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Screening for library-assisted identification and fully validated quantification of 22 beta-blockers in blood plasma by liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization

Hans H. Maurer*, Oliver Tenberken, Carsten Kratzsch, Armin A. Weber, Frank T. Peters

Department of Experimental and Clinical Toxicology, Institute of Experimental and Clinical Pharmacology and Toxicology, University of Saarland, D-66421 Homburg (Saar), Germany

Dedicated to Prof. Dr. Karl Pfleger, Homburg/Saar, at the occasion of his 80th birthday.

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Abstract

A liquid chromatographic–mass spectrometric assay with atmospheric pressure chemical ionization (LC–APCI–MS) is presented for screening for, library-assisted identification (both in scan mode) and quantification (selected-ion mode) of the beta-blockers acebutolol, diacetolol, alprenolol, atenolol, betaxolol, bisoprolol, bupranolol, carazolol, carteolol, carvedilol, celiprolol, esmolol, labetalol, metoprolol, nadolol, nebivolol, oxprenolol, penbutolol, propranolol, sotalol, talinolol and timolol in blood plasma after mixed-mode (HCX) solid-phase extraction (SPE) and separation by reverse-phase liquid chromatography with gradient elution. The validation data were within the required limits. The assay was successfully applied to authentic plasma samples allowing confirmation of diagnosis of overdose situations as well as monitoring of patients' compliance.

Keywords: β-Blockers; Validation; Plasma

1. Introduction

β-Adrenoceptor antagonists briefly called beta-blockers are drugs mainly used for treatment of hypertension, angina pectoris, and cardiac dysrhythmias as well as in the follow-up treatment of myocardial infarctions. They can also be used for treatment of glaucoma, thyreotoxicosis (as an adjunct), anxiety states and benign essential tremor. Serious side-effects of beta-blockers include bradycardia, hypotension, aggravation of cardiac failure, bronchoconstriction, hypoglycemia, and fatigue. Overdose of beta-blockers may lead to life-threatening situations [1–3]. The International Olympic Committee prohibits the use of these drugs in several sports because they reduce heart rate and tremor and improve performance in sports that are not physiologically challenging but require accuracy, e.g. shooting [4,5].

Although there is no strong correlation between plasma concentrations of beta-blockers and their pharmacological and toxic effects [6], suitable analytical procedures are necessary for toxicological screening, identification and quantification in clinical toxicology (CT) and forensic toxicology (FT). Determination of plasma levels allows to check for non-compliance concerning beta-blocker medication in patients with persistent hypertonia, to confirm the diagnosis of a beta-blocker poisoning, to assess the prognosis of such a poisoning, and to monitor the efficiency of detoxification. In addition, therapeutic drug monitoring (TDM) of plasma concentrations of sotalol, which is not only a beta-blocker but mainly an increasingly popular class III antiarrhythmic drug, may be of value, especially in difficult situations like severe

* Corresponding author. Tel.: +49 6841 16 26050; fax: +49 6841 16 26051.
E-mail address: hans.maurer@uniklinik-saarland.de (H.H. Maurer).

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renal dysfunction [6]. In doping control, urinalysis is still mandatory for which mainly GC–MS or LC–MS procedures have been published [5,7,8].

In CT and FT, it is mostly unknown which drug has been taken so that the drugs have to be screened for and identified before quantification in plasma can be performed. For this reason, multi-analyte assays are especially suitable for such purposes. Although screening can also be performed in urine using, e.g. the authors' systematic toxicological screening procedure [9–12] or other particular urinalysis procedures [5,7,8,13–19], it is comfortable to use the same blood sample and extract for both, screening and identification as well as for quantification. This is especially important when no urine sample is available, which is often the case in FT.

So far, only few papers have dealt with screening and/or quantification of several beta-blockers in blood plasma or serum. Kataoka et al. used LC–MS for determination of nine beta-blockers in serum [13], Black et al. GC–MS for nine beta-blockers in post mortem whole blood and in urine [14], Siren et al. ion-pair chromatography and GC–MS for six beta-blockers in serum [20], and Witek and Przyborowski thin-layer chromatography for six beta-blockers in plasma [21]. However, none of these procedures allowed comprehensive screening for, reliable identification and validated quantification of most of the marketed beta-blockers. Therefore, the aim of the presented study was to develop and validate an LC–APCI–MS procedure which fulfills these demands and to check its applicability in CT and FT.

2. Experimental

2.1. Chemicals and reagents

The reference substances of the studied analytes were kindly supplied by the following manufacturers: talinolol by Arzneimittelwerk Dresden (Dresden, Germany), atenolol and metoprolol by AstraZeneca (Wedel, Germany), penbutolol by Aventis Pharma (Frankfurt, Germany), esmolol by Baxter (Unterschleißheim, Germany), acebutolol and diacetolol by Bayer Vital (Leverkusen, Germany), nebivolol by Berlin-Chemie (Berlin, Germany), carvedilol by Boehringer Mannheim/Roche (Mannheim, Germany), sotalol and nadolol by Bristol-Myers Squibb (München, Germany), carteolol by Madaus (Köln, Germany), labetalol by GlaxoSmithKline (Durham, England), bisoprolol by Hexal (Holzkirchen, Germany), atenolol and propranolol by ICI-Pharma (Plankstadt, Germany), carazolol by Klinge (München, Germany), oxprenolol and pindolol by Novartis Pharma (Nürnberg, Germany), celiprolol by Rorer (Bielefeld, Germany), bupranolol by Sanol (Monheim, Germany), betaxolol by Sanofi-Synthelabo (Berlin, Germany), mepindolol by Schering (Berlin, Germany), timolol by Sharp & Dohme (München, Germany). A methanolic solution (1 mg/ml) of trimipramine-d_3 (internal standard, IS) was obtained from Promochem (Wesel, Germany). Isolute Confirm HCX cartridges (mixed-mode sorbent, C8 and cation exchanger; 130 mg, 3 ml) were obtained from Separtis (Grenzach-Wyhlen, Germany). Ammonium formate (analytical grade) was obtained from Fluka (Neu-Ulm, Germany). Acetonitrile (HPLC grade) and all other chemicals (analytical grade) were obtained from Merck (Darmstadt, Germany).

