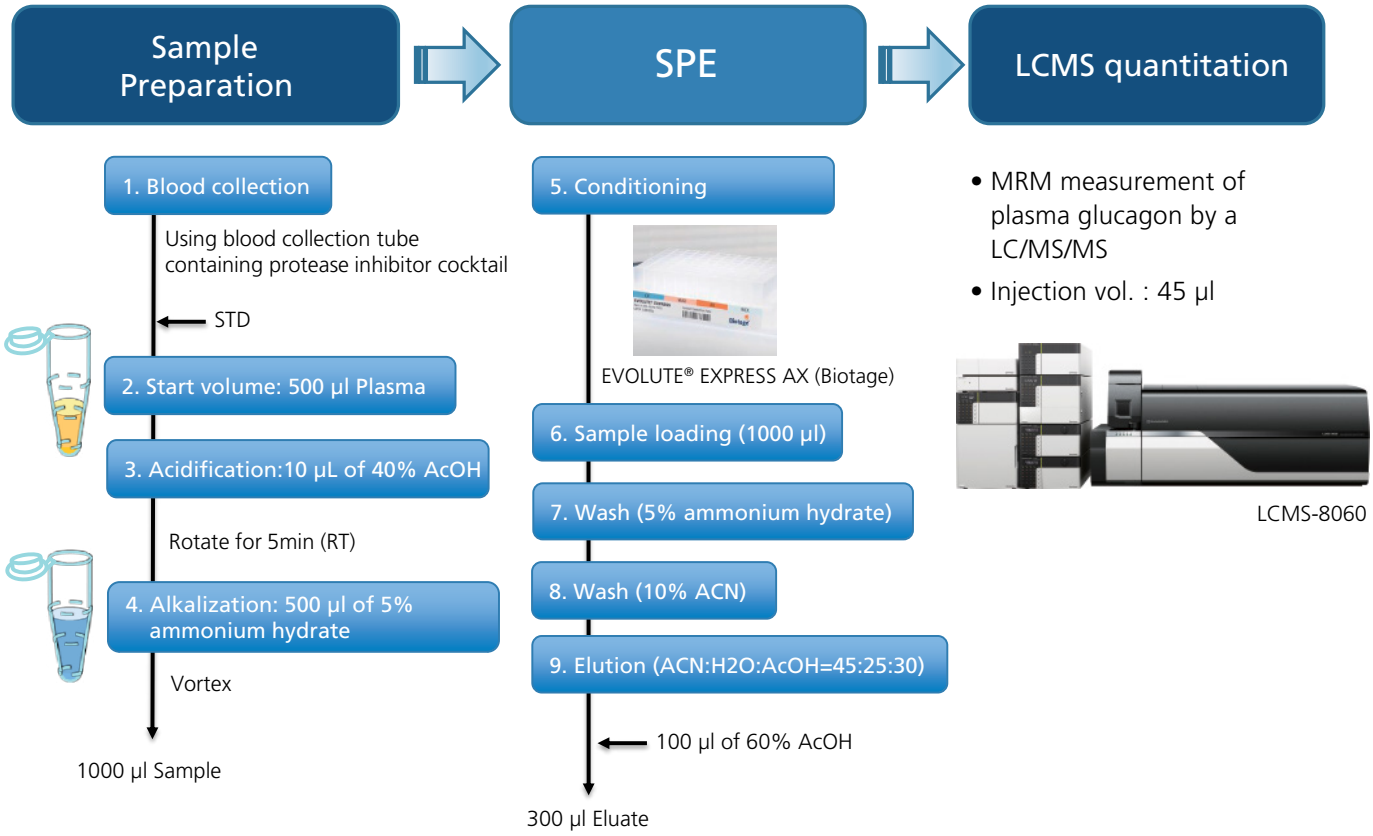


Figure 5. Procedure of sample preparation for glucagon in plasma sample

Selective and sensitive quantification of glucagon and glucagon-related peptide hormones in human plasma using conventional LC/MS/MS

Quantitative analysis of plasma sample spiked with glucagon

The lower limit of detection for glucagon was estimated as 2.5 pM (Figure 6). According to previous reports using conventional immunoassay, the normal level of plasma glucagon is approximately 10-50 pM. Thus, our results indicate that the method described here is potentially useful for quantification of endogenous glucagon.

- Sample: Glucagon spiked in pooled plasma
- 45 µL injection (n=2)
- Averaged accuracy was 99.8% (104.9% at 2.5 pM)

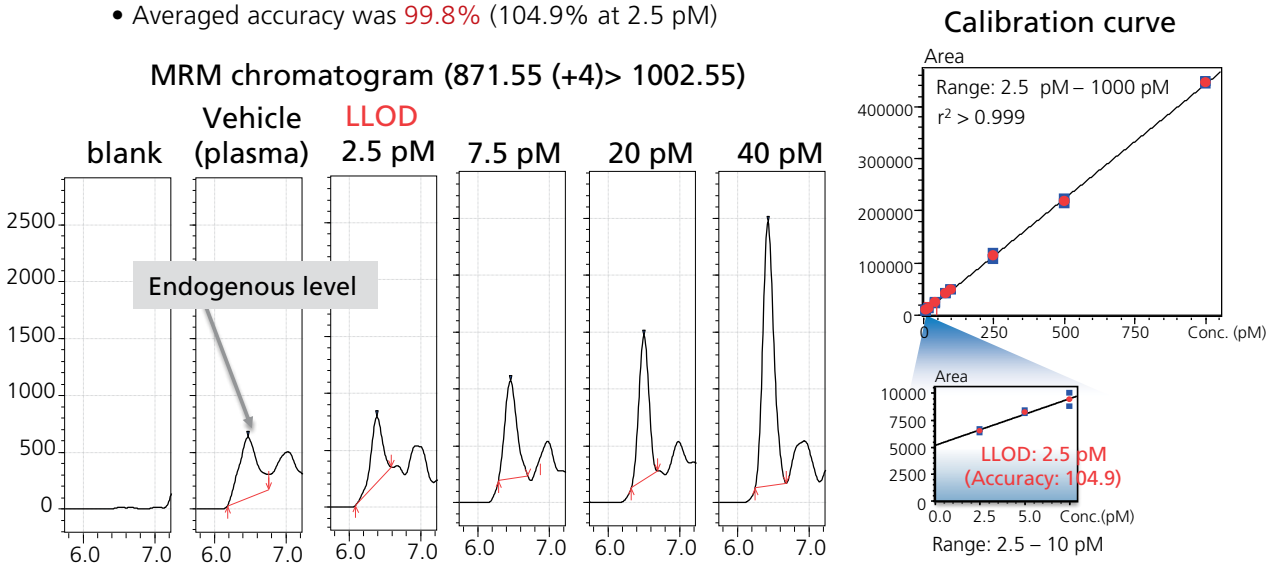


Figure 6. Performance of developed method using plasma sample spiked with glucagon.

Quantitative analysis of endogenous glucagon in healthy volunteers

Blood glucagon levels in fasting plasma is two-folds higher than casual plasma (Figure 7). From this result, enhanced secretion of glucagon under the fasting condition was confirmed since glucagon secretion is reported to upregulated in fasting state of healthy subjects.

Selective and sensitive quantification of glucagon and glucagon-related peptide hormones in human plasma using conventional LC/MS/MS

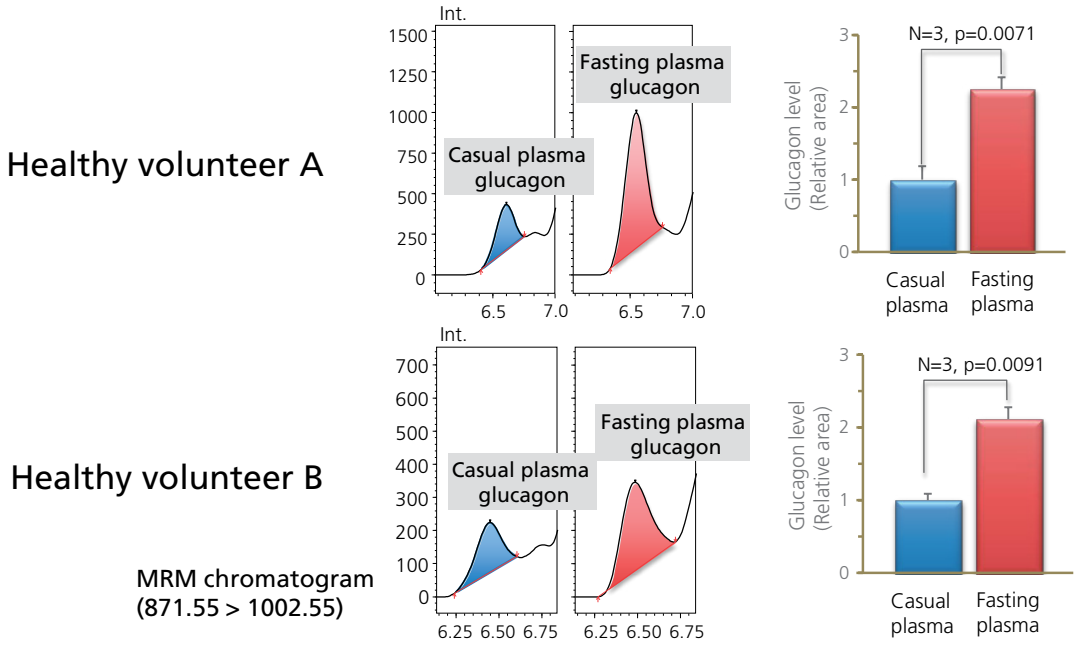


Figure 7. Glucagon levels in casual and fasting blood of healthy volunteers.

Conclusions

- Endogenous glucagon was successfully detected by the optimized sample preparation protocol and the sensitivity of the developed method.
- Under the fasting conditions in healthy subjects, glucagon secretion is known to be increased to maintain the blood glucose level. Developed methods clearly figured out this physiological change.

Disclaimer : LCMS-8060 is intended for Research Use Only (RUO). Not for use in diagnostic procedures.

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CHROMATOGRAPHICALLY RESOLVED-SENSITIVE METHOD FOR ESTIMATION OF OCTREOTIDE IN HUMAN PLASMA USING SHIMADZU LCMS-8060

Medha Wagh, Chaitanya Atmakuri, Dr. Avinash Gaikwad - Application Development Centre (SAIP)

INTRODUCTION

Octreotide is acetate salt of long-acting octapeptide with pharmacologic properties mimicking those of the natural hormone somatostatin[1]. Octreotide suppresses the secretion of serotonin and gastroenteropancreatic peptides (eg, gastrin, insulin, glucagon, secretin, motilin) along with growth hormone[2]. Octreotide is used for long-term maintenance therapy in acromegaly patients, in patients with metastatic carcinoid tumors and vasoactive intestinal peptide tumors (Vipomas)[3].

Long acting octreotide release therapy results in very low plasma levels and requires a sensitive method for estimation. Octreotide exhibits matrix interference due to the presence of residual phospholipids and the analytical method used for evaluation of analyte should ensure proper separation from such interferences.

We have therefore developed a method with high chromatographic resolution and ample sensitivity giving lowest limit of quantification (LLOQ) of 3 pg/mL for octreotide in human plasma using LCMS-8060. Method was developed keeping some key criteria in focus - namely simpler extraction process, highly optimized chromatography and enhanced sensitivity. These factors enable selective and high-throughput analysis of octreotide for the pharmacokinetic investigation.

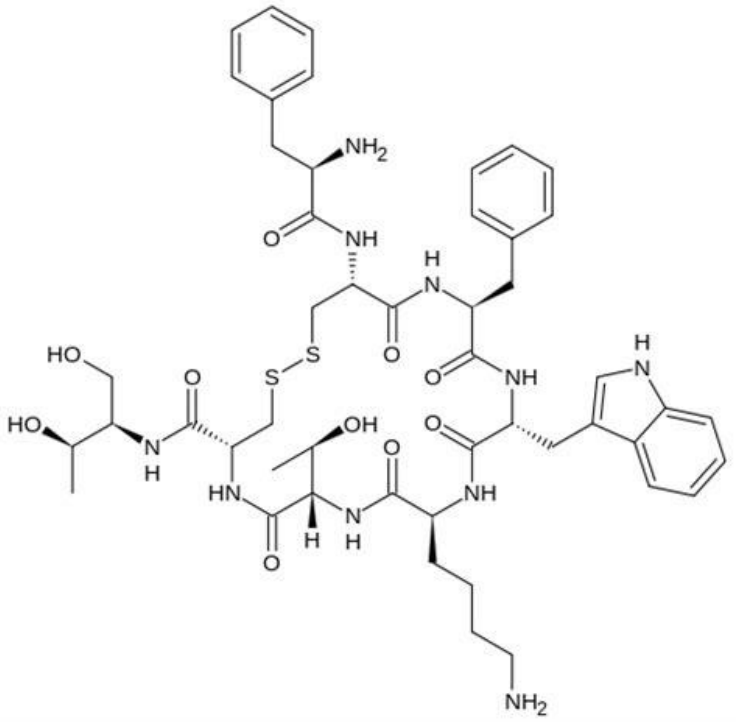

Figure 1. Structure of octreotide

SALIENT FEATURES

1- **Quantitative method** for estimation of **octreotide** in human plasma was developed. Method was **validated** as per USFDA Guidelines (results are presented in table 1).

2- Effective **throughput** for quantitative assessment is increased by use of a quick **single step extraction** procedure along with fast analysis of 5.5 min contributed by **Ultra-fast technologies** of **LCMS-8060**.

3- **Heated ESI** along with **New UF-Qarray** ion guide technology contributes by **increasing ion production and enhancing transmission respectively.** This ensures **sensitive and selective** quantification of octreotide at 3 pg/mL.

4- **Lesser plasma volume** (500 µL) avoided unnecessary wastage of plasma samples and at the same time increased the life of the mass spectrometer.

5- **Isocratic method**, gave consistent and reproducible peak area and retention time for the analyte and internal standard.

6- Method was validated for the following criteria:

   a. Selectivity
   b. Linearity
   c. Inter-day and intra-day precision and accuracy (PA)
   d. Matrix effect
   e. Recovery
   f. Carry-over effect
   g. Stability

Table 1. Method validation summary

Calibration curve range			3.001 pg/mL to 250.050 pg/mL	
Intraday precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)		100.91	
	Precision (% CV)		5.64	
Intraday precision and accuracy (For LQC, MQC, HQC)	Accuracy (% Nominal)		99.72 to 103.92	
	Precision (% CV)		3.01 to 6.07	
Global precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)		98.43	
	Precision (% CV)		18.96	
Global precision and accuracy (For LQC, MQC, HQC)	Accuracy (% Nominal)		100.71 to 102.78	
	Precision (% CV)		2.59 to 8.52	
Global % recovery	% Recovery		83.80	
	Precision (% CV)		5.82	
Matrix effect	Matrix Factor		1.01	
	IS normalized Matrix Factor		0.98	
Bench top stability in matrix (6.0 hrs.)	% Change		LQC=-1.19	HQC=0.10
	Precision (% CV)	Stability	LQC=4.45	HQC=2.50
		Comparison	LQC=3.51	HQC=1.68
	Accuracy (% Nominal)	Stability	LQC=99.80	HQC=104.05
		Comparison	LQC=101.00	HQC=103.95
Auto sampler stability in matrix (72.0 hrs.)	% Change		LQC=1.45	HQC=3.55
	Precision (% CV)	Stability	LQC=6.07	HQC=3.14
		Comparison	LQC=3.51	HQC=1.55
	Accuracy (% Nominal)	Stability	LQC=102.47	HQC=107.81
		Comparison	LQC=101.00	HQC=104.12
Freeze thaw stability in matrix (Fifth Cycle)	% Change		LQC=-5.56	HQC=-2.18
	Precision (% CV)	Stability	LQC=5.37	HQC=1.68
		Comparison	LQC=5.42	HQC=2.32
	Accuracy (% Nominal)	Stability	LQC=94.98	HQC=98.79
		Comparison	LQC=100.57	HQC=100.99
Long term matrix stability at -80 °C (12 days)	% Change		LQC=-7.21	HQC=-2.40
	Precision (% CV)	Stability	LQC=5.32	HQC=2.89
		Comparison	LQC=5.42	HQC=2.32
	Accuracy (% Nominal)	Stability	LQC=93.32	HQC=98.57
		Comparison	LQC=100.57	HQC=100.99
Extended batch (130 samples)	Precision (%)	LQC=10.17	MQC=2.15	HQC=2.00
	Accuracy (%)	LQC=107.33	MQC=100.10	HQC=103.83

METHOD VALIDATION RESULTS:

LINEARITY

Linearity experiments were conducted for octreotide quantitation in human plasma. Calibration curve was plotted from 3.001-250.050 pg/mL (refer table 2) with r^2> 0.997 as shown in figure 2. Signal to noise ratio (s/n) for 3.001 pg/mL was more than 13, across 5 PA batches. Representative chromatograms are shown in figure 3.

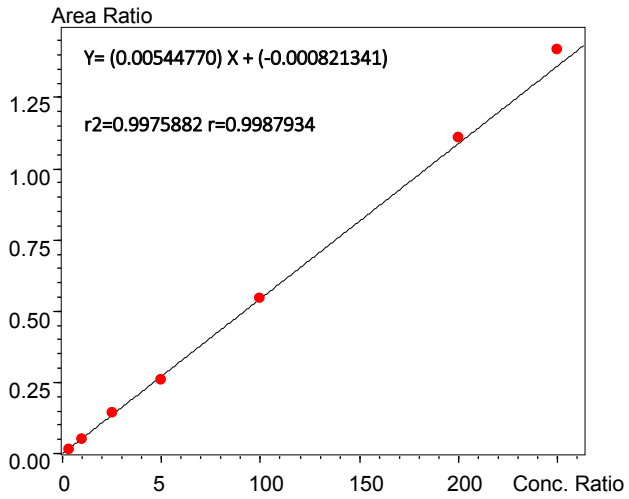

Figure 2. Calibration curve

Table 2.

Calibration curve range

Level	Nominal Conc
CC1	3.001
CC2	10.002
CC3	25.005
CC4	50.010
CC5	100.020
CC6	200.040
CC7	250.050

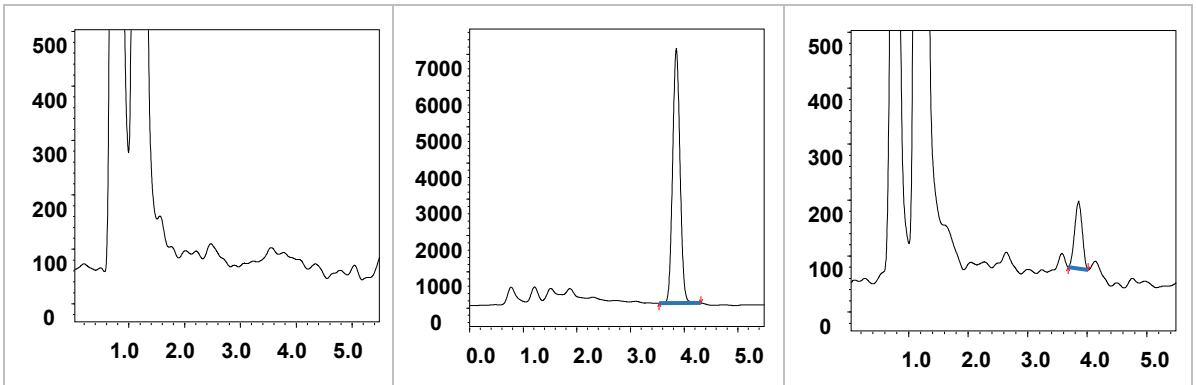

Figure 3. Chromatograms of octreotide (extracted blank, extracted IS, extracted LLOQ)

SELECTIVITY

Selectivity of this method, with respect to octreotide, was assessed in different lots of plasma. Interference from blank matrix was assessed for both octreotide and octreotide-D8 (refer figure 3) and no significant interference was detected as shown in table 3 below.

Table 3. Percentage interference in blank with respect to LLOQ

Plasma Lot no.	Octreotide			Octreotide -D8		
	Area in blank matrix	LLOQ area	% Interference	Area in blank matrix	IS area	% Interference
P0002	0	974	0	36	49,093	0.07
P0003	0	1,007	0	65	73,897	0.09
P0005	0	1,267	0	106	57,711	0.18
P0006	0	833	0	29	56,194	0.08
P0007	0	785	0	108	51,582	0.21
P0008	0	813	0	1,381	47,112	2.93

INTRA DAY PRECISION AND ACCURACY

Intraday precision and accuracy was conducted using 6 replicates of LLOQ-QC, LQC, MQC and HQC over one P&A batch. Figure 4 represents the stability of QC samples (X-axis represents number of QC injections and Y-axis represents calculated concentration in log scale). Quantitative data is summarised in table 4.

Table 4. Quantitative tabulation of intra-day QC assessment

QC (n=6)	LLOQQC	LQC	MQC	HQC
Nominal concentration	3.001	15.003	110.022	225.045
PA batch observed concentration (pg/mL)	3.039	17.198	111.027	228.809
	2.869	16.233	112.529	238.356
	2.775	14.933	112.151	245.809
	3.164	14.758	111.66	226.708
	3.195	15.208	105.49	231.757
	3.128	15.145	105.453	231.756
Mean	3.0283	15.5792	109.7183	233.8658
Standard deviation	0.1708	0.9451	3.3278	7.0499
% CV	5.64	6.07	3.03	3.01
% Nominal	100.91	103.84	99.72	103.92

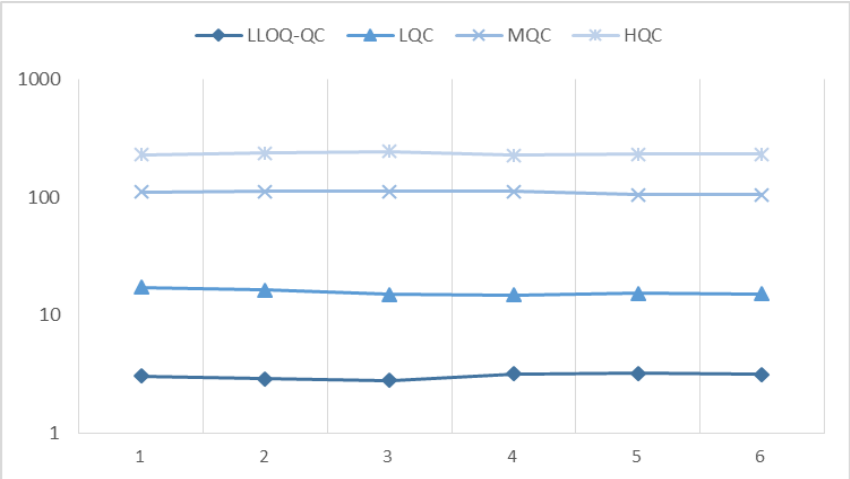

Figure 4. Trend plot of intra-day QC assessment.

GLOBAL PRECISION AND ACCURACY

Precision and accuracy experiments were conducted in 5 batches. Excellent accuracy and repeatability was observed with % CV < 8.52 % and % accuracy between 100.71- 102.78 % for LQC, MQC and HQC. At LLOQQC, the % CV was found to be 18.96 % and % accuracy as 98.43 %. The results are presented in table 5. Figure 5 shows trend plots of 30 QC at each level, analysed over 5 batches.

Table 5. Quantitative tabulation of inter-day QC assessment

QC level (n=30)	Mean Conc. (pg/mL)	% CV	% Accuracy
LLOQ QC (3.001 pg/mL)	2.9539	18.96	98.43
LQC (15.003 pg/mL)	15.1446	8.52	100.94
MQC (110.022 pg/mL)	110.8011	3.40	100.71
HQC (225.045 pg/mL)	231.2936	2.59	102.78

Note: % CV is calculated including outliers

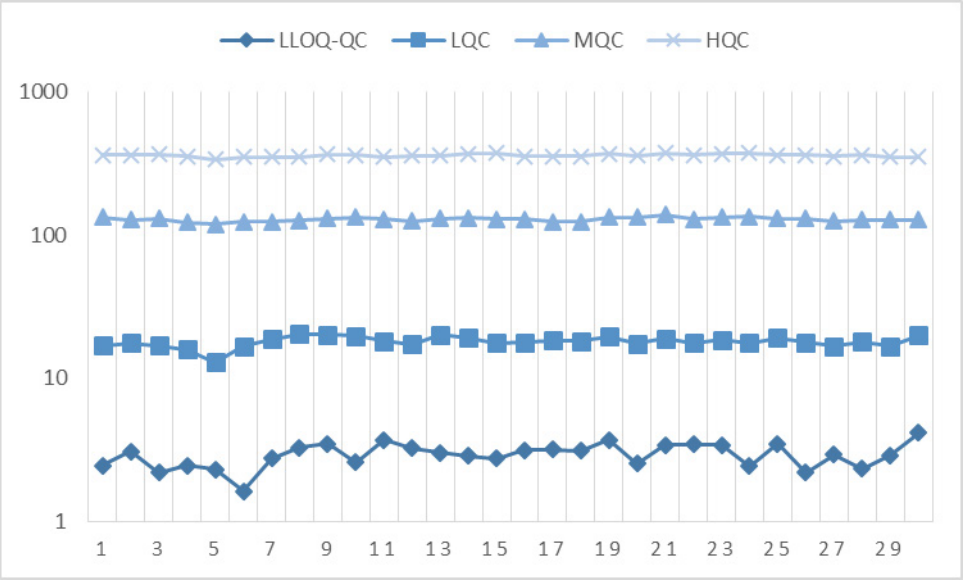

Figure 5. Trend plot of inter-day QC assessment.

X-axis represents the number of injections and Y-axis represents calculated concentration (log scale)

MATRIX EFFECT

Matrix effect was studied for both octreotide and octreotide-D8 using LQC and HQC samples. Mean matrix factor and IS normalised matrix factor was found to be 1.01 and 0.98 respectively. Representative data of matrix effect is shown in table 6. The results confirm the suitability of method for quantitative estimation in human plasma.

Table 6. Quantitative tabulation of matrix effect

	Response of aqueous standard	Response of post extracted spike sample	Matrix factor
LQC	8,483	7,689	0.91
	8,008	9,048	1.13
	7,200	7,219	1.00
	6,668	7,229	1.08
	7,132	6,778	0.95
	7,143	6,347	0.89
HQC	113,944	111,067	0.97
	115,937	117,556	1.01
	113,723	108,219	0.95
	108,817	112,321	1.03
	107,868	116,258	1.08
	103,137	119,258	1.16
Mean matrix factor of octreotide			1.01
Standard deviation			0.09
% CV			8.41

RECOVERY

Recovery experiments were conducted to judge combined effect of sample extraction efficiency and instrument method. Average recovery was found to be 83.80 % as shown in table 7.

Table 7. Quantitative tabulation of % recovery

LQC	78.17
MQC	86.76
HQC	86.48
Mean	83.80
SD	4.88
% CV	5.82

CARRY OVER EFFECT

Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was present/observed at the retention time and MRM transition of the analyte in the extracted blank sample following the highest standard calibrator.

