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Technical Report

# A Fatal Case of High-concentrated Bisoprolol in Blood

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## Abstract:

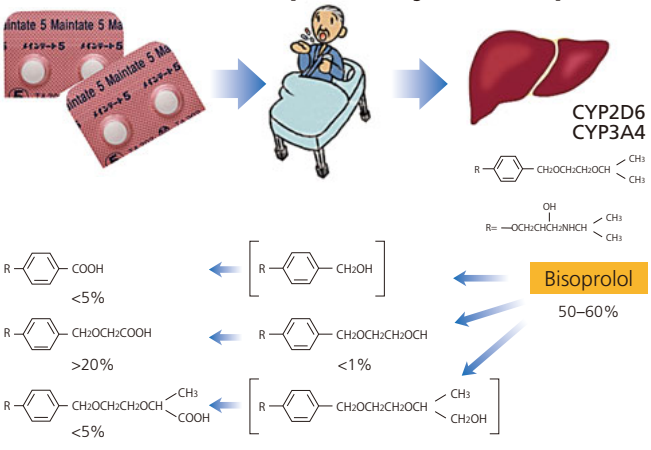
A woman in her seventies was estimated to be died of overdose of Maintate® (bisoprolol fumarate). A large amount of bisoprolol (230 ng/mL) was detected in her blood using LC-MS/MS equipment. By the genotyping analysis of metabolic enzyme, she had *CYP2D6*10/*10* and *CYP3D4*1/*1* homozygote alleles. One should check not only genotyping of metabolic enzyme but also the expression level of the metabolic enzyme in the liver if case is highly suspicious of drug overdose.

**Keywords: Bisoprolol, LC-MS/MS, Cytochrome P450 isozyme**

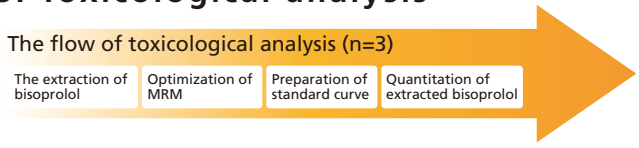
## 1. Case profile

A woman in her seventies was admitted to hospital with a chief complaint of dyspnea. She received medical treatment for irregular heart-beat. However, she was prescribed 10 mg Maintate® (bisoprolol fumarate) instead of 1.25 mg, accidentally. Five hours later, she had bradycardia and died eight hours after taking Maintate®. Autopsy revealed left pulmonary fibrosis and eccentric hypertrophy of the right ventricle.

## 2. The metabolic pathway of bisoprolol




## 3. Toxicological analysis




### Extraction the bisoprolol from blood sample

Extracted from cardiac blood using a QuEChERS method developed in our laboratory.

### The authentic preparation of bisoprolol for calibration curve

6 points: 1, 5, 10, 50, 100, 500 ng/mL in methanol

### Internal standard

D5-Diazepam 50 ng/mL in ACN

### HPLC conditions (Nexera UHPLC System)

| | |
|---|---|
| Column | : Shim-pack XR-ODS II [30 mmL. × 1.5 mmI.D., 2.2 µm] |
| Mobile phase | : A: 95% 10 mM ammonium formate + 5% MetOH<br>B: 5% 10 mM ammonium formate + 95% MetOH |
| Flow rate | : 0.3 mL/min |
| Time program | : B conc. 20% (0 min) -95% (3.0–4.0 min) -20% (4.01–6.0min) |
| Injection volume | : 1 µL |
| Column temperature | : 40°C |

### MS conditions (LCMS-8030; modified ion optics)

| | |
|---|---|
| Ionization | : ESI |
| Polarity | : Positive |
| Measurement mode | : MRM |

| Compounds | Quantitative ion : CE | | Conformation ion : CE | |
|---|---|---|---|---|
| Bisoprolol | 326.20>116.15 | –16 | 326.20>74.10 | –25 |
| Diazepam-d5 | 290.15>198.20 | –35 | 290.20>154.05 | –34 |

Dwell Time 100 msec, Pause time 3 msec

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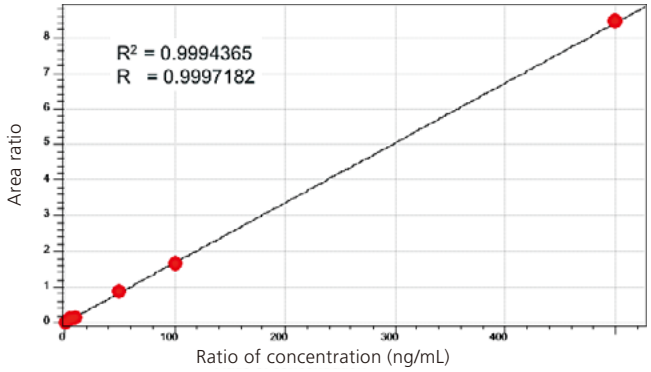
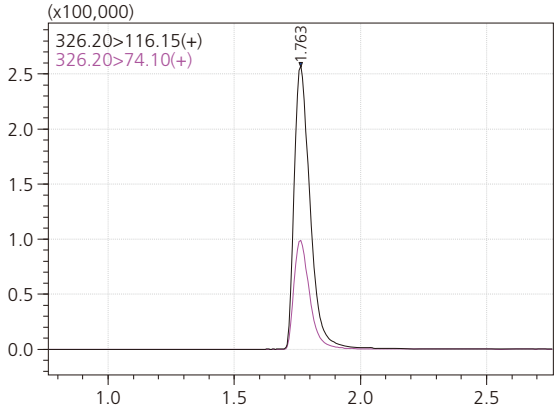
## Preparation of standard curve




