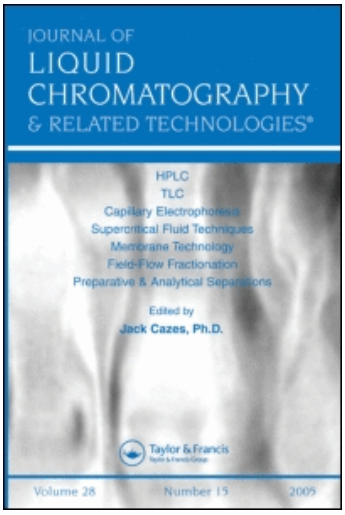

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Microextraction in Packed Syringe/Liquid Chromatography/Electrospray Tandem Mass Spectrometry for Quantification of Acebutolol and Metoprolol in Human Plasma and Urine Samples

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Abstract: The aim of the present investigation was to develop a simple, fast, and sensitive method for the determination of acebutolol and metoprolol in human plasma and urine samples. The determination of acebutolol and metoprolol in plasma and urine was performed using micro extraction in packed syringe (MEPS) as a sample preparation method, online with high performance liquid chromatography and tandem mass spectrometry (LC-MS/MS). In MEPS the sampling sorbent was 1 mg polystyrene polymer, which was inserted in a 250 μL syringe. The lower limits of quantification (LLOQ) for acebutolol and metoprolol were set to 1.0 ng/mL. The accuracy of quality control samples (QC) varied by ±10%, and precision (R.S.D.) had a deviation of 1.4–12% for plasma and urine samples. The calibration curve was obtained within the concentration range 1.0–100 ng/mL in both plasma and urine. The regression correlation coefficients (R^2) for plasma and urine samples were ≥0.999 for all runs. The present method is miniaturized, fully automated, robust, and

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can be easily used for pharmacokinetic and pharmacodynamic studies of acebutolol and metoprolol.

Keywords: Microextraction in packed syringe, Sample preparation, Polystyrene sorbent, Acebutolol, Metoprolol, Plasma, Urine, LC-MS/MS

INTRODUCTION

The measurement of drug levels in biological fluids is of crucial importance for drug discovery and development. In addition, it allows the optimization of pharmacotherapy and provides a basis for studies of patient compliance, bioavailability, pharmacokinetics, and the influences of comedications. Sample preparation is frequently done off-line and, in fact, this is often a limiting step to perform fast bioanalysis; the introduction of on-line sample pretreatment would greatly speed up the analyses. Further, as the number of samples increases, high throughput and fully automated analytical techniques becomes required. Current developments of sample handling techniques are directed toward automatization and online coupling of sample preparation units and detection systems. In addition, there is a trend toward development of more selective sorbents for sample clean up and enrichment. MEPS is a new technique for miniaturized solid-phase extraction that can be connected on-line to GC or LC without any modifications.[1–6] In MEPS, approximately 1 mg of the solid packing material is inserted into a syringe (100–250 μL) as a plug. The plasma sample (50–250 μL) is withdrawn through the syringe by an autosampler. When the plasma has passed through the solid support, the analytes are adsorbed to the solid phase. The solid phase is then washed once by water to remove the proteins and other interfering materials. The analytes are then eluted with an organic solvent such as methanol or the LC mobile phase (20–50 μL) directly into the instrument's injector. The process is fully automated. Many different types of absorption materials such as silica based (C_2, C_8, C_{18}), restricted access material (RAM), or molecular imprinted polymers (MIPs) can be used.

Acebutolol and metoprolol are beta-adrenoceptor blocking drugs (β-blockers) that are widely used as effective antihypertensive and antianginal agents. They are used for the treatment of various cardiovascular disorders such as hypertension, angina pectoris, and cardioarrhythmia. Thus, the International Olympic Committee and International Sports Federations have banned the use of β-blockers because of their activity as anabolic agents. Therefore, screening and determination of β-blockers in biological samples are required in many circumstances, such as clinical control for diagnosis and treatment of diseases, doping control, forensic analysis, and toxicology.[7–11] Therefore, the primary objective of the present study was to develop, optimize, and validate an innovative analytical method for

determination of acebutolol and metoprolol in plasma and urine samples, employing online sample preparation and LC-MS/MS analysis.