2.2. Biosamples

Pooled human blank plasma samples were used for development and validation of the procedure and were obtained from a local blood bank. Authentic blood samples were submitted to the authors' laboratory by various hospitals for toxicological analysis.

2.3. Extraction procedure

Plasma (0.5 ml) was diluted with 2 ml of purified water. After the addition of 0.05 ml of IS solution (0.01 mg/ml trimipramine-d_3 in methanol) the samples were mixed (15 s) on a rotary shaker and loaded on solid-phase extraction (SPE) cartridges previously conditioned with 1 ml of methanol and 1 ml of purified water. After extraction, the cartridges were washed with 1 ml of purified water, 1 ml of 0.01 M hydrochloric acid, and 2 ml of methanol. Vacuum was applied until the cartridges were dry and the analytes were eluted with 1.5 ml of methanol–aqueous ammonia (98:2, v/v) into autosampler vials. The eluate was evaporated to dryness under a gentle stream of nitrogen at 56 °C. The residue was dissolved in 50 μl of methanol and 5 μl of this solution were injected into the LC–MS system.

2.4. LC–MS procedure

The studied analytes were separated and quantified in plasma using an Agilent Technologies (AT, Waldbronn, Germany) AT 1100 atmospheric pressure chemical ionization (APCI) electrospray LC–MSD, SL version, including an AT 1100 Series HPLC system which consisted of a degasser, a binary pump and an autosampler.

Gradient elution was performed on a Merck LiChroCART column (125 mm × 2 mm internal diameter) with Superspher 60 RP Select B as stationary phase and a LiChroCART 10-2 Superspher 60 RP Select B guard column. The mobile phase consisted of 5 mM aqueous ammonium formate adjusted to pH 3 with formic acid (eluent A) and acetonitrile (eluent B). Before use, the mobile phases were degassed for 30 min in an ultrasonic bath. During use, the mobile phase was degassed by the integrated AT 1100 series degasser. Until the beginning of the analysis, the HPLC system was flushed with an 80:20 mixture of the two eluents. The gradient and the flow rate were programmed as follows: 0–2.20 min 20% B (flow: 0.4 ml/min), 2.21–5.50 min 40% B (flow: 0.4 ml/min), 5.51–8.00 min 90% B (flow: 0.7 ml/min), 8.01–9.50 min 20% B (flow:

Table 1
Analyte groups for quantification process with target ions (*m*/z), gain values and fragmentor voltages

	Gain value	Fragmentor voltage (V)	Time windows	Analyte	Target ions (*m*/z)
Group 1			Window 1.1 (0–2.40 min)	Carteolol	293
			Window 1.2 (2.41–5.50 min)	Timolol	317
	8.0	100	Window 1.3 (5.51–7.00 min)	Bisoprolol	326
				Carazolol	299
				Propranolol	260
				Betaxolol	308
				Alprenolol	250
			Window 1.4 (7.01–8.50 min)	Propranolol	260
				Betaxolol	308
				Alprenolol	250
				Nebivolol	406
Group 2			Window 2.1 (0–2.50 min)	Atenolol	267
			Window 2.2 (2.51–5.20 min)	Metoprolol	268
	4.0	100	Window 2.3 (5.21–8.50 min)	Metoprolol	268
				Celiprolol	380
				Esmolol	296
				Oxprenolol	266
				Labetalol	329
				Talinolol	364
				Bupranolol	272
				Carvedilol	407
				Penbutolol	292
				Trimipramine-d_3	298
Group 3	1.0	100	Window 3.1 (0–2.60 min)	Sotalol	273
				Diacetolol	309
			Window 3.2 (2.61–5.00 min)	Acebutolol	337
Group 4	8.0	200	Window 4.1 (0.00–3.00 min)	Nadolol	254

0.65 ml/min) 9.51–10.00 min 20% B (flow: 0.4 ml/min). Subsequently, re-equilibration of the HPLC column had been achieved and the autosampler could begin with the next injection.

For screening and identification purposes, the following APCI inlet conditions were selected: drying gas, nitrogen (7 L/min, 300 °C) and nebulizer gas, nitrogen (172.5 kPa); capillary voltage, 4000 V; vaporizer temperature, 400 °C; corona current, 5.0 μA; positive scan mode with a scan range from *m*/*z* 50–550, fragmentor voltage 100 and 200 V.

For quantification, the following APCI inlet conditions were applied: drying gas, nitrogen (7 L/min, 300 °C) and nebulizer gas, nitrogen (172.5 kPa); capillary voltage, 4000 V; vaporizer temperature, 400 °C; corona current, 5.0 μA; SIM mode for quantification, fragmentor voltage 100 or 200 V.

The HPLC effluent entered the APCI chamber only in the time window between 0 and 8.2 min. Tuning of the MS was performed with the help of the autotune feature of the LC–MS ChemStation software (rev. A.08.03) using the APCI acetonitrile solution tuning mix supplied with the apparatus.

