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A Rapid Screening Method for the Analysis of Beta-Blockers Using Electrospray Ionization-Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Cardiovascular drugs, when overdosed, may present serious symptoms of intoxication and can even be more severe than the underlying diseases themselves. The aim of this study was to develop and validate a sensitive electrospray ionization (ESI)-liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for the simultaneous analysis of 17 beta-blockers (Method A), beta-blockers (metoprolol, propranolol) and their metabolites (*α*-hydroxy metoprolol, *O*-desmethyl metoprolol, and 4-hydroxy propranolol) (Method B) in serum and urine. The extraction of beta-blockers and their metabolites from human serum and urine was performed by a solid-phase extraction method using Oasis® PRiME HLB columns. Chromatographic separation was performed on a Mightysil-RP-18 MS column. For gradient elution, two solvents were used: a) 10 mM acetic ammonium buffer and b) acetonitrile. Separation and sensitivity for the detection of beta-blockers and their metabolites by LC-MS/MS were sufficient, and the precursor $[M + H]^+$ ion was detected in the mass spectrum of each drug (S/N = 3). These methods had a total chromatographic run time of 15 min. The calibration curves were linear over the concentration range of 20 - 400 ng/mL for the 17 beta-blockers (Method A) and 2 - 200 ng/mL (Method B). The extraction yields for human serum samples (17 beta-blockers) ranged from 66.1% - 93.5%, and the accuracy and precision were within 7.0% CV values, while the extraction yields for human serum or urine samples ranged from 93.8% to 106.5%, and the accuracy and precision were within 5.9% CV values, which were acceptable for these methods. The limits of quantification (LOQ) can cover the therapeutic blood concentration range in which beta-blockers are clinically used (Method A and

Method B). The presently established method is useful for the simultaneous measurement of beta-blockers and their metabolites in human serum and urine by LC-MS/MS in clinical and forensic investigations.

Keywords

Beta Blocker, Analysis, LC-MS/MS, Intoxication, Solid-Phase Extraction

1. Introduction

In recent years, various drugs responsible for acute drug intoxication in the elderly have been identified, including benzodiazepines, phenobarbital, digitalis preparations, phenytoin, beta-blockers, calcium antagonists, and antidepressants in Japan. A number of analyses of various pharmaceuticals using LC-MS/MS have been reported [1]-[12]. Cardiovascular drugs (beta-blockers) are clinically used in the management of a range of disorders, including hypertension, heart failure, arrhythmias, migraines, headaches, and tremors. Beta-blockers are likely to be misused owing to their widespread use. Side effects of beta-blockers include bradycardia derived from the β_1 receptor, heart failure, atrioventricular block, bronchial asthma, and dyspnea derived from the β_2 receptor [13] [14]. Furthermore, they can cause fluctuations in blood levels owing to interactions with other drugs and are likely to cause toxic symptoms [15]. Therefore, beta-blockers are important targets for the analysis of drugs for acute intoxication. Examples of past beta-blocker toxic symptoms include cardiac arrest owing to an overdose of propranolol [16], manifestation of cardiovascular toxicity due to metoprolol [17], refractory cardiogenic shock, and complete heart failure after atenolol overdose [18]. These reports describe cases in which acute toxic effects on the heart derived from β_1 receptors have emerged from pharmacological effects.

Recently, several methods have been developed for the determination of beta-blockers in human serum, including gas chromatography-mass spectrometry (GC-MS) [19], high-performance liquid chromatography (HPLC) [20]-[23], liquid chromatography-mass spectrometry (LC-MS), and electrospray ionization (ESI)-LC-MS/MS [22] [24]-[30]. However, a method for the simultaneous analysis of beta-blockers, which are frequently used clinically in Japan, using solid-phase extraction from human serum has not yet been reported.

In this paper, we investigated a selective and sensitive method to simultaneously determine 17 beta-blockers (atenolol, carteolol, nadolol, pindolol, timolol, acebutolol, arotinolol, metoprolol, esmolol, celiprolol, labetalol, bisoprolol, propranolol, alprenolol, betaxolol, bevantolol, and carvedilol) in serum (Method-A) (**Figure 1**) [1] [2].

In addition, we developed analytical methods for metoprolol, propranolol [31] [32] and their main metabolites α-hydroxy metoprolol, O-desmethyl metoprolol, and 4-hydroxy propranolol in serum and urine (Method-B) (**Figure 2**).

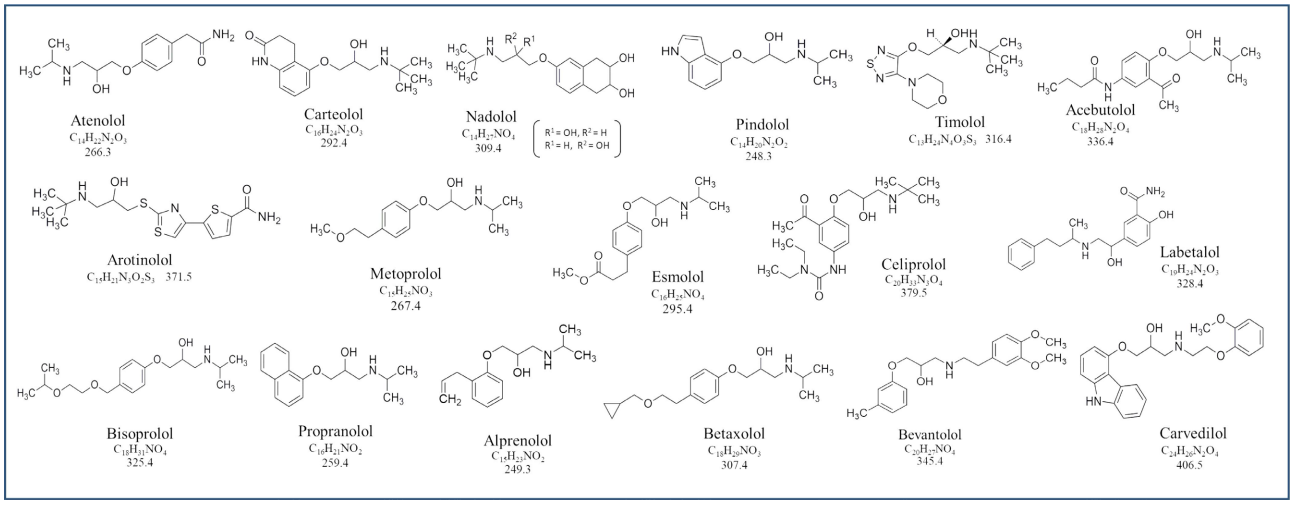


Figure 1. Structure of beta-blockers.

