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Simultaneous determination of ten antiarrhythic drugs and a metabolite in human plasma by liquid chromatography—tandem mass spectrometry

Shuijun Li [a], Gangyi Liu [a], Jingying Jia [a], Yun Liu [a], Cheng Pan [b], Chen Yu [a,*], Yongbao Cai [a], Jianying Ren [b]

[a] *Central Laboratory, Shanghai Xuhui Central Hospital, Shanghai 200031, China*
[b] *Department of Cardiology, Shanghai Xuhui Central Hospital, Shanghai 200031, China*

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Abstract

A simple, accurate and selective LC–MS/MS method was developed and validated for simultaneous quantification of ten antiarrhythic drugs (diltiazem, amiodarone, mexiletine, propranolol, sotalol, verapamil, bisoprolol, metoprolol, atenolol, carvedilol) and a metabolite (norverapamil) in human plasma. Plasma samples were simply pretreated with acetonitrile for deproteinization. Chromatographic separation was performed on a Capcell C_{18} column (50 mm × 2.0 mm, 5 μm) using a gradient mixture of acetonitrile and water (both containing 0.02% formic acid) as a mobile phase at flow rate of 0.3 ml/min. The analytes were protonated in the positive electrospray ionization (ESI) interface and detected in multiple reaction monitoring (MRM) mode. Calibration curves were linear over wide ranges from sub- to over-therapeutic concentration in plasma for all analytes. Intra- and inter-batch precision of analysis was <12.0%, accuracy ranged from 90% to 110%, average recovery from 85.0% to 99.7%. The validated method was successfully applied to therapeutic drug monitoring (TDM) of antiarrhythic drugs in routine clinical practice.

Keywords: Antiarrhythic drugs; LC–MS/MS; Therapeutic drug monitoring

1. Introduction

Arrhythmias, commonly observed as atrial fibrillation, atrial flutter, atrial tachycardia, ventricular tachycardia and premature beats, are the consequences of abnormal autorhythmicity or conduction disturbance of heart [1–3]. Generally, antiarrhythic drugs therapy is preferred for patients with cardiac arrhythmia [4]. Antiarrhythic drugs, as discussed by many researchers [1,5–7], are conventionally divided into four major classes, based on their effect on the cardiac action potential. The Vaughan Williams classification and examples of these drugs are listed as follows. Class I, Na^+ channel blocker, which can be further subdivided into three subgroups: IA (quinidine and procainamide); IB (lidocaine and mexiletine); and IC (propafenone). Class II, β-adrenergic blocker, includes propranolol, atenolol, bisoprolol and metoprolol. Class III, drugs for prolongs action potentional duration, include amiodarone and sotalol. Class IV, Ca^{2+} channel blocker, represents by verapamil and diltiazem. At present, most of the antiarrhythic drugs listed above have been the national essential drugs in China and widely used in clinical practice [8].

However, a number of these drugs exhibit a narrow therapeutic window and marked inter-individual variability in their pharmacokinetics. Optimal therapy with antiarrhythic drugs requires therapeutic drug monitoring (TDM) in order to avoid adverse effects and obtain the desired clinical benefit [9,10]. Many literatures have reported bioanalytical methods for antiarrhythic drugs determination, but the majority of these methods only focused on individual drugs mainly detected with HPLC-UV or LC–MS [11–24]. LC–MS/MS has proved to be an extremely important analytical technique that couples high sensitive and specific detection with high-resolution chromatographic separation. It is probably the most powerful technique currently available for pharmaceutical analysis [25].

The present paper described and validated, for the first time, a universal method for simultaneous quantification of ten

* Corresponding author. Tel.: +86 21 54037810 2120; fax: +86 21 5403 0254.
E-mail address: chen-yu@online.sh.cn (C. Yu).

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antiarrhythic drugs (diltiazem, amiodarone, mexiletine, propranolol, sotalol, verapamil, bisoprolol, metoprolol, atenolol, carvedilol) and an active metabolite (norverapamil) in human plasma. The method was based on a simple sample preparation, rapid LC separation and selective MS/MS detection. The applicability of the proposed method was demonstrated for routine TDM of drugs used in clinical antiarrhythmic treatment.

2. Experimental

2.1. Chemicals

The reference standards, including hydrochlorides of diltiazem, amiodarone, mexiletine, propranolol, sotalol, verapamil, and bisoprolol fumarate, metoprolol tartrate, atenolol were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA), carvedilol from J&K Chemical Ltd. (Shanghai, China). Norverapamil, the active metabolite of verapamil, was kindly donated by Dr. Margarete Fischer–Bosch Institute (Stuttgart, Germany). Sulfamethoxydiazine, sulfadimethoxine, sulfisomedine (used as internal standards) were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade acetonitrile, formic acid were supplied by Tedia Company Inc. (Fairfield, OH, USA). All other reagents were of analytical grade. Double distilled water was used throughout the study.