The presence of the analytes was screened for in the full scan mode by mass chromatography with the ions (*m*/*z*, in order of appearance in the chromatogram) 267, 273, 309, 293, 317, 337, 268, 380, 296, 266, 326, 329, 364, 299, 260, 308, 250, 272, 407, 406, 292, 298 (IS) in the 100 V trace and *m*/*z* 254 in the 200 V trace of the same run. Positive peaks in the recorded traces were identified by library search comparing the underlying APCI mass spectra with the reference spectra of the authors' LC–MS library of drugs, poisons, pesticides and their metabolites created for the NIST98 search algorithm [22].

For quantification, SIM mode at fragmentor voltages 100 and 200 V with different gain values was used. The analytes were divided into four different groups according to their therapeutic concentration ranges and each group was assigned to one of four separately recorded traces with specific gain values as given in Table 1. Within the four groups, the target ions of the analytes were monitored in different time windows which are also given in Table 1.

Quantification was carried out by comparing the peak area ratios (analyte versus IS) obtained from the samples with weighted least squares ($1/x^2$) calibration curves in which the peak area ratios (analytes versus IS) of the

calibration standards had been plotted versus their concentrations.

2.5. Method validation

2.5.1. Preparation of stock solutions, calibration standards and control samples

Methanolic stock solutions of each analyte were prepared in duplicate at a concentration of 1 mg/ml by separate weighings. Working solutions of each analyte were prepared by dilution from each stock solution at the following concentrations: 0.001, 0.01 and 0.1 mg/ml. The calibration standards were prepared using pooled blank plasma and spiking solutions prepared from the stock solutions as mixtures of the analytes in methanol at concentrations 10 times higher than those of the corresponding calibrators. The quality control (QC) samples (concentrations see Table 2) were prepared using pooled blank plasma and independently prepared mixtures of the 22 analytes at concentrations ten times higher than the concentrations of the corresponding QC samples. All solutions were stored at 4 °C.

2.5.2. Selectivity

Ten blank plasma samples from different sources were analyzed for peaks interfering with the detection of the analytes or the IS. In addition, to check for possible interferences from other common drugs and/or their metabolites, plasma samples routinely submitted to the authors' laboratory for drug monitoring or toxicological analysis, which contained psychotropic, analgesic and/or cardiovascular drugs but none of the analytes, were analyzed by the described procedure.

2.5.3. Linearity of calibration

Aliquots of blank plasma (0.5 ml) were spiked with 0.05 ml of the corresponding analytical standard solutions and 0.05 ml of IS solution to obtain calibrators at six concentration levels equally distributed over the linearity ranges given in Table 2. Replicates (n = 6) at each concentration level were analyzed as described above. Daily calibration curves using the same concentrations (single measurements per level) were prepared with each batch of validation and authentic samples.

2.5.4. Accuracy and precision

QC samples (LOW, MEDIUM, HIGH, concentrations see Table 2) were analyzed according to the procedure described above in duplicate on each of eight days. The concentrations of the analytes in the QC samples were calculated via the daily calibration curves. Accuracy was calculated for each analyte in terms of bias as the percent deviation of the mean of all calculated concentration values at a specific level from the corresponding nominal concentration. The data for within-day (repeatability) and total precision (combined within- and between-day effects) of the method were calculated as relative standard deviations (R.S.D.) using analysis of variance according to [23].

2.5.5. Bench top stability/processed sample stability

For estimation of stability of processed samples under the conditions of LC–MS analysis, LOW and HIGH QC samples (n = 5, each) were extracted as described above. The resulting extracts at each concentration level were pooled. Aliquots of these pooled extracts at each concentration level were transferred to autosampler vials and injected under the conditions of a routine analytical run at time intervals of 2 h. Stability of the extracted analytes was tested by regression analysis plotting absolute peak areas of each analyte at each concentration versus injection time. Instability of processed samples would be indicated by a negative slope significantly different from zero ($P \leq 0.05$).

2.5.6. Freeze/thaw stability

For evaluation of freeze/thaw stability, QC samples (LOW and HIGH) were analyzed prior to (control samples, n = 6) and after three freeze/thaw cycles (stability samples, n = 6). For each freeze/thaw cycle, the samples were frozen at −20 °C for 21 h, thawed and kept at ambient temperature for 3 h. The experiments were carried out together with the accuracy and precision experiments and the concentrations of the control and stability samples were calculated via daily calibration curves. Stability was tested against an acceptance interval of 90–110% for the ratio of the means (stability samples versus control samples) and an acceptance interval of 80–120% from the control samples mean for the 90% confidence interval of stability samples.

2.5.7. Long-term stability

The experimental design and procedure for evaluation of long-term stability were similar to those used for freeze/thaw stability. Analyte stability for long-term storage was tested by analyzing spiked samples at two concentrations of the analytes (LOW and HIGH) before (control samples, n = 6) and after storage for one month at −20 °C (stability samples, n = 6).

2.5.8. Limits

For determination of the limit of detection (LOD, signal to noise ratio 3:1) in the full scan mode, spiked samples were assayed. To establish the limit of quantification (LOQ) in the SIM mode, the fulfillment of the requirements of LOQ (signal to noise ratio 10:1) was checked for the lowest calibrators. The noise data from the assay of blank matrices was taken from the selectivity experiments.