OTHER EXPERIMENTATION

Based on validation guidelines, method was assessed for following criteria.

1- Bench top stability

2- Auto sampler stability

3- Freeze thaw stability

4- Long term stability at -80 °C

5- Extended batch verification

CONCLUSION

LCMS-8060, along with special sample preparation and optimized chromatography provides a very selective and sensitive method for bioanalytical assay of octreotide. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple isocratic mobile phase at a minimal flow rate of 0.5 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavours.

REFERENCES

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3. https://www.rxlist.com/sandostatin-lar-drug.htm#indications_dosage (accessed Jan 15, 2018).

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Application News

No. SAIP/ADC/B-007

Pharma CRO / LCMS-8060

SELECTIVE AND SENSITIVE METHOD FOR ESTIMATION OF LIRAGLULTIDE IN HUMAN PLASMA USING SHIMADZU LCMS-8060

Yogesh Arote, Chaitanya Atmakuri, Dr. Avinash Gaikwad

❑ Introduction

Liraglutide is a glucagon-like peptide-1 receptor agonist (GLP-1 receptor agonist) also known as incretin mimetics. It works by increasing insulin release from the pancreas and decreases excessive glucagon release [(1)]. Liraglutide is a medication used for treatment of type 2 diabetes or obesity. The prolonged action of liraglutide is achieved by attaching a fatty acid molecule at one position of the GLP-1-(7-37) molecule, enabling it to both self-associate and bind to albumin with in the subcutaneous tissue and blood stream. The active GLP-1 is then released from albumin at a slow, consistent rate. Albumin binding also results in slower degradation and reduced renal elimination compared to GLP-1-(7-37).

Following subcutaneous administration, a mean C max of 35 ng/mL was achieved after 8-12 hours of dosing with an absolute bioavailability of 55 %. It indicates that the method required for pharmacokinetic evaluations need to achieve a sensitivity limit of 0.50 ng/mL.

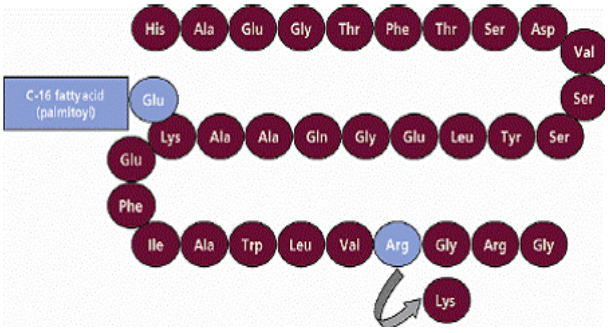

Figure 1. Structure of liraglutide [(2)]

Such method should address many problems posed by peptides viz., poor ionization, non-specific adsorption, carry-over and low extraction recovery.

We have therefore developed a method with high chromatographic resolution and ample sensitivity giving lowest limit of quantification (LLOQ) of 0.50 ng/mL for liraglutide in human plasma using LCMS-8060. Method was developed keeping some key criteria in focus- namely simpler extraction procedure, highly optimized chromatography and enhanced sensitivity. These factors enable selective and high-throughput analysis of liraglutide for the pharmacokinetic investigation.

❑ Salient features

- Quantitative method for estimation of liraglutide in human plasma was developed and validated as per USFDA Guidelines (except matrix effect and stability experiment). For validation results refer table 1
- Effective throughput for quantitative assessment is increased by use of a quick single step extraction procedure.
- Heated ESI along with New UF-Qarray ion guide technology contributes by increasing ion production and enhancing transmission respectively. This ensures sensitive and selective quantification of liraglutide at 0.50 ng/mL.
- Less plasma volume (200 µL) avoided unnecessary wastage of plasma samples and at the same time increased the life of the mass spectrometer.
- Customised gradient method enhanced the chromatographic resolution of liraglutide with consistent and reproducible peak area and retention time
- Method was validated for the following criteria:
  - ✓ Selectivity
  - ✓ Linearity
  - ✓ Inter-day and intra-day precision and accuracy (PA)
  - ✓ Recovery
  - ✓ Carry-over effect

Table 1 Method validation summary

Calibration curve range		0.50 ng/mL to 202.70 ng/mL
Intraday precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)	102.63
	Precision (% CV)	14.52
Intraday precision and accuracy (For LQC, MQC-2, MQC-1 and HQC)	Accuracy (% Nominal)	96.10 to 101.01
	Precision (% CV)	2.85 to 12.79
Global precision and accuracy (For LLOQ-QC)	Accuracy (% Nominal)	109.98
	Precision (% CV)	18.52
Global precision and accuracy (For LQC, MQC-2, MQC-1 and HQC)	Accuracy (% Nominal)	102.40 to 106.78
	Precision (% CV)	7.45 to 13.09
Global % recovery	Recovery (%)	50.92
	Precision (% CV)	13.06

❑ Results and Discussion

A. Linearity

Linearity was assessed by 3 calibration curves in human plasma on 3 consecutive days. Calibration curve was for liraglutide was linear from 0.5 - 200 ng/mL (refer table 2). Correlation coefficient (r^2) of liraglutide was found to be > 0.98 as shown in figure 2. Signal to noise ratio (s/n) at 0.5 ng/mL was greater than 10:1, across 3 PA batches. Representative chromatograms are shown in Figure 3.

Table 2 Calibration curve range

Level	Back Cal. Conc.
CC1	0.52
CC2	0.77
CC3	1.46
CC4	3.80
CC5	10.26
CC6	27.12
CC7	50.50
CC8	103.41
CC9	151.04
CC10	202.83

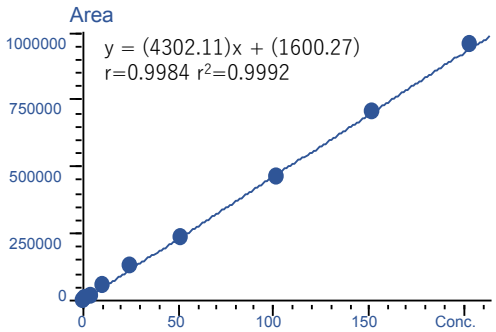

Fig.2 Calibration curve

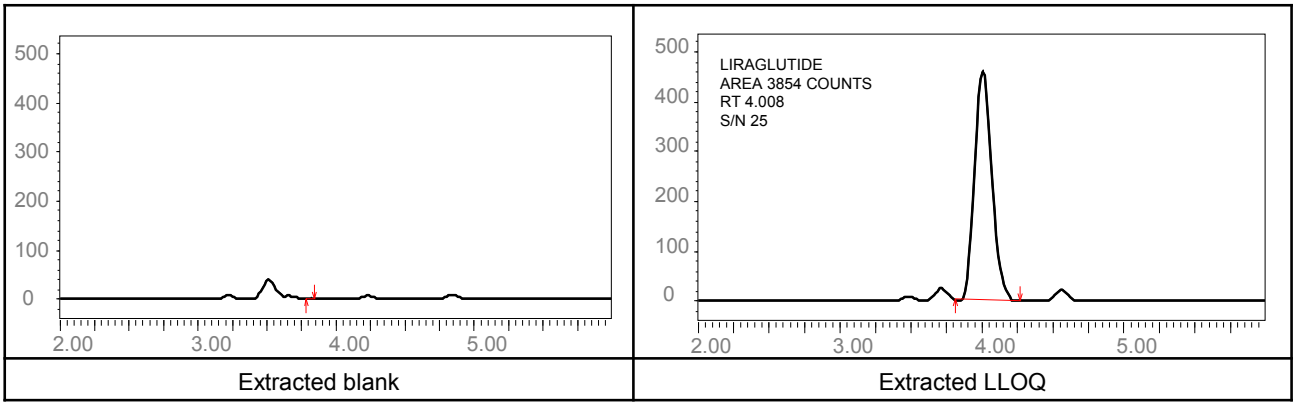

Fig.3 Chromatograms of liraglutide (extracted blank and extracted LLOQ)

B. Selectivity

Selectivity of the method was evaluated by processing and analyzing 6 different lots of blank human plasma and blank human plasma spiked with liraglutide at LLOQ level. No significant interference was observed at the retention time and MRM transition of analyte (refer figure 3).

Table 3. Percentage interference in blank with respect to LLOQ

	Liraglutide		
Plasma lot no	Area in blank matrix	LLOQ area	Interference (%)
V2533	335	1,699	19.72
V2528	169	3,597	4.70
P8072	54	2,880	1.88
V2534	510	2,842	17.95
V2537	87	2,049	4.25
P8200	189	3,961	4.77

C. Intra-day precision and accuracy

Intraday precision and accuracy was evaluated on 6 replicates samples at LLOQ-QC, LQC, MQC and HQC in one P&A batch. Figure 4 represents the consistency of QC samples (X-axis represents number of QC injections and Y-axis represents calculated concentration in log scale). Quantitative data is summarized in table 4.

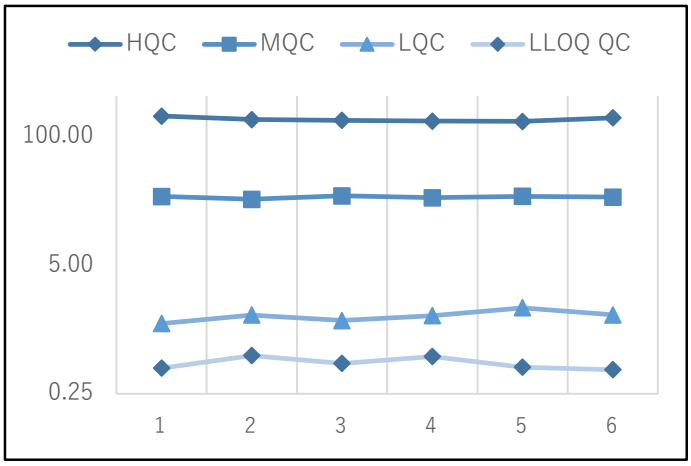

Fig 4. Trend plot of intra-day QC assessment

Table 4. Statistical calculation for liraglutide intra-day precision and accuracy batch

QC (n=6)	LLOQQC	LQC	MQC	HQC
Nominal concentration	0.50	1.52	25.39	152.03
PA batch observed concentration (ng/mL)	0.46	1.29	24.79	161.22
	0.61	1.57	23.20	148.50
	0.51	1.38	25.12	146.16
	0.60	1.55	24.05	143.41
	0.47	1.86	24.84	142.15
	0.44	1.57	24.41	154.97
Mean	0.51	1.54	24.40	149.40
Standard deviation	0.07	0.20	0.70	7.35
% CV	14.52	12.79	2.85	4.92
% Nominal	102.63	101.01	96.10	98.27

D. Global precision and accuracy

Global precision and accuracy were evaluated on 3 batches. Excellent precision and accuracy were observed with % CV < 13.09 % and % accuracy between 102.40 to 106.78 % at LQC, MQC and HQC level. At LLOQQC level, the % CV was found to be 18.52 % and % accuracy was found to be 109.98 %. The results are presented in table 5. Figure 5 shows trend plots of 18 QC at each level, analysed over 3 batches.

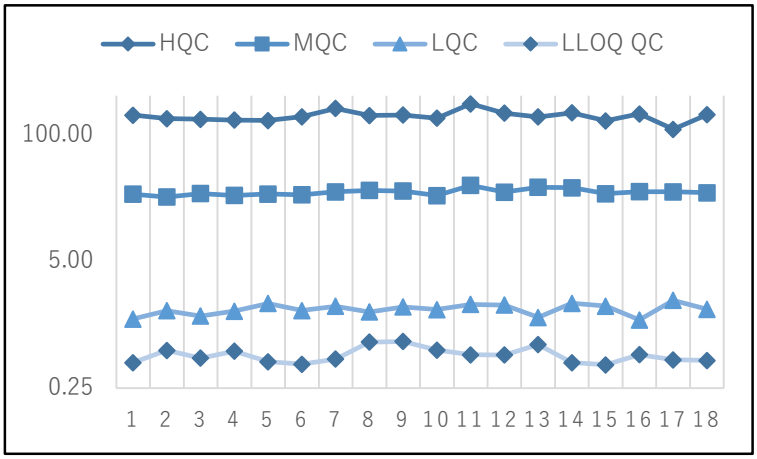

Fig 5. Trend plot of inter-day QC assessment.
X-axis represents the number of injections and Y-axis represents calculated concentration (log scale)

Table 5. Statistical calculation for liraglutide inter-day precision and accuracy batch

QC level (n=18)	Mean Conc. (ng/mL)	% CV	% Accuracy
LLOQ QC (0.50 ng/mL)	0.55	18.52	109.98
LQC (1.52 ng/mL)	1.62	13.09	106.78
MQC (25.39 ng/mL)	26.00	7.45	102.40
HQC (152.03 ng/mL)	158.18	12.87	104.04

E. Recovery

Recovery experiments was conducted at LQC, MQC and HQC level. Mean recovery of liraglutide is found to be 50.92%. Recovery of liraglutide is found to be precise, consistent and reproducible at all QC levels.

F. Carry-over effect

Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was present/observed at the retention time and MRM transition of the analyte in the extracted blank sample following the highest standard calibrator.

❑ Conclusion

LCMS-8060, along with special sample preparation, optimized chromatography provides a very selective and sensitive method for bioanalytical assay of liraglutide. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple mobile phase at a minimal gradient flow rate of 0.25 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavours.

❑ References

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Application News

No. SAIP/ADC/B-008

Pharma CRO / LCMS-8045

SIMPLE AND SENSITIVE METHOD FOR ESTIMATION OF LEUPROLIDE IN HUMAN PLASMA USING SHIMADZU LCMS-8045

Yogesh Arote, Chaitanya Atmakuri, Dr. Avinash Gaikwad

❑ Introduction

Leuprolide acetate is a synthetic nonapeptide analog of naturally occurring gonadotropin releasing hormone (GnRH or LH-RH). The analog possesses greater potency than the natural hormone. The chemical name is 5-oxo-L-prolyl-L-histidyl-L-tryptophyl-L-seryl-L-tyrosyl-D-leucyl-L-leucyl-L-arginyl-N-ethyl-L-prolinamide acetate (salt) and the structure is provided in figure 1 [(1)].

Leuprolide also known as Leuprorelin acetate, is orally inactive and generally given subcutaneously or intramuscularly. Leuprorelin may be used in the treatment of hormone-responsive cancers such as prostate cancer and breast cancer. It may also be used for estrogen-dependent conditions such as endometriosis or uterine fibroids. It may be used for precocious puberty in both males and females, and to prevent premature ovulation in cycles of controlled ovarian stimulation for in vitro fertilization. It may be used to reduce the risk of premature ovarian failure in women receiving cyclophosphamide for chemotherapy [(2)].

The peptide drug is released from the depot formulations at a functionally constant daily rate for 1, 3 or 4 months, depending on the polymer type [polylactic/glycolic acid (PLGA) for a 1-month depot and polylactic acid (PLA) for depot of >2 months], with doses ranging between 3.75 and 30mg.

Mean peak plasma leuprorelin concentrations of 13.1, 20.8 to 21.8, 47.4, 54.5 and 53 µg/L occur within 1 to 3 hours of depot subcutaneous administration of 3.75, 7.5, 11.25, 15 and 30 mg, respectively, compared with 32 to 35 µg/L at 36 to 60 min after a subcutaneous injection of 1mg of a non-depot formulation. Sustained drug release from the PLGA microspheres maintains plasma concentrations between 0.4 and 1.4 µg/L over 28 days after single 3.75, 7.5 or 15mg depot injections [(3)]. It indicates that the method required for pharmacokinetic evaluations need to achieve a sensitivity limit of sub-picogram level as low as 400 pg/mL.

Such method should address many problems posed by peptides viz., poor ionization, non-specific adsorption, carry-over and low extraction recovery.

We have therefore developed a method with high chromatographic resolution and ample sensitivity giving lowest limit of quantification (LLOQ) of 25 pg/mL for leuprolide in human plasma using LCMS-8045. Method was developed keeping some key criteria in focus- namely simpler extraction procedure, highly optimized chromatography and enhanced sensitivity. These factors enable selective and high-throughput analysis of leuprolide for the pharmacokinetic investigation.

❑ Salient features

- Quantitative method for estimation of leuprolide in human plasma was developed. Method was validated as per USFDA Guidelines (except stability experiment), results are presented in table 1.
- Effective throughput for quantitative assessment is increased by use of a quick simple extraction procedure.
- Heated ESI along with New UF-Qarray ion guide technology contributes by increasing ion production and enhancing transmission respectively. This ensures sensitive and selective quantification of leuprolide at 25 pg/mL.
- Lesser plasma volume (200 µL) avoided unnecessary wastage of plasma samples and at the same time increased the life of the mass spectrometer.
- Customised gradient method enhanced the chromatographic resolution of leuprolide with consistent and reproducible peak area and retention time.
- Method was validated for the following criteria:
  - ✓ Selectivity
  - ✓ Linearity
  - ✓ Inter-day and intra-day precision and accuracy (PA)
  - ✓ Recovery
  - ✓ Matrix effect
  - ✓ Carry over effect

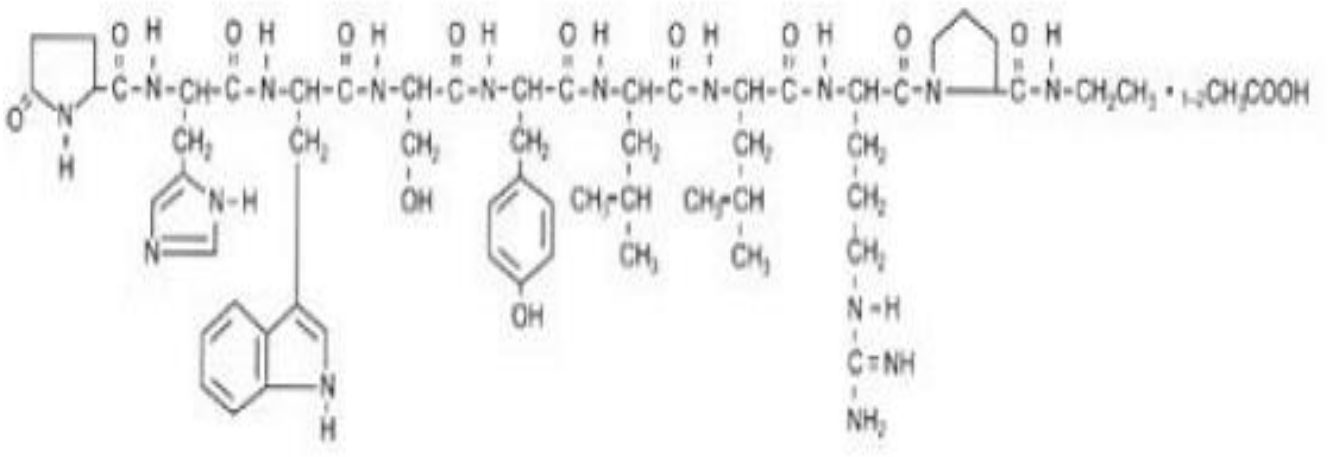

Fig.1 Structure of leuprolide

Table 1. Method validation summary

Calibration curve range		0.025 ng/mL to 30.000 ng/mL
Intraday precision and accuracy (For LLOQ QC)	Accuracy (% Nominal)	117.33
	Precision (% CV)	15.50
Intraday precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	104.64 to 107.45
	Precision (% CV)	1.97 to 6.08
Global precision and accuracy (For LLOQ QC)	Accuracy (% Nominal)	111.11
	Precision (% CV)	16.13
Global precision and accuracy (For LQC, MQC and HQC)	Accuracy (% Nominal)	102.17 to 105.27
	Precision (% CV)	3.00 to 6.37
Global % recovery	Recovery (%)	73.50
	Precision (% CV)	5.08
Matrix effect	IS normalized Matrix Factor	0.92

❑ Results and Discussion

A. Linearity

Linearity was assessed by 3 calibration curves in human plasma on 3 consecutive days. Calibration curve for leuprolide was linear from 0.025 - 30.000 ng/mL (refer table 2). The correlation coefficient (r^2) for leuprolide was found to be > 0.98 as shown in figure 2. Signal to noise ratio (s/n) for 0.025 ng/mL was more than 15:1, across 3 PA batches. Representative chromatograms are shown in figure 3.

Table 2. Calibration curve range

Level	Cal. Conc
CC1	0.025
CC2	0.100
CC3	0.200
CC4	1.020
CC5	3.070
CC6	5.120
CC7	10.800
CC8	20.410
CC9	30.000

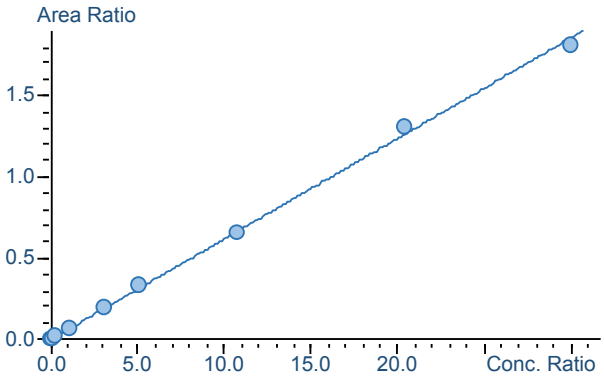

Fig.2 Calibration curve

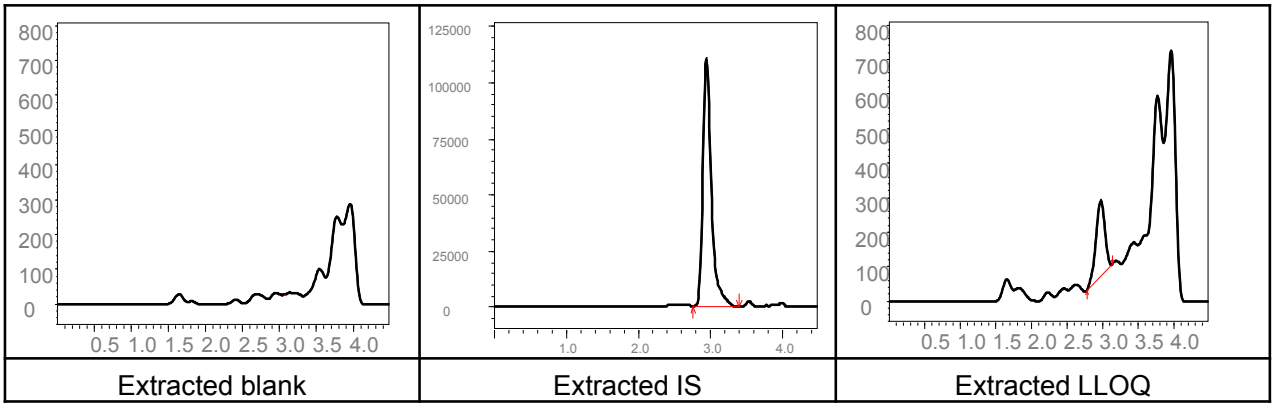

Fig.3 Chromatograms of leuprolide (extracted blank, extracted IS, extracted LLOQ)

B. Selectivity

Selectivity of the method was evaluated by processing and analyzing 6 different lots of blank human plasma and blank plasma spiked with leuprolide at LLOQ level. No significant interference was observed at the retention time and MRM transition of analyte (refer figure 3).

Table 3. Percentage interference in blank with respect to LLOQ

	Leuprolide			Deuterated IS		
Plasma lot no.	Area in blank matrix	LLOQ area	% Interference	Area in blank matrix	IS area	% Interference
V3071	159	1,362	11.67	205	93,692	0.22
V1889	267	1,416	18.86	243	86,611	0.28
V1789	208	1,912	10.88	638	89,217	0.72
V1166	0	1,371	0.00	690	89,838	0.77
V3074	374	2,051	18.24	574	91,397	0.63
V3077	168	1,267	13.26	864	89,865	0.96

C. Intra-day precision and accuracy

Intraday precision and accuracy was conducted on 6 replicates samples at LLOQQC, LQC, MQC and HQC over one P&A batch. Figure 4 represents the consistency of QC samples (X-axis represents number of QC injections and Y-axis represents calculated concentration in log scale). Quantitative data is summarized in table 4.

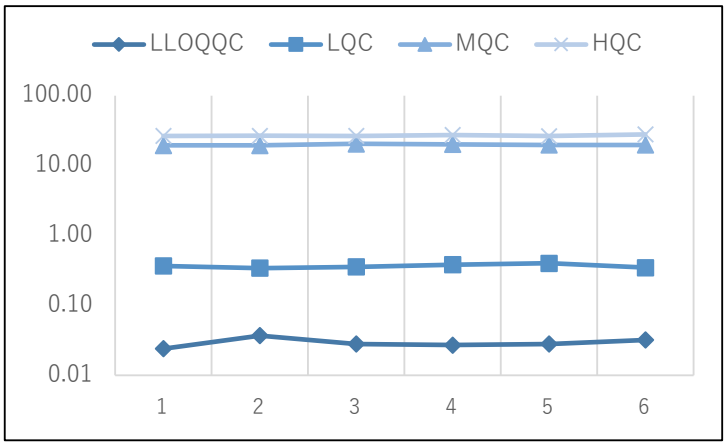

Fig 4. Trend plot of intra-day QC assessment

Table 4. Statistical calculation for leuprolide intra-day precision and accuracy batch

QC (n=6)	LLOQQC	LQC	MQC	HQC
Nominal concentration (ng/mL)	0.03	0.34	18.90	25.05
PA batch observed concentration (ng/mL)	0.02	0.37	19.42	26.55
	0.04	0.34	19.39	26.63
	0.03	0.36	20.39	26.56
	0.03	0.38	20.08	27.32
	0.03	0.40	19.74	26.36
	0.03	0.35	19.65	27.94
Mean	0.03	0.37	19.78	26.89
Standard deviation	0.00	0.02	0.39	0.61
% CV	15.50	6.08	1.97	2.27
% Nominal	117.33	107.45	104.64	107.36

D. Global precision and accuracy

Global precision and accuracy were evaluated on 3 batches. Excellent precision and accuracy was observed with % CV < 6.37 % and percent accuracy between 102.17 % to 105.27% at LQC, MQC and HQC level. At LLOQ QC level, the % CV was found to be 16.13 % and percent accuracy 111.11 %. The results are presented in table 5. Figure 5 shows trend plots of 18 QC at each level, analyzed over 3 batches.