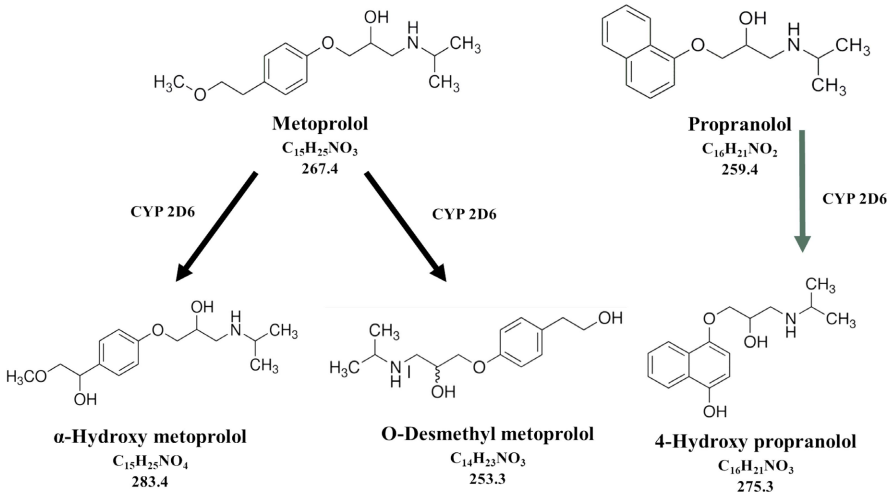


Figure 2. Structure of metoprolol, propranolol, and their metabolites.

2. Materials and Methods

2.1. Chemicals and Reagents

The drug standards for atenolol, nadolol, bisoprolol, carvedilol, acebutolol, labetalol, propranolol hydrochloride, and metoprolol tartrate were purchased from Sigma-Aldrich (Tokyo, Japan). Timolol maleate, arotinolol, and betaxolol hydrochloride were purchased from the Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Carteolol hydrochloride, esmolol hydrochloride, celiprolol hydrochloride, alprenolol hydrochloride, bevantolol hydrochloride, and atenolol-d7 (internal standard: IS) were purchased from Toronto Research Chemicals (Ontario, Canada). Pindolol, acetonitrile (LC-MS grade, >99.9% purity), methanol (LC-MS grade, 99.7% purity), ultrapure water (LC-MS grade), acetic acid (analytical grade), and ammonium acetate (analytical grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Drug standards for *α*-hydroxy metoprolol, O-desmethyl metoprolol, and rac metoprolol-d7 (internal

standard: IS) were purchased from Toronto Research Chemicals (Ontario, Canada). 4-hydroxy propranolol hydrochloride was purchased from Cayman Chemical (Wisconsin, USA). Pooled normal human serum and urine samples were purchased from Millipore (Billerica, MA, USA) [1] [2]. The Oasis® PRiME HLB extraction cartridges were purchased from Waters (Milford, MA, USA). All other chemicals were commercially available and were of reagent grade [1] [2].

2.2. Method

2.2.1. Preparation of Standard Solutions and Calibration Standards

(Method-A): Individual stock solutions (1 mg/mL concentration) of the compounds to be analyzed were prepared with methanol (stable for at least three months when stored at −20˚C). Standard serum solutions with concentrations of 20, 100, 200, and 400 ng/mL of each compound were prepared by diluting appropriate aliquots of the stock solution with drug-free serum (normal human serum). Human serum (0.5 mL) was added to water (0.5 mL) containing 10, 50, 100, or 200 ng of each of the 17 beta-blockers and 100 ng of IS. Calibration curves were obtained using simple linear regression analysis of the concentration of each drug and the corresponding peak area ratio. The regression equations for the 17 beta-blockers extracted from the human serum were based on the ratio of the peak area of each drug to that of the IS [1] [2].

(Method-B): Serum or urine standards were prepared with concentrations of 2, 10, 100, and 200 ng/mL of each compound (metoprolol, propranolol, and *α*-hydroxy metoprolol) and 10, 50, 100, and 200 ng/mL for 4-hydroxy propranolol by diluting appropriate aliquots of the stock solution with drug-free serum (normal human serum) or urine (normal human urine). In our study, 0.5 mL of human serum or urine was added to 0.5 mL of water containing 1, 5, 50, or 100 ng of metoprolol, propranolol, *α*-hydroxy metoprolol, O-desmethyl metoprolol, 5, 25, 50, or 100 ng of 4-hydroxy propranolol, and 50 ng metoprolol-d7 (IS). The calibration curves were prepared in the same manner as Method-A.

2.2.2. Apparatus and Chromatographic Conditions

(Method-A): LC-MS/MS experiments were performed using an HPLC system equipped with Shimadzu LC-20AD pumps (Shimadzu, Kyoto, Japan), a SIL-20AC autosampler (Columbia, MD, USA), and a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA).

Chromatographic separation was performed using a Mightysil-RP-18 MS column (2.0 mm × 150 mm, particle size 5 μm; Kanto Chemical Co., Inc., Tokyo, Japan). Two solvents were used for gradient elution: a) 10 mM ammonium acetate buffer and b) acetonitrile. The mobile phase composition was initially held at 90% (A) for 0.1 min and then changed during linear gradient elution from 90% (A) to 40% (A) over 10 min. The mobile phase was returned to 90% (A) for 5 min before the next run. The flow rate was set to 0.20 mL/min and 10 μL of sample was injected for each analysis. The column and autosampler were maintained at 37˚C and 4˚C, respectively.

All experiments were conducted in the positive-ion electrospray mode. The TurboIonSpray source was operated at 600˚C with a capillary voltage of 5500 V. Nitrogen was used as the nebulizer, curtain (40 psi), and collision (4 psi) gases. The optimal chromatographic and mass spectrometric conditions for the analysis of all compounds were determined by injecting pure standard solutions into the LC-MS/MS system. Simultaneous analysis was performed in the dMRM mode for 1 min before and after the retention time of each compound [1] [2] [10]-[12].

(Method-B): An Agilent 1290 liquid chromatograph, equipped with a Mightysil-RP-18 MS column (2.0 mm × 150 mm, particle size 5 μm; Kanto Chemical Co., Inc., Tokyo, Japan), in combination with a 6460 triple quadrupole mass spectrometer with a Jet Stream ESI source (Agilent Technologies, Santa Clara, CA, USA), was used for the analysis.

Mobile phase A consisted of 10 mM ammonium acetate and 0.1% acetic acid in ultrapure water, and mobile phase B was acetonitrile. The gradient elution program was as follows: the mobile phase composition was held initially at 90% (A) for 0.1 min and then changed during the linear gradient elution from 90% (A) to 40% (A) over 10 min. The mobile phase was then returned to 100% (A) for 5 min for the next analysis. The flow rate was 0.2 mL/min. The autosampler was maintained at 4˚C. The column oven temperature was 37˚C. The ESI parameters were capillary voltage, 4000 V; nebulizer gas (N2) pressure, 50 psi; drying gas (N2) flow rate, 10 L/min at 300˚C; sheath gas (N2) flow rate, 12 L/min at 350˚C. The optimal chromatographic and mass spectrometric conditions for the analysis of all the compounds were obtained by injecting pure standard solutions into the LC/MS/MS system. All experiments were measured in product ion scan mode to confirm the retention time. Simultaneous analysis was performed in dMRM mode for 1 min before and after the retention time of each compound. The determination of the MS/MS parameters and data acquisition was performed using MassHunter Workstation Software (version B.07.00, Agilent Technologies).

2.2.3. Extraction

(Method-A): The drugs were extracted from human serum using an Oasis® PRiME HLB cartridge column. These samples were analyzed to determine possible endogenous interferences and were used as "blanks." An Oasis® PRiME HLB cartridge column with a 1 mL capacity was placed in an Agilent Vac Elut system (Agilent Technologies).