2.5.9. Recoveries

Recoveries were tested at low and high concentration levels (n = 5 each, concentrations, see Table 2). Methanolic solutions (0.05 ml) containing the 22 analytes as a mixture at concentrations resulting in the low and high plasma levels, respectively, were spiked to 0.5 ml of blank plasma. The samples were extracted according to the procedure described above. The residue was dissolved in 0.05 ml of IS solution (0.01 mg/ml trimipramine-d_3 in methanol). As controls

Table 2
Therapeutic concentration ranges, LODs, linearity ranges, coefficients of determination (R^2), nominal concentrations, accuracy (in terms of bias), precision, and recovery data of the LC–MS assay for beta-blockers

Drug	Therapeutic range (mg/l)	LOD (mg/l)	Linearity range (mg/l)	R^2	Nominal concentration (LOW, MEDIUM, HIGH) (mg/l)	Bias (%)[a]	Precision [23], R.S.D. (%)		Recovery mean ± S.D. (%)
							Within-day (repeatability)	Total	
Acebutolol	0.2–2.0	0.01	0.1–2.5	0.993	0.20	+3.1	8.4	8.6	78.2 ± 4.0
					1.00	+3.2	7.2	11.9	
					2.00	+7.2	4.2	8.2	74.0 ± 5.3
Diacetolol	0.65–4.5	<0.01	0.325–5.625	0.990	0.65	−1.2	4.5	7.5	75.6 ± 3.7
					2.25	−0.3	2.7	7.7	
					4.50	+6.6	3.3	5.5	74.3 ± 5.3
Alprenolol	0.025–0.14	0.01	0.0125–0.175	0.989	0.025	−2.3	6.0	10.3	48.8 ± 5.2
					0.070	+3.0	3.4	6.7	
					0.140	+0.5	11.6	10.6	46.9 ± 2.8
Atenolol	0.1–1.0	0.01	0.05–1.25	0.992	0.10	+4.1	8.4	8.6	89.2 ± 4.8
					0.50	+7.8	6.7	6.8	
					1.00	+3.6	8.5	8.8	89.3 ± 5.2
Betaxolol	0.005–0.05	0.0025	0.0025–0.0625	0.996	0.0050	−1.2	2.2	8.4	71.2 ± 4.8
					0.0250	+3.0	8.6	8.9	
					0.050	−1.6	4.8	7.3	63.1 ± 5.5
Bisoprolol	0.01–0.1	<0.005	0.005–0.125	0.993	0.010	−0.6	7.3	10.8	68.6 ± 5.8
					0.050	+1.6	4.2	6.3	
					0.10	+4.4	6.7	9.8	65.9 ± 3.3
Bupranolol	0.1–1.6	0.01	0.1–2.0	0.991	0.10	+0.3	3.9	10.0	59.2 ± 5.4
					0.80	+2.9	3.2	4.5	
					1.60	+4.7	2.2	9.4	69.8 ± 3.9
Carazolol	0.007–0.015	0.0035	0.0035–0.01875	0.992	0.0070	+2.9	4.6	6.4	69.8 ± 6.2
					0.075	−0.7	4.4	7.8	
					0.150	+4.2	8.6	12.6	66.4 ± 4.9
Carteolol	0.01–0.1	<0.005	0.005–0.125	0.996	0.010	−5.4	5.7	7.9	78.0 ± 7.0
					0.050	+1.1	4.8	7.3	
					0.10	+6.3	4.5	9.6	80.0 ± 9.3
Carvedilol	0.05–0.5	<0.01	0.025–0.625	0.990	0.050	−3.8	5.2	10.6	61.3 ± 5.9
					0.250	−2.6	6.4	7.8	
					0.50	−1.6	3.8	8.4	69.5 ± 7.2
Celiprolol	0.05–0.5	0.001	0.025–0.625	0.994	0.050	+0.3	3.6	8.8	87.2 ± 5.3
					0.250	−2.3	3.2	7.1	
					0.50	+5.4	3.9	8.0	94.5 ± 3.0
Esmolol	0.2–1.2	<0.01	0.1–1.5	0.993	0.20	−0.3	3.2	10.3	70.5 ± 9.7
					0.70	+3.1	6.8	13.0	
					1.20	+5.4	6.4	10.6	78.2 ± 5.4
Labetalol	0.08–0.65	0.01	0.04–0.8125	0.993	0.080	+3.3	5.0	9.0	91.5 ± 5.7
					0.30	−2.2	5.4	9.9	
					0.650	+5.8	2.5	6.0	91.8 ± 6.3
Metoprolol	0.035–0.5	0.01	0.0175–0.625	0.995	0.035	−1.8	4.1	10.1	71.2 ± 5.8
					0.250	−2.6	3.2	8.0	
					0.50	+4.2	2.5	7.1	67.1 ± 3.2
Nadolol	0.01–0.25	0.025	0.005–0.3125	0.994	0.010	−2.6	6.3	9.8	74.5 ± 8.5
					0.150	+5.1	10.8	12.6	
					0.250	+1.7	7.7	9.9	64.4 ± 6.2
Nebivolol	0.005–0.03	0.0025	0.0025–0.0375	0.990	0.0050	+4.5	7.8	10.2	60.1 ± 5.4
					0.0150	+2.3	7.4	9.8	
					0.030	+3.8	5.0	10.3	51.4 ± 4.9
Oxprenolol	0.05–1.0	0.01	0.02–1.25	0.992	0.050	−2.9	10.0	13.6	76.5 ± 3.6
					0.50	−3.2	7.5	10.1	
					1.00	+4.2	5.0	7.9	81.9 ± 3.0

Table 2 (*Continued*)