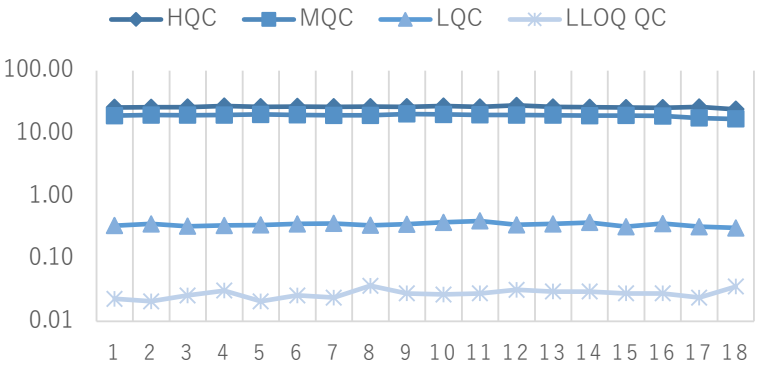

Fig 5. Trend plot of inter-day QC assessment.
X-axis represents the number of injections and Y-axis represents calculated concentration (log scale)

Table 5. Statistical calculation for leuprolide inter-day precision and accuracy batch

QC level (n=18)	Mean Conc. (pg/mL)	% CV	% Accuracy
LLOQ QC (0.025 ng/mL)	0.03	16.13	111.11
LQC (0.34 ng/mL)	0.35	6.37	103.42
MQC (18.90 ng/mL)	19.31	4.24	102.17
HQC (25.05 ng/mL)	26.37	3.00	105.27

E. Matrix effect

Matrix effect was studied for both leuprolide and deuterated internal standard at LQC and HQC levels. Mean matrix factor and IS normalised matrix factor was found to be 1.05 and 1.06 respectively.

Representative data for matrix effect is shown in Table 6. The results confirm the suitability of the method for quantitative estimation of leuprolide in human plasma.

Table 6. Quantitative tabulation for checking of matrix effect

	Response ratio of aqueous standard	Response ratio of post extracted spike sample	Matrix factor
LQC	0.019	0.020	1.01
	0.020	0.018	0.90
	0.017	0.017	0.96
	0.019	0.018	0.95
	0.018	0.018	1.04
	0.020	0.019	0.93
HQC	1.294	1.167	0.90
	1.305	1.158	0.89
	1.364	1.156	0.85
	1.364	1.152	0.84
	1.335	1.149	0.86
	1.360	1.165	0.86
Mean matrix factor of leuprolide			0.92
Standard deviation			0.06
% CV			6.94

F. Recovery

Recovery experiment was evaluate at LQC, MQC and HQC level. Mean recovery of leuprolide is found to be 73.50%. Recovery of leuprolide is found to be precise, consistent and reproducible at all QC levels.

G. Carry-over effect

Carryover was evaluated by injecting extracted samples in the sequence of extracted blank, extracted highest calibrator, extracted blank and extracted lowest calibrator. No carryover was present/observed at the retention time and MRM transition of the analyte in the extracted blank sample following the highest standard calibrator.

❑ Conclusion

LCMS-8045, along with special sample preparation, optimized chromatography provides a very selective and sensitive method for bioanalytical assay of leuprolide. Ultra-high speed and high-separation analysis was achieved on Nexera X2 UHPLC by using a simple mobile phase at a minimal gradient flow rate of 0.5 mL/min. By providing these ready to use solutions, we partner with your labs to achieve desired results in your scientific endeavours.

❑ References

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Application News

No. AD-0078

LCMS-8040 UFMS

LC/MS/MS Method for Quantitative Determination of Ethinyl Estradiol in Human Plasma

Manikandan Venkateswaran, Vinod Pillai. S.L, Venkat Manohar and Arvind Thyagarajan
*Indian Institute of Chromatography & Mass Spectrometry (IICMS)**

Abstract

An ultra sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for quantitative analysis of oral contraceptive Ethinyl estradiol (EE) has been developed in human plasma using UHPLC Nexera coupled to LCMS-8040 triple quadrupole mass spectrometer and described here. A two stage sample clean-up and also off-line derivatization using Dansyl chloride enabled ultra sensitivity up to 1.0 pg/mL using 750 µL of human plasma. The method was found to be linear in the range of 1 - 200 pg/mL.

❑ Introduction

Ethinyl estradiol (EE) is a potent synthetic estrogen that is widely used therapeutically as an oral contraceptives primarily because of its high estrogenic activity. It is also used for treatment of menopausal and post menopausal symptoms, treatment of female hypogonadism, osteoporosis and as a palliative care treatment in malignant neoplasms of breast and prostrate. A very sensitive method for estimation of EE from human plasma using the UHPLC Nexera connected to a LCMS-8040 triple quadrupole mass spectrometer is described here. Ethinyl estradiol (EE) and the internal standard (Ethinyl estradiol-D4) were extracted from plasma matrix using hexane: methyl-tert-butyl ether (MTBE) mixture (50:50 v/v), derivatized with Dansyl chloride, then further cleaned up using Sola CX (10 mg/mL) SPE cartridge and injected onto LC/MS/MS system. Chromatographic separation of EE from other related estrogenic peaks were achieved using a Purospher star RP18, 100 x 2.1mm, 2.0µ column with a 0.1% Formic acid in water and ACN as mobile phase. The offline derivatization procedure using the Dansyl chloride lead to the introduction of easily ionizable tertiary amino group in the EE moiety that greatly improved the analyte sensitivity in the electrospray ionization and enabled achieving the desired lower limit of quantitation of 1.0 pg/mL.

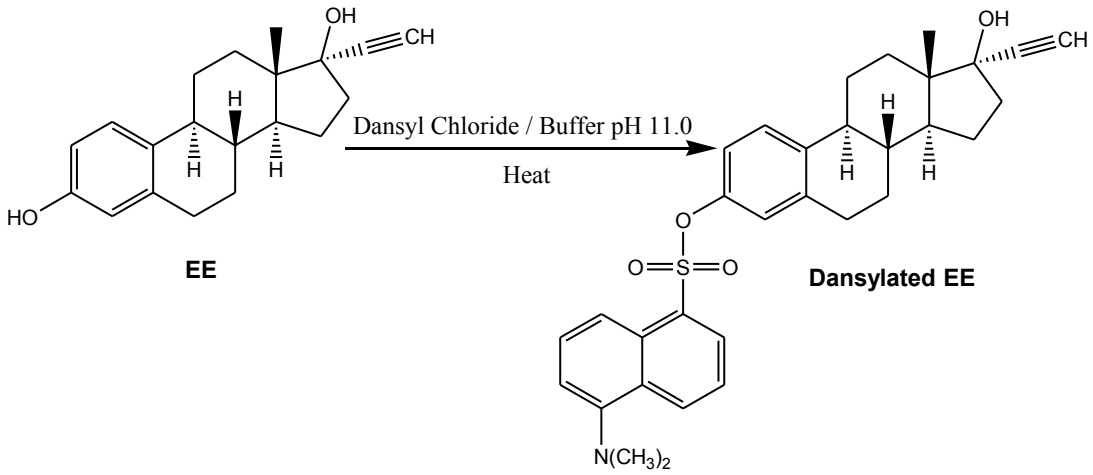

Figure 1: Chemical structures of Ethinyl Estardiol (EE) and its dansylated product

❑ Experimental

Preparation of Aqueous Standards: Stock solutions of EE was prepared separately at 1.0 mg/mL concentration in methanol. The solution was further diluted with water : methanol (50:50) mixture to get intermediate standards at various concentrations ranging from 20-4000 pg/mL. Similarly, stock solution of Ethinyl estradiol-D4 (IS) was prepared at 1.0 mg/mL concentration in methanol. This solution was further diluted with water : methanol (50:50) mixture to get intermediate standard at concentration of 2000 pg/mL.

Preparation of Plasma Calibration Standards (CC): 713 µL of human plasma was spiked with 37.5 µL of each aqueous EE standard solution and 50 µL of IS solution in a polypropylene (PP) tube with cap, vortexed for 15 seconds to obtain plasma calibration standard whose concentration ranged from 1.0 – 200.0 pg/mL. Each of these samples was then extracted according to the procedure as described under sample preparation. Care was taken to make sure that each vial was immediately capped to avoid contamination.

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Preparation of Plasma Quality Control Standards (QC): The quality control standard solutions were prepared at three intermediate concentrations of CC standards namely 3.0, 90.0 and 180.0 pg/mL (LQC, MQC and HQC respectively). Six individual preparations of each of the QC standards were prepared to evaluate precision and recovery. Each of these sample preparation was then extracted according to the procedure as described under sample preparation.

Sample Preparation: Ethinyl estardiol has only a limited ionization in the ESI source. Hence, in order to enhance ionization, a derivatization with dansyl chloride has been suggested in the literature. A liquid-liquid extraction was used first to extract the drug from the plasma matrix followed by derivatization with dansyl chloride in a water bath. After dansylation, the sample was cleaned up through additional SPE step and then introduced onto the LC/MS/MS system. By way of a two stage sample cleanup, most of the phospholipids from the plasma matrix was removed and other closely related estrogenic compounds that are present were either removed or well resolved from the EE.

To 750 µL of plasma sample in a PP vial, 100 µL of 0.1M hydrochloric acid was added and mixed briefly for 10s. Then, 2.5 mL of mixture of Hexane and MTBE in the ratio of 50:50 v/v was added. The drug was extracted by vortexing for 15 minutes followed by centrifugation for 5 minutes at 4000 rpm. Two mLs of the top organic layer was removed and evaporated using an Xcelvap solvent evaporator at 45˚C under stream of nitrogen. To the residue, 100 µL of sodium carbonate solution (100mM) was added followed by 150 µL of dansyl chloride solution (0.1 mg/mL in acetone). The mixture was heated in a water bath at 60˚C for 15 minutes. The extracts were mixed with 500 µL of water and then transferred to a Sola CX (10 mg/mL) cartridge that has been already pre-conditioned and equilibrated with 1000 µL each of methanol and water. The cartridge was washed with 1000 µL each of 5% methanol in water (two times) followed by 20% methanol in water and eluted with 2 x 200 µL of Acetonitrile and 2-propanol mixture in the ratio of 90:10 v/v. The eluate was directly injected into the LC/MS/MS system.

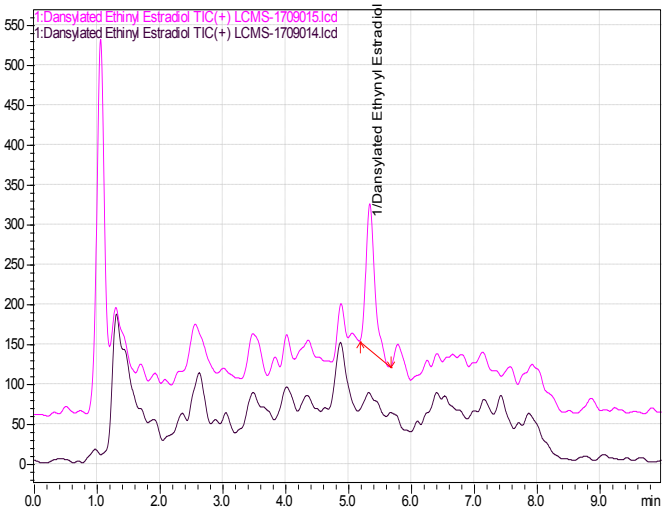

Figure 2: Representative overlay chromatograms of EE at LLOQ with blank

The LC-MS conditions are summarized in Table 1

Table 1: Analytical conditions

Column	: Puropsher star RP18, 100 x 2.1mm, 2.0µm		
Mobile phase-A	: 0.1% v/v Formic acid in water		
Mobile phase-B	: Acetonitrile		
Gradient	: 65%B initial to 85%B in 2 min, ramp to 90%B in 2.5 min, ramp to 95%B in 1.0 min, hold at 95%B for 0.5 min, back to 65% in 0.1 min, equilibrate at 65%B for 3.9 minutes.		
Flow rate	: 300 µL/min	DL temp	: 250 ^{0}C
Column temp	: 35 ^{0}C	Heat block	: 400 ^{0}C
Drying gas	: 20 L/min	Interface	: ESI
Nebulizing gas	: 3.0 L/min	Interface volt	: 4.5 kV
Injection volume	: 15.0 µL	Run time	: 10 min.
For EE			
MRM	: 529.90 → 171.10	Polarity	: Positive
Dwell time	: 180 ms	CE	: -39.0 V
Q1 pre-bias	: -20.0 V	Q3 pre-bias	: -32.0 V
For IS			
MRM	: 534.00 → 171.10	Polarity	: Positive
Dwell time	: 180 ms	CE	: -41.0 V
Q1 pre-bias	: -40.0 V	Q3 pre-bias	: -32.0 V

❑ Results and Discussion

LLOQ

The concentration of EE at lower limit of quantitation (LLOQ) was determined to be 1.0 pg/mL. This was confirmed from the coefficient of variance (%CV) of 15.08% for the six replicate injections of EE at this concentration. A representative mass chromatogram of EE at its LLOQ concentration and the blank is presented in Figure 2.

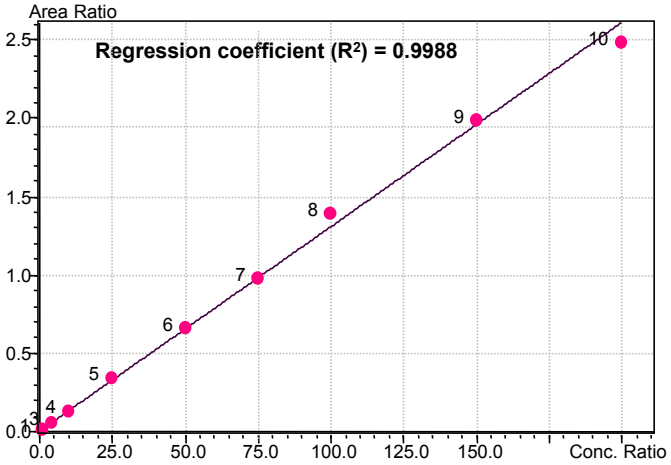

Figure 3 : Calibration curve of EE

Linearity

The CC standards were used to construct a calibration curve by plotting the area ratio of EE with respect to IS versus the concentration of CC standards. Linear curve fit type was used and weighted ($1/x^2$). A linear dynamic range of 1.0 to 200.0 pg/mL was achieved for EE with R^2 value of 0.9988 that meets the acceptance criteria. Figure 3 shows a representative calibration curve of EE in plasma using Ethinyl estradiol-D4 as internal standard. Table 2 summarizes the back calculated concentrations obtained for the calibration standards.

Table 2: Accuracy of EE in CC samples

Nominal Concentration (pg/mL)	Measured Concentration (pg/mL)	Accuracy*
1.0	1.0	100.5
4.0	4.0	98.8
10.0	9.7	97.2
25.0	25.5	102.0
50.0	49.9	99.8
75.0	74.3	99.1
100.0	106.5	106.5
150.0	151.9	101.3
200.0	189.8	94.9

* Expressed as Bias = (mean concentration / nominal concentration) x 100

Precision & Accuracy of QC Samples

Low, middle and high QC samples containing EE were prepared at concentrations of 3.0, 90.0 and 180.0 pg/mL in plasma. The precision (%CV, n=6) of the QCs for EE varied from 3.3 to 9.8 % while the average percent accuracy for QC samples were 96.6% (Table 3). A representative mass chromatogram of MQC and HQC are shown in Figure 4 and 5.

Table 3: Precision and accuracy of EE in QC samples

Nominal Conc. (pg/mL)	Measured conc. (pg/mL)	Accuracy*	Precision (n=6)
3.0	2.7	90.0	9.8
	3.4	113.3	
	2.7	90.0	
	2.9	96.7	
	2.7	90.0	
	2.7	90.0	
90.0	89.6	99.6	3.3
	86.8	96.4	
	82.3	91.4	
	87.7	97.4	
	86.8	96.4	
	83.1	92.3	
180.0	183.0	101.7	3.6
	186.8	103.8	
	176.5	98.0	
	176.4	98.0	
	180.3	100.2	
	168.2	93.4	

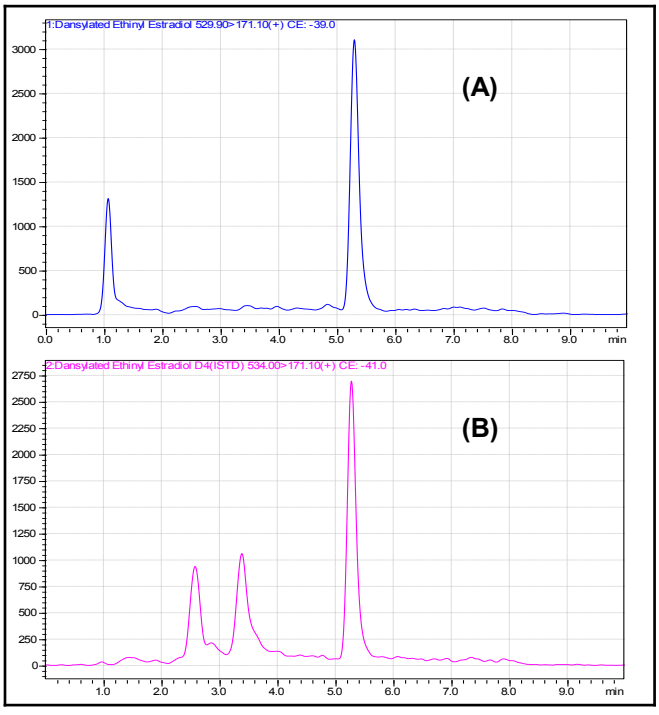

Figure 4: Mass chromatogram of EE (A) and IS (B) at MQC

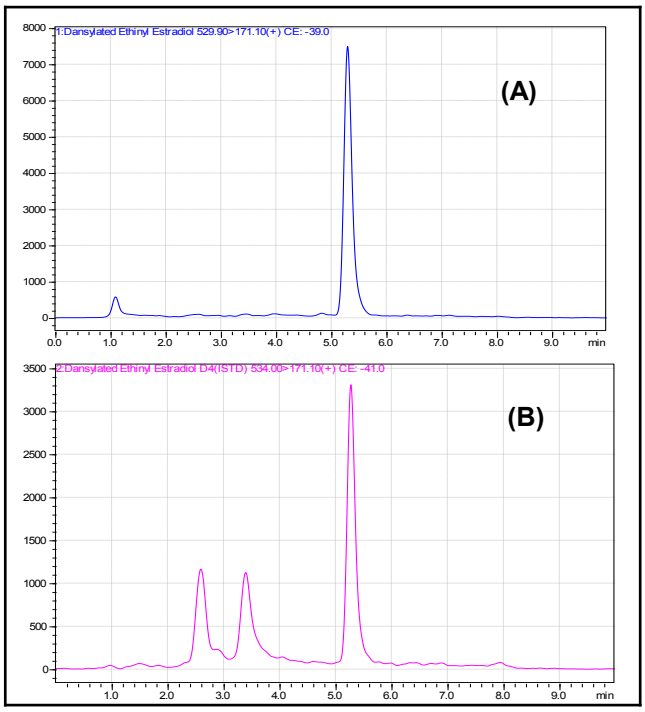

Figure 5 : Mass chromatogram of EE (A) and IS (B) at HQC

Recovery of QC Samples

The recovery of EE was calculated by comparing the peak area obtained for QC samples that were subjected to extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations. The average recovery across QC concentration is 64.5% and is found to be consistent across all the three levels.

❑ Conclusions

An ultra sensitive method capable of detecting Ethinyl Estradiol to as low as 1.0 pg/mL has been developed and evaluated using the LCMS-8040 triple quadrupole mass spectrometer. An important aspect of this method is the sample preparation which involves a liquid-liquid extraction, derivatization followed by solid-phase extraction. The method focuses on the clean up of the analyte from the biological matrix whereby the sensitivity of the method is enhanced greatly. A linear range of 1.0 – 200.0 pg/mL has been established with a regression value of 0.9988. The average percent accuracy for EE was 100.0% for the standard curve samples and 96.6% for the QC samples. No interference peak was observed in the plasma blank demonstrating effective removal of all biological matrix and other related endogenous compounds during extraction. Hence, the developed method shows significant promise for applications that need ultra-low level detection of EE in plasma samples.

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Application News

No. AD-0098

Quantitative Bioanalysis / LCMS-8050

Development of 2D-LC/MS/MS Method for Quantitative Analysis of 1α,25-Dihydroxylvitamin D3 in Human Serum

ASMS 2014 WP449

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Development of 2D-LC/MS/MS Method for Quantitative Analysis of 1α,25-Dihydroxylvitamin D3 in Human Serum

Introduction

Developments of LC/MS/MS methods for accurate quantitation of low pg/mL levels of 1α,25-dihydroxy vitamin D2/D3 in serum were reported in recent years, because their levels in serum were found to be important indications of several diseases associated with vitamin D metabolic disorder in clinical research and diagnosis [1]. However, it has been a challenge to achieve the required sensitivity directly, due to the intrinsic difficulty of ionization of the compounds and interference from matrix [2,3]. Sample extraction and clean-up with SPE and immunoaffinity methods were applied to remove the interferences [4] prior to LC/MS/MS analysis. However, the amount of serum required was normally rather high from 0.5mL to 2mL, which is not favourite in the clinical applications. Direct analysis methods with using smaller amount of serum are in demand. Research efforts have been reported in literatures to enhance ionization efficiency by using different interfaces such as ESI, APCI or APPI and ionization reagents to form purposely NH3 adduct or lithium adduct [4,5]. Here, we present a novel 2D-LC/MS/MS method with APCI interface for direct analysis of 1α,25-diOH-VD3 in serum. The method achieved a detection limit of 3.1 pg/mL in spiked serum samples with 100 uL injection.

Experimental

High purity 1α,25-dihydroxyl Vitamin D3 and deuterated 1α,25-dihydroxyl-d6 Vitamin D3 (as internal standard) were obtained from Toronto Research Chemicals.
Charcoal-stripped pooled human serum obtained from Bioworld was used as blank and matrix to prepare spiked samples in this study. A 2D-LC/MS/MS system was set up on LCMS-8050 (Shimadzu Corporation) with a column switching valve installed in the column oven and controlled by LabSolutions workstation. The details of columns, mobile phases and gradient programs of 1st-D and 2nd-D LC separations and MS conditions are compiled into Table 1. The procedure of sample preparation of spiked serum samples is shown in Figure 1. It includes protein precipitation by adding ACN-MeOH solvent into the serum in 3 to 1 ratio followed by vortex and centrifuge at high speed. The supernatant collected was filtered before standards with IS were added (post-addition). The clear samples obtained were then injected into the 2-D LC/MS/MS system.

Table 1: 2D-LC/MS/MS analytical conditions

LC condition

Column	1st D: FC-ODS (2.0mmI.D. x 75mm L, 3μm) 2nd D: VP-ODS (2.0mmI.D. x 150mm L, 4.6μm)
Mobile Phase of 1st D	A: Water with 0.1% formic acid B: Acetontrile
Mobile Phase of 2nd D	C: Water with 0.1% formic acid D: MeOH with 0.1% formic acid
1st D gradient program & flow rate	B: 40% (0 to 0.1min) → 90% (5 to 7.5min) → 15% (11 to 12min) → 40% (14 to 25min); Total flow rate: 0.5mL/min
2nd D gradient program & flow rate	D: 15% (0min) → 80% (20 to 22.5min) → 15% (23 to 25min); Peak cutting: 3.15 to 3.40; Total flow rate: 0.5 mL/min
Oven Temp.	45°C
Injection Vol.	100 uL

MS Interface condition

Interface	APCI, 400°C
MS mode	Positive, MRM
Heat Block & DL Temp.	300°C & 200°C
CID Gas	Ar (270kPa)
Nebulizing Gas Flow	N2, 2.5 L/min
Drying Gas Flow	N2, 7.0 L/min

Development of 2D-LC/MS/MS Method for Quantitative Analysis of 1α,25-Dihydroxylvitamin D3 in Human Serum

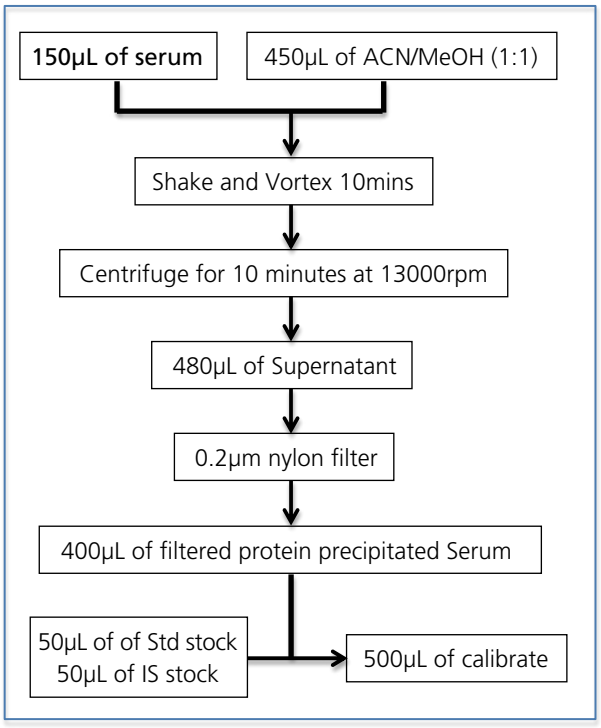

Figure 1: Flow chart of serum sample pre-treatment method

Results and Discussion

Development of 2D-LC/MS/MS method

An APCI interference was employed for effective ionization of 1α,25-diOH-VitD3 ($C_{27}H_{44}O_3$, MW 416.7). A MRM quantitation method for 1α,25-diOH-VitD3 with its deuterated form as internal standard (IS) was developed. MRM optimization was performed using an automated MRM optimization program with LabSolutions workstation. Two MRM transitions for each compound were selected (Table 2), the first one for quantitation and the second one for confirmation. The parent ion of 1α,25-diOH-VitD3 was the dehydrated ion, as it underwent neutral lost easily in ionization with ESI and APCI [2,3]. The MRM used for quantitation (399.3>381.3) was dehydration of the second OH group in the molecule.

Table 2: MRM transitions and CID parameters of 1α,25-diOH-VitD3 and deuterated IS

Name	RT[1] (min)	Transition (m/z)	CID Voltage (V)		
			Q1 Pre Bias	CE	Q3 Pre Bias
1α,25-dihydroxyl Vitamin D3	22.74	399.3 > 381.3	-20	-13	-14
		399.3 > 157.0	-20	-29	-17
1α,25-dihydroxyl-d6 Vitamin D3 (IS)	22.71	402.3 > 366.3	-20	-12	-18
		402.3 > 383.3	-20	-15	-27

1, Retention time by 2D-LC/MS/MS method

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Development of 2D-LC/MS/MS Method for Quantitative Analysis of 1α,25-Dihydroxylvitamin D3 in Human Serum

The reason to develop a 2-D LC separation for a LC/MS/MS method was the high background and interferences occurred with 1D LC/MS/MS observed in this study and also reported in literatures. Figure 2 shows the MRM chromatograms of 1D-LC/MS/MS of spiked serum sample. It can be seen that the baseline of the quantitation MRM (399.3>381.3) rose to a rather high level and interference peaks also appeared at the same retention time.
The 2-D LC/MS/MS method developed in this study involves "cutting the targeted peak" in the 1st-D separation precisely (3.1~3.4 min) and the portion retained in a stainless steel sample loop (200 uL) was transferred into the 2nd-D column for further separation. The operation was accomplished by switching the 6-way valve in and out by a time program. Both 1st-D and 2nd-D separations were carried out in gradient elution mode. The organic mobile phase of 2nd-D (MeOH with 0.1% formic acid) was different from that of 1st-D (pure ACN). The interference peaks co-eluted with the analyte in 1st-D were separated from the analyte peak (22.6 min) as shown in Figure 3.