Human serum (0.5 mL) was added to water (0.5 mL) containing 10 - 200 ng of each of the 17 beta-blockers and 100 ng of IS. The mixture was loaded onto an Oasis® PRiME HLB cartridge column and allowed to pass through it. The column was then washed with 1 mL of a 5% aqueous methanol solution. Beta-blockers were eluted with 1 mL of 90% methanol and 10% acetonitrile. The eluent was then dried under N2 gas at 40˚C. The residue was dissolved in 200 μL of the initial mobile phase composition (A:B = 90:10), and an aliquot (10 μL) was analyzed using a chromatographic system (**Figure 3**) [1] [2] [10]-[12].

(Method-B): 0.5 mL of human serum or urine was added to 0.5 mL of water containing 1 - 200 ng of each of the beta blockers, their metabolites, and 50 ng metoprolol-d7 (IS). The extraction methods were performed in the same manner as Method-A, and an aliquot (1 μL) was then analyzed by the chromatographic system (Figure 3).

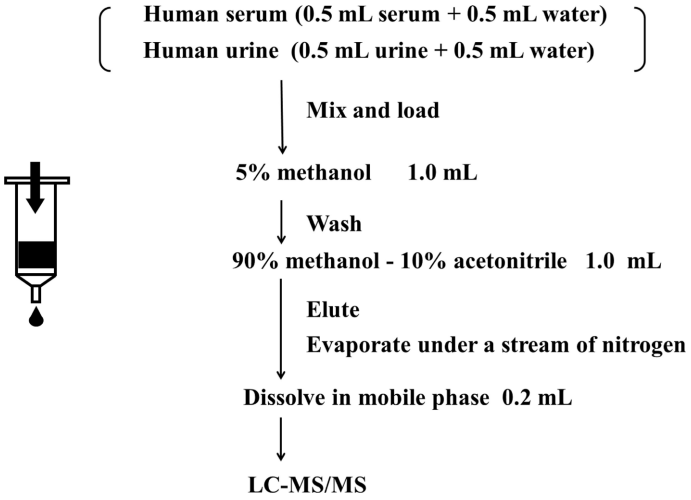


Figure 3. Extraction method of beta-blockers and their metabolites using an Oasis® PRiME HLB column.

2.2.4. Linearity, Accuracy, Precision, and Recovery

(Method-A): Analyses of the 17 beta-blockers in serum samples were performed on three separate days using freshly prepared samples and solutions to evaluate the linearity of the standard calibration curve. Calibration curves were prepared over a linear range of 20 - 400 ng/mL using four serum concentrations (20, 100, 200, and 400 ng/mL). Linearity was evaluated by analyzing six replicates.

Intraday assay precision and accuracy were obtained by analyzing six aliquots of quality control samples in duplicate using a calibration curve constructed on the same day. Interday assay precision and accuracy were evaluated using six replicate determinations for each concentration with solutions prepared on different days. Matrix effects and extraction recovery were verified. The matrix effect and extraction recovery were estimated at four analyte concentrations (20, 100, 200, and 400 ng/mL for beta-blockers, n = 6) in the serum. The recoveries were calculated by comparing the chromatographic peak areas obtained from the extracts of the serum samples (containing water) with those obtained by adding beta-blockers to the extracted solution of blank serum [1]-[12].

(Method-B): The calibration curves were prepared over a linear range of 2 - 200 ng/mL using four concentrations (2, 10, 100, and 200 ng/mL for metoprolol, propranolol, *α*-hydroxy metoprolol, and O-desmethyl metoprolol; 10, 50, 100, and 200 ng/mL for 4-hydroxy propranolol) in serum or urine.

The intraday assay precision, accuracy, the matrix effect, and extraction recovery were performed in the same manner as Method-A. The matrix effect and ex-

traction recovery were estimated at three analyte concentrations (10, 100, and 200 ng/mL, n = 5) for the beta blockers and their metabolites in serum or urine. The recoveries were calculated as in Method-A.

2.2.5. Stability

The stability of the 17 beta-blockers (Method-A), 2 major metabolites (Method-B), and IS in serum and urine was investigated at various concentrations. Freshly prepared samples were evaluated at room temperature, 4˚C, and −20˚C over 12 days. Freeze/thaw stability was determined after three freeze/thaw cycles. Stability tests were performed by analyzing two replicates for each sample [1] [2] [10]-[12].

2.2.6. Limits of Detection and Quantification (LOD and LOQ)

The LOD was determined as the injection volume required to produce a peak with a signal-to-noise ratio of at least 3:1. The LOQ was defined as the lowest concentration on the standard curve that could be measured with acceptable accuracy (coefficient of variation, CV < 10.0%). The LOQ for the 17 beta-blockers in serum, as well as the beta-blockers and their metabolites in serum or urine, were determined using LC-MS/MS with ESI after solid-phase extraction (Oasis® PRiME HLB cartridge column) [1] [2] [10]-[12].

3. Results and Discussion

3.1. Selectivity and Chromatography

In Method A, a 4000 QTRAP mass spectrometer (Applied Biosystems) system was used to analyze 17 beta-blockers, and in Method B, a 6460 triple quadrupole mass spectrometer (Agilent Technologies) was used to analyze two types of drugs and their major metabolites.

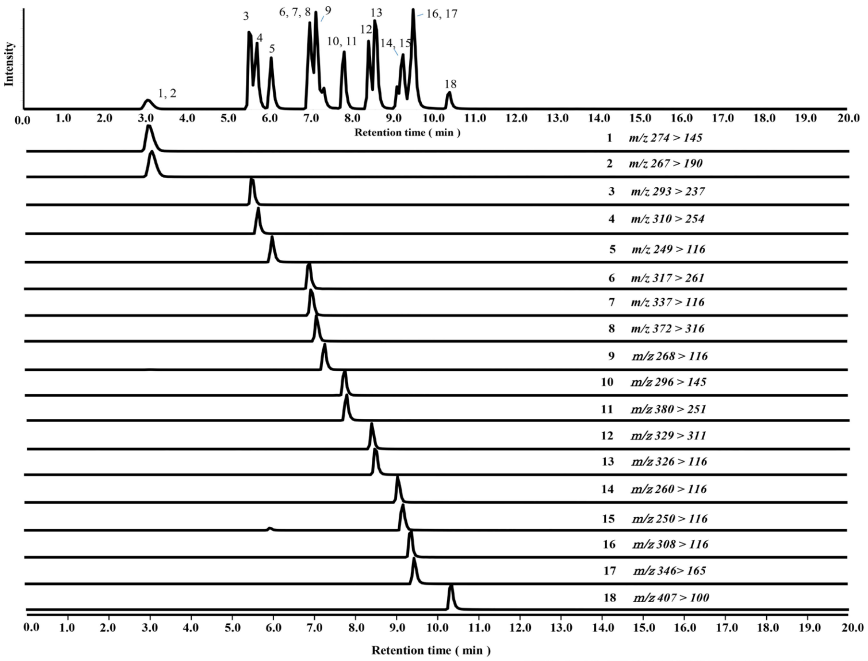
(Method-A): The positive-ion electrospray mode was selected, and the collision energies were optimized for each drug. All the precursor ions (Q1) of the 17 beta-blockers were $(M + H)^+$. The $(M + H)^+$ ion peak was clearly detected, and the product ions were obtained from the mass spectrum of each drug with sufficient detection sensitivity (**Table 1**). Good separation of the 17 beta-blockers is observed using LC-MS/MS, as shown in the chromatograms (**Figure 3**). The positive-ion electrospray mode is selected, and the collision energies are optimized for each drug (**Figure 2**). All the precursor ions (Q1) of the 17 beta-blockers were $(M + H)^+$. The $(M + H)^+$ ion peak is clearly detected and the product ions are obtained from the mass spectrum of each drug with sufficient detection sensitivity (**Table 1**, **Figure 4**) [1] [2].