Drug	Therapeutic range (mg/l)	LOD (mg/l)	Linearity range (mg/l)	R^2	Nominal concentration (LOW, MEDIUM, HIGH) (mg/l)	Bias (%)[a]	Precision [23], R.S.D. (%)		Recovery mean ± S.D. (%)
							Within-day (repeatability)	Total	
Penbutolol	0.01–1.0	0.005	0.005–1.25	0.993	0.010	+3.3	9.4	10.5	44.5 ± 2.6
					0.50	−7.1	6.6	8.0	
					1.00	−0.4	4.8	12.0	52.3 ± 3.9
Propranolol	0.02–0.3	0.01	0.01–0.375	0.995	0.020	+2.0	3.5	6.3	75.5 ± 1.2
					0.150	+1.4	7.7	9.6	
					0.30	+6.3	6.0	8.8	83.7 ± 2.1
Sotalol	0.5–3.0	<0.1	0.25–3.75	0.992	0.50	−2.6	3.4	11.7	76.2 ± 4.9
					1.50	+3.3	2.3	9.0	
					3.00	+2.1	5.4	9.1	68.1 ± 3.6
Talinolol	0.1–0.5	0.01	0.05–0.625	0.996	0.10	+5.8	7.5	13.0	94.8 ± 4.2
					0.250	+5.3	3.9	9.9	
					0.50	+6.3	5.1	7.9	90.6 ± 1.9
Timolol	0.005–0.05	0.001	0.0025–0.0625	0.994	0.0050	−1.9	3.9	4.6	71.2 ± 3.4
					0.0250	−1.7	9.9	9.9	
					0.050	+2.7	5.6	8.5	63.4 ± 3.9

[a] Bias = ((mean calculated concentration − nominal concentration)/nominal concentration) × 100.

($n = 5$) corresponding to 100% recovery, a mixture of 0.05 ml of a mixture the above mentioned solutions of the 22 analytes in methanol at the low and high concentration levels, respectively, and 0.05 ml of IS solution was carefully evaporated to dryness. The residues were then dissolved in 0.05 ml of methanol. Recoveries were calculated by comparing the peak area ratios (analytes versus IS) of spiked plasma samples and controls.

2.5.10. Proof of applicability

Various plasma samples from CT cases were assayed with the described method.

3. Results and discussion

3.1. Extraction procedure

In the early development stages of the presented assay, it was intended to isolate the 22 analytes by the authors' standard plasma liquid–liquid extraction procedure [24,25] using trimipramine-d_3 as routine IS. This extraction procedure has proved to be very versatile for GC–MS and LC–MS analysis in clinical toxicology and drug monitoring in the authors' laboratory [25–28]. Unfortunately, with this broadly applicable standard plasma liquid-liquid extraction procedure only unsatisfactory recoveries were obtained for sotalol. Because of the growing importance of this drug as an antiarrhythmic agent and the possible value of TDM of this drug [6], another extraction procedure had to be developed. It was based on a previously published mixed-mode SPE procedure, developed for the determination of drugs of abuse in plasma [29,30] and also effectively applied to the determination of neuroleptics [31]. However, for extraction of beta-blockers the volume of elution solvent had to be increased from 1.0 to 1.5 ml in order to achieve reproducible recoveries, especially for the relatively high-dosed beta-blockers. As given in Table 2, the mean recovery values ranged from 46.9 to 94.8% with standard deviations well below 10% which shows the suitability of the described procedure for extraction of beta-blockers from plasma samples. Originally, mepindolol and pindolol had also been included in procedure, but only very low and erratic recoveries had been observed and other validation data were also unacceptable for these two drugs. The most probable explanation for this phenomenon is degradation of these two analytes during SPE because both are known to be sensitive to diluted mineral acids [9,32]. Nevertheless, preliminary experiments had shown that, if necessary, pindolol and mepindolol can be isolated from plasma using the above mentioned standard liquid–liquid extraction procedure and that the resulting extract can be analyzed using the described LC–MS system.

3.2. LC–MS screening, identification and quantification

The presence of the beta-blockers was successfully screened for in the full scan mode by mass chromatography with selected ions followed by library search of the underlying APCI mass spectra with the authors' LC–MS reference library [22,25,28,31]. The authors preferred the APCI mode over the electrospray ionization (ESI) mode, because APCI is much less susceptible to ion suppression, which might lead to false negative results [12,33–36]. Moreover, the used type of LC–MS apparatus showed higher sensitivity for the studied analytes when operated in the APCI mode. In Fig. 1, APCI electrospray mass spectra recorded at 100 and 200 V fragmentor voltages and the structures of the studied analytes and the IS trimipramine-d_3 are shown. As can be seen from the spectra, the different beta-blockers resulted in spectra of various significance at different fragmentor voltages. Therefore,