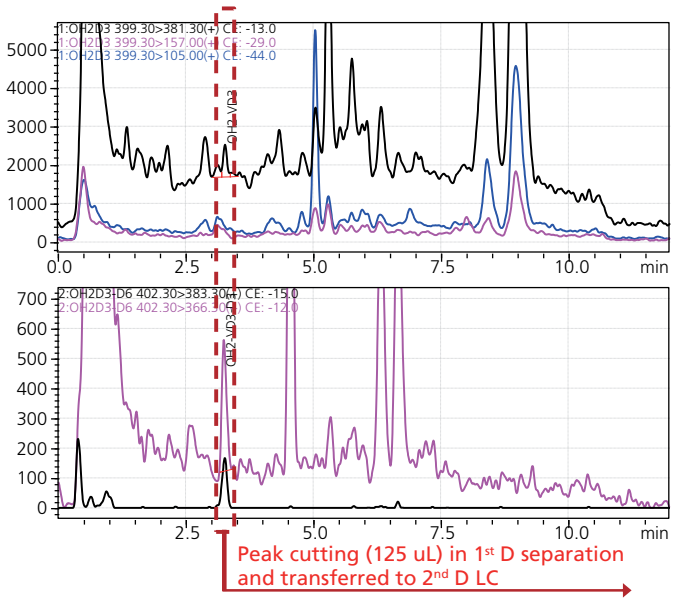

Figure 2: 1D-LC/MS/MS chromatograms of 22.7 pg/mL 1α,25-diOH-VitD3 (top) and 182 pg/mL internal standard (bottom) in serum (injection volume: 50uL)

Calibration curve (IS), linearity and accuracy

Two sets of standard samples were prepared in serum and in clear solution (diluent). Each set included seven levels of 1α,25-diOH-VitD3 from 3.13 pg/mL to 200 pg/mL, each added with 200 pg/mL of IS (See Table 3). The chromatograms of the seven spiked standard samples in serum are shown in Figure 3. A linear IS calibration curve (R2 > 0.996) was established from these 2D-LC/MS/MS analysis results, which is shown in Figure 4. It is worth to note that the calibration curve has a non-zero Y-intercept, indicating that the blank (serum) contains either residual 1 α,25-diOH-VitD3 or other interference which must be deducted in the quantitation method. The peak area ratios shown in Table 3 are the results after deduction of the background peaks. The accuracy of the method after this correction is between 92% and 117%.

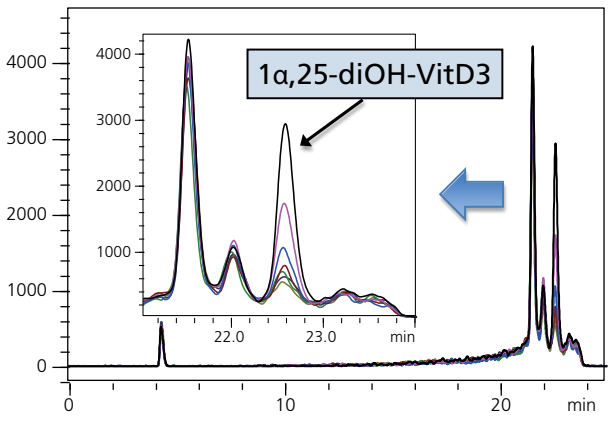

Figure 3: Overlay of 2nd-D chromatograms of 7 levels from 3.13 pg/mL to 200 pg/mL spiked in serum.

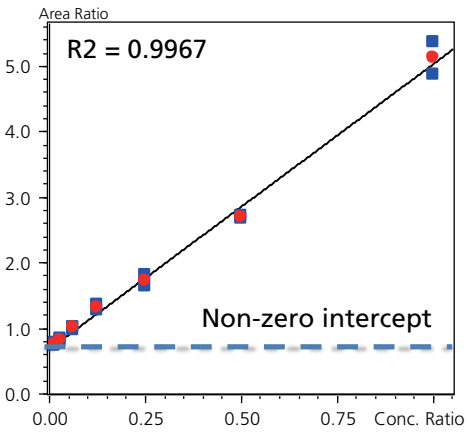

Figure 4: Calibration curves of 1α,25-diOH VD3 in serum by IS method.

Development of 2D-LC/MS/MS Method for Quantitative Analysis of 1α,25-Dihydroxylvitamin D3 in Human Serum

Table 3: Seven levels of standard samples for calibration curve and performance evaluation

Conc. Level of Std.	1α,25-diOH VD3 (pg/mL)	Conc. Ratio[1] (Target/IS)	Area Ratio[2] (in serum)	Area Ratio[2] (in clear solu)	Accuracy[3] (%)	Matrix Effect (%)
L1	3.13	0.0156	0.243	0.414	103.8	58.7
L2	6.25	0.0313	0.321	0.481	100.0	66.8
L3	12.5	0.0625	0.456	0.603	117.3	75.6
L4	25.0	0.1250	0.757	0.914	115.9	82.9
L5	50.0	0.2500	1.188	1.354	95.5	87.7
L6	100.0	0.5000	2.168	2.580	92.15	84.0
L7	200.0	1.0000	4.531	4.740	102.0	95.6

1, Target = 1α,25-diOH VD3; 2, Area ratio = area of target / area of IS; 3, Based on the data of spiked serum samples

Matrix effect, repeatability, LOD/LOQ and specificity

Matrix effect of the 2D-LC/MS/MS method was determined by comparison of peak area ratios of standard samples in diluent and in serum at the seven levels. The results are compiled into Table 3. The matrix effect of the method are between 58% and 95%. It seems that the matrix effect is stronger at lower concentrations than at higher concentrations. Repeatability of peak area of the method was evaluated with L2 and L3 spiked serum samples for both target and IS. The Results of RSD (n=6) are displayed in Table 4.

The MRM peaks of 1α,25-diOH VD3 in clear solution and in serum are displayed in pairs (top and bottom) in Figure 5. It can be seen from the first pair (diluent and serum blank) that a peak appeared at the same retention of 1α,25-diOH VD3 in the blank serum. As pointed out above, this peak is from either the residue of 1α,25-diOH VD3 or other interference present in the serum. Due to this background peak, the actual S/N ratio could not be calculated. Therefore, it is difficult to determine the LOD and LOQ based on the S/N method. Tentatively, we propose a reference LOD and LOQ of the method for 1α,25-diOH VD3 to be 3.1 pg/mL and 10 pg/mL, respectively.

The specificity of the method relies on several criteria: two MRMs (399>381 and 399>157), their ratio and RT in 2nd-D chromatogram. The MRM chromatograms shown in Figure 5 demonstrate the specificity of the method from L1 (3.1 pg/mL) to L7 (200 pg/mL). It can be seen that the results of spiked serum samples (bottom) meet the criteria if compared with the results of samples in the diluent (top).

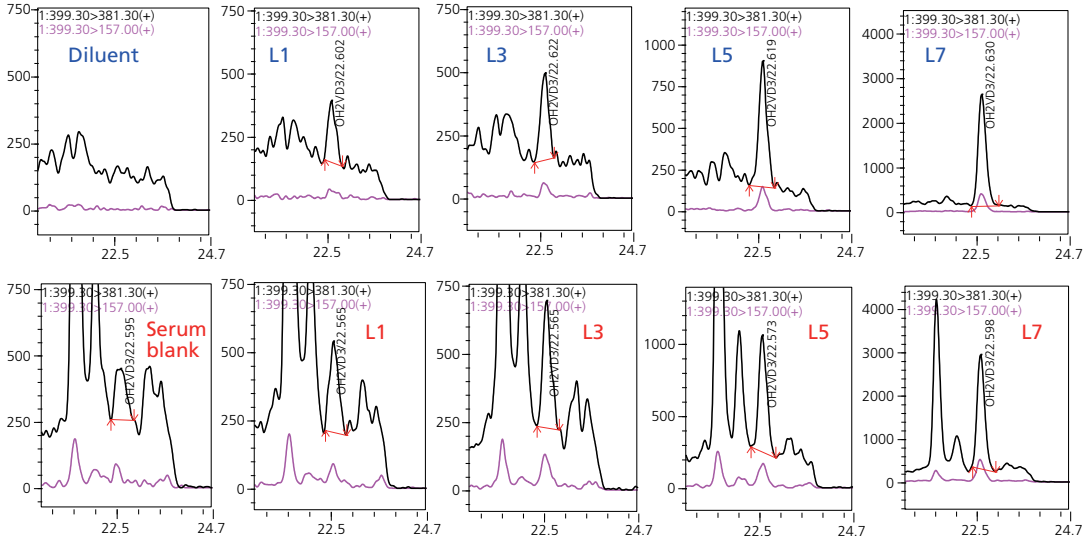

Figure 5: MRM peaks of 1α,25-diOH-VitD3 spiked in pure diluent (top) and in serum (bottom) of L1, L3, L5 and L7 (spiked conc. refer to Table 3)

Development of 2D-LC/MS/MS Method for Quantitative Analysis of 1α,25-Dihydroxylvitamin D3 in Human Serum

Table 4: Repeatability Test Results (n=6)

Sample	Compound	Spiked Conc. (pg/mL)	%RSD
L2	1α,25-diOH VD3	6.25	10.10
	IS	200	7.66
L3	1α,25-diOH VD3	12.5	9.33
	IS	200	6.28

Conclusions

A 2D-LC/MS/MS method with APCI interface has been developed for quantitative analysis of 1α,25-dihydroxylvitamin D3 in human serum without offline extraction and cleanup. The detection and quantitation range of the method is from 3.1 pg/mL to 200 pg/mL, which meets the diagnosis requirements in clinical applications. The performance of the method was evaluated thoroughly, including linearity, accuracy, repeatability, matrix effect, LOD/LOQ and specificity. The results indicate that the 2D-LC/MS/MS method is sensitive and reliable in detection and quantitation of trace 1α,25-dihydroxylvitamin D3 in serum. Further studies to enable the method for simultaneous analysis of both 1α,25-dihydroxylvitamin D3 and 1α,25-dihydroxylvitamin D2 are needed.

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Application News

No. AD-0099

Quantitative Bioanalysis / LCMS-8050

A Direct LC/MS/MS Method for Quantitative Determination of 25-Hydroxyvitamin D_2 and D_3 in Human Plasma

ASMS 2015 MP316

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A Direct LC/MS/MS Method for Quantitative Determination of 25-Hydroxyvitamin D_2 and D_3 in Human Plasma

Introduction

Vitamin-D is an essential nutrient for human health. Vitamin-D deficiency has been reported among populations, which is potentially a factor to develop chronic diseases. The level of vitamin-D is maintained by its metabolite 25OH-vitamin-D2/D3 in the bloodstream. Vitamin-D deficiency is normally defined as its serum level below 50~75nmol/mL. Therefore, accurate measurement of vitamin D status is required in clinical screening test. In recent year, LC/MS/MS has been used for quantitative analysis of vitamin-D metabolites. However, tedious sample preparation is often required such as extraction, derivatization and clean-up with SPE or immunoaffinity columns. This is to remove matrix interferences and increase sensitivity due to poor ionization of the compounds [1, 3]. Furthermore, the amount of serum/plasma required was rather high at 0.5~2 mL per analysis, which is not favourable in the clinical applications. Here, we present a direct high sensitivity LC/MS/MS method for quantitative determination of 25OH-vitamin-D2/D3 in plasma, achieving LLOQ of 3ng/mL with 10uL injection. The method exhibits good accuracy, reproducibility, linearity and specificity over the concentration range from 1-100 ng/mL.

Experimental

Stock solution of 25-hydroxyvitamin D2 (25-OH VD2) and 25-hydroxyvitamin D3 (25-OH VD3) were purchased from Sigma Aldrich (Cerilliant). The deuterated forms, 25-hydroxyvitamin D2-d6 (25-OH VD2-d6) and 25-hydroxyvitamin D3-d6 (25-OH VD3-d6) were used as internal standards, obtained from TRC (Toronto Research Chemicals). Pooled human plasma was purchased from i-DNA Biotechnology. A Shimadzu LCMS-8050 system was used in this work. The details of column, mobile phases and gradient program of LC separations and MS conditions are compiled into Table 1. The procedure of sample preparation for spiked human plasma samples (pre-spiked) is illustrated in Figure 1. It includes spiking of standards and IS into the plasma and protein precipitation by adding ACN-MeOH solvent into the human plasma followed by vortex and centrifugation at high speed. The supernatant collected was filtered and injected directly into the LC/MS/MS system.

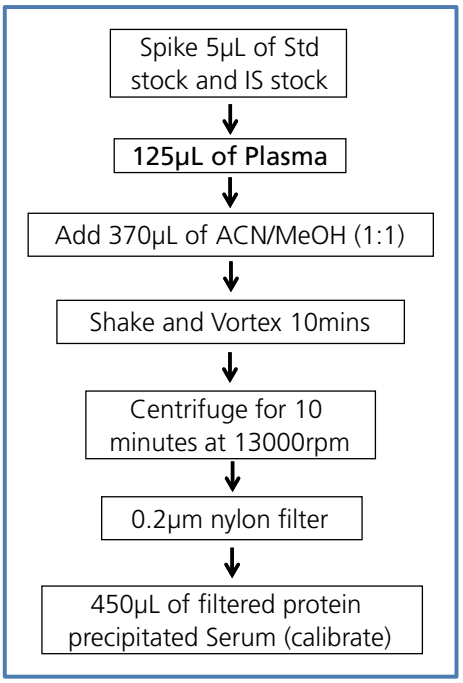

Figure 1: Flow chart of plasma sample pre-treatment by protein precipitation

A Direct LC/MS/MS Method for Quantitative Determination of 25-Hydroxyvitamin D_2 and D_3 in Human Plasma

Table 1: Analytical conditions of direct analysis of 25-OH VD2/VD3 on LCMS-8050

LC condition	
Column	Kinetex 1.7u C18 100A (100x2.1mm) P/N:00D-4475-AN
Flow Rate	0.5 mL/min
Mobile Phase	A: 0.1% formic acid in Water B: 0.1% formic acid in Methanol
Elution Mode	Gradient elution, LC program 15 minute: 10% (0.0 to 0.5min) → 70% (1.5min) → 80% (6.5 to 9.0min) → 90% (9.1min to 11.0min) → 10% (12.0 to 15.0min)
Oven Temperature	45 °C
Injection Volume	10uL

MS Interface condition	
Interface	APCI
MS mode	Positive
Block Temperature	200 °C
Interface Temp.	400 °C
DL Temperature	200 °C
CID Gas	Ar (270kPa)
Nebulizing Gas Flow	N_2, 2.5L/min
Drying Gas Flow	N_2, 5.0L/min

Results and Discussion

Development of Direct LC/MS/MS method

A MRM quantitation method for 25-OH-VD2 and 25-OH-VD3 with their deuterated forms as internal standard (IS) was developed on LCMS-8050 using an APCI interface. The MRM optimization was performed in positive mode using an automated MRM optimization program with LabSolutions workstation. Three MRM transitions for each compound were selected (Table 2), and the transition with highest intensity for quantitation and the second & third ones for confirmation. The MRM used for quantitation was actually the dehydration of the OH group in the molecule. The plasma samples after protein precipitation and centrifugation were injected onto the LC/MS/MS directly without any further clean-up step.

Table 2: MRM transitions and CID parameters of 25-OH-VD2/VD3 and deuterated internal standards

Name	RT (min)	MRM Transition (m/z)		CID Voltage (V)		
		Precursor $[M+H]^+$	Product	Q1 Pre Bias	CE	Q3 Pre Bias
25-OH VD2	8.388	413.3	395.3	-20	-9	-28
			355.3	-20	-11	-26
			377.3	-20	-12	-24
25-OH VD2-d6	8.340	419.4	401.4	-20	-12	-28
			355.3	-21	-12	-25
			337.3	-21	-13	-24
25-OH VD3	7.927	401.4	383.2	-20	-11	-18
			365.2	-20	-13	-25
			257.2	-21	-15	-29
25-OH VD3-d6	7.884	407.4	389.4	-12	-11	-27
			371.3	-20	-14	-26
			263.2	-20	-17	-27

A Direct LC/MS/MS Method for Quantitative Determination of 25-Hydroxyvitamin D_2 and D_3 in Human Plasma

Calibration curve (IS), linearity and accuracy

Three sets of standard samples were prepared by pre-spiking and post-spiking in plasma, as well as spiking in the diluent. Each set includes seven levels (triplicate) from 1 ng/mL to 100 ng/mL with IS of 25 ng/mL (Table 3). The processing of the calibration curves includes setting up the weighing method to 1/C. Linear IS calibration curves with R2 greater than 0.996 were established as shown in Figure 2. The MRM chromatograms of pre-spiked samples are shown in Figure 3.

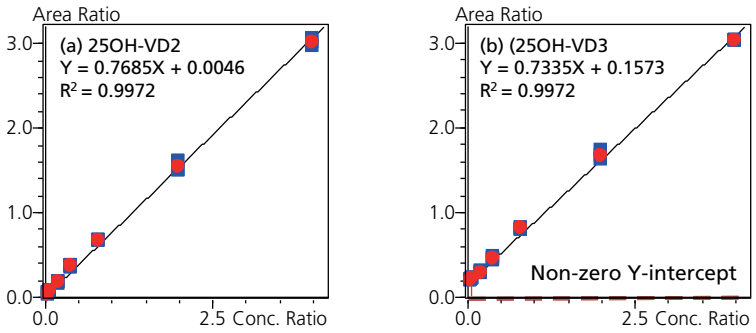

Figure 2: Internal standard calibration curves of 25-OH VD2 (a) and 25-OH VD3 (b) with pre-spiked plasma samples

It is worth to note that the calibration curve of 25-OH-VD3 has a non-zero Y-intercept, indicating that the blank (plasma) contains residual 25-OH-VD3. The level of the residue in the blank was determined to be at 5.14 ~ 5.36 ng/mL (the two values were obtained from post-spiked samples and pre-spiked samples, respectively). This corresponds to 15.42 ~ 16.08 ng/mL of residual 25-OH-VD3 present in the original plasma (the dilution factor of sample pre-treatment is 3.0). The peak area ratios shown in Table 3 are the results after deduction of the background peaks of 25-OH-VD3. The accuracy of the calibration curves are between 78.70% and 119.07%.

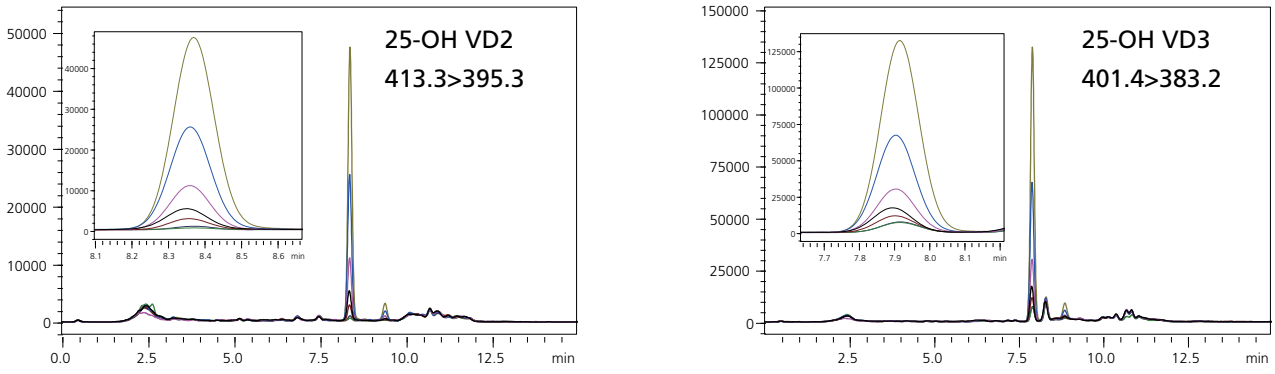

Figure 3: MRM chromatograms of 25-OH VD2 (left) & 25-OH VD3 (right) in pre-spiked in plasma L1 ~ L7

A Direct LC/MS/MS Method for Quantitative Determination of 25-Hydroxyvitamin D_2 and D_3 in Human Plasma

Method Performance validation

Matrix effect of the MRM method was evaluated by comparison of the peak area ratios of post-spiked in plasma to the standard in diluent. Recovery efficiency of the simple pre-treatment method (protein precipitation) was evaluated by comparison of peak area ratios of pre-spiked to post-spiked in plasma. Process efficiency of the whole process is evaluated by comparison of peak area ratios of pre-spiked in plasma to standard spiked in diluent. The results are compiled into Table 3. The matrix effects, recovery efficiency and process efficiency of the method are within the ±15% except for a few data points out of the range. One data (the P.E of 25-OH-VD3 at 1 ng/mL) is greater than 20% (126.5%).
Repeatability of the method was evaluated with standard samples in solvent and in spiked plasma samples (pre-spiked and post-spiked of both target and IS). The results of %RSD (n=7) are below 7.5% for L3 and above. The %RSD of L1 (1 ng/mL) are 7% ~ 22%.

Table 3(a): Summary of method performance evaluation of 25-OH-VD2 spiked in plasma

Std Conc. Level	25-OH-VD2 (ng/mL)	Conc. Ratio (Target/IS)	Area ratio			Accuracy (%)			M.E (%)	R.E (%)	P.E (%)
			Neat	Pre-spiked	Post-spiked	Neat	Pre-spiked	Post-spiked			
L1	1	0.04	0.0329	0.0319	0.0307	78.7	88.7	99.1	93.3	103.9	96.9
L2	2	0.08	0.0677	0.0578	0.0574	99.3	86.5	91.5	84.7	100.7	85.3
L3	5	0.2	0.1602	0.1681	0.1583	103.3	106.4	100.0	98.8	106.2	104.9
L4	10	0.4	0.3374	0.3567	0.3355	112.5	114.5	105.7	99.4	106.3	105.7
L5	20	0.8	0.6612	0.6646	0.6789	111.9	107.4	106.8	102.7	97.9	100.5
L6	50	2.0	1.4035	1.5251	1.5525	95.8	98.9	97.6	110.6	98.2	108.7
L7	100	4.0	2.8774	3.0074	3.1653	98.6	97.7	99.5	110.0	95.0	104.5

Table 3(b): Summary of method performance evaluation for 25-OH-VD3 spiked in plasma

Std Conc. Level	25-OH-VD3 (ng/mL)	Conc. Ratio (Target/IS)	Area ratio			Accuracy (%)			M.E (%)	R.E (%)	P.E (%)
			Neat	Pre-spiked	Post-spiked	Neat	Pre-spiked	Post-spiked			
L1	1	0.04	0.0276	0.0349	0.0318	101.1	119.1	110.0	115.1	109.9	126.5
L2	2	0.08	0.0549	0.0509	0.0509	94.0	86.7	88.3	92.9	99.8	92.7
L3	5	0.2	0.1495	0.1264	0.1402	97.9	86.2	97.1	93.8	90.2	84.6
L4	10	0.4	0.3114	0.2877	0.2805	100.5	98.1	97.1	90.1	102.6	92.4
L5	20	0.8	0.6709	0.6434	0.6161	107.5	109.5	106.6	91.8	104.4	95.9
L6	50	2.0	1.5744	1.5041	1.4937	100.6	102.5	103.4	94.9	100.7	95.5
L7	100	4.0	3.0835	2.8683	2.8185	98.4	97.7	97.6	91.4	101.8	93.0

The MRM peaks of 25-OH VD3 in clear solution and plasma matrix are displayed in Figure 4. As mentioned above, due to the present of residual 25-OH VD3 in the plasma, the actual S/N ratio could not be obtained directly. As a result, LOD and LOQ of 25-OH VD3 could not be determined.

A Direct LC/MS/MS Method for Quantitative Determination of 25-Hydroxyvitamin D_2 and D_3 in Human Plasma

Table 4: Summary of repeatability of IS method (n=7)

Cali. Level	Compound	Conc. (ng/mL)	RSD% (Conc.)		
			Clear Soln	Pre Spiked	Post Spiked
L1	25-OH VD2	1	21.7	20.1	15.6
	25-OH VD3	1	10.2	14.2	7.0
L3	25-OH VD2	5	5.2	3.7	6.4
	25-OH VD3	5	2.7	6.1	6.6
L4	25-OH VD2	10	4.9	4.2	3.9
	25-OH VD3	10	4.2	7.4	3.7
L5	25-OH VD2	20	4.1	3.6	3.6
	25-OH VD3	20	4.0	4.1	3.1

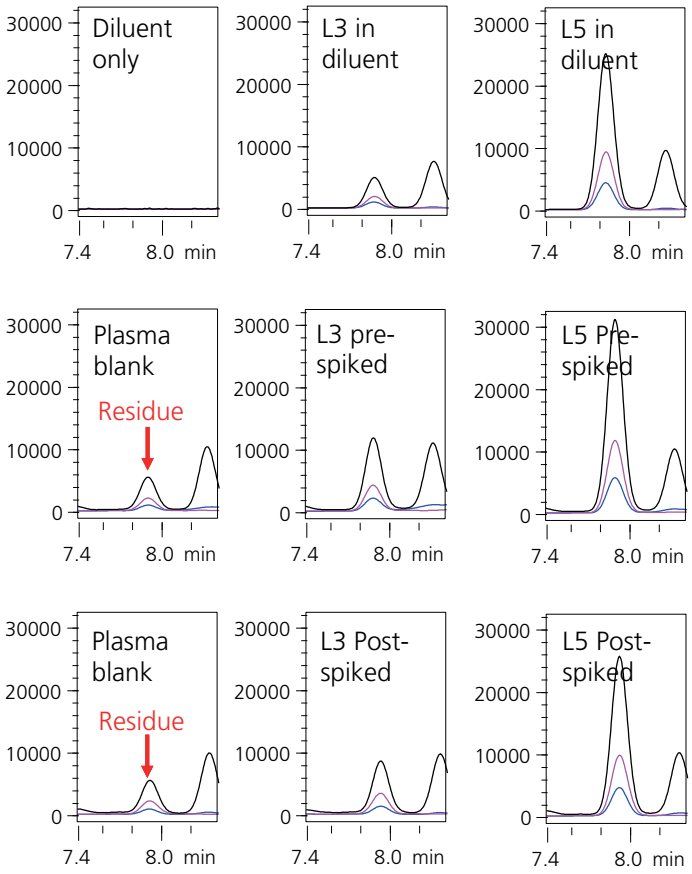

Figure 4: MRM peaks of 25-OH-VD3 in diluent (top row) and pre-spiked in plasma (middle row) & post-spiked in plasma (bottom row)

A Direct LC/MS/MS Method for Quantitative Determination of 25-Hydroxyvitamin D_2 and D_3 in Human Plasma

They were estimated from extrapolation of S/N values after deduction of the residual level (5.1 ~ 5.4 ng/mL). Hence, we propose a reference LOD and LOQ of the method for 25-OH VD3 to be equal or better than 1 ng/mL and 3 ng/mL respectively. As for the LOD and LOQ of 25-OH VD2, it was determined directly from pre-spiked plasma samples to be 0.9 ng/mL and 2.7 ng/mL (average of three injections) respectively. The specificity of the method relies on several criteria: three MRM transitions (m/z), their intensity ratios and RT of the analytes. It can be seen in Figure 4 that the criteria (MRMx3, ratio and RT) could ensure a high level of reliability for the analysis in confirmation and quantitation of residual vitamin in blank plasma.