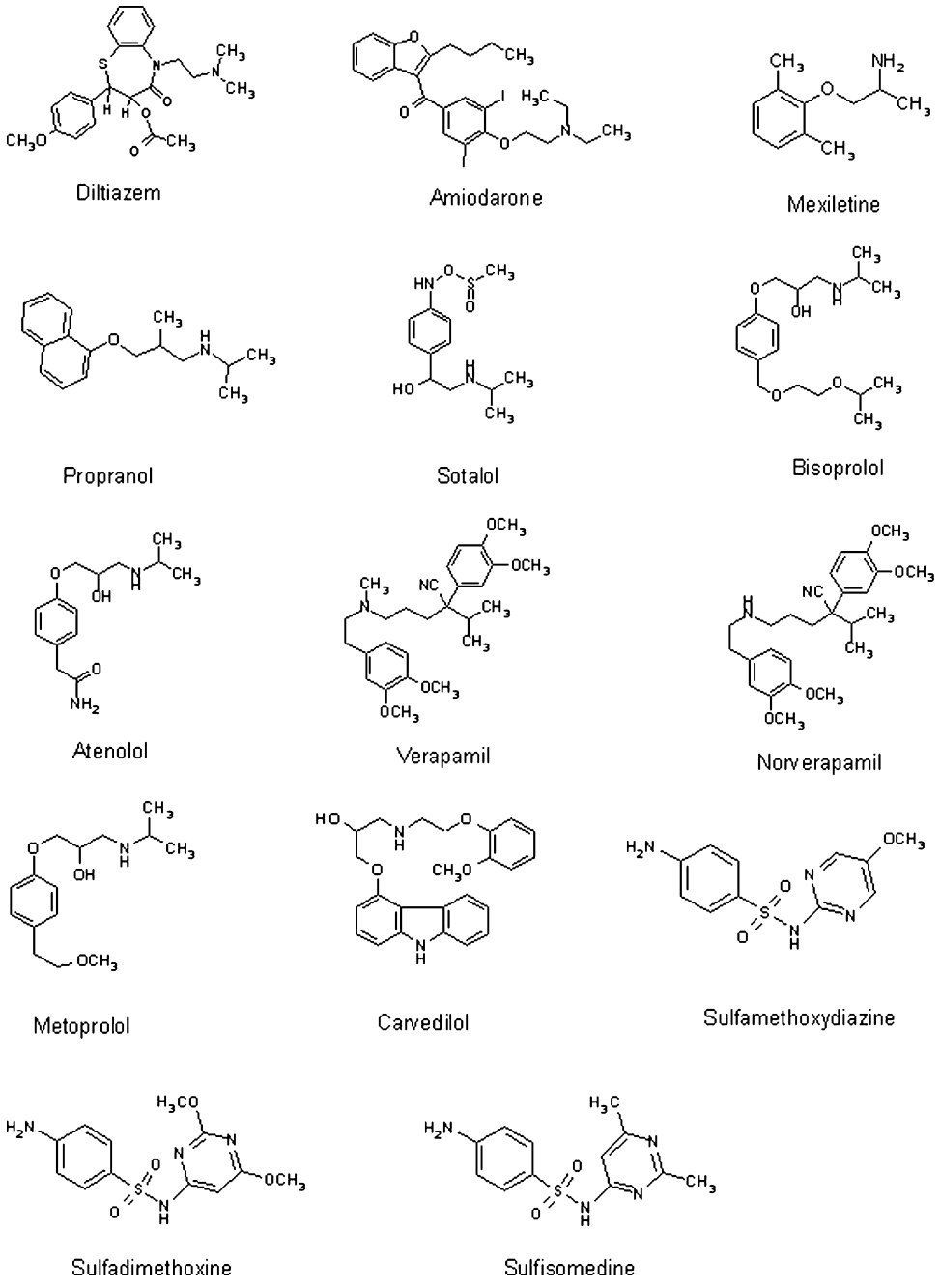

Fig. 1. Chemical structure of antiarrhythic drugs and internal standards.