- This standard curve has the good linearity ($R^2$>0.999) within 1 ng/mL to 500 ng/mL.
- The average of S/N was calculated as 12.44 in 1 ng/mL.
- The detection limit was calculated as 0.241 ng/mL in case of S/N: 3.

## The quantitation of extracted bisoprolol from blood sample

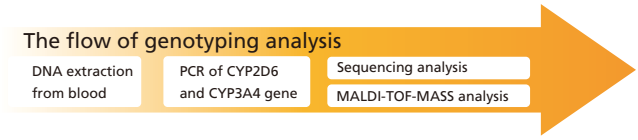



## The results of quantitation of bisoprolol in blood sample (n=3)

| Data file | Retention time | Height | Area | Concentrate (ng/mL) |
|---|---|---|---|---|
| f_sample_20.lcd | 1.763 | 256,334 | 1,101,215 | 228.943 |
| f_sample_21.lcd | 1.791 | 258,749 | 1,116,106 | 232.393 |
| f_sample_22.lcd | 1.752 | 251,702 | 1,089,046 | 228.413 |
| average | 1.768 | 255,595 | 1,102,122 | 229.916 |
| %RSD | 1.139621 | 1.401142 | 1.229695 | 0.939979 |

The total-ion-chromatogram by LC-MS/MS analysis showed a peak at 1.77 min retention-time and the product-ion-spectrum divided from the peak was accurately-matched with the one from bisoprolol. The concentration of bisoprolol in the blood was 230 ng/mL.

# 4. Genotyping of cytochrome P450 isozyme




## The flow of genotyping analysis

### DNA extraction from blood

- Extraction Kit

### PCR of CYP2D6 and CYP3A4 gene

- Sistonen J. *et.al*. CYP2D6 genotyping by a multiplex primer extension reaction. *Clinical Chemistry*. 2005 Jul; 51 (7):1291-5.
- Beer B. *et al*. CYP2D6 genotyping by liquid chromatography-electrospray ionization mass spectrometry. *Analytical and Bioanalytical Chemistry*. 2011 Jun; 400 (8):2361-70

### Sequencing analysis

- DNA Sequencer
- AXIMA® Confidence™ (SHIMADZU)

## The flow of CYP2D6 genotyping

### Step 1. Long range PCR

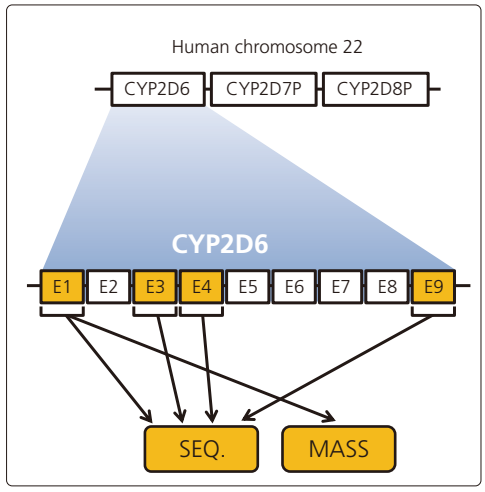
Specific amplification of the CYP2D6 gene has to be done because this region involves two pseudogenes, CYP2D7P and CYP2D8P. All three are disposed in tandem and share a nucleotide homology of 92–97%. In addition, CYP2D6 gene deletion and duplication were identified according to long PCR protocols. No gene deletion and duplication were found in our sample (data not shown).