(Method-B): The positive ion electrospray mode was selected, and optimal collision energies were optimized for each drug. All precursor ions (Q1) of metoprolol, propranolol, and their metabolites (*α*-hydroxy metoprolol, O-desmethyl metoprolol, and 4-hydroxy propranolol) showed the $[M + H]^+$. The $[M + H]^+$ ion peak was detected clearly, and product ions were obtained in the mass spectrum of each drug, with sufficient detection sensitivity (**Table 2**). In addition, the separation of metoprolol, propranolol, *α*-hydroxy metoprolol, O-desmethyl metopro-

lol, and 4-hydroxy propranolol by LC-MS/MS was good, as seen on the dMRM chromatograms (Table 2, Figure 5).

Table 1. Retention time and tandem mass spectrometry parameters of beta-blockers.

Drugs	Monoisotopic mass	Retention time (min)	Collision Energy (V)	Precursor Ion (m/z)	Production (m/z)
Atenolol	266.16	3.04	30	267	190, 116, 98, 165, 145, 208, 178, 74
Carteolol	292.18	5.47	30	293	237, 202, 74, 164, 190
Nadolol	309.19	5.64	30	310	254, 201, 236, 74, 183, 171, 158, 57
Pindolol	248.15	5.99	25	249	116, 172, 146, 98, 134, 74, 144, 118
Timolol	316.16	6.90	30	317	261, 244, 74, 188, 57, 243
Acebutolol	336.20	6.94	30	337	116, 98, 72, 319, 260, 222, 235
Arotinolol	371.08	7.08	25	372	316, 299, 290, 268, 356, 74, 313
Metoprolol	267.18	7.26	30	268	116, 98, 74, 159, 121, 133, 72
Esmolol	295.18	7.74	30	296	145, 219, 98, 116, 74, 72, 254, 119
Celiprolol	379.25	7.75	33	380	251, 307, 306, 233, 324, 74, 289
Labetalol	328.18	8.35	25	329	311, 294, 162, 207, 179, 91, 190
Bisoprolol	325.23	8.51	30	326	116, 74, 98, 72, 147, 133, 91, 162
Propranolol	259.16	9.04	35	260	116, 155, 74, 72, 157, 56, 98, 58
Alprenolol	249.17	9.17	30	250	116, 72, 74, 98, 56, 91, 147, 131
Betaxolol	307.21	9.36	30	308	116, 98, 161, 72, 121, 177, 133, 74
Bevantolol	345.19	9.43	35	346	165, 150, 133, 105
Carvedilol	406.19	10.29	40	407	100, 222, 224, 194, 180, 210, 283



1. Atenolol-d7 (IS), 2. Atenolol, 3. Carteolol, 4. Nadolol, 5. Pindolol, 6. Timolol, 7. Acebutolol, 8. Arotinolol, 9. Metoprolol, 10. Esmolol, 11. Celiprolol, 12. Labetalol, 13. Bisoprolol, 14. Propranolol, 15. Alprenolol, 16. Betaxolol, 17. Bevantolol, 18. Carvedilol. Injection volume: 5 ng of each beta blocker and Atenolol-d7 (IS).

Figure 4. TIC and tandem mass chromatograms of beta-blockers and Atenolol-d7 (IS) extracted from human serum.

Table 2. Retention time and tandem mass spectrometry parameters of metoprolol, propranolol, and their metabolites.

Drugs	Monoisotopic mass	Retention time (min)	Precursor Ion (m/z)	Production (m/z)
α-Hydroxy metoprolol	283.6	3.99	284	74, 116, 56, 98, 133, 163, 207, 91, 189
O-Desmethyl metoprolol	253.34	4.18	254	177, 72, 116, 98, 159, 56, 212, 133, 86, 236
Metoprolol	267.18	6.22	268	74, 56, 116, 133, 98, 159, 191, 148, 176, 91, 218
4-Hydroxy propranolol	275.15	6.23	276	58, 72, 173, 116, 100, 153, 181, 199, 84
Propranolol	259.16	6.24	260	116, 74, 56, 98, 157, 165, 86, 130, 218

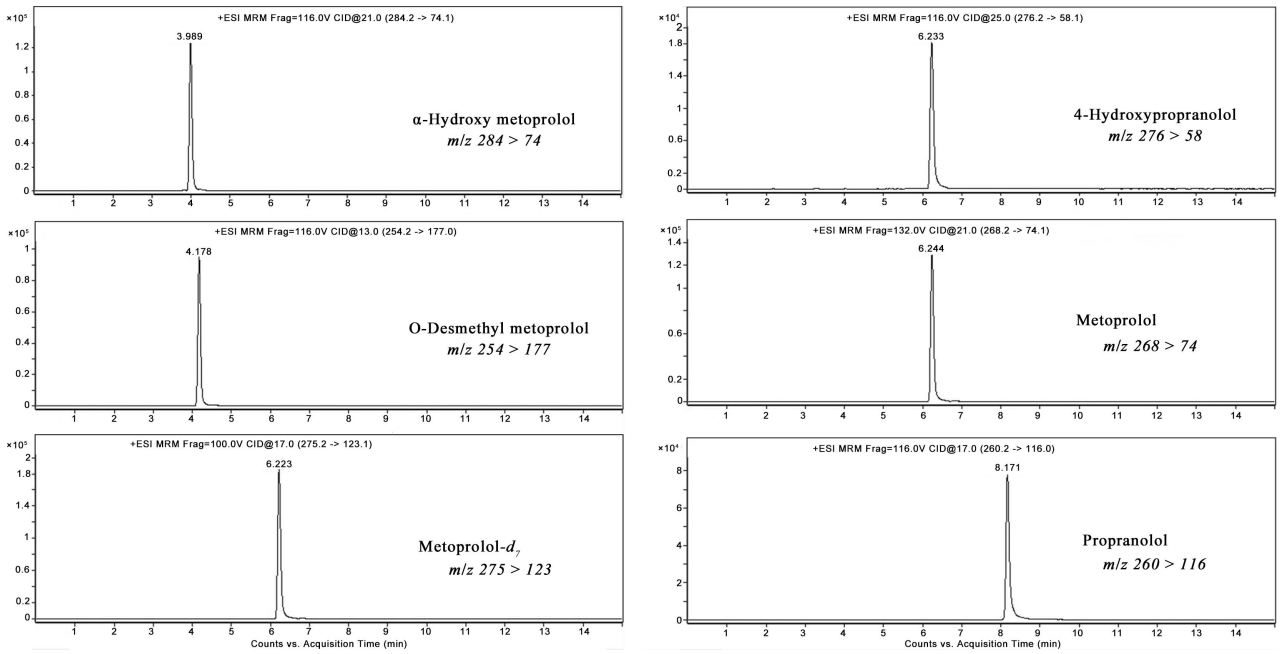


Figure 5. Dynamic multiple reaction monitoring chromatograms of metoprolol, propranolol, their metabolites, and metoprolol-d7 (IS) extracted from serum. Column: Mightysil-RP-18 MS column (2.0 mm × 150 mm, particle size 5 μm). Injection volume: 0.25 ng of each drug.