2.2. LC–MS/MS analysis

A Shimadzu LC20AD system (Kyoto, Japan), equipped with two pumps, a vacuum degasser and an auto-sampler, a controller module, was used in the study. Chromatographic separation was performed on Capcell Pak C_{18} column (50 mm × 2.0 mm, 5 μm, Shiseido, Japan) at room temperature. The mobile phase was consisted of acetonitrile (A) and water (B), both containing 0.02% formic acid, for gradient elution. The gradient program, with a total run time of 7.5 min, was eluted with 95–50% (B) during 3.5 min, followed by 50–5% (B) during 0.5 min, then back to 95% (B) during 0.5 min followed by re-equilibration for 3 min. The flow rate remained at 0.3 ml/min throughout the run. The auto-sampler was kept at 4 °C and 5 μl samples were injected.

A triple quadrupole tandem mass spectrometer API 3000 instrument (ABI-SCIEX, Toronto, Canada) was equipped with Turbo Ionspray source and operated in positive ionization mode. Analyst 1.4 software package was used for instrument control and data acquisition. The ion spray voltage was set at 2.5 kV and source temperature at 450 °C. The collision activated dissociation (CAD) was set at 12, using nitrogen as the collision gas.

2.3. Sample preparation

To 1.5 ml polypropylene centrifuge tube, 100 μl of plasma sample and 200 μl of acetonitrile (containing three internal standards with concentration of 200 ng/ml) were added for