EXPERIMENTAL

Chemicals

Acebutolol and metoprolol (Figure 1A) were purchased from Sigma-Aldrich (Stockholm, Sweden) and [$^2H_7$] ropivacaine (IS), was supplied by the Department of Medicinal Chemistry, AstraZeneca (Södertälje, Sweden). Acetonitrile, methanol, formic acid, and ammonium hydroxide were obtained from Merck (Darmstadt, Germany). All chemicals were of analytical grade.

Apparatus

A high performance liquid chromatography (HPLC) instrument included a Shimadzu LC-10Advp pump, Shimadzu (Kyoto, Japan), an autosampler, CTC-Pal, Crelab (Knivsta, Sweden), and a 20 μL sample loop. A Zorbax (50 × 2.1 mm, SB-C18, 3.5 μm) column obtained from Agilent (CA, USA) was used as the analytical column connected to an Optiguard (C_8, 10 × 1 mm) as a guard column. A Valco C4W valve, Valco Instruments (Houston, TX, USA) was used as the gate valve between the liquid chromatograph and the mass spectrometer. The water used was purified using a Reagent Grade Milli-Q Plus water purification system from Millipore Corporation (Bedford, MA, USA). A centrifuge, Hettich Rotanta/AP (Tuttlingen, Germany), was used for plasma centrifugation.

Mobile Phase was 0.1% formic acid in acetonitrile/methanol/water (15:15:70, v/v). The flow rate was 200 μL/min and sample volume (loading) was 50 μL.

All experiments were conducted using a triple quadrupole mass spectrometric instrument Micromass QII Z-spray (Manchester, UK), equipped with a Z-electrospray interface operated in positive ion mode. Nitrogen was used both as drying (400 L h^{-1}), and nebulizing gases (20 L h^{-1}), the vacuum was 2×10^{-5} mbar in the mass analyzer and 2×10^{-3} mbar in the collision cell (argon was used as collision gas). The gases were from ScanGas (Stockholm, Sweden). Source block and desolvation temperatures were set to 150°C and 300°C, respectively. The data were collected and processed using MassLynx version 3.4, and all calculations were based on peak area ratios.

The scan mode was multiple reaction monitoring (MRM) using a precursor ion at (M + 1) (*m/z:* 337, 268, and 282), and after collisional dissociation the product ions 116, 116, and 133 were used for quantification of acebutolol, metoprolol, and [$^2H_7$] ropivacaine (IS), respectively. Different