3.2. Linearity, Accuracy, Precision, and Recovery

(Method-A): The calibration curve (ratio of the peak area to the concentration of each drug) was linear over the entire concentration range of 20 - 400 ng/ml. The coefficients of determination (r) from the regression analyses of the drugs are in the range of 0.9686 - 0.9997 (**Table 3**). In our LC-MS/MS with ESI method for the simultaneous determination of 17 beta-blockers, the precision was satisfactory, with CV values always lower than 10.0% for intraday, interday, and total assay reproducibility. The recoveries of the 17 beta-blocker drugs were in the range of 66.1% - 93.5%, and a precision within 7.0% CV was acceptable for this method. However, the extraction yields of bevantolol and carvedilol were low (37.5% and 26.2%, respectively) (**Table 4**). No significant interference was observed at the retention times of the analytes and the IS, indicating acceptable selectivity. However, the matrix effect is insignificant (**Figure 6**) [1] [2]. The primary objective of this

study is to conduct a broad-scale screening of low-concentration beta-blockers. While the extraction recovery rate may be low for some compounds, the overall sensitivity, linearity, and ability of this method to detect these compounds within the acceptable error range make it suitable for initial-stage screening.

Table 3. Retention time and tandem mass spectrometry parameters of metoprolol, propranolol, and their metabolites.

Drugs	Range (ng/mL)	Curve	r
Atenolol	20.0 - 400.0	y = 0.0116x+0.306	r = 0.9970
Carteolol	20.0 - 400.0	y = 0.109x+0.995	r = 0.9911
Nadolol	20.0 - 400.0	y = 0.0605x+0.164	r = 0.9982
Pindolol	20.0 - 400.0	y = 0.0593x+0.447	r = 0.9978
Timolol	20.0 - 400.0	y = 0.069x+1.63	r = 0.9948
Acebutolol	20.0 - 400.0	y = 0.0449x+0.362	r = 0.9980
Arotinolol	20.0 - 400.0	y = 0.0529x+0.35	r = 0.9971
Metoprolol	20.0 - 400.0	y = 0.0367x+1.12	r = 0.9978
Esmolol	20.0 - 400.0	y = 0.0154x+0.231	r = 0.9851
Celiprolol	20.0 - 400.0	y = 0.03x+0.354	r = 0.9863
Labetalol	20.0 - 400.0	y = 0.101x+0.225	r = 0.9997
Bisoprolol	20.0 - 400.0	y = 0.154x+1.62	r = 0.9748
Propranolol	20.0 - 400.0	y = 0.029x+1.34	r = 0.9709
Alprenolol	20.0 - 400.0	y = 0.0367x+0.39	r = 0.9817
Betaxolol	20.0 -400.0	y = 0.0392x+2.25	r = 0.9963
Bevantolol	20.0 - 400.0	y = 0.0433x+0.514	r = 0.9776
Carvedilol	20.0 -400.0	y = 0.00904x+0.0646	r = 0.9686

Table 4. Extraction yields, limits of detection, and limits of quantification of beta-blockers using LC-MS/MS.

Drugs	Recovery (n = 6)			LOD (ng/mL) (S/N = 3)	LOQ (ng/mL)
	Amount added (ng)	Average (%)	C.V. (%) (Coefficient of Variation)		
Atenolol	100.0	90.2	2.0	0.048	20.0
Carteolol	100.0	90.3	1.4	0.016	20.0
Nadolol	100.0	89.4	1.0	0.044	20.0
Pindolol	100.0	84.3	1.0	0.0034	20.0
Timolol	100.0	90.6	1.3	0.14	20.0
Acebutolol	100.0	88.5	0.7	0.040	20.0
Arotinolol	100.0	66.1	0.5	0.040	20.0
Metoprolol	100.0	85.7	1.9	0.017	20.0

Continued

Esmolol	100.0	76.7	4.8	1.6	20.0
Celiprolol	100.0	78.1	6.1	0.013	20.0
Labetalol	100.0	85.0	2.9	0.11	20.0
Bisoprolol	100.0	93.5	0.9	0.026	20.0
Propranolol	100.0	85.1	3.6	0.036	20.0
Alprenolol	100.0	73.3	4.1	0.033	20.0
Betaxolol	100.0	85.9	1.2	0.22	20.0
Bevantolol	100.0	37.5	2.9	0.03	20.0
Carvedilol	100.0	26.2	7.0	0.040	20.0

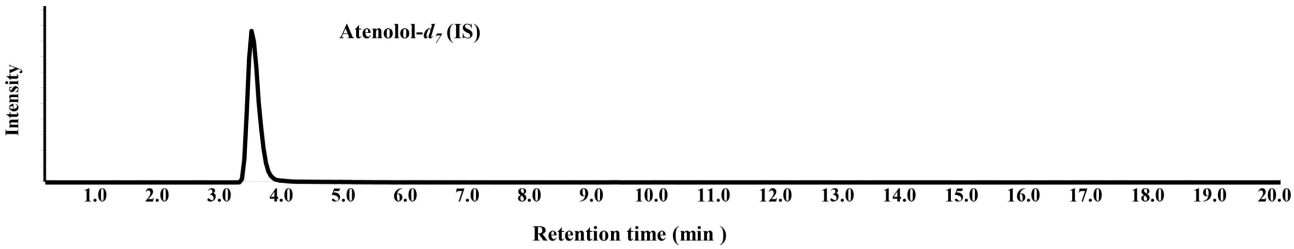


Figure 6. Tandem mass chromatograms of Atenolol-d_7 (IS) extracted from human serum. Injection volume: 5 ng.

(Method-B): The calibration curve (the ratio of the peak area to the concentration of each drug) was linear over the concentration range of 2 - 200 ng/mL (metoprolol, propranolol, *α*-hydroxy metoprolol, and O-desmethyl metoprolol). The calibration curve of 4-hydroxy propranolol was linear over the concentration range of 10 - 200 ng/mL. The coefficients of determination (r) from the regression analyses of the drugs were between 0.9246 and 0.9997 (n = 5: **Table 5**).

Table 5. Calibration curves of metoprolol, propranolol, and their metabolites.

Drugs	Range (ng/mL)	Curve and r (Serum)	Curve and r (Urine)
α-Hydroxy metoprolol	2.0 - 200.0	y = 0.0115x + 0.0314 r = 0.9986	y = 0.0109x + 0.0283 r = 0.9986
O-Desmethyl metoprolol	2.0 - 200.0	y = 0.0087x + 0.0266 r = 0.9976	y = 0.0078x + 0.0253 r = 0.9964
Metoprolol	2.0 - 200.0	y = 0.0137x + 0.0362 r = 0.9986	y = 0.0129x + 0.0367 r = 0.9981
4-Hydroxy propranolol	10.0 - 200.0	y = 0.0008x + 0.0092 r = 0.9246	y = 0.0019x + 0.0166 r = 0.9285
Propranolol	2.0 - 200.0	y = 0.0113x + 0.0135 r = 0.9997	y = 0.009x + 0.0171 r = 0.9989

In our LC-MS/MS with ESI method for simultaneous determination of the beta blockers and their metabolites, the precision was satisfactory, with CV values al-

ways lower than 10.0% for the intraday, interday, and total assay reproducibility. The recoveries at 50 ng of metoprolol, propranolol, *α*-hydroxy metoprolol, O-desmethyl metoprolol, and 4-hydroxy propranolol in serum ranged from 96.8 to 106.5%, with precision within 5.9% CV values; in urine, the recoveries ranged from 93.8% to 99.0%, with precision within 4.1% CV values, which were acceptable for this method. However, the extraction yields of 4-hydroxy propranolol were low (in serum 54.4%, in urine 54.1%). Currently, we are experimenting to improve the extraction yields of 4-hydroxy propranolol (Table 6, Table 7). No significant interferences were observed at the retention times of the analytes and IS, indicating acceptable method selectivity. The matrix effect was insignificant.