Conclusions

A direct LC/MS/MS method with APCI has been developed for quantitative determination of 25-hydroxyvitamin D2/D3 in human plasma. The performance of the method was evaluated thoroughly, including linearity, accuracy, repeatability, LOD/LOQ and specificity. The detection and quantitation range of the MRM-based method was 1 ng/mL to 100 ng/mL with 10ul of injection volume. The LOD and LOQ of the method was proposed to be 1ng/mL and 3ng/mL respectively. Furthermore, matrix effect, recovery efficiency and process efficiency were also obtained and the results indicated that the LC/MS/MS method with simple sample pre-treatment is highly sensitive and reliable in detection and quantitation of 25-hydroxyvitamin D2 and D3 in human plasma.

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Note: The method and data shown in the poster are for research use only, Not for Use in Diagnostic Procedures.

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LC-MS/MS quantitative method development of Herceptin based on selective hydrolysis (nSMOL) technology and Skyline software

ASMS 2017 TP089

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LC-MS/MS quantitative method development of Herceptin based on selective hydrolysis (nSMOL) technology and Skyline software

Introduction

Herceptin is approved for the treatment of early-stage breast cancer that is Human Epidermal growth factor Receptor 2-positive (HER2+) and has spread into the lymph nodes, or is HER2-positive and has not spread into the lymph nodes. With the wide use of Herceptin in clinic, the requirement is increasing for precision and accuracy in quantitative analysis of Herceptin in human plasma.
With the development of high performance liquid phase, mass spectrometry and separation technology in biological samples, LC-MS/MS technology has been used for protein quantity study. Relative to conventional analytical technology ELISA, LC-MS/MS quantitative method improved the precision and accuracy of protein analysis. For the close combination of protein quantitative technology and drug development, Shimadzu combined LC-MS/MS analysis platform and proteomics software "Skyline". And we also developed nSMOL pretreatment technology for selective enzymolysis of Fab zone in monoclonal antibody. Thus, LC-MS/MS combining with Skyline software and nSMOL technology can provide useful tool for the accurate quantity of monoclonal antibody drugs.

Methods and Materials

Pretreatment: 1, peptide screening, 10 µg/mL Herceptin standard sample was hydrolyzed according to nSMOL technology, and the hydrolysis product was analyzed by LC-MS/MS; 2, quantitative analysis of Herceptin in plasma, Herceptin standards with 0.122, 0.244, 0.488, 0.975, 1.95, 3.90, 7.80, 15.6, 31.3, 62.5, 125 µg/mL in plasma were prepared, and then pretreated with nSMOL kit according to the operation manual, and then the hydrolysis products were analyzed by LCMS-8060.

Instrument : As an LC-MS/MS system, UHPLC was coupled to triple quadrupole mass spectrometer (Nexera MP with LCMS-8060, Shimadzu Corporation, Kyoto, Japan).
LC-MS/MS with electrospray ionization was operated in multiple-reaction-monitoring (MRM) mode.
Separation: A BEH Peptide C18 column (2.0 mm I.D.× 150 mm L., 1.7 µm) was used for method developing and an Inertsil Sustain Swift C18 column (2.1 mm I.D.×50 mm L., 1.9 µm) was used for quantitative analysis.

Table 1 MRM parameters of Herceptin

Peptides	MRM [m/z]	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)	Peptides function
GLEWVAR	415.70 → 660.30*	-16.0	-15.0	-34.0	Qualitative peptide
	415.70 → 531.30	-16.0	-16.0	-40.0	
	415.70 → 345.20	-16.0	-20.0	-24.0	
FTISADTSK	485.15 → 721.30*	-19.0	-18.0	-38.0	Qualitative peptide
	485.15 → 608.30	-19.0	-20.0	-32.0	
	485.15 → 521.20	-19.0	-21.0	-26.0	
IYPTNGYTR	542.80 → 404.70*	-20.0	-18.0	-30.0	Quantitative peptide
	542.80 → 808.40	-20.0	-18.0	-28.0	
	542.80 → 610.30	-20.0	-25.0	-22.0	
P14R (IS)	512.10 → 292.30*	-38.0	-20.0	-20.0	IS
	512.10 → 389.30	-38.0	-16.0	-28.0	
	512.10 → 757.50	-38.0	-19.0	-38.0	
	512.10 → 660.40	-38.0	-17.0	-24.0	
	512.10 → 563.30	-38.0	-17.0	-40.0	

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LC-MS/MS quantitative method development of Herceptin based on selective hydrolysis (nSMOL) technology and Skyline software

High Speed Mass Spectrometer

Ultra Fast Polarity Switching
- 5 msec
Ultra Fast MRM
- Max. 555 transition /sec

Figure 1 LCMS-8060 triple quadrupole mass spectrometer

Result

Characteristic peptides screening of Herceptin

The nSMOL technology is designed as solid–solid proteolysis on the nanoparticle surface with retaining protease activity. Antibody Fc was immobilized onto the resin via protein A/G, such that the Fab is oriented outward to the solution, and Fab-selective limited proteolysis was occurred. In this work, firstly, Herceptin was selectively hydrolyzed using nSMOL technology, and then characteristic peptides of Herceptin were predicted by Skyline software, and analyzed by LC-MS/MS (Figure2). There were 10 peptides which had good sensitivity and good shape after deleting the peptides with no signal and weak response (Figure 3). Among these 10 peptides, 8 peptides belonged to Fab zone, which indicated that nSMOL technology can selectively hydrolyzed Fab zone of monoclonal antibodies.

UHPLC conditions (Nexera MP system)	
Column	: BEH Peptide C18 column (2.0 mm I.D.× 150 mm L., 1.7 µm)
Mobile phase A	: 0.1% formic acid in H_2O
B	: 0.1% formic acid in acetonitrile
Flow rate	: 0.4 mL/min
Time program	: B conc. 0.0-3.0 min, 5% ; 3.0-35.0 min, 5%~40% ; 35.0-37.5 min, 40%~95% ; 37.5~45.0 min, 95% ; 45.5-50.0 min, 5%
Injection vol.	: 10 uL
Column temperature	: 40°C
MS conditions (LCMS-8060)	
Ionization	: ESI, Positive MRM mode

LC-MS/MS quantitative method development of Herceptin based on selective hydrolysis (nSMOL) technology and Skyline software

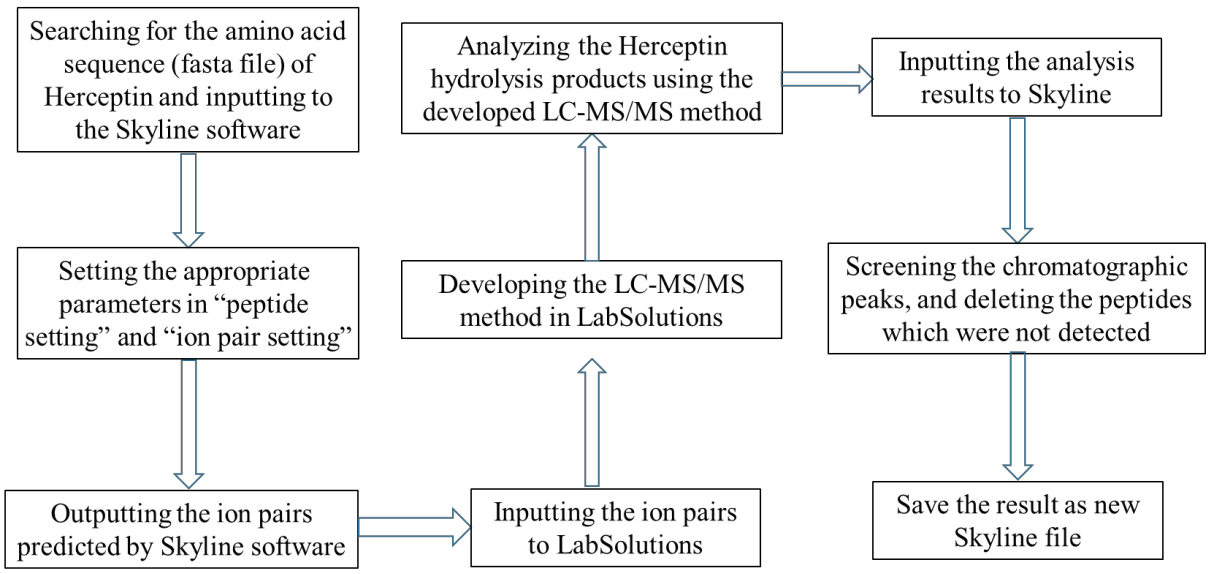

Figure 2　Operational procedure of characteristic peptide screening

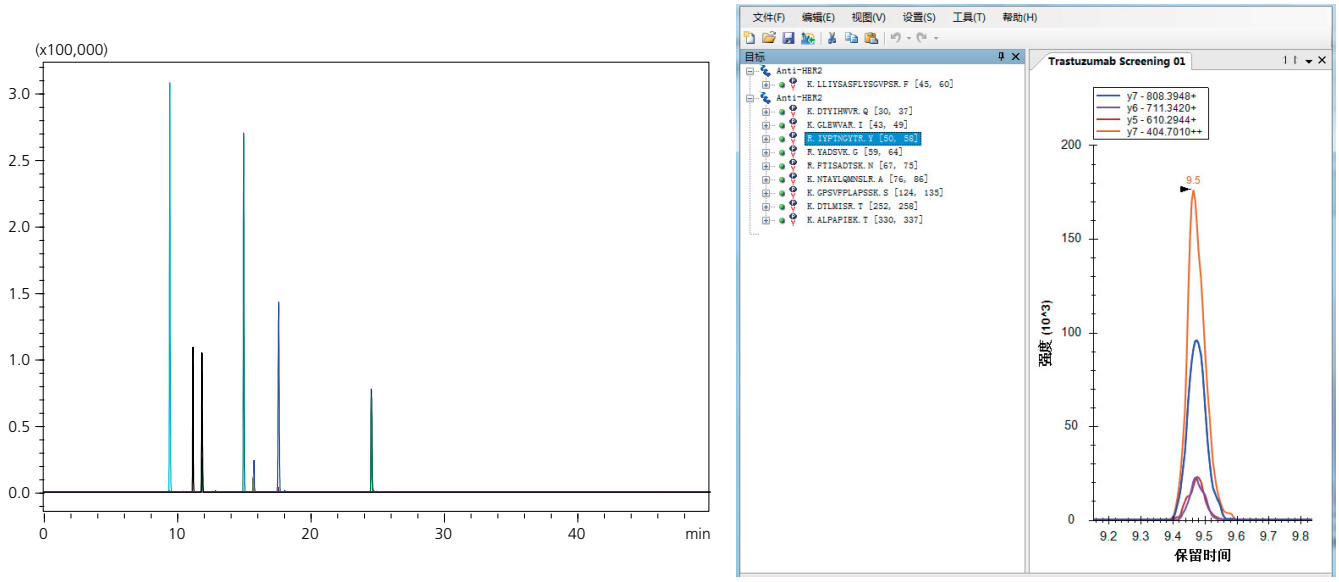

Figure 3　Screened characteristic peptide chromatogram and the corresponding result in Skyline

LC-MS/MS quantitative method development of Herceptin based on selective hydrolysis (nSMOL) technology and Skyline software

Characteristic peptides screening of Herceptin

For establishing good quantitative method, MRM collision energies for 8 peptides (LLIYSASFLYSGVPSR, DTYIHWVR, IYPTNGYTR, GLEWVAR, YADSVK, FTISADTSK, NTAYLQMNSLR, GPSVFPLAPSSK) which belonged to Fab zone were synchronously optimized based on the high speed scan of LCMS-8060. An optimizing LC-MS/MS method for 8 peptides was developed by exporting ion pairs setting in Skyline, and then the optimized results by LC-MS/MS were imported to Skyline for getting the optimization result (Figure 4). According to the optimized result, three peptides GLEWVAR, FTISADTSK, IYPTNGYTR which belonged to CDR zone were selected as characteristic peptides for quantitative and qualitative analysis.

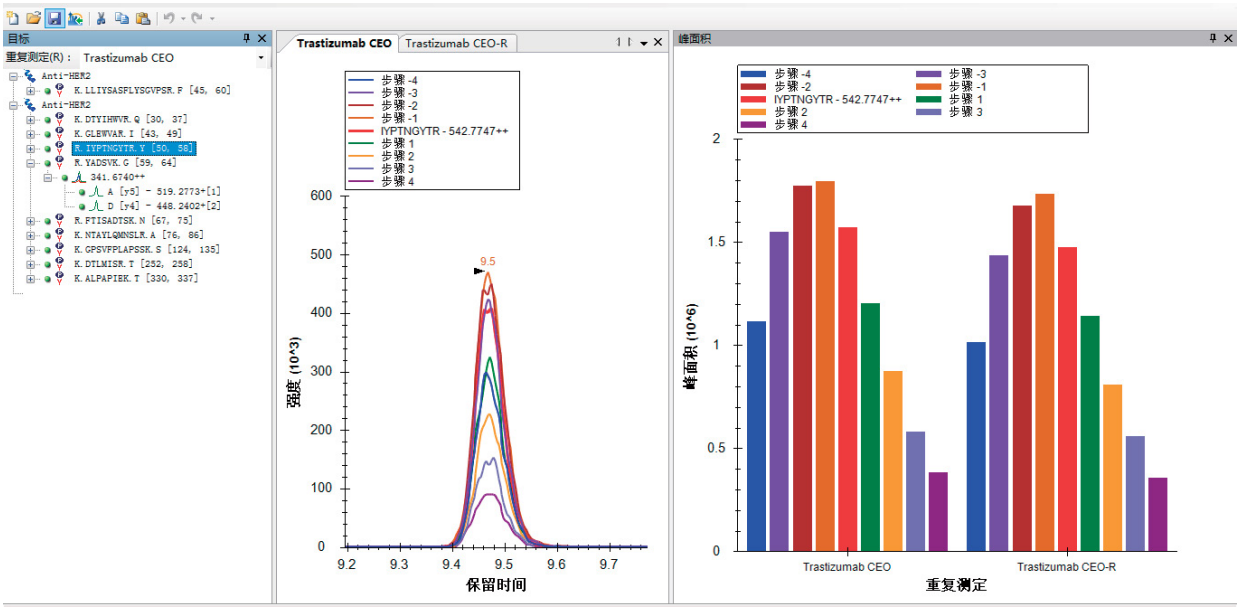

Figure 4　The optimized results by LC-MS/MS which were imported to Skyline

Quantitative analysis of Herceptin in plasma

Based on the optimized CE energies for the characteristic peptides, a complete LC-MS/MS method was established. The peptide IYPTNGYTR (542.80>404.70) which have high intensity and no matrix interference was selected as quantitative characteristic peptide, and the peptides GLEWVAR and FTISADTSK were selected as qualitative characteristic peptides.

For establishing LC-MS/MS quantitative method, firstly, different concentrations of Herceptin standards (0.122-125 μg/mL) in plasm were prepared, and then these standard samples were pretreated with nSMOL kit to get selectively hydrolyzed products for LC-MS/MS analysis. As a result, a calibration curve (Figure 5) was obtained with correlation coeffient 0.9995 and accuracy 91.5-114.0% (Table 2).

LC-MS/MS quantitative method development of Herceptin based on selective hydrolysis (nSMOL) technology and Skyline software

UHPLC conditions (Nexera MP system)	
Column	: Inertsil Sustain Swift C18 Column (2.1 mm I.D.×50 mm L., 1.9 µm)
Mobile phase A	: 0.1% formic acid in H_2O
B	: 0.1% formic acid in acetonitrile
Flow rate	: 0.4 mL/min
Time program	: B conc. 0.00-1.50 min, 1% ; 1.50-5.00 min, 1%~30% ; 5.02-5.83 min, 95% ; 5.85~7.00 min, 1%
Injection vol.	: 10 uL
Column temperature	: 40°C
MS conditions (LCMS-8060)	
Ionization	: ESI, Positive MRM mode

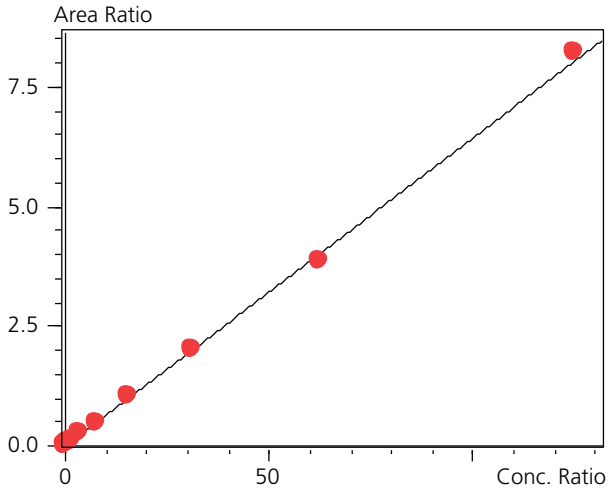

Figure 5 The calibration curve of the characteristic peptide IYPTNGYTR

Table 2 The related information about the calibration curve

Drug name	Peptide for quantitative	Calibration curve	Linear (µg/mL)	Correlation coefficient	Accuracy (%)
Herceptin	IYPTNGYTR	Y = (0.0644864)X + (-0.00317900)	0.122~125	0.9995	91.5~114.0

LC-MS/MS quantitative method development of Herceptin based on selective hydrolysis (nSMOL) technology and Skyline software

Conclusions

There were 8 peptides belonging to Fab zone among the 10 peptides which screened by Skyline and LC-MS/MS, which indicated that the peptides in Fab zone were selectively hydrolyzed by nSMOL technology. For establishing good quantitative method, MRM collision energies were optimized. Based on these results, the peptide IYPTNGYTR (542.80>404.70) was selected as characteristic peptide for quantity. The calibration curve range was from 0.122 to 125 mg/L and the correlation coefficient was 0.9995.

Disclaimer: Shimadzu LCMS-8060 CL is registered in the U.S. as a Class I device and is not specifically cleared for TDM. Nexera X2 UHPLC system is intended for Research Use Only (RUO). Not for use in diagnostic procedures.

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Application News

No. LC-31-ADI-54

LC/MS
nSMOL and Liquid Chromatography Mass Spectrometry

Bioanalytical assessment of Cetuximab in Wistar rat plasma using nSMOL and LCMS-8060

Introduction

Cetuximab is a human monoclonal antibody (mAb) for the treatment of metastatic colorectal carcinoma, head and neck cancer. Cetuximab was first approved by U.S. FDA in 2004.

Generally bioanalysis of mAB drugs is based on Ligand Binding Assay (LBA). However LBA tends to show larger variability and development time for assay can be longer. Hence in this work, we present bioanalysis of Cetuximab in Wistar rat plasma using nSMOL™ (nano surface molecular orientation limited proteolysis) reagent kit and LCMS-8060, triple quadrupole from Shimadzu Corporation.

nSMOL™ Antibody BA kit features

nSMOL is Shimadzu's completely new and breakthrough technology that enables selective proteolysis of the Fab region of monoclonal antibodies. This technique facilitates method development independent of a variety of antibody drugs and achieves a paradigm shift in the bioanalysis of antibody drugs.

Furthermore, numerous bioanalytical method validation have been reported using nSMOL technique as per the criteria of "Guideline on Bioanalytical Method Validation in Pharmaceutical Development" for low MW drug compounds issued by the Japanese Ministry of Health, Labour and Welfare. Shimadzu also offers optimization methods and protocols, and nSMOL can be applied to clinical research at various institutions.

nSMOL principle

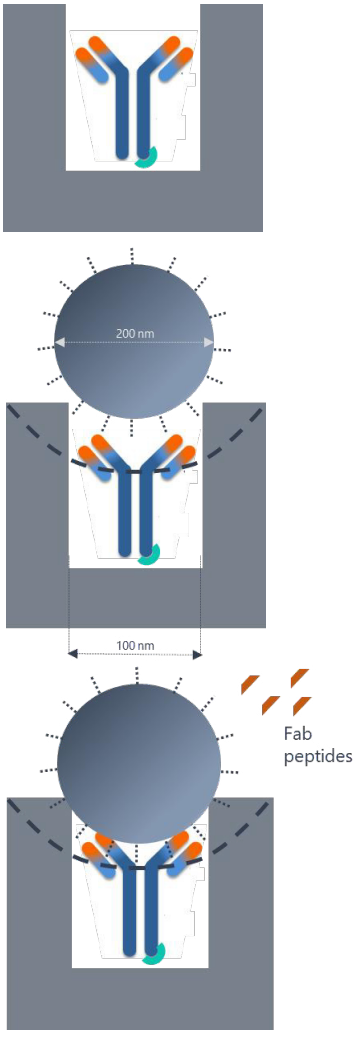
nSMOL works on selective proteolysis of Fab by making use of the difference in size of the protease nanoparticle diameter (200 nm) and the antibody resin pore size (100 nm). With the use of nSMOL one can maintain the specificity of the antibody sequences while minimizing the sample complexity as well as the elimination of extra protease. This approach leads to the shortening of analytical time, LCMS robustness, wide dynamic range, and considerable improvement in sensitivity using LCMS-8060.

Sample Preparation

The standard samples of Cetuximab with concentration between 0.295 to 150 ug/mL in rat plasma were prepared for calibration curve and QC samples with concentration of 0.295 and 0.59 ug/mL were prepared to validate the analysis results. Standards and QC samples were processed based on nSMOL, which involves selective cleavage of Fab region from mAb using trypsin nano patricles. P14 was used as internal standard.

Selection of signature peptide and MRM optimization

Unique peptide for Cetuximab was selected by sequence alignment using skyline. 'GPSVFPLAPSSK' peptide selectively represents Cetuximab from rat plasma. However, this peptide is generic signature peptide. Therefore, this method can be selectively used for only assessment of a singular chimeric mAb in rat plasma. For analysis of Cetuximab from human plasma, peptide sequence of 'SQVFFK' should be used. MRM was optimized using Skyline and MRM optimization tool from Labsolutions.



| Capture

Fab region is captured in collection resin and oriented towards solution

| Digestion

Limited proteolysis by trypsin immobilized on nano particle

| Analysis

Fab derived peptides are harvested and injected in LCMS-8060

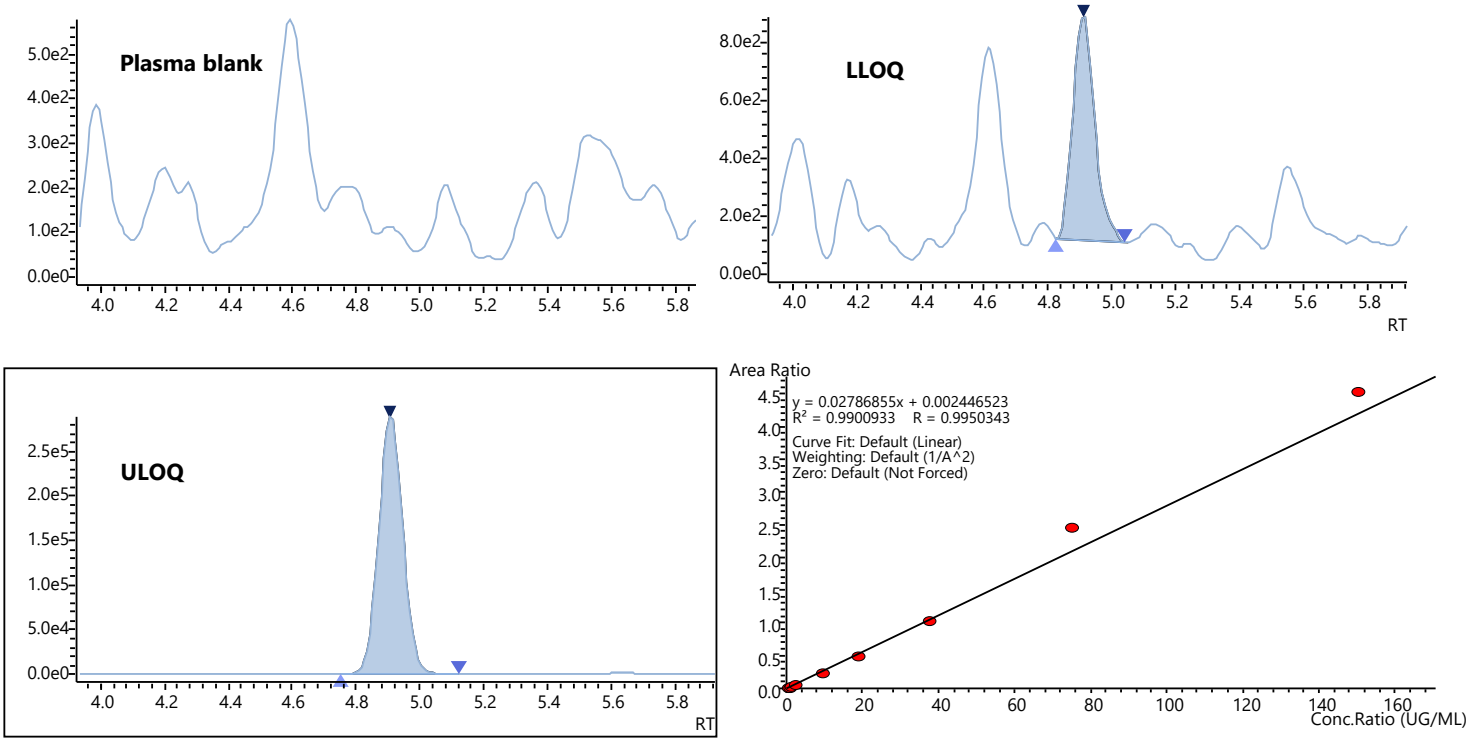


Fig. 1: MRM chromatograms of blank rat plasma, LLOQ level (0.295 ug/mL) and ULOQ (150 ug/mL) in rat plasma for Cetuximab

Fig. 2: Calibration curve for Cetuximab in rat plasma from 0.295 to 150 ug/mL

Results and Discussions

Chromatograms for blank rat plasma, LLOQ and ULOQ level of Cetuximab in rat plasma are shown in **Fig. 1** and the calibration curve is shown in **Fig. 2**. Good linearity was obtained over the concentration range of 0.295 to 150 ug/mL of the Cetuximab in rat plasma with a correlation coefficient of 0.9900. The accuracy of linearity and QC samples for Cetuximab in rat plasma is shown in **Table 3**. For all the linearity and QC samples accuracy was within acceptance criteria.