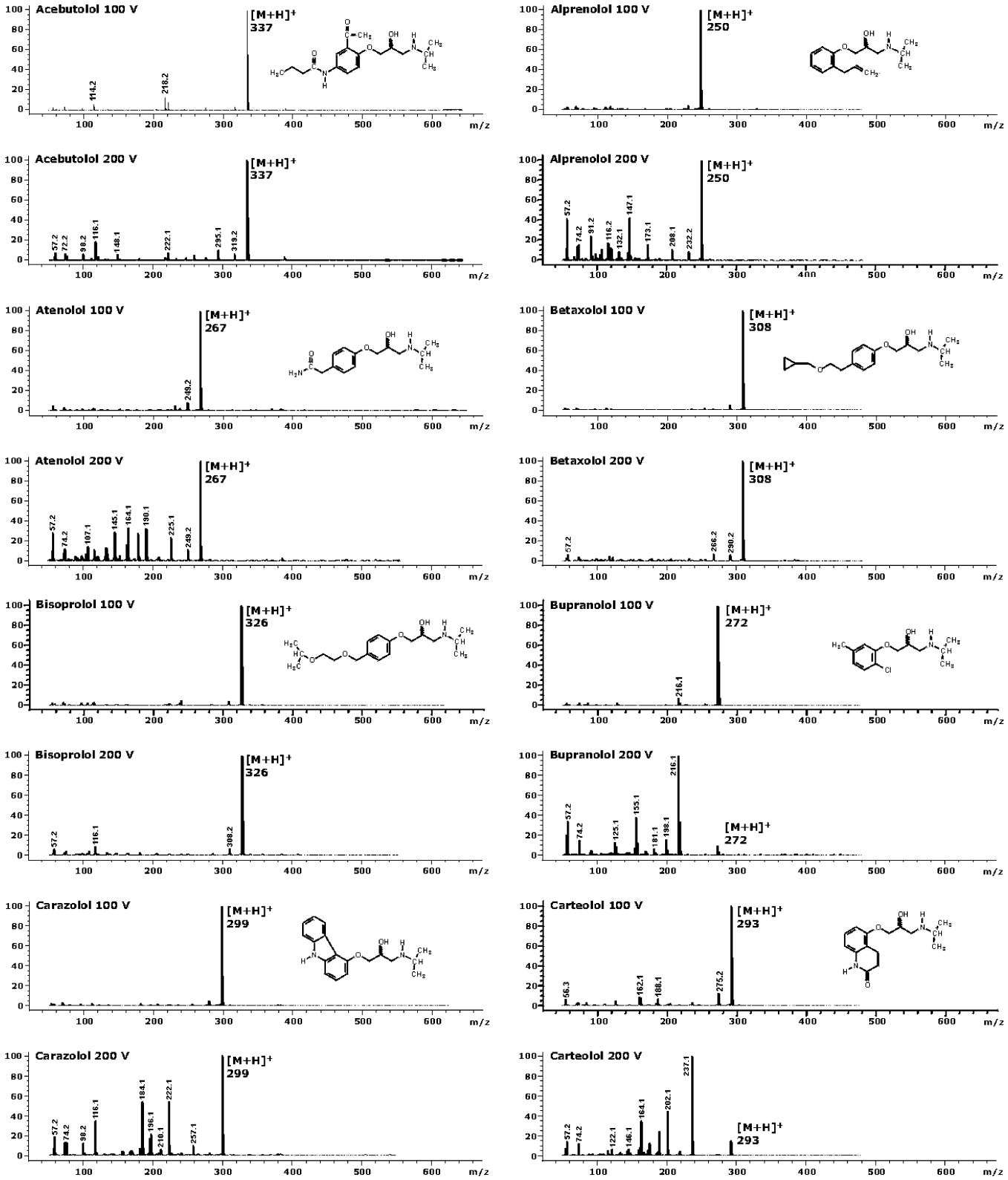

Fig. 1. APCI electrospray mass spectra recorded at 100 and 200 V fragmentor voltages and structures of the studied beta-blockers as well as of the internal standard trimipramine-d_3. The abscissa represents the *m*/*z* value (u) and the ordinate the relative abundances of the fragment ions (%).

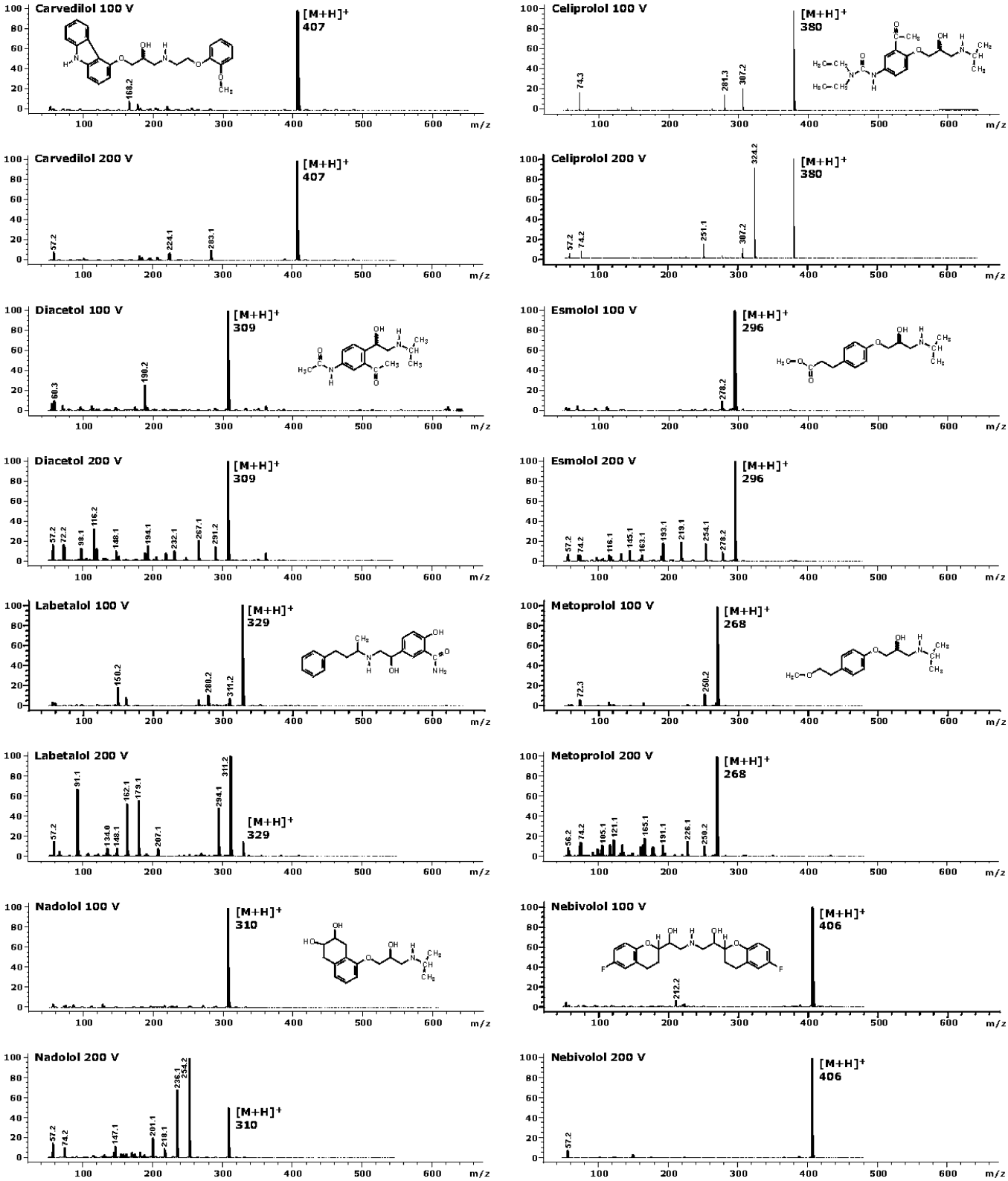

Fig. 1. (*Continued*)