Table 6. Extraction yields, limits of detection, and limits of quantification of metoprolol, propranolol, and their metabolites from serum using LC-MS/MS.

Drugs	Extraction yield (n = 5)				
	Amount add (ng)	Average (%)	C.V. (%) (Coefficient of Variation)	LOD (ng/mL)	LOQ (ng/mL)
α-Hydroxy metoprolol	50.0	106.0	4.0	0.69	2.0
O-Desmethyl metoprolol	50.0	106.5	3.4	1.28	2.0
Metoprolol	50.0	105.3	1.9	0.50	2.0
4-Hydroxy propranolol	50.0	54.4	5.9	0.54	10.0
Propranolol	50.0	96.8	3.9	0.68	2.0

Table 7. Extraction yields, limits of detection, and limits of quantification of metoprolol, propranolol, and their metabolites from urine using LC-MS/MS.

Drugs	Extraction yield (n = 5)				
	Amount add (ng)	Average (%)	C.V. (%) (Coefficient of Variation)	LOD (ng/mL)	LOQ (ng/mL)
α-Hydroxy metoprolol	50.0	98.7	1.4	1.00	2.0
O-Desmethyl metoprolol	50.0	98.8	1.6	0.09	2.0
Metoprolol	50.0	99.0	0.6	0.65	2.0
4-Hydroxy propranolol	50.0	55.1	4.1	0.71	10.0
Propranolol	50.0	93.8	1.4	0.17	2.0

3.3. Stability

(Method-A): A stability study was conducted to determine the optimal storage

temperature for serum samples. The results indicated that the 17 beta-blockers and IS were stable for up to 24 h at room temperature. Furthermore, these drugs were stable for 10 days at 4˚C and −20˚C and for at least two weeks. Therefore, all extracted samples were refrigerated at 4˚C for same-day analysis, whereas serum samples were frozen at −20˚C or −80˚C until LC-MS/MS analysis [1] [2].

(Method-B): A stability study was conducted to determine the best storage temperature for the serum or urine samples. The results indicated that metoprolol, propranolol, *α*-hydroxy metoprolol, O-desmethyl metoprolol, 4-hydroxy propranolol, and the IS were stable up to 12 h at room temperature. Furthermore, these drugs were stable up to 12 days when stored at 4˚C and −20˚C. Therefore, all extracted samples were stored refrigerated at 4˚C for same-day analysis, whereas serum and urine samples were frozen at −20˚C until their analysis by LC-MS/MS.

3.4. LOD and LOQ

(Method-A): The LODs of atenolol, carteolol, nadolol, pindolol, timolol, acebutolol, arotinolol, metoprolol, esmolol, celiprolol, labetalol, bisoprolol, propranolol, alprenolol, betaxolol, bevantolol, and carvedilol were 0.048, 0.016, 0.044, 0.0034, 0.14, 0.040, 0.040, 0.017, 1.6, 0.013, 0.11, 0.026, 0.036, 0.033, 0.22, 0.03, and 0.040 ng/mL, respectively, and the LOQs of the 17 beta-blockers were 20.0 ng/mL (**Table 4**) [1] [2].

These results suggest that the detection sensitivity of this analytical method is acceptable for measuring the therapeutic concentrations [33] of these drugs. Therefore, our analytical method can be used to determine the therapeutic range of beta-blocker concentrations.

For the GC-MS analysis, it is necessary to gasify the sample, and the analyzable samples are limited to relatively low-molecular-weight gases, volatile compounds, or thermally stable compounds. In LC-MS/MS, it is possible to analyze volatile and thermally unstable compounds as long as they are dissolved in the mobile phase, which has the advantage that the application range of the analytical samples is wider than that of GC-MS. Brunetto M.d.R., *et al.* [19] developed a method for the determination of atenolol and propranolol in human plasma samples by GC-MS. The LOQ was approximately 6.0 ng/mL. Phyo Lwin E.M., *et al.* [25] developed a method for determining atenolol levels in human plasma and milk samples using LC-MS/MS. The LOD and LOQ values were in the range of 1.0 - 5.0 ng/mL for atenolol. Elmongy H. *et al.*, [27] developed a method for determining metoprolol levels in human plasma using LC-MS/MS. The LOQ was approximately 1.5 ng/mL. Our LC-MS/MS with ESI method could measure compounds at approximately the same concentrations as the analytical methods of Brunetto M.d.R. *et al.*, Phyo Lwin *et al.*, and Elmongy *et al.* In our experiments, the LOD values were in the range of 0.0034 - 1.6 ng/mL, and the LOQ values were 20 ng/mL for the 17 beta-blockers, respectively. The matrix effect on the LOQ values is insignificant, and both accuracy and precision were sufficient. Therefore, our ana-

lytical method can measure beta-blockers in a manner similar to the methods mentioned above.

(Method-B): The LODs of metoprolol, propranolol, *α*-hydroxy metoprolol, O-desmethyl metoprolol, and 4-hydroxy propranolol were 0.50, 0.68, 0.69, 1.28, and 0.54 ng/mL, respectively, in the serum (Table 6). The LODs of metoprolol, propranolol, *α*-hydroxy metoprolol, O-desmethyl metoprolol, and 4-hydroxy propranolol were 0.65, 0.17, 1.0, 0.09, and 0.71 ng/mL, respectively, in the urine (Table 7). The LOQs of metoprolol, propranolol, *α*-hydroxy metoprolol, and O-desmethyl metoprolol were 2.0 ng/mL. The LOQ of 4-hydroxy propranolol was 10 ng/mL (Table 6, Table 7).

These results suggest that the detection sensitivity of this method is acceptable for the determination of therapeutic concentrations [33] of these drugs (for example, the therapeutic concentration of propranolol is 20 - 100 ng/mL).

In GC-MS analysis, it is necessary to gasify the sample, and the analyzable samples are limited to relatively low molecular gases, volatile compounds, or thermally stable compounds. In LC-MS/MS, it is possible to analyze compounds that are difficult to volatilize and thermally unstable compounds as long as they are dissolved in the mobile phase, which has the advantage that the application range of analytical samples is wider than that of GC-MS. Brunetto M.d.R., *et al.* [19] developed a method for the determination of atenolol and propranolol in human plasma samples by GC-MS. The LOQ was approximately 6.0 ng/mL. Elmongy H, *et al.* [27] developed a method for the determination of metoprolol in human plasma by LC-MS/MS. The LOQ was approximately 1.5 ng/mL. Our LC-MS/MS with ESI method was able to measure compounds at approximately the same concentrations as the analytical methods of Brunetto M.d.R., *et al.*, Phyo Lwin E.M., *et al.*, and Elmongy H, *et al.* In our experiments, the LOD and LOQ were in the range of 0.09 - 1.28 ng/mL and were 2.0 ng/mL for metoprolol, propranolol, *α*-hydroxy metoprolol, O-desmethyl metoprolol, and 4-hydroxy propranolol (the LOQ of 4-hydroxy propranolol was 10 ng/mL).