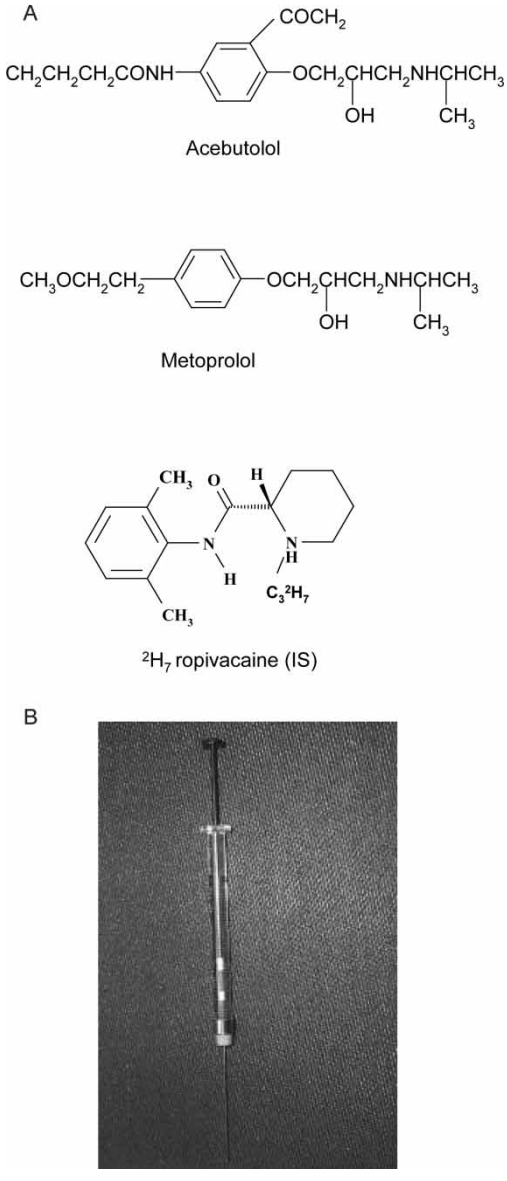

Figure 1. (A) Chemical structure of acebutolol, metoprolol, and internal standard; (B) Packed syringe.

parameters such capillary voltage, cone voltage, and collision gas energy were optimized to get a maximum signal in MS and MS-MS. After optimization, the parameter settings were: capillary voltage at 3.1 kV, cone voltage at 38 V, extractor at 5 V, RF lens at 0.2 V. The collision energy was 25 eV.

Preparation of Samples

Stock solutions of acebutolol and metoprolol (10 mg/mL) and [$^2H_7$] Ropivacaine (internal standard, 3 μg/mL) were dissolved in methanol. From the stock solution, stepwise dilution series were made in water. Spiked plasma and urine samples were prepared by adding 10–50 μL of stock solutions to 1.0 mL of plasma or urine. Twenty μL of internal standard were added. After vortex mixing, the samples were extracted and analyzed. The concentration range of the standard curve was between 1.0–100 ng/mL (1, 2, 5, 10, 20, 40, 50, 80, and 100 ng/mL). Quality control (QC) samples concentrations were 15, 30, and 60 ng/mL. All standard and control solutions were stored at −20°C.

MEPS Conditions

MEPS was performed using a 250 μL gas-tight syringe (Figure 1B). The sorbent used was a polystyrene polymer. This sorbent has irregular particles with average size of 50 μm and nominal 60 Å porosity. One milligram of the solid material was manually inserted inside the syringe as a plug. The sorbent material was tightened by filters (polyethylene, obtained from Varian, CA, USA) to avoid moving inside the syringe.

Before using for the first time, the sorbent was manually conditioned with 50 μL methanol followed by 50 μL of water/methanol (90:10, v/v). After that, the syringe was connected to the autosampler and the spiked plasma or urine sample (50 μL) was withdrawn into the syringe by the autosampler. It is important that the plasma samples are withdrawn slowly (20 μL s^{-1}) and with caution to obtain good percolation of the sample through the solid support bed. The sorbent was then washed once with 100 μL of water/methanol (90:10, v/v) for plasma, and pure water for urine sample to remove proteins, salts, and other interferences. The analytes were then desorbed by 25 μL methanol/water (95:5, v/v) containing 0.2% ammonium hydroxide directly into a gate valve, which was situated between the liquid chromatograph and the tandem mass spectrometer. Cleaning of the sorbent was carried out using a 5 × 50 μL elution solution followed by 5 × 50 μL of the washing solution between every extraction. This step decreased memory effects, but also functioned as a conditioning step before the next extraction. The same packing bed was used for about 100 extractions before it was discarded.

Method Validation

The plasma and urine used were collected and pooled from different objects. The peak area ratios for acebutolol or metoprolol and internal standard were

measured and a standard curve without zero concentration was constructed. The calibration curves were described by the equation:

$$y = ax^2 + bx + c$$

Where y is peak area ratio, x is the concentration, a is the curvature, b is the slope, and c is the intercept. The calibration curves were quadratic and the weight was 1/x. The accuracy and precision were calculated for the QC samples at three different assays. The method was validated at optimized conditions.