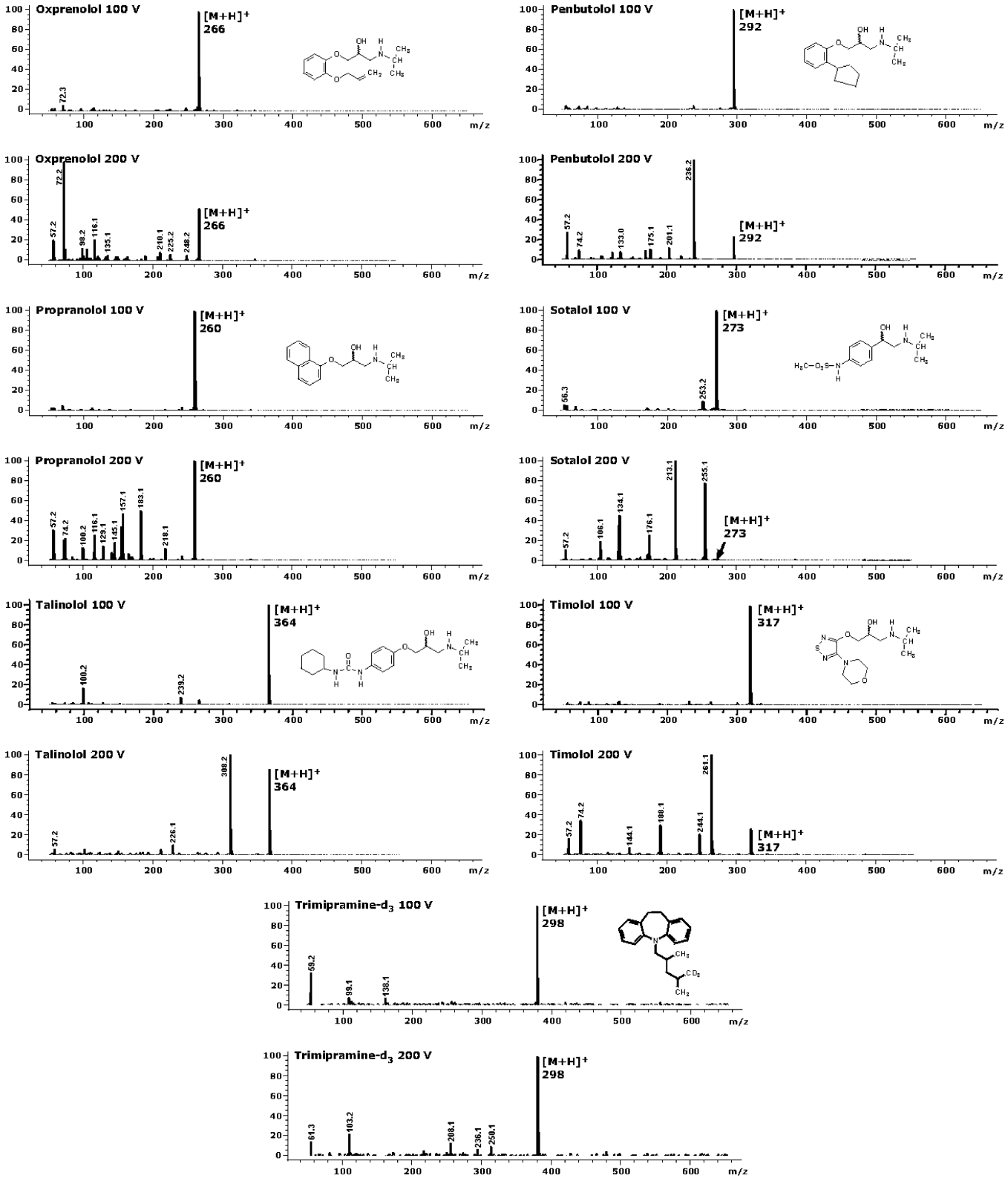

Fig. 1. (*Continued*).