Table 3: Results of linearity and QC samples for analysis of Cetuximab from rat plasma

Sample Name	Conc. (ug/mL)	Accuracy
BLK	-----	-----
CS 01	0.295	95.73
CS 02	0.59	111.82
CS 03	1.17	99.99
CS 04	2.34	96.09
CS 05	4.69	80.09
CS 06	9.35	88.53
CS 07	18.75	95.41
CS 08	37.5	98.67
CS 09	75	117.84
CS 10	150	108.39
QC 1	0.295	98.69
QC 2	0.59	99.79

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First Edition: May 2018

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Application News

No. XXXX

nSMOL and Liquid chromatograph triple quadrupole mass- spectrometer

Bioanalysis of Denosumab in human plasma using nSMOL and LCMS-8060

Introduction

Denosumab is a human monoclonal antibody (mAb) for the treatment of osteoporosis, treatment-induced bone loss, metastases to bone, and giant cell tumor of bone.

Denosumab was first approve by U.S. FDA in June 2010.

Generally bioanalysis of mAb drugs is based on ligand bindind assay (LBA). However LBA tends to show larder variability, and development time for assay can be very long. Hence in this work, we present methodology for bioanalysis of denosumab in human plasma using nSMOL™ reagent kit and triple quadrupole LCMS, LCMS-8060 from Shimadzu Corporation.

Due to the lack of established industry guidance for bioanalysis of large therapeutic molecules on LCMS, we applied small molecule guidelines criteria, set by FDA.

Experimental

The standard samples of Denosumab with concentration of 2, 5, 10, 50, 100, and 250 ug/mL in plasma were prepared for calibration curve and QC samples with concentration of 2, 15, 75 and 150 ug/mL were prepared to validate the analysis results. Standards and QC were processed based on nSMOL™ (nano surface molecular orientation limited proteolysis), which involves selective cleavage of Fab region from mAb using trypsin nano patricles. P14 is used as IS

Signature peptide used was LEPEDFAVFYCQQYGSSPR, which is part of light chain on Denosumab.

Analysis was carried out using reverse phased HPLC in gradient mode and ionization was done using electro spray ionization (ESI) positive mode. Shimpack Kinetix column was used for analysis.

Instrument configuration used is given in **Table 1**. Parameters such as collision energy, nebulizing gas flow rate, desolvation line (DL) temperature and heat block temperature were optimized for LCMS-8060. The parameters are given in **Table 2**. Blank (mobile phase) and Blank+I.S. were injected so as to confirm absence of any interferences at retention time of selected signature peptides. For linearity studies, calibration curve was plotted over a concentration range of 2 ug/mL to 250 ug/mL of Denosumab

Table 1: Instrument Configuration

HPLC	Nexera X2
System Controller	CBM-20A
Delivery pumps	2 x LC-30AD
Auto sampler	SIL-30AC
Column oven	CTO-30A
MS	LCMS-8060

Table 2: LCMS/MS Analytical Conditions

LC Run Time	20 min
Mobile Phase	A: 0.1% Formic acid in Water B: 0.1% Formic acid in ACN
Gradient:	B. Conc. 2% (0-3.0 min) → 50% (3.0-12.0 min) → 95% (12.0-13.0 min) → 95% (13.0-16.0 min) → 2% (16-17 min) → 2% (17-20 min)
Flow Rate	0.3 mL/min
Oven Temp.	50 ^{0}C
Nebulizing gas	3.0 L/min
Heating gas	18 L/min
Interface Temp.	400 ^{0}C
DL Temp.	150 ^{0}C
Heat block Temp.	400 ^{0}C
Drying gas	10 L/min

Result and Discussion

The calibration curve for Denosumab in plasma is shown in **Fig. 1** and overlay of chromatograms for blank and LLOQ samples is shown in **Fig. 2.** Good linearity was obtained over the concentration range of 2 to 250 ug/mL of the samples in plasma with a correlation coefficient of 0.995.

The accuracy of each point on the calibration curve for Denosumab concentration in plasma is shown in **Table 3.** Good results were obtained for all points on the calibration curve with accuracy within acceptance criteria. QC sample measurement results are also shown in Table 3. The results obtained were sufficiently accurate, with accuracy within acceptance criteria.

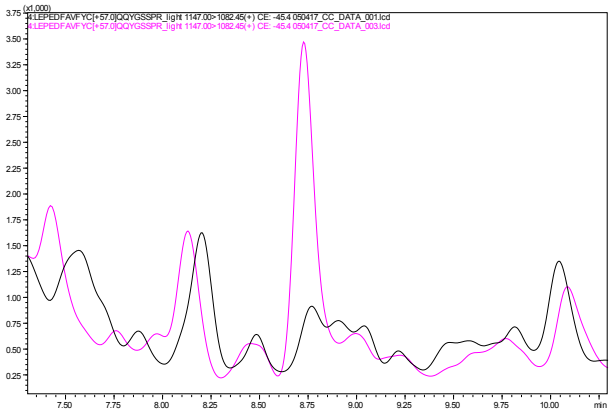

Fig. 1 Overlay of blank and LOQ levels of Denosumab

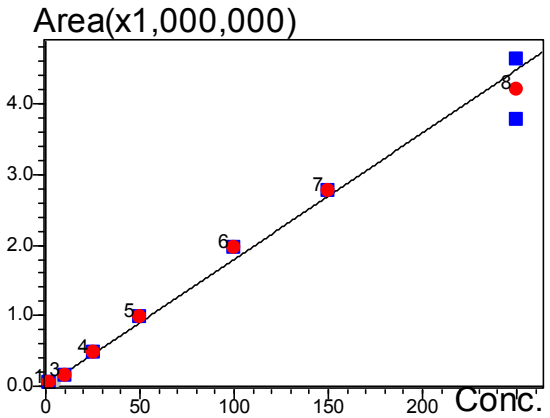

Fig. 2 Calibration curve of Denosumab

Table 3: Calibration point and QC information

SAMPLE NAME	AREA COUNTS	STD CONC.	CALCULATED CONC.	ACCURACY	S/N
BLK	2,004	-----	-0.037	-----	1.21
BLK	1,897	-----	-0.049	-----	0.79
2 UG/ML	21,196	2	2.156	107.8	12.8
10 UG/ML	74,264	10	8.22	82.2	40.43
25 UG/ML	235,509	25	26.645	106.6	84.29
50 UG/ML	480,722	50	54.664	109.3	155.69
100 UG/ML	954,754	100	108.83	108.8	389.54
150 UG/ML	1,369,268	150	156.194	104.1	444.77
250 UG/ML	2,262,156	250	258.221	103.3	803.13
250 UG/ML	1,828,080	250	208.621	83.4	564.89
2 QC UG/ML	16,477	2	1.617	80.9	8.65
2 QC UG/ML	17,536	2	1.738	86.9	8.53
15 QC UG/ML	120,641	15	13.519	90.1	82.32
15 QC UG/ML	110,673	15	12.38	82.5	57.02
75 QC UG/ML	603,178	75	68.657	91.5	289.95
75 QC UG/ML	580,263	75	66.038	88.10	263.01
175 C UG/ML	1,537,139	175	175.376	100.2	741.36
175 C UG/ML	1,406,392	175	160.436	91.7	625.73

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Application Note

LCMS-8040

Quantification of Immunosuppressant drugs in whole blood by LCMS/MS.

No. - LC-08-ADI-029

❑ Introduction

Cyclosporine A, tacrolimus, sirolimus, and everolimus are four of the most commonly administered immunosuppressant drugs which play a central role in the success of tissue and organ transplants. Immunosuppressants are a class of drugs that inhibit the body's immune response and are typically administered to prevent the rejection of transplanted organs (e.g. kidney) or tissue (e.g. bone marrow), and may also be used to treat various autoimmune disorders such as Crohn's Disease or rheumatoid arthritis. The first effective immunosuppressant drug was cyclosporine A (or CsA), an undecapeptide initially discovered by researchers. Since the development of CsA, many other immunsuppressant drugs have been developed, including the macrolides tacrolimus (FK506), sirolimus (also known as rapamycin), and everolimus. Because of their potent immunosuppressant effects and relatively narrow therapeutic index, therapeutic drug monitoring of patients is required in order to ensure the efficacy of the treatment, and also to minimise toxic side effects. Liquid chromatography coupled to tandem mass spectrometry (LCMS/MS) has become the analytical method of choice for the analysis of immunosuppressants. These drugs must be monitored from whole blood, which poses a sample preparation challenge as matrix effects can confound analyses through ion suppression and/or enhancement, and can also affect the reproducibility and accuracy of analytical methods.
Presented is a simple and rapid method for the analysis of immunosuppressants from whole blood that utilises a simple protein precipitation step followed online sample cleaning directly by LCMS/MS[1, 2, 3, 4 and 5]. This fast, simple method shows excellent precision and accuracy down to the µg/L concentration range.

❑ Experimental

Instrumentation

HPLC : NEXERA
MS : LCMS-8040
Degasser : DGU-20A5
Pump : LC-30AD x 2
Autosampler : SIL-30AC
Column Oven : CTO-30A

Analytical Conditions

HPLC conditions
Run time : 5min
Column : Phenomenex C8 50X4.6 mmX 2.6um
Mobile phase : A – Water
: B – Methanol 90:10 (Water: ACN) + 0.1 % Acetic Acid + 10 mM Ammonium acetate.
Flow rate : 1.0 mL/min
Column oven : 60 ^{0}C

MS Condition
Ionization mode : Electro Spray Ionization (ESI) + ve
Acquisition mode : MRM (Multiple Reaction Monitoring)
Injection volume : 5 µL

Table 1:Gradient Program

Time	Mode	Command	% conc. or Valve position
0.01	Valve position	CTO.RVL	1
0.01	Pumps	Pump B Conc.	20
1.5	Pumps	Pump B Conc.	20
1.55	Pumps	Pump B Conc.	90
1.6	Valve position	CTO.RVL	0
3.5	Pumps	Pump B Conc.	90
3.6	Pumps	Pump B Conc.	20
4	Valve position	CTO.RVL	1
5	Controller	Stop	

Table 2:MRM Transition

No.	Compound Name	Transitions
1	Sirolimus	931.50 > 864.30
2	Tacrolimus	821.40 > 768.35
3	Cyclosporine A	1219.80 > 1202.60
4	Everolimus	975.55 > 908.40

Application Note **No. LC-08-ADI-029**

Preparation for Analysis

Standards were weighed in order to get linearity concentration in the range which covers required therapeutic range. Concentration levels prepared from the stock solution and used for plotting calibration graph[1, 2, 3, 4 and 5]. Calibration solutions were prepared in the range of 0.2-25 ng/ml of Everolimus, Tacrolimus and Sirolimus.6.2-800 ng/ml of Cyclosporin.

Sample Preparation

- Taken 300 uL of whole blood sample in 2 ml centrifuge tube
- Added 210 uL of $ZnSO_4$ and 390 uL of methanol.
- Vertex for 2 min and kept it for 5 min at room temp.
- Centrifuged at 13000 rpm at 4^0C for 10 min.
- Taken 500 uL of supernunt layer in HPLC vial for LCMS analysis.

Sample Analysis

Sample Code	Drug	Concentration
269333	Tacrolimus	3.29
135734	Cyclosporine A	20.37
128457	Cyclosporine A	23.17
268672	Tacrolimus	2.99

❑ Results and Discussion

LCMS-8040 has feature of ' Optimization of method' in which mass spectrometer automatically selects the best precursor ion and product ion(s) and optimizes voltage and collision energy. The optimized parameters are shown in Table 2. Calibration curved was plotted as per the concentrations prepared. No matrix interference was observed in blank at the retention time of analyte.(Figure 1A, Figure 1B). Plotted calibration curve in the concentration level given above.

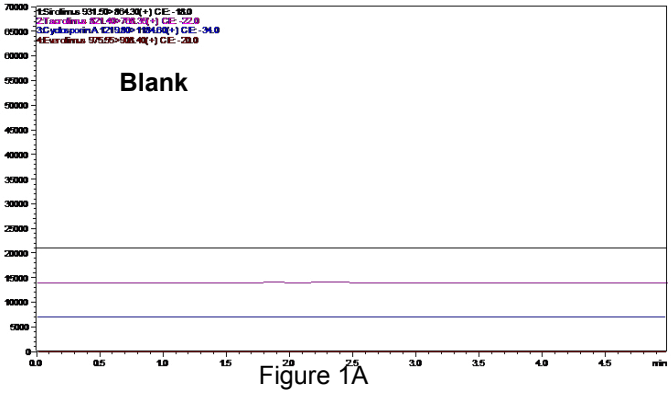

Figure 1A

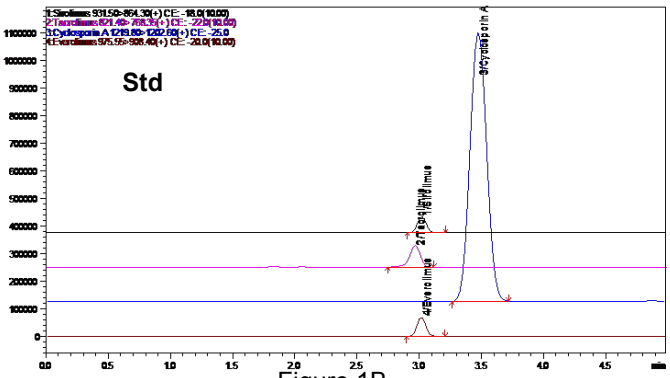

Figure 1B

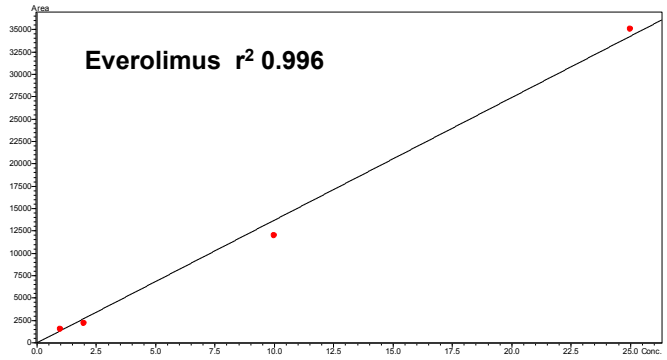

Figure 2A

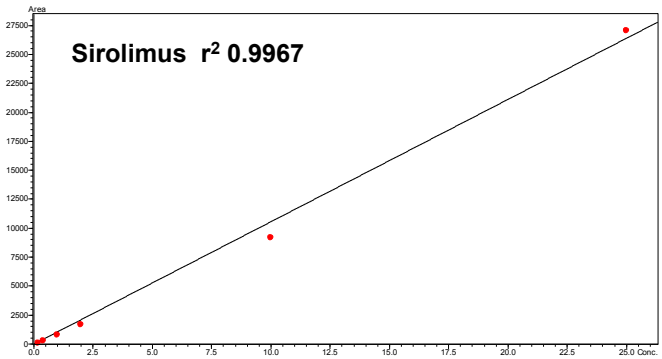

Figure 2B

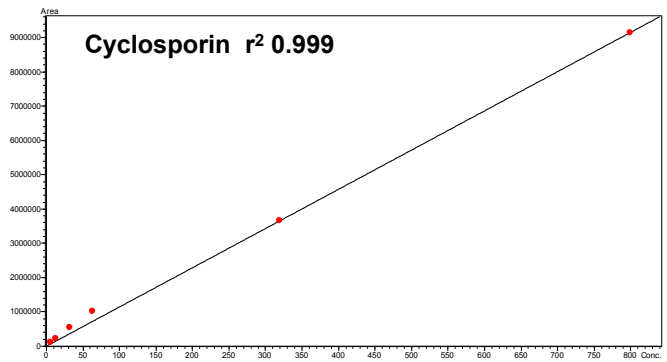

Figure 2D

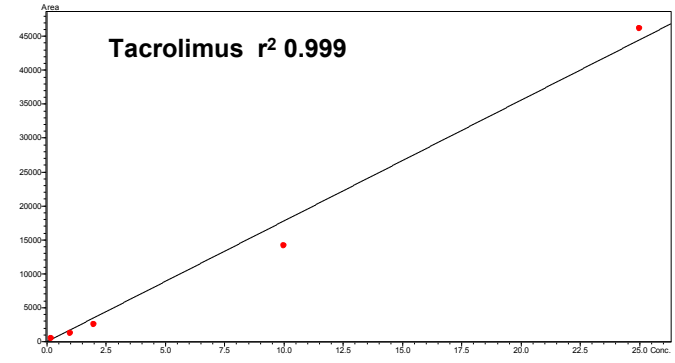

Figure 2C

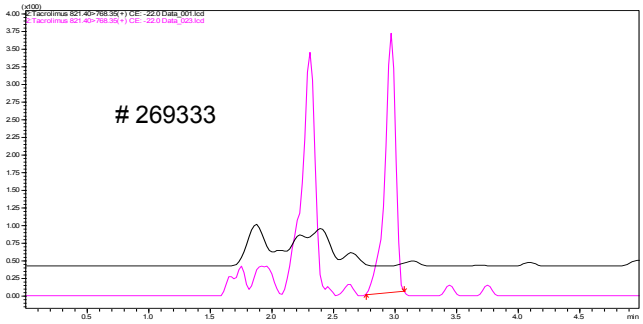

Figure 3A

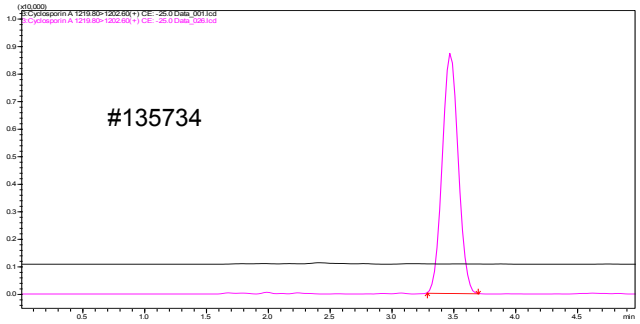

Figure 3B

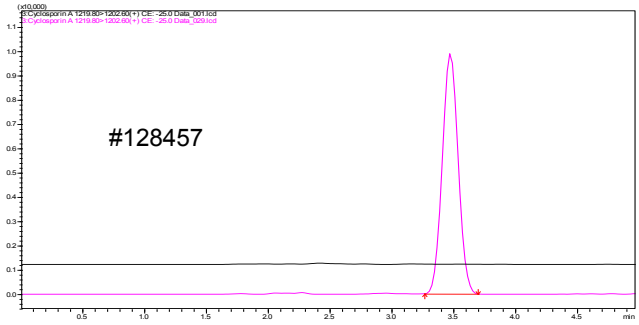

Figure 3C

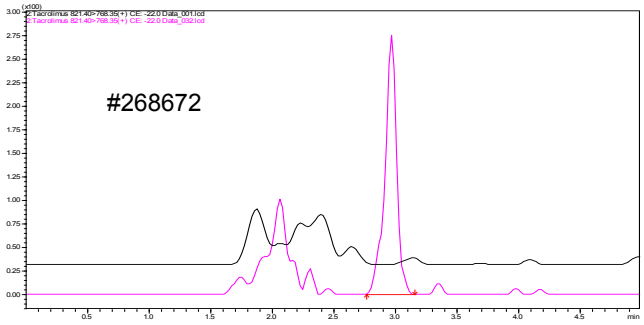

Figure 3D

❑ Conclusion

This method developed on LCMS/MS for simultaneous quantification of 4 immunosuppressant drugs in whole blood with the ultra high sensitivity and accuracy.

Online sample cleanup process was used which reduces the efforts of sample preparation.

High throughput ***five minute method for quantifying four*** immunosuppressive drugs Cyclosporin A, everolimus, sirolimus and tacrolimus

The high specificity and ease of maintenance of LCMS-8040 system, enables less to use simple sample preparation and handling more number of samples.

❑ References

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Accelerated and robust monitoring for immunosuppressants using triple quadrupole mass spectrometry

ASMS 2014 WP628

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Accelerated and robust monitoring for immunosuppressants using triple quadrupole mass spectrometry

Introduction

Immunosuppressants are drugs which lower or suppress activity of the immune system. They are used to prevent the rejection after transplantation or treat autoimmune disease. To avoid immunodeficiency as adverse effect, it is recommended to monitor blood level of therapeutic drug with high throughput and high reliability. There are several analytical technique to monitor drugs, LC/MS is superior in terms of cross-reactivity at low level and throughput of analysis. Therefore, it is important to analyze these drugs in blood by using ultra-fast mass spectrometer to accelerate monitoring with high quantitativity. We have developed analytical method for four immunosuppressants (Tacrolimus, Rapamycin, Everolimus and Cyclosporin A) with two internal standards (Ascomycin and Cyclosporin D) using ultra-fast mass spectrometer.

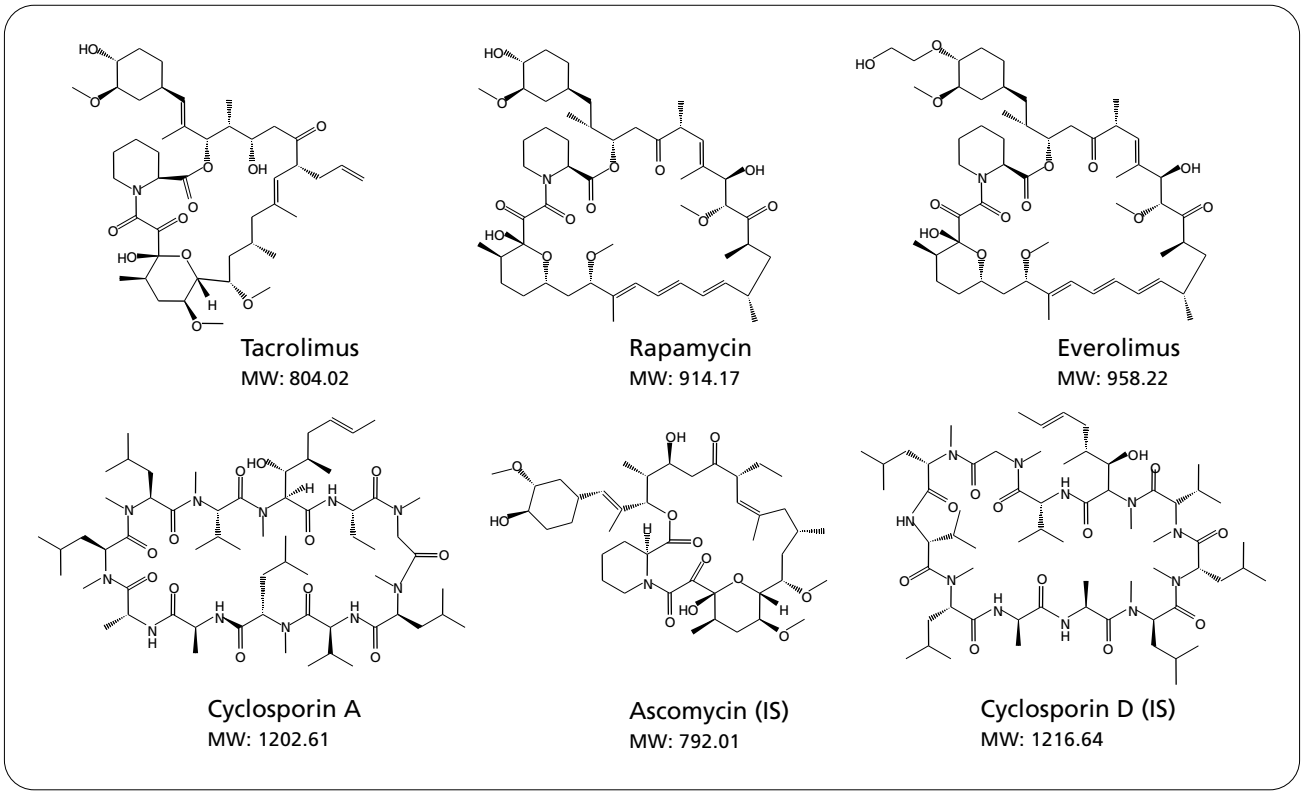


Figure 1 Structure of immunosuppressants and internal standards (IS)

Accelerated and robust monitoring for immunosuppressants using triple quadrupole mass spectrometry

Methods and Materials

Standard samples of each compound were analyzed to optimize conditions of liquid chromatograph and mass spectrometer. Whole blood extract was prepared based on liquid-liquid extraction described bellow.

2.7 mL of Whole blood and 20.8 mL of Water
↓
Vortex for 15 seconds
↓
Add 36 mL of MTBE/Cyclohexane (1:3)
↓
Vortex for 15 seconds and Centrifuge with 3000 rpm at 20 °C for 10 minutes
↓
Extract an Organic phase
↓
Evaporate and Dry under a Nitrogen gas stream
↓
Redissolve in 1.8 mL of 80 % Methanol solution with 1 mmol/L Ammonium acetate
↓
Vortex for 1 minute and Centrifuge with 3000 rpm at 4 °C for 5 minutes
↓
Filtrate and Transfer into 1 mL glass vial

Table 1 Analytical conditions

UHPLC

Liquid Chromatograph	: Nexera (Shimadzu, Japan)
Analysis Column	: YMC-Triart C18 (30 mmL. × 2 mmI.D.,1.9 µm)
Mobile Phase A	: 1 mmol/L Ammonium acetate - Water
Mobile Phase B	: 1 mmol/L Ammonium acetate - Methanol
Gradient Program	: 60 % B. (0 min) – 75 % B. (0.10 min) – 95 % B. (0.70 – 0.90 min) – 60 % B. (0.91 – 1.80 min)
Flow Rate	: 0.45 mL/min
Column Temperature	: 65 °C
Injection Volume	: 1.5 µL

MS

MS Spectrometer	: LCMS-8050 (Shimadzu, Japan)
Ionization	: ESI (negative)
Probe Voltage	: -4.5 ~ -3 kV
Nebulizing Gas Flow	: 3.0 L/min
Drying Gas Flow	: 5.0 L/min
Heating Gas Flow	: 15.0 L/min
Interface Temperature	: 400 °C
DL Temperature	: 150 °C
HB Temperature	: 390 °C

Accelerated and robust monitoring for immunosuppressants using triple quadrupole mass spectrometry

Result

Immunosuppressants, which we have developed a method for monitoring of, has been often observed as ammonium or sodium adduct ion by using positive ionization. In general, protonated molecule (for positive) or deprotonated molecule (for negative) is more preferable for reliable quantitation than adduct ions such as ammonium, sodium, and potassium adduct. In this study, each compound was detected as deprotonated molecule in negative mode by using heated ESI source of LCMS-8050 (Table 2).

The separation of all compounds was achieved within 1.8 min, with a YMC-Triart C18 column (30 mmL. × 2 mmI.D.,1.9 µm) and at 65 °C of column oven temperature.