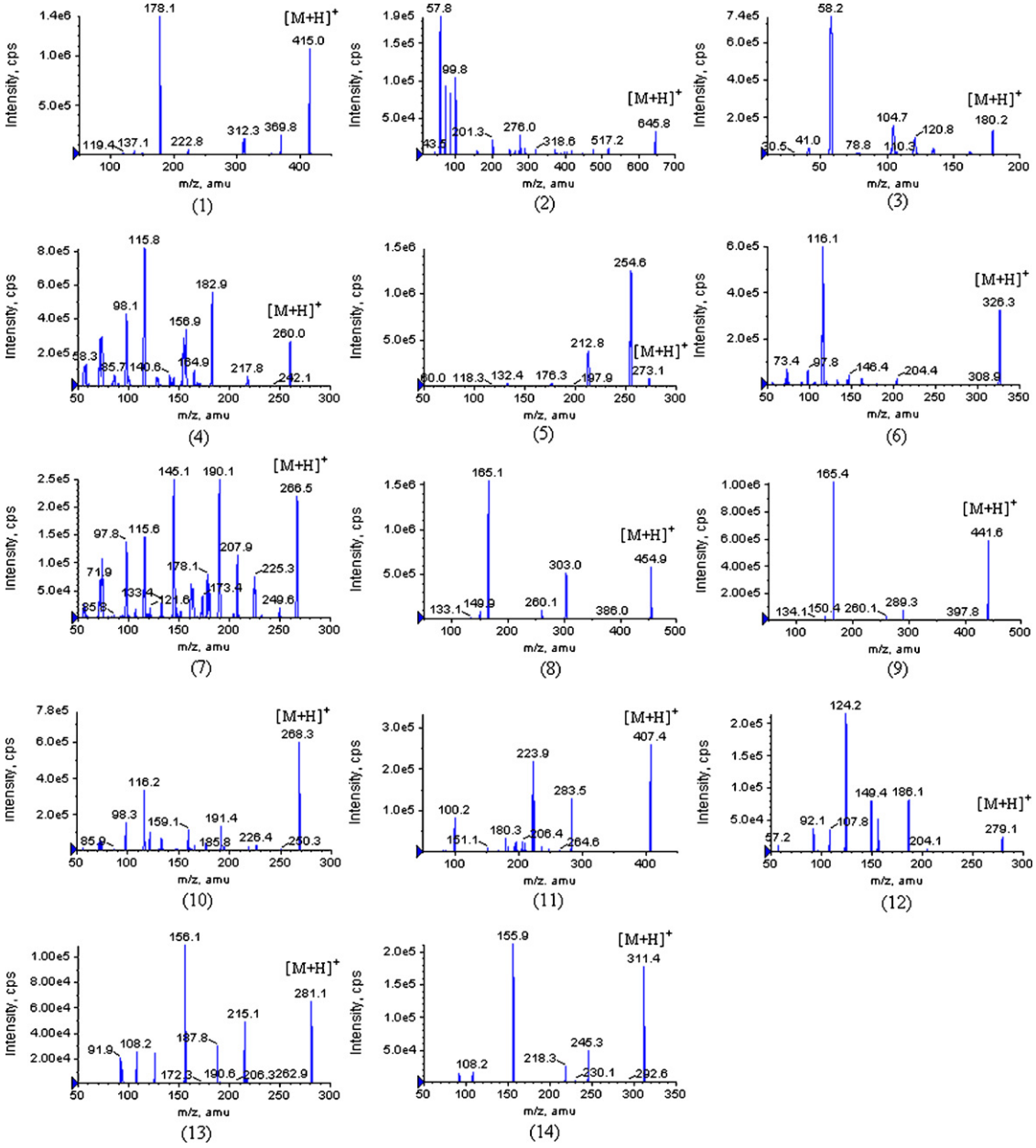

Fig. 2. Product ion spectra of antiarrhythic drugs and internal standards (1) diltiazem, (2) amiodarone, (3) mexiletine, (4) propranolol, (5) sotalol, (6) bisoprolol, (7) atenolol, (8) verapamil, (9) norverapamil, (10) metoprolol, (11) carvedilol, (12) sulfisomedine, (13) sulfamethoxydiazine, (14) sulfadimethoxine.

deproteinization. The mixture was briefly vortex-mixed for 10 s and followed by centrifugation at 16,000 × *g* for 3 min. Fifty microlitres of supernatant was added to 150 μl of water and 5 μl was injected into LC–MS/MS system.

2.4. Stock solutions, calibrators and quality control standards

Stock solutions, separately prepared in methanol, were spiked together to obtain a mixture of working solution followed by serial dilution with methanol–water (50/50, v/v). All the stock and working solutions were stored at −20 °C. The working solutions were used to prepare seven calibrators in blank plasma. QC standards were separately prepared in blank plasma in the same way at low, middle and high concentrations. The calibrators and QCs were treated as in Section 2.3 with unknown samples.

2.5. Assay validation

All assay validation steps were carried out according to the 2001 version of the FDA guidance for bioanalytical method validation [26]. Linearity was evaluated using a 1/*x* weighted linear regression method between wide ranges from sub- to over-therapeutic concentration in plasma at clinical practice. The sensitivity of the analytical procedure was expressed as the lower limit of quantification (LLOQ) or the lowest concentration on the calibration curve that can be quantitatively determined with acceptable accuracy and precision, and should be at least 10 times the response compared to blank response. The specificity of assay was determined by analysis of six blank plasmas from different subjects. There should be no interference from endogenous or exogenous materials observed at the retention time in each analyte channel.

The accuracy and precision were assessed by determining QC samples at three concentration levels on three different validation batches. The QC samples were prepared for six duplicates together with calibration samples.

The stock solution stability was determined by placing the stock solution mixture at −20 °C for a month. The freeze–thaw, short-term, autosampler, and long-term stability studies were evaluated. For freeze–thaw stability, QC plasma samples were subjected to 3 cycles from −20 °C to room temperature. Short-term benchtop stability was performed by placing samples on the benchtop at room temperature for 24 h. The autosampler stability was assessed by placing processed QC samples in an autosampler at 4 °C for 24 h, and long-term stability was evaluated by freezing QC samples at −20 °C for a month, then comparing the concentrations with those of QCs before the storage period.

2.6. Matrix effect and recovery

As described in detail by Matuszewski et al. [27], the matrix effect and recovery were assessed by comparing the peak areas of the neat analyte standards, standards spiked before and after extraction in six different lots of plasma at three concentration levels.

3. Results and discussion

3.1. LC–MS/MS optimization

Antiarrhythic drugs (Fig. 1), with numerous methyl and amino groups in their chemical structures, gave higher MS responses in positive ionization mode. When tuned with flow injection analysis (FIA) using single standard solution, obvious protonated molecules $[M+H]^+$ were observed in Q1 full-scan. Then fragments of protonated molecules were obtained in product ion scan at collision cell. Fig. 2 presented the product ion scan spectra of the analytes and internal standards. Reaction monitoring mode (MRM) scan was used for quantitation of all analytes. In order to obtain the most intense signal, a prominent product ion was selected for optimization of compound dependant parameters including declustering potential (DP), focusing potential (FP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP). Detailed parameters were summarized in Table 1. A dwell time of 100 ms for each MS/MS transition was used.