Accuracy was defined as the degree of deviation of the determined value and the nominal value: [(measure value-nominal value)/nominal value]*100. Precision (C.V.%) was defined as the percentage of standard deviation of the observed values divided by their mean values: [(standard deviation)/mean value]*100.

RESULTS AND DISCUSSION

Washing and Elution Solvents

The effect of different washing solutions on the recovery was investigated. The recovery was measured as the response of a processed spiked plasma sample expressed as peak area and calculated as a mean of three different experiments. The use of methanol in the washing mixture slightly affected the loss and the recovery of the analyte. The use of methanol in the washing mixture increased the leakage and decreased the recovery; however, a quite clean extract was obtained. The lowest amount of leakage, with no interferences and the highest recovery, was obtained with the use of 100 μL of water/methanol (90:10, v/v) as washing solution.

To study the recovery, solutions containing methanol, water, formic acid, and ammonium hydroxide were investigated as elution solutions. After introduction of the sample (50 μL) into the syringe and washing with 100 μL of water/methanol (90:10, v/v), the elution efficiency was measured and compared to that of the pure standard solution (100 ng/mL). The eluting efficiency increased as the methanol content in the eluent increased. Acceptable recovery (~50%) was obtained when using a solution of methanol/water (95:5, v/v) containing 0.2% ammonium hydroxide, and this was used as the elution solution for validation of the method.

Selectivity

The method selectivity was defined as non-interference with the endogenous substances in the regions of interest. LC–MS/MS analysis of the blank

plasma and urine samples showed no presence of an endogenous interference peak with the quantification of acebutolol and metoprolol. Representative chromatograms of blank human plasma and acebutolol and metoprolol spiked plasma are presented in Figures 2A and 2B. Representative chromatograms of blank human urine and acebutolol and metoprolol spiked urine, are shown in Figures 3A and 3B.

Calibrations

The constructed calibration curve consisted of nine concentration levels of human plasma and urine spiked with acebutolol and metoprolol. The R^2 for plasma and urine samples were ≥ 0.999 for all runs. The back calculated values of the calibration points showed good agreement with the theoretical concentrations. Deviation between -4% and $+8\%$ of the nominal concentrations was observed ($n = 3$) for plasma, and between -4% and $+5\%$ for urine samples (Tables 1 and 2) .

Accuracy and Precision

The accuracy for quality control (QC) samples varied from 94% to 104% for plasma, and from 91% to 107% for urine ($n = 12$). The precisions (R.S.D.) were 9.5–12% for plasma samples and 1.4–9.9% for urine samples ($n = 12$). The accuracy and precision data are summarized in Table 3. The accuracy and the precision of the method were within the internationally accepted limits.[12]

Limit of Quantification (LOQ) and Carry Over

The carry over was investigated by injecting the elution solution after the highest standard concentration; the carry over was lower than 0.1%.

The LOQ was set to 1.0 ng/mL. At this concentration, the accuracy of LOQ was between 97% and 104% and the precision had a maximum deviation of 3% ($n = 6$) for plasma samples.

CONCLUSIONS

In the present work, an LC-MS/MS assay for the determination of acebutolol and metoprolol in plasma and urine samples has been developed and validated. Our data showed that the method is selective and accurate. The acceptance criteria for the method validation were in accordance with