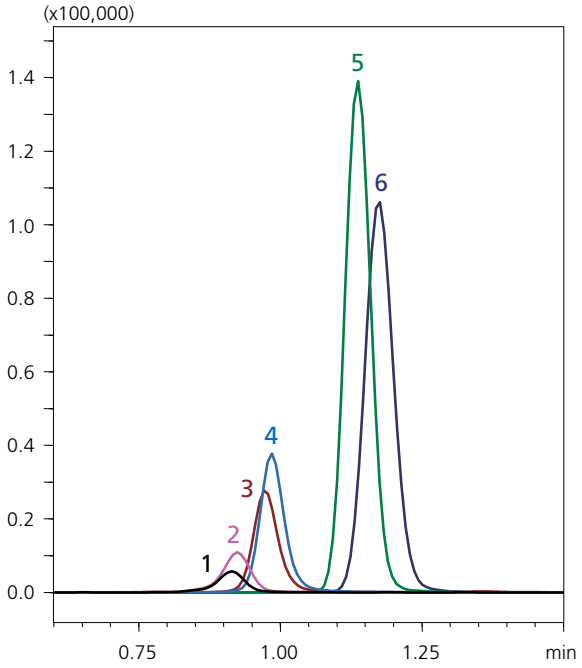


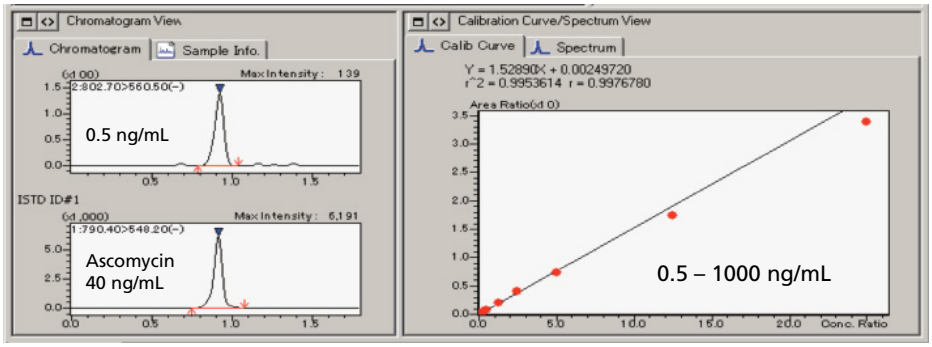
Figure 2 MRM chromatograms of immnosuppresants in human whole blood (50 ng/mL)

Table 2 MRM transitions

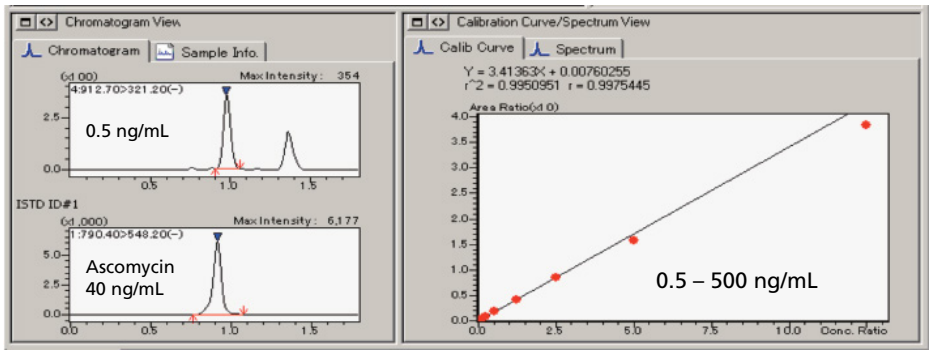
Peak No.	Compound	Porality	Precursor ion (*m/z*)	Product ion (*m/z*)
1	Ascomysin (IS)	neg	790.40	548.20
2	Tacrolimus	neg	802.70	560.50
3	Rapamycin	neg	912.70	321.20
4	Everolimus	neg	956.80	365.35
5	Cyclosporin A	neg	1200.90	1088.70
6	Cyclosporin D (IS)	neg	1215.10	1102.60

Accelerated and robust monitoring for immunosuppressants using triple quadrupole mass spectrometry

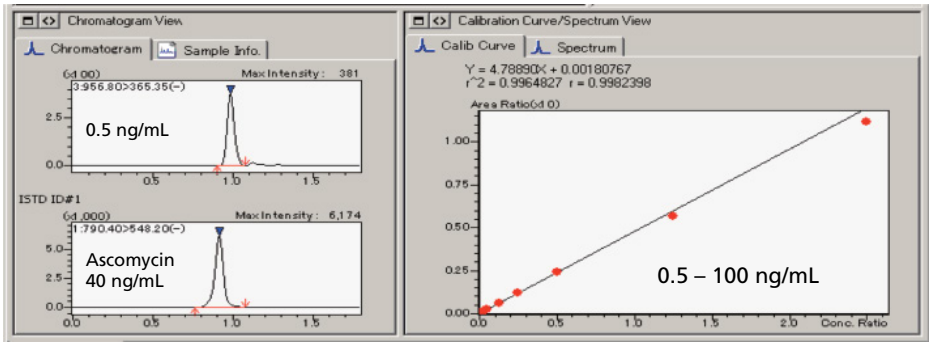
a) Tacrolimus



b) Rapamycin



c) Everolimus



d) Cyclosporin A

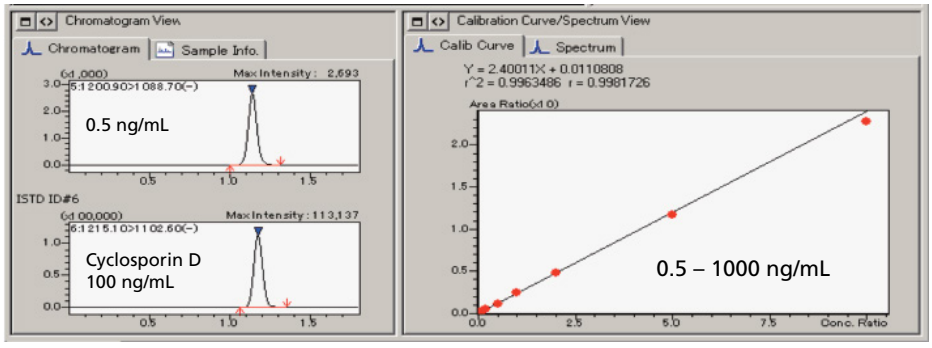


Figure 3 MRM chromatograms at LLOQ and ISTD (left), and calibration curves (right) for four immnosuppresants in human whole blood

Figure 3 illustrates both a calibration curve and chromatogram at the lowest calibration level for all immunosuppressants analyzed. Table 3 lists both the reproducibility and accuracy for each immunosuppressant that has been simultaneously measured in 1.8 minutes.

Table 3 Reproducibility and Accuracy

Compound	Concentration	CV % (n = 6)	Accuracy %
Tacrolimus	Low (0.5 ng/mL) Low-Mid (2 ng/mL) High (1000 ng/mL)	18.0 13.0 2.87	99.4 99.5 88.7
Rapamycin	Low (0.5 ng/mL) Low-Mid (5 ng/mL) High (500 ng/mL)	6.87 2.88 3.41	95.6 109.3 90.0
Everolimus	Low (0.5 ng/mL) Low-Mid (5 ng/mL) High (100 ng/mL)	10.4 5.11 2.26	95.3 104.4 93.3
Cyclosporin A	Low (0.5 ng/mL) Low-Mid (10 ng/mL) High (1000 ng/mL)	7.31 2.36 2.67	95.1 99.9 94.9

In high speed measurement condition, we have achieved high sensitivity and wide dynamic range for all analytes. Additionally, the accuracy of each analyte ranged from 88 to 110 % and area reproducibility at the lowest calibration level of each analyte was less than 20%.

Conclusions

- Monitoring with negative mode ionization permitted more sensitive, robust and reliable quantitation for four immunosuppressants.
- A total of six compounds were measured in 1.8 minutes. The combination of Nexera and LCMS-8050 provided a faster run time without sacrificing the quality of results.
- Even with a low injection volume of 1.5 µL, the lower limit of quantitation (LLOQ) for all compounds was 0.5 ng/mL.
- In this study, it is demonstrated that LCMS-8050 is useful for the rugged and rapid quantitation for immunosuppressants in whole blood.

Acknowledgement

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High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

ASMS 2016 ThP 682

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High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

Introduction

In this paper, we introduce four research use only LC/MS/MS methods for therapeutic drug monitoring (TDM), mycophenolic acid, sunitinib and axitinib, voriconazole, itraconazole. TDM is indispensable for managing drug dosage based on the drug concentration in blood in order to conduct a rational and efficient drug therapy. Liquid chromatography coupled with tandem quadrupole mass spectrometry (LC/MS/MS) is increasingly used in TDM because it can perform selective and sensitive analysis by simple sample pretreatment. In the field of TDM, it is necessary to measure the specimen such as plasma or serum quickly after suitable pretreatment and report the precise result. LC/MS/MS system with a simple and user-friendly interface can provide a streamlined workflow and reduce the load of analysts. We developed high-throughput LC/MS/MS methods for TDM with a new data acquisition and processing software.

Method

Instruments and LC/MS/MS analytical conditions

For LC/MS/MS analysis, a LCMS-8050 triple quadrupole mass spectrometer coupled to a Nexera X2 UHPLC system with mobile phase switching unit (Shimadzu corporation, Japan) was used. In all the methods, the compounds were separated by a reversed phase mode using a common column, Shim-pack GIS (Shimadzu corporation, Japan). All data acquisition and processing were performed by Open Solution QuantAnalytics (Shimadzu corporation, Japan), a software package for acquiring and reviewing quantitative LC/MS/MS data with ease.

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

Table 1 LC/MS/MS consitions

	Method 1	Method 2
Target	Mycophenolic acid (Immuno-suppressant)	Voriconazole (Triazole antifungal agent)
Column	Shim-pack GIS (2.1 mmI.D. x 75mmL., 3um)	
Column Oven	40°C	
Flow rate	0.3 mL/min	0.4 mL/min
Mobile phase A	1% Acetic acid-water	10 mM Ammonium acetate-water
Mobile phase B	Acetonitrile	Methanol
Gradient	A/B = 1/1, isocratic	Bconc. 30% (0 - 0.50min) → 100% (1.50 - 3.00min) → 30% (3.01 - 5.00min)
Injection volum	5 µL	1 µL
Ionization	ESI-positive	
MRM transition	321.40 > 207.30	350.20 > 281.20
Run time	4 min.	5 min.
	Method 3	**Method 4**
Target	Sunitinib and Axitinib (Anti-cancer drug)	Itraconazole (Triazole antifungal agent)
Column	Shim-pack GIS (2.1 mmI.D. x 75mmL., 3um)	
Column Oven	40°C	
Flow rate	0.3 mL/min	0.4 mL/min
Mobile phase A	10 mM Ammonium acetate-water	10 mM Ammonium acetate-water
Mobile phase B	Acetonitrile	Acetonitrile
Gradient	Bconc. 10% (0 - 0.25min) → 80% (2.00 - 3.00min) → 10% (3.01 - 5.00min)	Bconc. 65% (0 - 1.00 min) → 95% (1.50 - 2.50min) → 65% (2.51 - 4.50min)
Injection volum	5 µL	3 µL
Ionization	ESI-positive	
MRM transition	Sunitinib 399.40 > 283.30 Axitinib 387.40 > 356.30 SU12662 (Active metabolite of Sunitinib) 371.40 > 283.30	Itraconazole 705.40 > 392.40 Hydroxy Itraconazole (Active metabolite of Itraconazole) 721.40 > 408.40
Run time	5 min.	4.5 min.

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

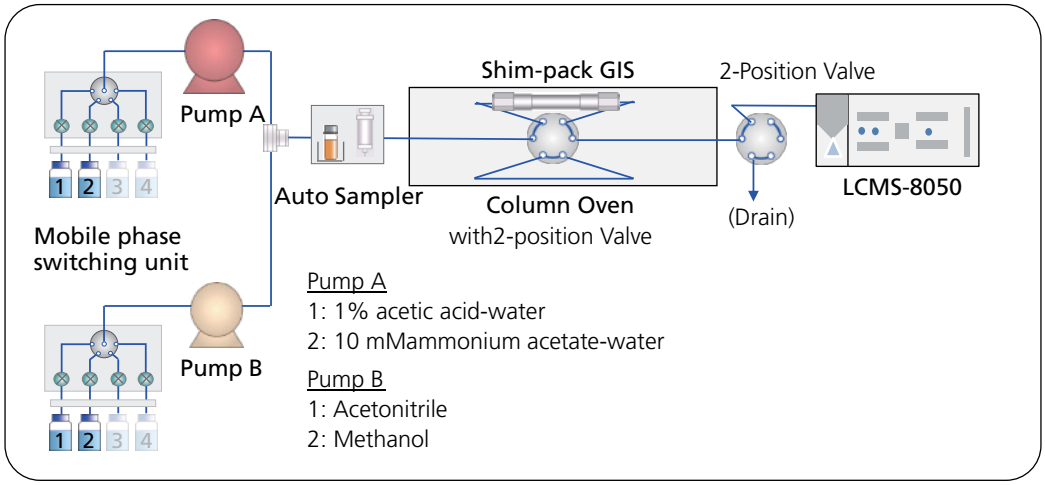


Figure 1 Nexera X2 UHPLC system with mobile phase switching unit

Calibration standards and QC samples

For each compound, more than five calibration standards and three QC samples were prepared. Samples were precipitated in a simple way of deproteination using organic solvent such as methanol or acetonitrile. The resulting supernatant was diluted and injected into LC/MS/MS without filtration.

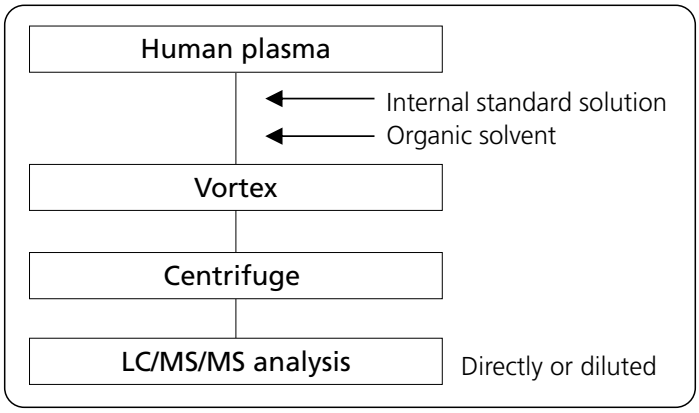


Figure 2 Work flow of the pretreatment

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

Result and discussion

Precision, accuracy and linearity

Table 2 illustrates linearity of all compounds and Table 3 illustrates accuracy and precision of the QC samples. Determination coefficient (r2) of all calibration curves was larger than 0.995, the precision (n=6) was within 15% RSD, and the accuracy (n=6) was within 80-120%.

Excellent linearity, accuracy and precision were obtained within a specific concentration range. Furthermore, All the methods took less than 5 minutes per one LC/MS/MS analysis, including column rinsing.

Table 2 Linear dynamic range for each compound

	Compound	Linearity (µg/mL)			r2
Method 1	Mycophenolic acid	0.2	~	20	0.999
Method 2	Voriconazole	0.1	~	10	0.999
Method 3	Sunitinib	3	~	300	0.999
	Axitinib	0.3	~	30	0.999
	SU12662	3	~	300	0.999
Method 4	Itoraconazole	10	~	1000	0.999
	Hydoroxy Itorazonazole	10	~	1000	0.999

Table 3 Precision and accuracy for analysis of QCs

	Compound	Concentrations of QC samples (µg/mL)			Precision (%)			Accuracy (%)		
		Low	Middle	High	Low	Middle	High	Low	Middle	High
Method 1	Mycophenolic acid	0.5	5	15	1.6	3.66	2.55	108.5	104.9	103.5
Method 2	Voriconazole	0.2	4	8	1.74	1.56	1.7	98.6	103.6	99
Method 3	Sunitinib	5	50	250	4.05	1.36	2.24	100.7	95.5	96
	Axitinib	0.5	5	25	8.26	4.56	4.43	85.3	88.5	91.2
	SU12662	5	50	250	4.07	1.26	3.17	97.2	96.5	97.8
Method 4	Itoraconazole	25	250	750	0.65	0.82	0.38	98.7	98.8	103.3
	Hydoroxy Itorazonazole				3.27	4.36	3.1	81.3	90.4	86.9

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

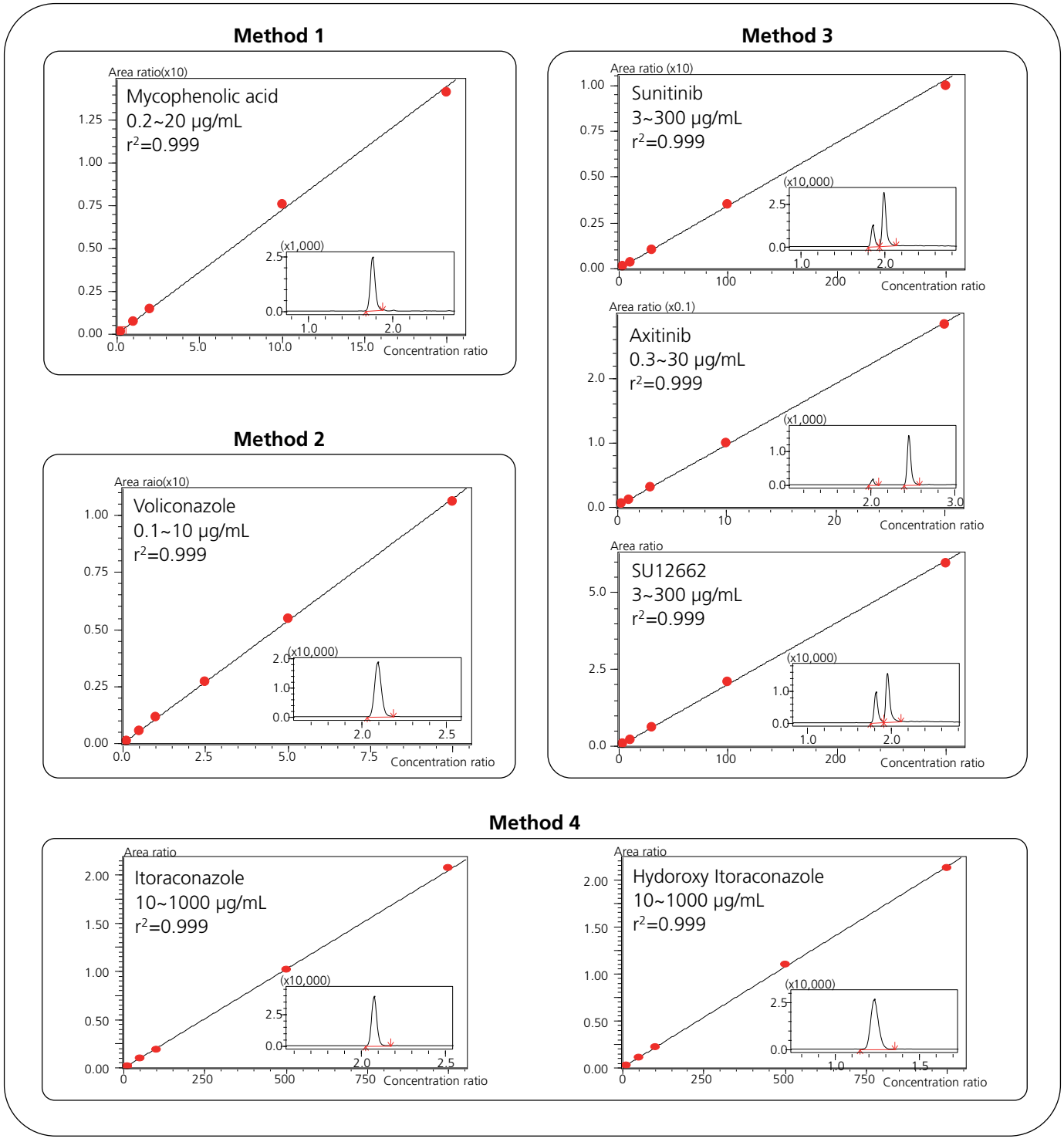
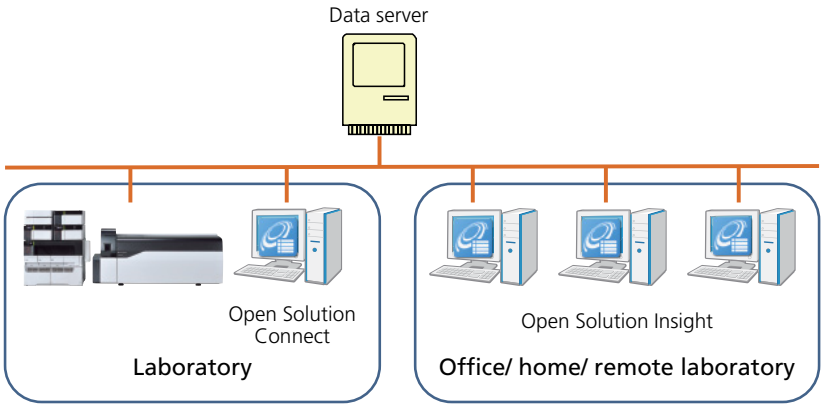


Figure 3 Calibration curves for each compound

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

Easy data acquisition with Open access software

The developed platform was used one common column for all methods and mobile phase changeover could be automatically done by just selecting method file. Open Solution QuantAnalytics software enables users to submit sample queue and set the LC/MS/MS condition easily and quickly. Users can intuitively start LC/MS/MS measurement by just selecting the predefined method and placing sample vials in the specified autosampler plate positions guided by software. The resulting data can be reviewed in office as soon as it becomes available on the designated data server. This system enables easy and quick data acquisition without tedious manual operation such as replacement of a column and mobile phases.



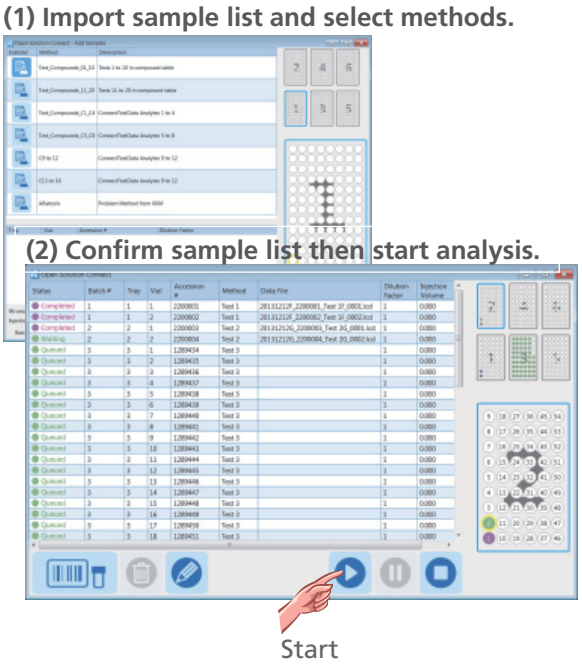


Figure 4 User interface for sample queue submission

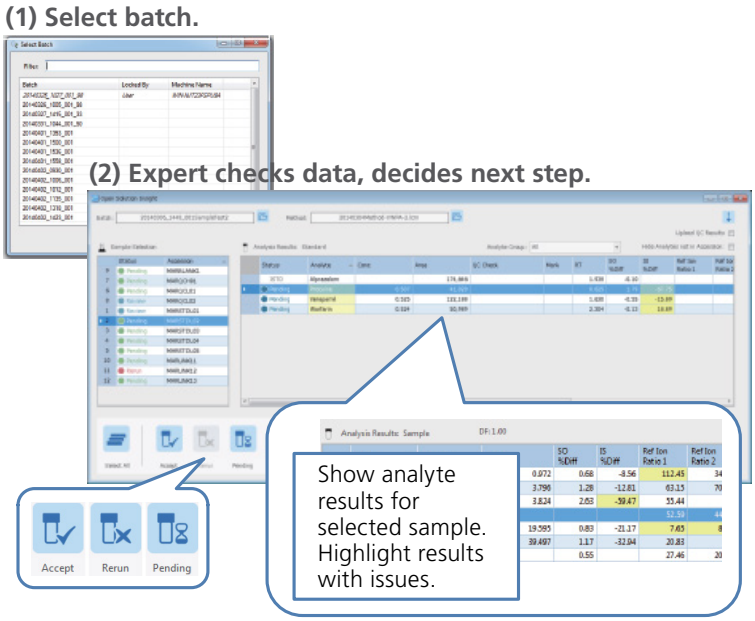


Figure 5 User interface for checking the result

High throughput quantitation for therapeutic drug monitoring with Open access LC/MS/MS system

Conclusions

- The combination of the Open Solution QuantAnalytics software and LC/MS/MS system with mobile phase switching unit enables easy sample submission and efficient data acquisition. The user only describes the vial position of samples, chooses a predefined method and the resulting data can be reviewed in office as soon as it becomes available on the designated data server.
- Saving time and effort for changing system conditions among each target compounds was achieved with mobile phase switching system and high through-put methods using a common column.

Disclaimer: Shimadzu LCMS-8050 CL and certain Nexera X2 UHPLC components are registered in the U.S. as a Class I device and is not specifically cleared for TDM. Other UHPLC components, Shim-pack GIS, and OpenSolution QuantAnalytics are intended for Research Use Only (RUO). Not for use in diagnostic procedures.

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Next generation plasma collection technology for the clinical analysis of temozolomide by HILIC/MS/MS

ASMS 2014 WP641

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Introduction

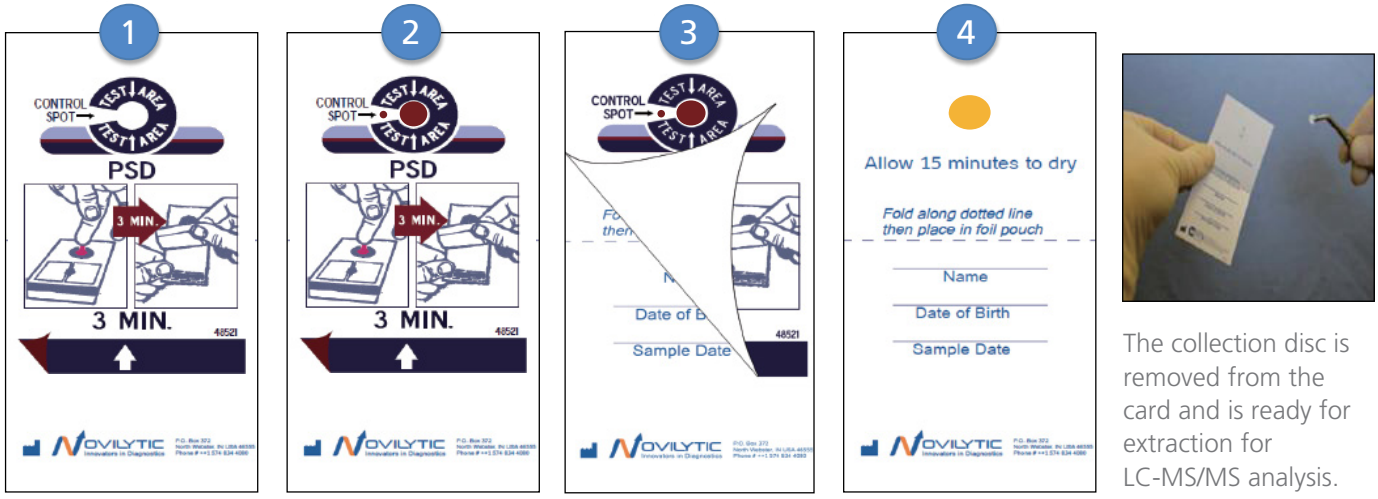
Plasma extraction technology is a novel technique achieved by applying a blood sample to a laminated membrane stack which allows plasma to flow through the asymmetric filter whilst retaining the cellular components of the blood sample.
Plasma separation card technology was applied to the quantitative analysis of temozolomide (TMZ); an oral imidazotetrazine alkylating agent used for the treatment of Grade IV astrocytoma, an aggressive form of brain tumour.