There was a great difference in polarity between various analytes investigated. Therefore, separation of these com-

Table 1
LC–MS/MS analysis conditions for the 11 antiarrhythic drugs and its internal standards

Compound	MW	MRM transition	DP (V)	CE (V)	Rt (min)
Diltiazem	414.5	415.4 → 178.2	34	35	4.1
Amiodarone	645.3	646.1 → 58.2	38	95	5.5
Mexiletine	179.3	180.2 → 58.1	20	22	3.4
Propranolol	259.3	260.4 → 116.2	32	27	3.9
Sotalol	272.4	273.4 → 213.0	35	26	2.0
Bisoprolol	325.4	326.6 → 116.2	28	26	3.6
Atenolol	266.3	267.6 → 145.2	28	36	2.1
Verapamil	454.6	455.4 → 165.2	30	38	4.3
Norverapamil	440.6	441.5 → 165.2	31	36	4.3
Metoprolol	267.5	268.5 → 116.2	33	27	3.2
Carvedilol	406.5	407.5 → 100.2	51	41	4.3
Sulfamethoxydiazine	280.1	281.2 → 156.2	35	23	3.9
Sulfadimethoxine	310.1	311.2 → 156.2	42	29	4.8
Sulfisomedine	278.1	279.2 → 124.2	38	35	2.8

pounds with single isocratic elution remained a difficult task. Acetonitrile–water (both containing 0.02% formic acid) were selected as mobile phase and eluted with gradient procedure. By using a short Capcell C18 column with dimension of 50 mm × 2.0 mm i.d., a total run time of 7.5 min for each sample was reached. Chromatograms of blank plasma extracted without the addition of internal standards and containing 11 analytes and its internal standards were shown in Fig. 3.

It is desirable to use isotope-labeled or structure-similar internal standards in a LC–MS/MS procedure. However, such compounds are not commercially available. In this study, a simple deproteinized procedure was employed to treat sample, therefore three sulfanilamides with similar chromatographic retention to its analyte were used as internal standards. According to its closeness to analytes in retention time, sulfisomedine was used as internal standards for determination of sotalol and atenolol, sulfamethoxydiazine for diltiazem, mexiletine, propranolol, verapamil, norverapamil, bisoprolol, metoprolol, and carvedilol, sulfadimethoxine for amiodarone to construct calibration curves.

3.2. Method validation

3.2.1. Linearity and LLOQ

Calibration curves were linear within the quantification ranges for all the assayed drugs using a linear regression with $1/x$ weighting. Quantification ranges were established according to effective blood concentration of respective antiarrhythic drug used in clinical practice. The seven-point calibration curves, used for all the calculations, gave acceptable results within linear ranges. Correlation coefficients (r) of above 0.99 were obtained in method validation. The lower limit of quantification for each analyte was the lowest concentrations of calibration curve with S/N > 10. The linearity results were listed in Table 2.

3.2.2. Precision and accuracy

The intra- and inter-batch precision and accuracy were evaluated by assaying the QC samples (Table 3). In this assay, the intra-batch precision was 10.3% or less, and the inter-batch precision was 12.0% or less, the accuracy was ranged from 90% to 110%, at low, medium and high QC levels for all investigated analytes. The results demonstrated that the values were within the acceptable range and the method was sufficiently accurate and precise.