4. Conclusions

We focused on various types of drug intoxication and studied the systematic simultaneous analysis of pharmaceuticals in biological samples using LC-MS/MS. We previously established a sensitive and specific LC-MS/MS method for the simultaneous detection and quantification of phenothiazines, nonsteroidal anti-inflammatory antipyretic analgesics, atypical antipsychotics, and local anesthetics in human serum [10]-[12]. In this study, we developed a sensitive and specific screening method for the simultaneous detection and quantification of 17 beta-blockers (atenolol, carteolol, nadolol, pindolol, timolol, acebutolol, arotinolol, metoprolol, esmolol, celiprolol, labetalol, bisoprolol, propranolol, alprenolol, betaxolol, bevantolol, and carvedilol) in human serum using LC-MS/MS. Various analytical methods reported thus far have used liquid-liquid extraction. The method is highly specific and sensitive and can detect and quantify multiple beta-blockers in

human serum samples with high accuracy. This makes LC-MS/MS highly useful for the simultaneous measurement of beta-blockers in human serum in clinical and forensic investigations.

We developed a sensitive and specific LC-MS/MS method for the simultaneous detection and quantification of the beta blockers (metoprolol, propranolol) and their metabolites (α-hydroxy metoprolol, O-desmethyl metoprolol, and 4-hydroxy propranolol) in human serum and urine. Many of the analytical methods reported so far use liquid-liquid extraction. In the method established in this study, human serum and urine samples were treated, prior to analysis, by solid-phase extraction with an Oasis® PRiME HLB cartridge column. By using this method, the separation of samples is achieved in a simple manner while providing sufficient sensitivity, precision, and accuracy, making it extremely useful for the simultaneous determination of beta-blockers and their metabolites in human serum and urine by LC-MS/MS in clinical and forensic investigations. This simultaneous analytical method is also applicable to the analysis of other beta-blockers.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Tonooka-Kubota K., Terada, M., Shinozuka, T., *et al.* (2019) Sensitive Liquid Chromatography/Tandem Mass Spectrometry Method for the Simultaneous Determination of Thirteen β Blockers. *The* 57*th Annual Meeting of the International Association of Forensic Toxicologists*, Birmingham, 2-6 September 2019.

[2] Tonooka-Kubota, K., Takanashi, K., Hosono, T., Terada, M. and Tatsuo, S. (2022) Sensitive Liquid Chromatography/Tandem Mass Spectrometry Method for the Simultaneous Determination of Seventeen β Blockers. *Toxicologie Analytique et Clinique*, **34**, S173. https://doi.org/10.1016/j.toxac.2022.06.298

[3] Yamaguchi, K., Ohno, Y. and Kanawaku, Y. (2022) Simultaneous Analysis of Zolpidem, Four Hydroxyzolpidems and Two Zolpidem Carboxylic Acids in Postmortem Urine Using Liquid Chromatography—Tandem Mass Spectrometry. *Journal of Analytical Toxicology*, **46**, 1059-1065. https://doi.org/10.1093/jat/bkac009

[4] Keung Chan, K., Moore, A.J., Hoofnagle, A.N., Baird, G.S. and Liao, H. (2023) Benzodiazepine Analysis by an Improved LC-MS/MS Method Illustrates Usage Patterns in Washington State. *Clinica Chimica Acta*, **543**, Article ID: 117274. https://doi.org/10.1016/j.cca.2023.117274

[5] Mastrovito, R.A., Papsun, D.M. and Logan, B.K. (2021) The Development and Validation of a Novel Designer Benzodiazepines Panel by LC-MS-MS. *Journal of Analytical Toxicology*, **45**, 423-428. https://doi.org/10.1093/jat/bkab013

[6] Romantsik, O., Barco, S., Bruschettini, M., Tripodi, G., Ley, D. and Cangemi, G. (2020) A LC-MS/MS Method for the Quantification of Caffeine, Betamethasone, Clonidine and Furosemide in Cerebrospinal Fluid of Preterm Infants. *Journal of Pharmaceutical and Biomedical Analysis*, **179**, Article ID: 112996. https://doi.org/10.1016/j.jpba.2019.112996

[7] Mafra, G., *et al.* (2016) A Straightforward and Semiautomated Membrane-Based Method as Efficient Tool for the Determination of Cocaine and Its Metabolites in

Urine Samples Using Liquid Chromatography Coupled to Quadrupole Time-Of-Flight-Mass Spectrometry. *Journal of Chromatography A*, **1621**, Article ID: 461088.

[8] Verplaetse, R., Cuypers, E. and Tytgat, J. (2012) The Evaluation of the Applicability of a High Ph Mobile Phase in Ultrahigh Performance Liquid Chromatography Tandem Mass Spectrometry Analysis of Benzodiazepines and Benzodiazepine-Like Hypnotics in Urine and Blood. *Journal of Chromatography A*, **1249**, 147-154. https://doi.org/10.1016/j.chroma.2012.06.023

[9] Dubreil-Chéneau, E., Pirotais, Y., Bessiral, M., Roudaut, B. and Verdon, E. (2011) Development and Validation of a Confirmatory Method for the Determination of 12 Non Steroidal Anti-Inflammatory Drugs in Milk Using Liquid Chromatography-Tandem Mass Spectrometry. *Journal of Chromatography A*, **1218**, 6292-6301. https://doi.org/10.1016/j.chroma.2011.06.006

[10] Tonooka, K., Yoshida, L., Tomobe, K., Kunisue, Y., Terada, M. and Shinozuka, T. (2018) Sensitive Liquid Chromatography/Tandem Mass Spectrometry Method for the Simultaneous Determination of Risperidone, Olanzapine, Quetiapine, Clozapine, Ziprasidone, Perospirone, Aripiprazole and Blonanserin in Human Serum. *American Journal of Analytical Chemistry*, **9**, 88-97. https://doi.org/10.4236/ajac.2018.92008

[11] Tonooka, K., Naruki, N., Honma, K., Agei, K., Okutsu, M., Hosono, T., *et al*. (2016) Sensitive Liquid Chromatography/Tandem Mass Spectrometry Method for the Simultaneous Determination of Nine Local Anesthetic Drugs. *Forensic Science International*, **265**, 182-185. https://doi.org/10.1016/j.forsciint.2016.02.044

[12] Shirako, J., Kawasaki, M., Komine, K., Kunisue, Y., Terada, M., Sasaki, C., *et al*. (2013) Simultaneous Determination for Oxicam Non-Steroidal Anti-Inflammatory Drugs in Human Serum by Liquid Chromatography-Tandem Mass Spectrometry. *Forensic Science International*, **227**, 100-102. https://doi.org/10.1016/j.forsciint.2012.11.016

[13] Drayer, D.E. (1987) Lipophilicity, Hydrophilicity, and the Central Nervous System Side Effects of Beta Blockers. *Pharmacotherapy*: *The Journal of Human Pharmacology and Drug Therapy*, **7**, 87-91. https://doi.org/10.1002/j.1875-9114.1987.tb04029.x