Under physiological conditions TMZ is rapidly converted to 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) which in-turn degrades by hydrolysis to 5-aminoimidazole-4-carboxamide (AIC). Storage of plasma has previously shown that both at -70C and 4C degradation still occurs. In these experiments, whole blood containing TMZ standard was applied to NoviPlex plasma separation cards (PSC). The aim was to develop a robust LC/MS/MS quantitative method for TMZ.

Materials and Methods

Plasma separation

TMZ spiked human blood calibration standards (50uL) were applied to the PSC as described below in figure 1.



A NoviPlex card is removed from foil packaging.

Approximately 50uL of whole blood is added to the test area.

After 3 minutes, the top layer is completely removed (peeled back).

The collection disc contains 2.5uL of plasma. Card is air dried for 15 minutes.

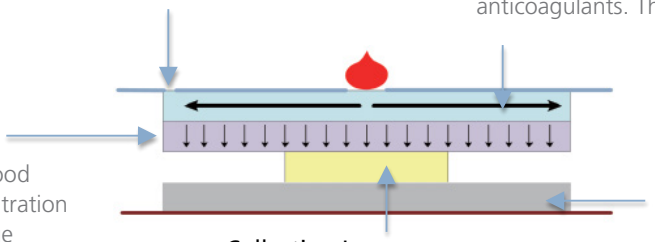
The collection disc is removed from the card and is ready for extraction for LC-MS/MS analysis.

Figure 1. Noviplex plasma separation card workflow

Next generation plasma collection technology for the clinical analysis of temozolomide by HILIC/MS/MS

Control Spot:
[Determines whether enough blood was placed on the card].

Spreading Layer
[Lateral spreading layer rapidly spreads blood so it will enter the filtration layer as a front while adding buffers and anticoagulants. The lateral spreading rate is 150um/sec].



Filtration Layer
[Filtration layer captures blood cells by a combination of filtration and adsorption. The average linear vertical migration rate is approximately 1um/sec].

Isolation Screen
[Precludes lateral wicking along the card surface].

Collection Layer
[Loads with a specific aliquot of plasma onto a 6.35mm disc]. Although flow through the filtration membrane is unlikely to be constant throughout the plasma extraction process, the average loading rate of the Collection Disc was 13 nL/sec. This corresponds to a volumetric flow rate into the Collection Disc of 400 pL/mm^2/sec.

Figure 1. Noviplex plasma separation card workflow (Cont'd)

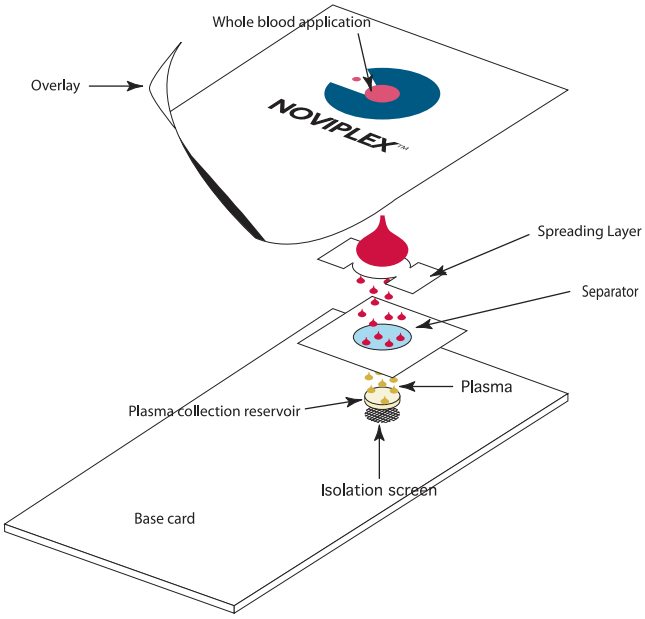


Figure 2. Applying a blood sample, either as a finger prick or by accurately measuring the blood volume, to the laminated membrane stack retains red cells and allows a plasma sample to be collected. The red cells are retained by a combination of adsorption and filtration whilst plasma advances through the membrane stack by capillary action. After approximately three minutes the plasma Collection Disc was saturated with an aliquot of plasma and was ready for LC/MS/MS analysis.

Sample preparation

TMZ was extracted from the dried plasma collection discs by adding 40uL acetonitrile + 0.1% formic acid, followed by centrifugation 16,000g for 5 min. 30uL supernatant was added directly to the LC/MS/MS sample vial for analysis.

As a control, conventional plasma samples were prepared by centrifuging the human blood calibration standards at 1000g for 10min. TMZ was extracted from 2.5uL of plasma using the same extraction protocol as applied for PSC.

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LC/MS/MS analysis

Ionisation	: Electrospray, positive mode MRM 195.05 >138.05 CE -10
Desolvation line	: 300°C
Drying/Nebulising gas	: 10L/min, 2L/min
Heating block	: 400°C

HPLC	: HILIC Nexera UHPLC system	Reverse Phase Nexera UHPLC system
Flow rate	: 0.5mL/min (0-7min), 1.8mL/min (7.5min-17.5min)	0.4mL/min
Mobile phase	: A= Water + 0.1% formic acid B= Acetonitrile + 0.1% formic acid	A= Water + 0.1% formic acid B= methanol + 0.1% formic acid
Gradient	: 95% B – 30%% B (6.5 min), 30% B (7.5 min), 95% B (18 min)	5% B – 100%% B (3 min), 100% B (7 min), 5% B (10 min)
Analytical column	: ZIC HILIC 150 x 4.6mm 5um 200ª	Phenomenex Kinetex XB C18 100 x 2.1mm 1.7um 100A
Column temperature	: 40°C	50°C
Injection volume	: 10uL	2µL

Results

HILIC LC/MS/MS

Temozolomide is known to be unstable under physiological conditions and is converted to 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide (MTIC) by a nonenzymatic, chemical degradation process. Previous studies have used HILIC to analyze the polar compound and to avoid degradation in aqueous solutions.

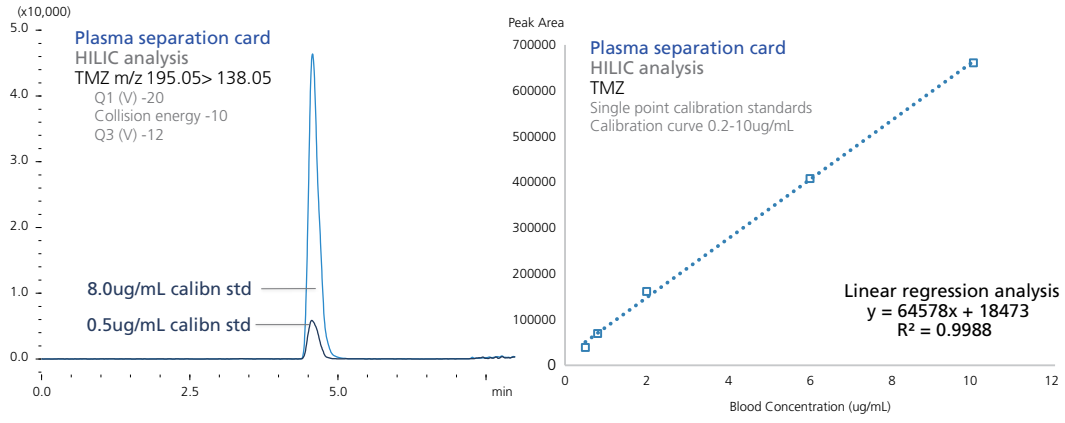

Figure 3. HILIC LC/MS/MS chromatograms for PSC TMZ analysis at 0.5 and 8ug/mL. The PSC calibration curve was linear between 0.2-10ug/mL (r2>0.99). HILIC was considered in response to previous published data and to minimize potential stability issues. However, to reduce sample cycle times a reverse phase method was also developed.

Next generation plasma collection technology for the clinical analysis of temozolomide by HILIC/MS/MS

Reversed Phase LC/MS/MS

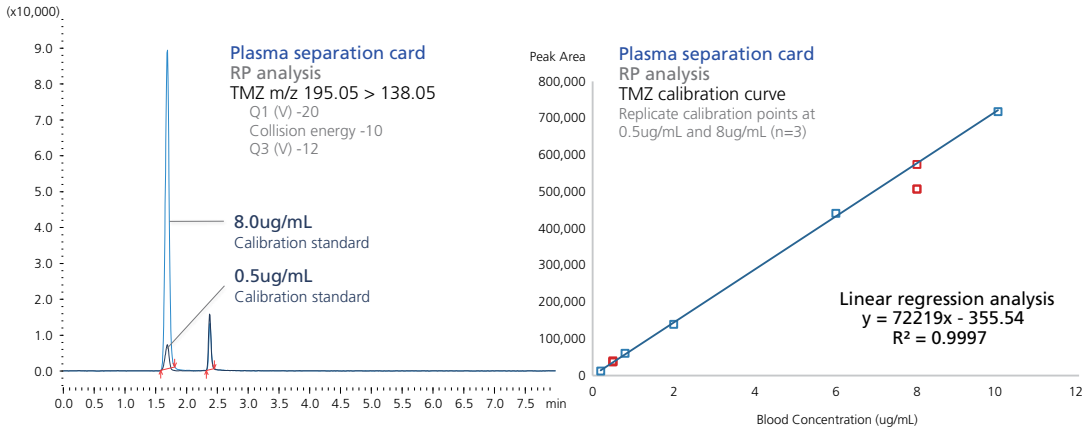

Figure 4. Reverse phase LC/MS/MS chromatograms for PSC TMZ analysis at 0.5 and 8ug/mL. The PSC calibration curve was linear between 0.2-10ug/mL (r2>0.99; replicate samples were included in the liner regression analysis at 0.5 and 8ug/mL; n=3).

Due to the relatively long cycle time (18 min), a faster reversed phase method was developed (10 min). Sample preparation was modified with PSC sample disk placed in 40uL methanol + 0.1% formic acid, followed by centrifugation 16,000g 5 min. 20uL supernatant was added directly to the LC/MS sample vial plus 80uL water + 0.1% formic acid. In addition to reversed phase being faster, the sample injection volume was reduced to just 2uL as a result of higher sensitivity from narrower peak width (reversed phase,13 sec; HILIC, 42 sec).

Comparison between PSC and plasma

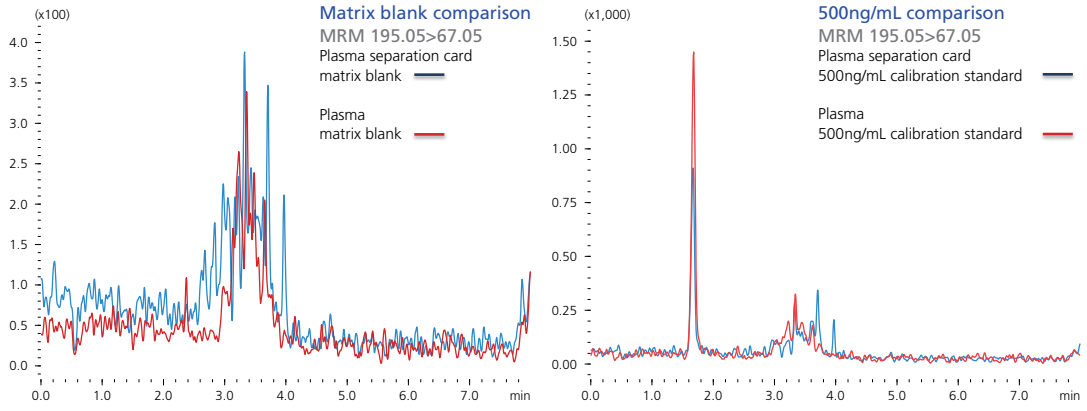

Figure 5. Human blood TMZ calibration standards were prepared using PSC and conventional plasma. Using the confirmatory ion transition 195.05>67.05 both the PSC and plasma sample are in broad agreement with regard to matrix ion signal distribution.

Next generation plasma collection technology for the clinical analysis of temozolomide by HILIC/MS/MS

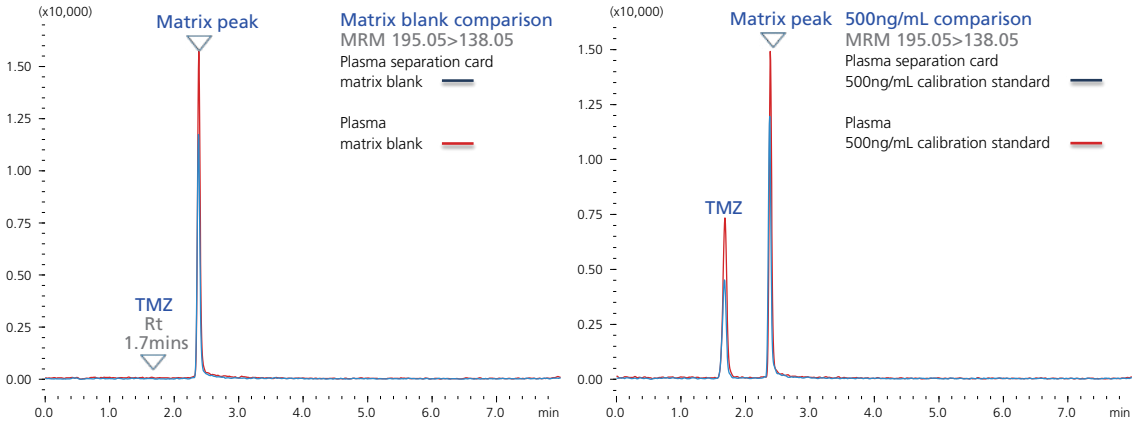

Figure 6. Human blood TMZ calibration standards were prepared using PSC and conventional plasma. Using the quantitation ion transition 195.05>138.05 both the PSC and plasma sample are in broad agreement in signal distribution and intensity including the presence of a matrix peak at 2.4mins.

Conclusions

This technology has the potential for a simplified clinical sample collection by the finger prick approach, with future work aimed to evaluate long term sample stability of PSC samples, stored at room temperature. Quantitation of drug metabolites MTIC and AIC also could help provide a measure of sample stability.

References

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Application News

No. AD-0069

LCMS-8040 UFMS

Fast LC/MS/MS Method for Quantitative Determination of Omeprazole in Human Plasma

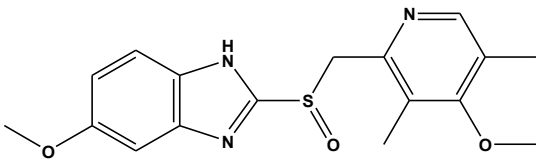
Manikandan Venkateswaran, Vinod Pillai. S.L, Venkat Manohar and Arvind Thyagarajan
*Indian Institute of Chromatography & Mass Spectrometry (IICMS)**

Abstract

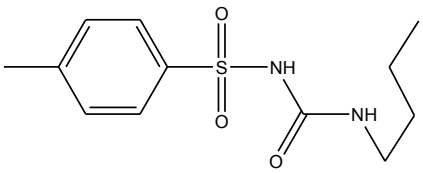
A sensitive and a fast LC/MS/MS method for quantification of Omeprazole in human plasma using UHPLC NEXERA coupled to LCMS-8040 Triple Quadrupole Mass Spectrometer was described. A simple liquid-liquid extraction method for extraction of Omeprazole and internal standard (Tolbutamide) from the biological matrix was employed. The new advanced LCMS-8040 enabled the quantification of Omeprazole from human plasma samples over a concentration range of 1.0 (LLOQ) to 2000ng/mL. The constructed calibration curve was linear with a regression of >0.99.

❑ Introduction

Omeprazole is a well-studied proton pump inhibitor, which inhibits the gastric parietal cell proton pump, dose-dependently reducing basal and stimulated gastric secretion and raising intragastric pH. It belongs to a class of anti-secretary compound used in treatment of peptic ulcer, gastro esophageal reflux, dyspepsia. Omeprazole is one of the most widely prescribed drugs internationally and is available over the counter in some countries. The chemical name for Omeprazole is (RS) -5-methoxy-2-((4-methoxy-3,5-dimethylpyridin-2-yl)methylsulfinyl)-5-methhoxy-1H-benzo[d]imidazole. For routine analysis of bio-fluids, simple sample preparation protocols that are sensitive and specific are preferred. LC/MS/Ms is then the method of choice for analytes extracted from biological matrices. In this note, a simple, fast and a sensitive method for quantitative determination of Omeprazole in human plasma with Tolbutamide as internal standard is described using the UHPLC Nexera coupled to LCMS-8040 instrument.



Omeprazole



Tolbutamide (IS)

Figure-1 : Chemical structure of Omeprazole and Tolbutamide (IS)

❑ Experimental

Preparation of Aqueous standards: A 1 mg/mL Stock solution of internal standard (IS) was prepared in acetonitrile. This solution was serially diluted using acetonitrile to obtain a working solution of internal standard containing 300µg/mL of Tolbutamide (IS). Similarly a 1mg/mL stock solution of Omeprazole was prepared using Methanol as diluent. This solution was further serially diluted using Methanol to obtain aqueous standards containing Omeprazole of concentration 10.0, 20.0, 200.0, 1000, 5000, 7500, 15000 and 20000 ng/mL respectively.

Preparation of Plasma calibration standards (CC): 180µL of human plasma was spiked with 20 µL of each aqueous Omeprazole standard solution and vortexed for 30 seconds to obtain plasma calibration standard whose concentration ranged from 1.0 – 2000 ng/mL. Each of these samples were then extracted according to the procedure as described under sample preparation.

Preparation of plasma quality control standards (QC): The Quality control standard solutions were prepared at three intermediate concentrations of that of CC standards namely 3.0, 900.0 and 1800.0 ng/mL (LQC, MQC and HQC respectively). Six individual preparations of each of the QC standards were prepared to evaluate precision and recovery. Each of these sample preparation were then extracted according to the procedure as described under

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sample preparation.

Sample preparation: 200 µL of Sodium bicarbonate buffer (pH 10.5) was added to each of the plasma sample taken in a centrifuge tube and vortexed briefly for a period of 60s. A 600 µL of Ethyl acetate was then added to each of the tubes and mixed for a period of 5 minutes. The samples were then centrifuged using a *minispin* at 10000 rpm for 10 minutes. A 100 µL of the upper layer was then mixed with 200 µL of acetonitrile and injected in to the LC/MS/MS system.

Table-1 : Analytical conditions

Column : Capcell pak C18, 50 x 2.0mm, 3.0µm
Mobile phase-A : 0.02m ammonium formate in water pH-3.0
Mobile phase-B : Acetonitrile: Methanol (50:50) v/v
Gradient (%B/T) : 5/0.01, 95/2.00, 5/2.01, & 5/3.5
Flow rate : 800 µL/min DL temp : 220 ^{0}C
Column temp : 35 ^{0}C Heat block : 275 ^{0}C
Drying gas : 15 L/min Interface : ESI
Nebulizing gas : 3.0 L/min Interface volt : 4.5 kV

For Omeprazole
MRM : 346.00 → 198.10 Polarity : Positive
Dwell time : 100 ms CE : - 13.0V
Q1 pre-bias : - 24.0V Q3 pre-bias : - 38.0V

For Tolbutamide
MRM : 269.00 → 170.10 Polarity : Negative
Dwell time : 100 ms CE : 17.0V
Q1 pre-bias : 28.0V Q3 pre-bias : 34.0V

The LC-MS conditions are as summarized as in Table-1. Precursor ions of Omeprazole and Tolbutamide (IS) were determined by injecting a solution containing these compounds in the Q1 scan mode. Under these conditions, the analyte and the IS yielded predominantly the quasi molecular ions of m/z 346 and m/z 269 respectively. Each of these precursor ions was subjected to collision induced dissociation (CID) in order to generate product ions. This operation was done automatically by the use of SSS (Synchronized Survey Scan) function in the software to obtain optimized parameters. Based on this, the ion transitions of m/z 346.00 → 198.10 and m/z 269.00 → 170.10 (Figure-2) were used in MRM mode for Omeprazole and Tolbutamide (IS) respectively.

❑ Results and Discussion

LLOQ

The concentration of Omeprazole at lower limit of quantitation (LLOQ) was determined to be 1.0 ng/mL. This was confirmed from the coefficient of variance (CV) being less than 20% for the six replicate injections of Omeprazole at this concentration. The overlay mass chromatograms corresponding to Omeprazole is as presented in Figure-3.

Linearity

The CC standards were used to construct a calibration curve by plotting the area ratio of Omeprazole with respect to IS versus the concentration of CC standards. Linear curve fit type was used and weighted ($1/x^2$). A linear dynamic range of 1.0 to 2000.0 ng/mL was achieved for

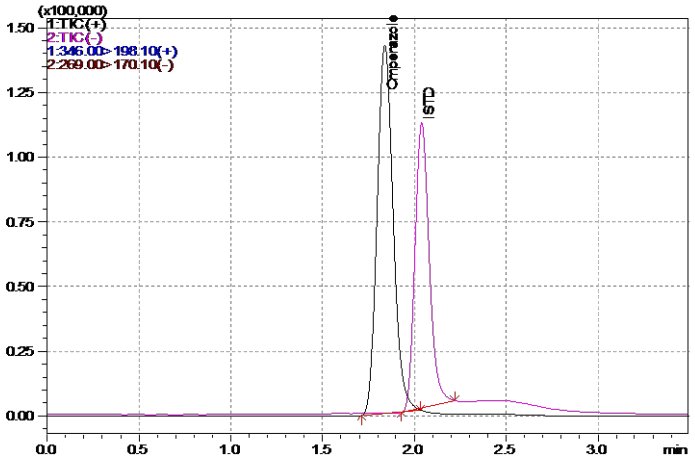

Figure-2 : MRM mass chromatogram of Omeprazole at MQC

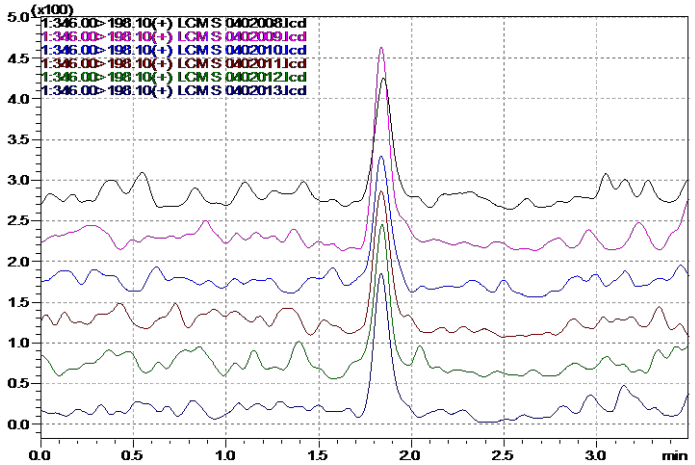

Figure-3 : Overlay chromatograms of Omeprazole at LLOQ

Omeprazole with a R^2 value of 0.99987 .

Figure-4 shows a representative calibration curve of Omeprazole in plasma using Tolbutamide as internal standard.

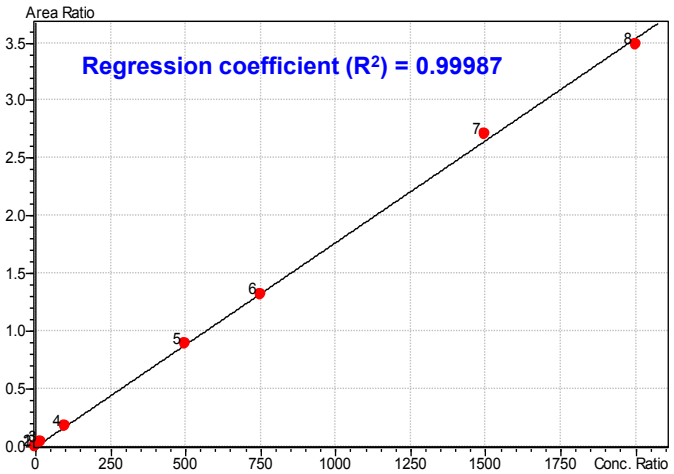

Figure-4 : Calibration curve of Omeprazole

Precision & Accuracy of QC samples

Low, middle and high QC samples containing Omeprazole were prepared at concentrations of 3, 900 and 1800 ng/mL in plasma. The precision (%CV, n=6) for the QCs for Omeprazole varied from 3.1 to 12.4% and accuracy from 80.0 to 113.3% of the nominal value (Table-2).

Table 2: Precision and accuracy of Omeprazole in QC samples

Nominal Conc. (ng/mL)	Measured conc. (ng/mL)	Accuracy*	Precision (n=6)
3.0	2.7	90.0	12.4
	2.5	83.3	
	3.4	113.3	
	3.2	106.7	
	2.4	80.0	
	2.7	90.0	
900.0	826.0	91.8	3.1
	802.9	89.2	
	771.4	85.7	
	842.7	93.6	
	828.2	92.0	
	824.5	91.6	
1800.0	1704.1	94.5	4.9
	1514.1	84.1	
	1536.5	85.4	
	1631.0	90.6	
	1554.2	86.3	
	1514.9	84.2	

* expressed as Bias = (mean concentration/nominal concentration) x 100

Table 3: Recovery of Omeprazole in QC samples

QC sample	Number of Preparations	% Recovery
		Omeprazole
LQC	1	103.9
	2	89.2
	3	114.4
	4	107.3
	5	103.0
	6	99.0
MQC	1	96.8
	2	95.8
	3	95.9
	4	96.5
	5	97.2
	6	98.4
HQC	1	95.0
	2	92.1
	3	91.7
	4	96.7
	5	93.8
	6	86.0

Recovery of QC samples

The recovery of Omeprazole was calculated by comparing the peak area obtained for QC samples that were subjected to extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations. Good recoveries were obtained (Table-3) for Omeprazole demonstrating the efficiency of analyte extraction in the presence of biological matrix.

❑ Conclusion

A simple, high throughput LC-MS/MS method for quantitative determination of Omeprazole in human plasma was developed. The LLOQ of the method using a 180 µL of plasma was determined as 1.0ng/mL. The linear dynamic range of the calibration curve was 1.0 – 2000 ng/mL with a regression of R^2 = 0.99987. Good recoveries (between 86.0 to 114.4%) were obtained were obtained at all the three levels of QC samples with repeatability .

❑ Reference

J. De Smet et al. J.Sep. Sci. 2010, 33, 939-947

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