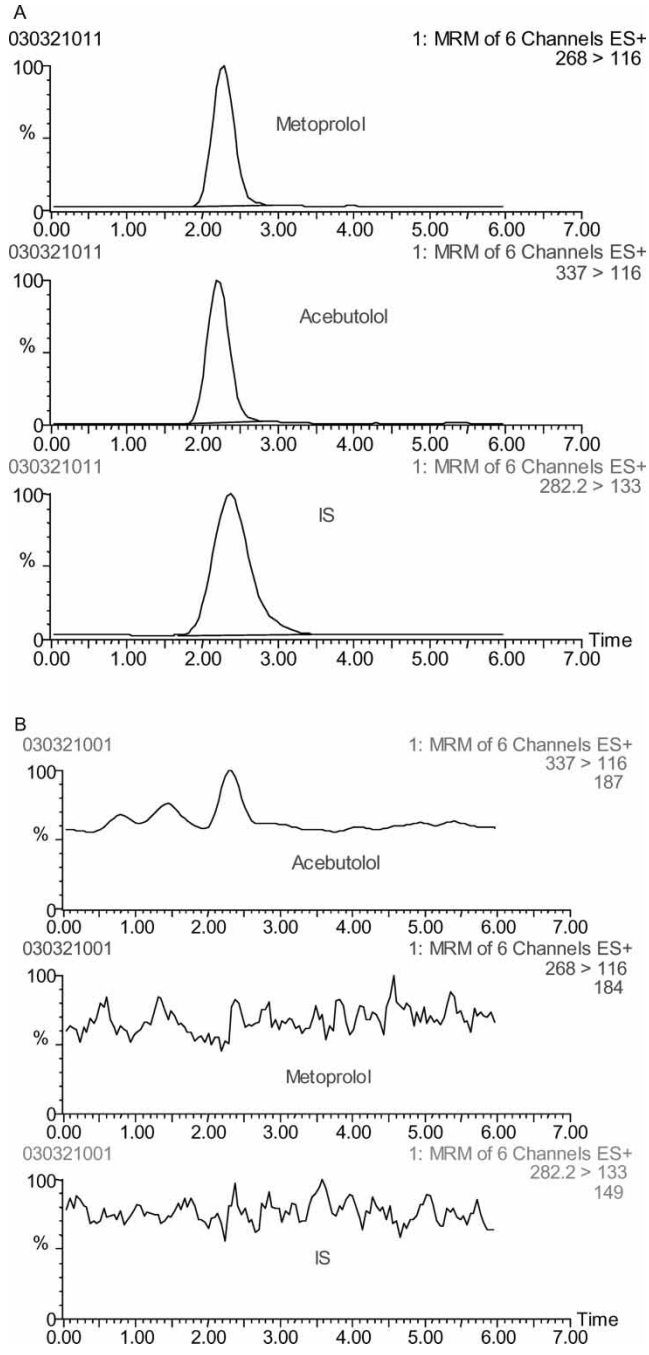

Figure 2. Mass chromatograms obtained from (A) human plasma spiked with acebutolol, metoprolol (100 ng/mL), and internal standard; (B) blank human plasma.