[14] McAinsh, J. and Cruickshank, J.M. (1990) Beta-Blockers and Central Nervous System Side Effects. *Pharmacology & Therapeutics*, **46**, 163-197. https://doi.org/10.1016/0163-7258(90)90092-g

[15] Sugimura, T., Muraishi, M. and Ariyoshi, K. (2015) Present Status of Acute Medication Intoxication in Elderly. *Nihon Kyukyu Igakukai Zasshi*: *Journal of Japanese Association for Acute Medicine*, **26**, 702-706. https://doi.org/10.1002/jja2.12070

[16] Sandroni, C., Cavallaro, F., Caricato, A., Scapigliati, A., Fenici, P. and Antonelli, M. (2006) Enoximone in Cardiac Arrest Caused by Propranolol: Two Case Reports. *Acta Anaesthesiologica Scandinavica*, **50**, 759-761. https://doi.org/10.1111/j.1399-6576.2006.01026.x

[17] Unverir, P., Topacoglu, H., Bozkurt, S. and Kaynak, F. (2007) Cardiovascular Toxicity Due to Metoprolol Poisoning in a Patient with Coronary Artery Disease. *British Journal of Clinical Pharmacology*, **64**, 694-697. https://doi.org/10.1111/j.1365-2125.2007.02930.x

[18] Frierson, J., Bailly, D., Shultz, T., Sund, S. and Dimas, A. (1991) Refractory Cardiogenic Shock and Complete Heart Block after Unsuspected Verapamil-SR and Atenolol Overdose. *Clinical Cardiology*, **14**, 933-935. https://doi.org/10.1002/clc.4960141114

[19] Brunetto, M.d.R., Clavijo, S., Delgado, Y., Orozco, W., Gallignani, M., Ayala, C., *et al*. (2015) Development of a MSFIA Sample Treatment System as Front End of GC-MS for Atenolol and Propranolol Determination in Human Plasma. *Talanta*, **132**, 15-

22. https://doi.org/10.1016/j.talanta.2014.08.056

[20] Šatínský, D., Havlíková, L. and Solich, P. (2013) HPLC Column-Switching Technique for Sample Preparation and Fluorescence Determination of Propranolol in Urine Using Fused-Core Columns in Both Dimensions. *Analytical and Bioanalytical Chemistry*, **405**, 6583-6587. https://doi.org/10.1007/s00216-013-7098-4

[21] Xu, T., Bao, S., Geng, P., Luo, J., Yu, L., Pan, P., *et al.* (2013) Determination of Metoprolol and Its Two Metabolites in Human Plasma and Urine by High Performance Liquid Chromatography with Fluorescence Detection and Its Application in Pharmacokinetics. *Journal of Chromatography B*, **937**, 60-66. https://doi.org/10.1016/j.jchromb.2013.08.017

[22] Baranowska, I., Adolf, W. and Magiera, S. (2015) Enantioselective Determination of Metoprolol and Its Metabolites in Human Urine High-Performance Liquid Chromatography with Fluorescence Detection (HPLC-FLD) and Tandem Mass Spectrometry (MS/MS). *Journal of Chromatography B*, **1004**, 79-84. https://doi.org/10.1016/j.jchromb.2015.09.019

[23] Gonçalves, V.M.F., Rodrigues, P., Ribeiro, C. and Tiritan, M.E. (2017) Quantification of Alprenolol and Propranolol in Human Plasma Using a Two-Dimensional Liquid Chromatography (2D-LC). *Journal of Pharmaceutical and Biomedical Analysis*, **141**, 1-8. https://doi.org/10.1016/j.jpba.2017.03.064

[24] Abdel-Rehim, M., Persson, C., Altun, Z. and Blomberg, L. (2008) Evaluation of Monolithic Packed 96-Tips and Liquid Chromatography-Tandem Mass Spectrometry for Extraction and Quantification of Pindolol and Metoprolol in Human Plasma Samples. *Journal of Chromatography A*, **1196**, 23-27. https://doi.org/10.1016/j.chroma.2008.04.078

[25] Phyo Lwin, E.M., Gerber, C., Song, Y., Leggett, C., Ritchie, U., Turner, S., *et al.* (2017) A New LC-MS/MS Bioanalytical Method for Atenolol in Human Plasma and Milk. *Bioanalysis*, **9**, 517-530. https://doi.org/10.4155/bio-2016-0304

[26] Shah, J.V., Patel, D.P., Shah, P.A., Sanyal, M. and Shrivastav, P.S. (2015) Simultaneous Quantification of Atenolol and Chlorthalidone in Human Plasma by Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry. *Biomedical Chromatography*, **30**, 208-216. https://doi.org/10.1002/bmc.3537

[27] Elmongy, H., Ahmed, H., Wahbi, A., Amini, A., Colmsjö, A. and Abdel-Rehim, M. (2016) Determination of Metoprolol Enantiomers in Human Plasma and Saliva Samples Utilizing Microextraction by Packed Sorbent and Liquid Chromatography-Tandem Mass Spectrometry. *Biomedical Chromatography*, **30**, 1309-1317. https://doi.org/10.1002/bmc.3685

[28] Santos, M.G., Tavares, I.M.C., Boralli, V.B. and Figueiredo, E.C. (2015) Direct Doping Analysis of β-Blocker Drugs from Urinary Samples by On-Line Molecularly Imprinted Solid-Phase Extraction Coupled to Liquid Chromatography/Mass Spectrometry. *The Analyst*, **140**, 2696-2703. https://doi.org/10.1039/c4an02066a

[29] Umezawa, H., Lee, X., Arima, Y., Hasegawa, C., Izawa, H., Kumazawa, T., *et al.* (2008) Simultaneous Determination of β-Blockers in Human Plasma Using Liquid Chromatography-Tandem Mass Spectrometry. *Biomedical Chromatography*, **22**, 702-711. https://doi.org/10.1002/bmc.987

[30] Partani, P., Modhave, Y., Gurule, S., Khuroo, A. and Monif, T. (2009) Simultaneous Determination of Propranolol and 4-Hydroxy Propranolol in Human Plasma by Solid Phase Extraction and Liquid Chromatography/Electrospray Tandem Mass Spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*, **50**, 966-976. https://doi.org/10.1016/j.jpba.2009.06.050

[31] Kriikku, P., Pelkonen, S., Kaukonen, M. and Ojanperä, I. (2021) Propranolol and Metoprolol: Two Comparable Drugs with Very Different Post-Mortem Toxicological Profiles. *Forensic Science International*, **327**, Article ID: 110978. https://doi.org/10.1016/j.forsciint.2021.110978

[32] Steenen, S.A., van Wijk, A.J., van der Heijden, G.J., van Westrhenen, R., de Lange, J. and de Jongh, A. (2015) Propranolol for the Treatment of Anxiety Disorders: Systematic Review and Meta-Analysis. *Journal of Psychopharmacology*, **30**, 128-139. https://doi.org/10.1177/0269881115612236

[33] Schulz, M., Schmoldt, A., Andresen-Streichert, H. and Iwersen-Bergmann, S. (2020) Revisited: Therapeutic and Toxic Blood Concentrations of More than 1100 Drugs and Other Xenobiotics. *Critical Care*, **24**, Article No. 195. https://doi.org/10.1186/s13054-020-02915-5