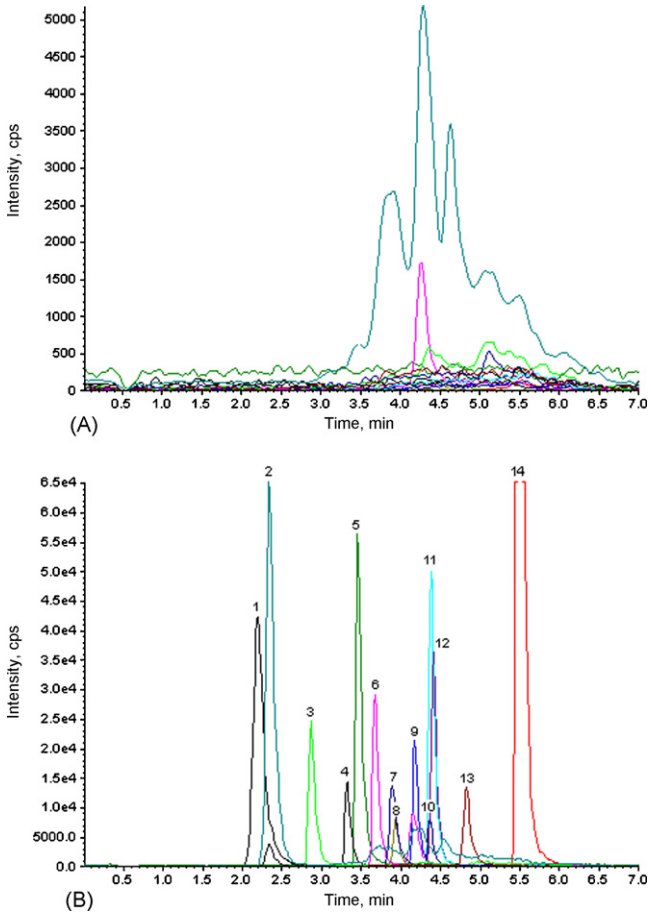

Fig. 3. LC–MS/MS chromatograms acquired from (A) blank human plasma and (B) blank plasma spiked with 200 ng/ml for sotalol (peak 1), sulfisomedine (IS, peak3) and sulfadimethoxine (IS, peak 13), 500 ng/ml for atenolol (peak 2), sulfamethoxydiazine (IS, peak 8) and amiodarone (peak 14), 100 ng/ml for metoprolol (peak 4), mexiletine (peak 5), bisoprolol (peak 6), propranolol (peak 7), diltiazem (peak 9), verapamil (peak 10), norverapamil (peak 11) and carvedilol (peak 12).

3.2.3. Selectivity, matrix effect and recovery

Selectivity was assessed by comparing the chromatograms of six different lots of blank human plasma with the correspond-

Table 2
Linear regression data from calibration curves

Drug	Range (ng/ml)	$y = ax + b$	r	Accuracy (%)
Diltiazem	1–200	$y = 0.023x + 0.000541$	0.9992	96.5–103.8
Amiodarone	50–10,000	$y = 0.0015x + 0.0022$	0.9982	91.3–113.2
Mexiletine	10–2000	$y = 0.00421x - 0.00436$	0.9989	95.3–103.7
Propranolol	2–400	$y = 0.00288x - 0.00153$	0.9983	94.3–109.0
Sotalol	20–4000	$y = 0.00175x + 0.00365$	0.9985	88.5–107.0
Bisoprolol	2–400	$y = 0.0122x - 0.00435$	0.9977	93.2–111.6
Atenolol	50–10,000	$y = 0.000893x - 0.00513$	0.9973	87.5–110.5
Verapamil	2–400	$y = 0.0198x + 0.00122$	0.9986	96.6–102.2
Norverapamil	2–400	$y = 0.0194x - 0.00209$	0.9986	94.2–105.1
Metoprolol	5–1000	$y = 0.00179x - 0.000117$	0.9992	96.4–106.2
Carvedilol	2–400	$y = 0.0236x + 0.000503$	0.9972	93.0–107.8

Table 3
Accuracy and precision for the assay of antiarrhythic drugs in human plasma ($n = 6$, for 3 days)

Drug	Spiked C (ng/ml)	Found C (ng/ml)	Intra-day RSD (%)	Inter-day RSD (%)	Drug	Spiked C (ng/ml)	Found C (ng/ml)	Intra-day RSD (%)	Inter-day RSD (%)
Diltiazem	2	2.2	4.3	7.4	Amiodarone	100	94.6	7.9	3.2
	80	82.6	4.4	5.9		4000	4182.3	6.7	4.2
	160	160.9	3.2	3.7		8000	7887.7	10.3	5.3
Mexiletine	20	20.2	5.5	6.1	Propranolol	4	4.2	3.0	5.4
	800	787.9	7.2	6.5		160	168.2	6.0	7.4
	1600	1600.1	6.6	5.3		320	317.6	5.5	4.4
Sotalol	40	41.2	7.7	6.8	Bisoprolol	4	4.1	2.9	5.3
	1600	1602.2	4.7	4.9		160	168.9	6.7	5.4
	3200	3196.0	7.1	6.0		320	338.9	4.4	4.3
Atenolol	100	106.9	2.4	6.9	Verapamil	4	4.3	8.2	6.0
	4000	4111.1	6.0	6.1		160	168.7	3.4	4.4
	8000	8113.9	9.8	5.1		320	337.0	7.2	5.9
Norverapamil	4	4.4	3.9	4.6	Metoprolol	10	9.7	7.9	12.0
	160	171.1	4.6	3.7		400	408.0	7.6	8.5
	320	343.9	6.6	6.1		800	786.6	5.5	6.1
Carvedilol	4	4.3	7.9	9.8					
	160	150.4	3.2	3.7					
	320	289.2	5.4	5.3					

ing spiked plasma. There were no significant interferences from endogenous substances observed at the retention times of the analytes and IS.