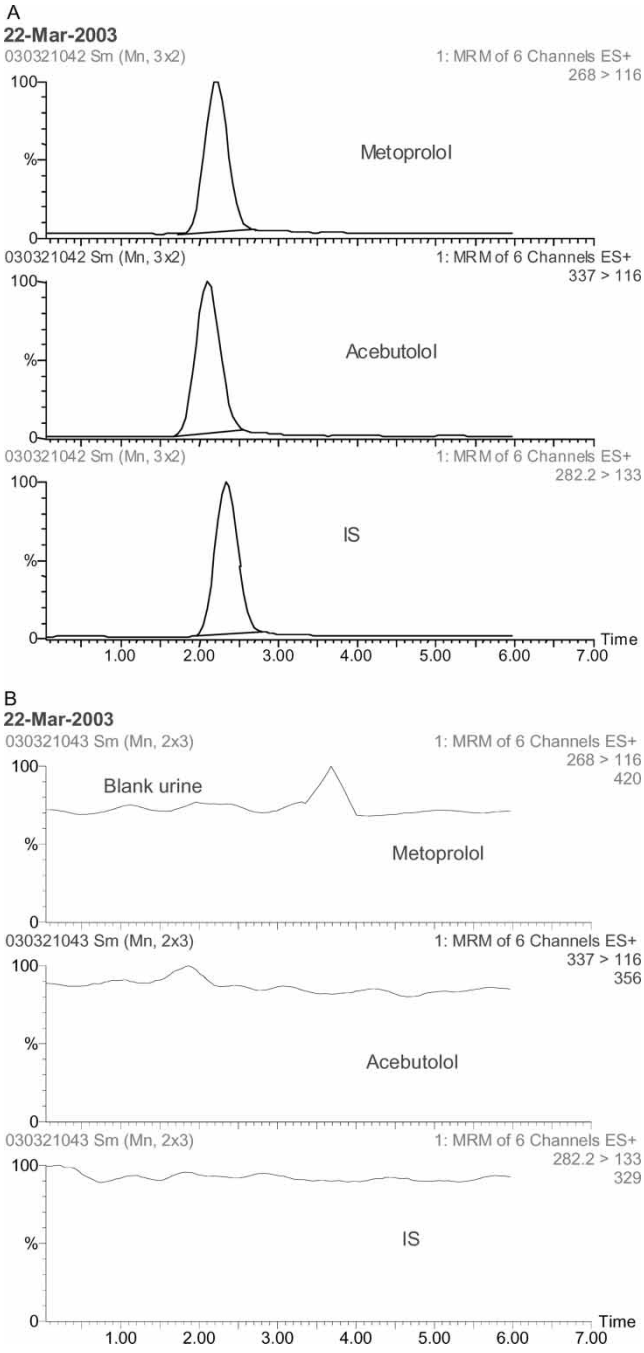

Figure 3. Mass chromatograms obtained from (A) human urine spiked with acebutolol, metoprolol (100 ng/mL), and internal standard; (B) blank human urine.

Table 1. Back-calculated values of the calibration points of the plasma samples

Compound	Concentration ng/mL	Mean conc (n = 6)	Mean accuracy (%)	RSD (%)
Acebutolol	1	1	101	7.6
	2	2	97	4.0
	5	5	107	2.8
	10	11	108	6.5
	20	20	98	4.7
	40	39	98	4.1
	50	48	96	7.3
	80	81	101	1.3
	100	100	100	1.2
Metoprolol	1	1	97	8.8
	2	2	101	2.0
	5	5	101	13
	10	10	97	5.4
	20	21	103	4.3
	40	39	98	7.8
	50	50	101	3.9
	80	80	100	4.5
	100	99	100	9.9

Table 2. Back-calculated values of the calibration points of the urine samples

Compound	Concentration ng/mL	Mean conc (n = 3)	Mean accuracy (%)	RSD (%)
Acebutolol	1	1	103	10.7
	2	2	101	1.2
	5	5	97	4.3
	10	10	97	4.9
	20	20	101	0.8
	40	41	101	1.9
	50	48	96	6.3
	80	84	105	6.0
	100	100	100	0.3
Metoprolol	1	1	103	3.7
	10	10.4	102	2.7
	20	20	102	2.6
	40	39	99	2.0
	50	44	90	18.8
	80	78	98	3.4
	100	100	100	0.4

Table 3. The accuracy and precision at various concentrations in urine and plasma samples

Compound	Conc. (ng/mL)	Mean conc. (n = 12)		Accuracy (%, n = 12)		RSD (%, n = 12)	
		Plasma	Urine	Plasma	Urine	Plasma	Urine
Acebutolol	15	15	15	104	103	11.5	2.8
	30	29	31	99	104	11	1.4
	60	59	64	98	107	10	1.4
Metoprolol	15	15	15	100	99	9.9	6.9
	30	20	24	99	91	9.5	6.4
	60	56	57	94	93	12	9.9

the international criteria. MEPS is a new sample preparation method suitable for the fully automated determination of analytes in complex matrices, in addition it requires only small sample volumes.

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