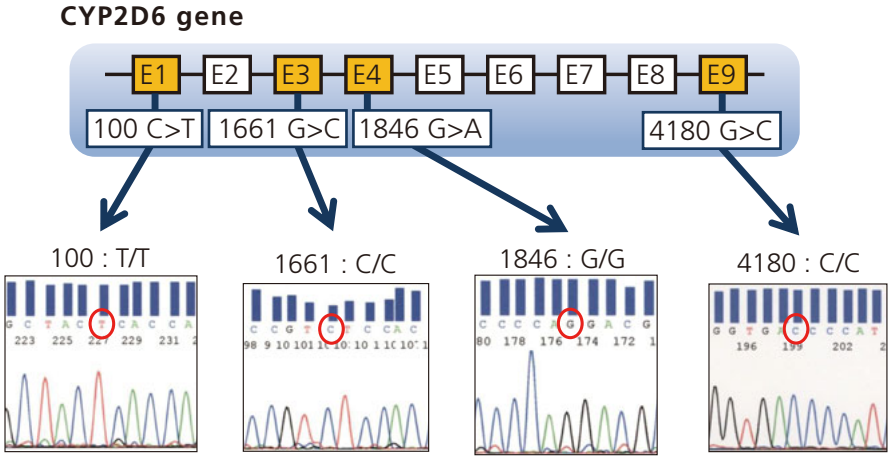
### Step 2. Amplification of each exons

We amplified 5 exons (E1, E3, E4 and E9) to identify the allele type of the CYP2D6 gene.

### Step 3. Sequencing or single-base extension

To determine the allele type, we used two methods, sequencing and MALDI-TOF MASS.

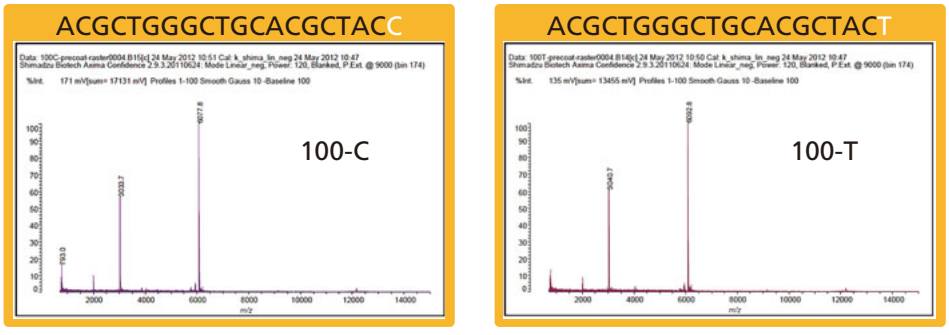





The sequence revealed that the sample was homozygote, *2D6*10/*10* which is caused by the SNPs.

## The MALDI-TOF-MASS analysis of the position 100 C>T in the CYP2D6 gene

Template DNA : 5'····CGGCGCAACGCTGGGCTGCACGCTAC $\begin{matrix}T\\C\end{matrix}$····3'
Primer : 5'-ACGCTGGGCTGCACGCTAC-3'
Single extension reaction : +ddCTP +ddTTP




We could not get clear SNP typings using the MALDI-TOF MASS method. Since the primers annealed not SNPs position but several unexpected positions, the single nucleotide extension reaction products showed the doubtful results. The primer sequences and the reaction condition were required to redefine.

## The flow of CYP3A4 genotyping

**Step 1. Amplification of each polymorphic site:**

We amplified the region including each seven SNPs which defined the alleles, *3A4*1G,*3,*5,*6,*11,*16A* and **18A*.

**Step 2. Sequencing:**

To determine the allele type, we sequenced the PCR products.

| Allele | Nucleotide changes | Allele | Nucleotide changes |
|---|---|---|---|
| *CYP3A*1G* | 20230 G>A | *CYP3A*11* | 21867 C>T |
| *CYP3A*3* | 23171 T>C | *CYP3A*16A* | 15603 C>G |
| *CYP3A*5* | 15702 C>G | *CYP3A*18A* | 20070 T>C |
| *CYP3A*6* | 17661 insA 17662 | | |

The Human Cytochrome P450 (CYP) Allele Nomenclature Database
http://www.cypalleles.ki.se/

We sequenced seven SNPs region which produced allele polymorphism, that is 3A4*1G, *3, *5, *6, *11, *16A and *18A.

All seven SNPs exhibited the same type of wild type (3A4*1) which means the homozygote, 3A4*1/1.

The cause of death was macroscopically diagnosed as circulatory insufficiency. Autopsy findings showed that she was in bad condition with the poor cardiorespiratory function.

She took two tablets of 5 mg. In this case, the question was the concentration of 230 ng/mL because the value was 4–5 times than we had expected. She was no information of renal diseases and microscopic findings supported it.

Genetic analysis showed she had *CYP2D6*10/*10*, homozygote alleles which decrease the activity of CYP2D6 metabolizing efficiency. This allele has relatively high frequency (about 20%) in Japanese and seemed to be less effect on bisoprolol metabolism. However, this is not just as valid for overdose case. It's not necessarily appropriate to suggest that the *CYP2D6*10* allele has no effect on bisoprolol metabolism.

The point is the expression level of metabolic enzyme, CYP2D6 and CYP3A4 in the liver.

The adverse drug reaction is mostly caused of increasing the drug concentration in the blood. However, the threshold amount and the onset time are mostly uncertain. This depends on many reasons such as genotype  and expression level of enzyme, effect on physical condition.

One should check not only genotyping of metabolic enzyme but also the expression level of the metabolic enzyme in the liver if case is highly suspicious of drug overdose.

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