Matrix effect was evaluated by comparing peak area of analyte and internal standards in blank plasma samples spiked after the sample preparation with those obtained by direct injection of chemical standards. Though the multiple clean-up procedures or stable-isotope-labeled internal standards were not employed in sample preparation procedure, sufficient chromatographic retention on the analytical column was achieved. As a result of it, the method almost showed no matrix effect from biological material. Table 4 presented acceptable matrix effect with mean peak area ratio of 89.8–100.0% obtained from post-extracted samples to standards, and recovery with that of 85.0–99.7% obtained from

Table 4
Matrix effect (ME) and recovery (RE) for the assay of 11 antiarrythic drugs in six different lots of human plasma

Drug	Spiked C (ng/ml)	Mean peak area			ME[a] (%)	RE[b] (%)	Drug	Spiked C (ng/ml)	Mean peak area			ME (%)	RE (%)
		Set 1	Set 2	Set 3					Set 1	Set 2	Set 3		
Diltiazem	4	8.71E3	8.12E3	7.40E3	93.6	91.2	Amiodarone	200	1.29E5	1.22E5	1.05E5	94.5	85.6
	80	1.36E5	1.28E5	1.21E5	94.0	94.3		4000	2.50E6	2.44E6	2.14E6	97.4	87.7
	160	2.55E5	2.37E5	2.08E5	93.0	87.6		8000	4.62E6	4.48E6	4.30E6	97.0	95.8
Mexiletine	40	2.25E4	2.08E4	2.02E4	92.3	97.1	Propranolol	8	3.70E3	3.50E3	3.30E3	94.7	94.5
	800	3.79E5	3.68E5	3.44E5	97.3	93.4		160	5.40E4	5.09E4	4.69E4	94.4	92.3
	1600	7.02E5	6.30E5	5.68E5	89.8	90.2		320	1.02E5	9.48E4	8.42E4	93.0	88.9
Sotalol	80	2.94E4	2.74E4	2.73E4	93.3	99.7	Bisoprolol	8	1.25E4	1.21E4	1.12E4	97.5	92.5
	1600	5.16E5	5.05E5	4.39E5	98.1	86.8		160	2.08E5	1.96E5	1.78E5	94.5	90.9
	3200	9.66E5	8.92E5	7.94E5	92.5	89.1		320	4.02E5	3.62E5	3.27E5	90.2	90.2
Atenolol	200	3.43E4	3.15E4	3.04E4	92.2	96.5	Verapamil	8	1.46E4	1.40E4	1.31E4	95.8	94.1
	4000	6.01E5	5.74E5	5.00E5	95.7	87.0		160	2.32E5	2.17E5	2.05E5	93.8	94.3
	8000	1.11E6	1.01E6	9.17E5	91.8	90.5		320	4.42E5	4.17E5	3.74E5	94.5	89.7
Norverapamil	8	2.13E4	2.02E4	1.91E4	95.0	94.3	Metoprolol	20	6.30E3	5.93E3	5.36E3	94.2	90.6
	160	3.41E5	3.16E5	2.92E5	93.0	92.4		400	9.27E4	9.29E4	7.89E4	100.0	85.0
	320	6.63E5	6.06E5	5.53E5	91.7	91.3		800	1.86E5	1.71E5	1.50E5	91.8	87.9
Carvedilol	8	3.19E3	2.99E3	2.70E3	93.8	90.3							
	160	5.30E4	4.87E4	4.81E4	92.1	98.8							
	320	1.00E5	8.98E4	8.24E4	89.9	91.9							

[a] Matrix effect expressed as the ratio of the mean peak area of analyte spiked postextraction (set 2) to the mean peak area of the same analyte standards (set 1) multiplied by 100.

[b] Recovery calculated as the ratio of the mean peak area of an analyte spiked before extraction (set 3) to the mean peak area of an analyte spiked postextraction (set 2) multiplied by 100.

Table 5
Plasma concentrations in arhythmia patients

Drug administered	*N* (M/F)[a]	Age (year)	Dose (mg/day)[b]	Concentration (ng/ml)[c]
Diltiazem	33 (21/12)	77.4 ± 9.4	90	70.1 ± 54.4
Amiodarone	87 (74/13)	81.2 ± 8.3	200	946.9 ± 852.2
Mexiletine	8 (4/4)	76.2 ± 7.8	400	1650 ± 1080
Sotalol	5 (3/2)	73.5 ± 9.1	120	2090 ± 873
Verapamil	14 (5/9)	69.5 ± 8.1	240	66.0 ± 46.0
Norverapamil				172.2 ± 117.4

[a] The number of samples analyzed, and the number of male to female was listed.
[b] The total dose administered orally within 24 h, from t.i.d. to q.i.d.
[c] Steady state plasma concentrations acquired after continuous dosage.

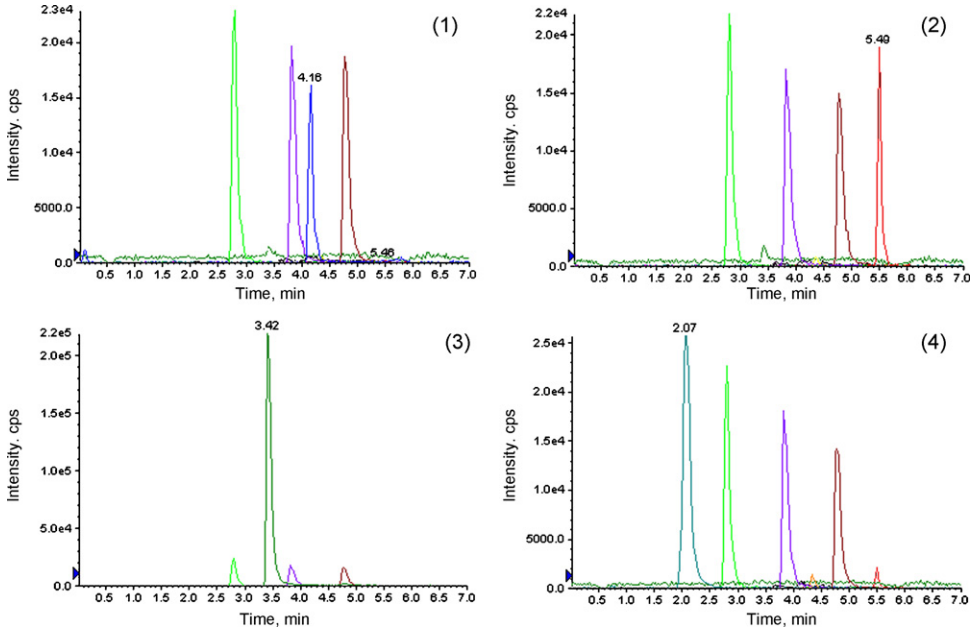

Fig. 4. Representative chromatograms acquired from patients plasma with (1) diltiazem 88.9 ng/ml, (2) amiodarone 769 ng/ml, (3) mexiletine 1.74 μg/ml, (4) sotalol 1.82 μg/ml.

standards spiked before and after extraction in six lots of human plasma.

3.2.4. Stability

The stock solutions in methanol were placed at −20 °C for a month and no significant degradation occurred. After exposed to room temperature for 24 h or underwent three freeze–thaw cycles, the plasma samples were stable with accuracies of 88.7–112.2% and CVs of 2.6–14.3%. The stability results for processed samples showed that analytes kept stable in autosampler at 4 °C for at least 24 h, with accuracies ranged from 93.2% to 103.6% and CVs from 1.2% to 8.9%. The results for long-term stability were obtained by comparing the concentration the QCs stored at −20 °C for a month with those obtained before the storage period. There was no significant change in the concentration of analytes investigated. The accuracies of QCs were ranged from 94.4% to 100.8% when compared concentrations post- to pre-storage. In addition, internal standards solution (200 ng/ml in acetonitrile) was also proved to be stable for at least a week at 4 °C by comparing the peak areas with those of freshly prepared.

3.3. Method application

The validated method was successfully applied to determine drug concentrations in plasma collected from arrhythmia patients after oral-administrated antiarrhythic drugs individually. For the purpose to evaluate the therapeutic effect, plasma samples reached steady state concentrations were collected from February, 2004 to April, 2006. Mean plasma concentrations of the five drugs commonly used in our hospital were shown in Table 5. Significant inter-individual differences were observed. Consequently, the TDM of antiarrhythic drugs is necessary in clinical practice to acquire the best treatment effect. Fig. 4 provided representative chromatograms in patients received diltiazem, amiodarone, mexiletine and sotalol therapy.

4. Conclusions

The described liquid chromatography-tandem mass spectrometry method enabled a simple, rapid and universal assay for the simultaneous determination of ten antiarrhythic drugs

(diltiazem, amiodarone, mexiletine, propranolol, sotalol, verapamil, bisoprolol, metoprolol, atenolol, carvedilol) and an active metabolite (norverapamil) in human plasma. Protein precipitation with acetonitrile was employed with 100 μl of plasma. The proposed method, proved to be accurate and selective, has met the standards of bioanalytical method validation acceptable by FDA. It was successfully applied to routine TDM of plasma samples from individuals received antiarrhythic drugs treatment in clinical practice. In addition, this method has the potential application to clinical research of drug combination, multi-drug pharmacokinetics and interaction.

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