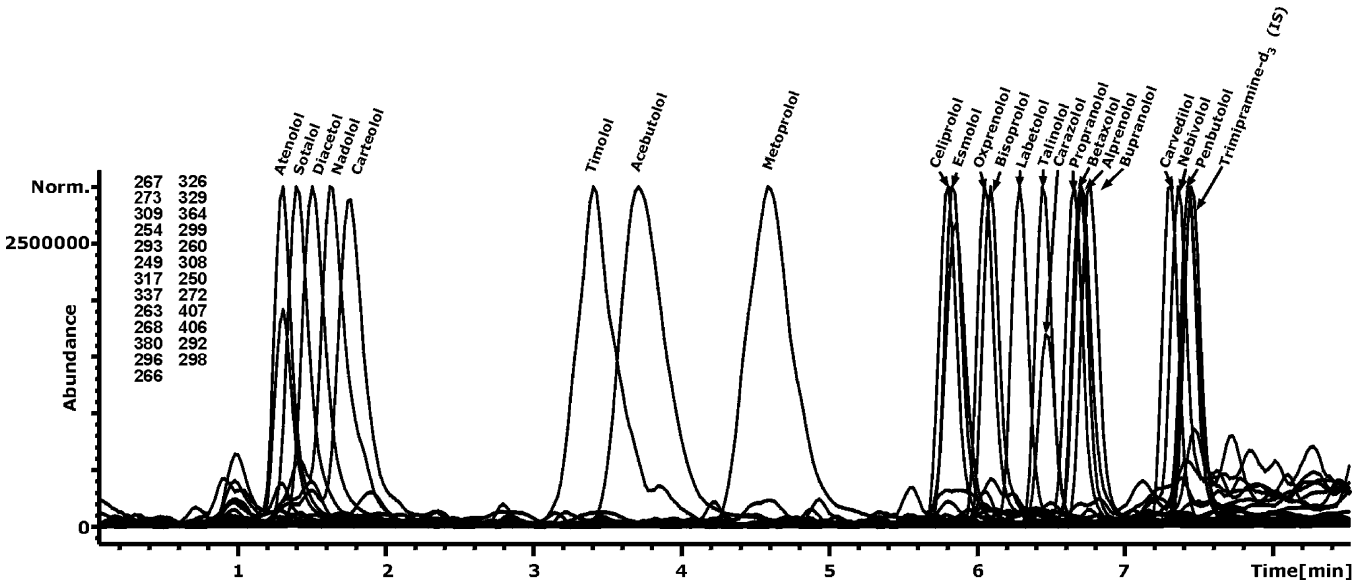

Fig. 2. Smoothed, normalized and merged mass chromatograms (scan mode) of a MEDIUM QC sample extract.

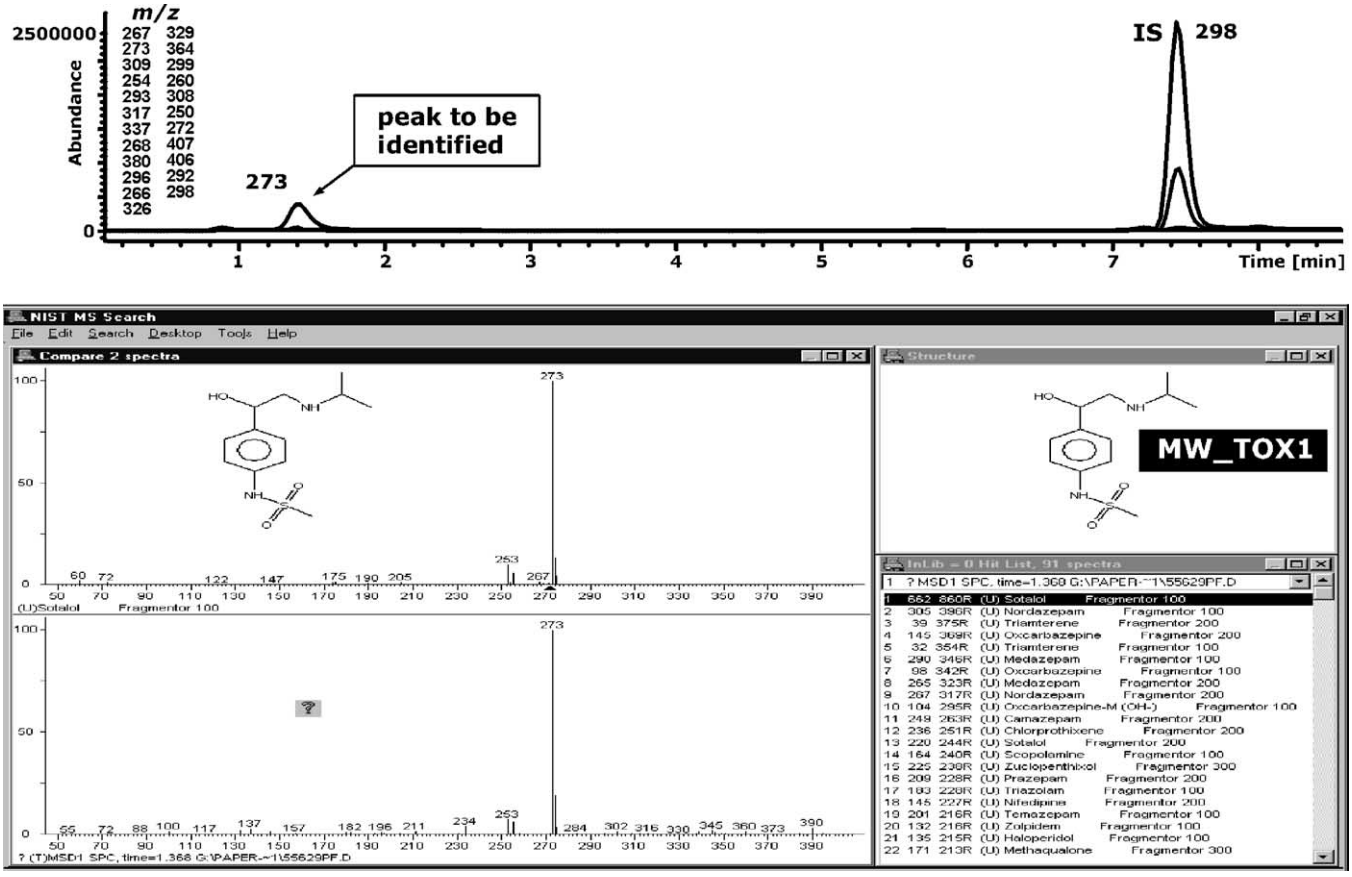

Fig. 3. Smoothed and merged mass chromatograms (scan mode) of an authentic plasma extract indicating beta-blockers (upper part). The mass spectrum underlying the marked peak (lower spectrum), the reference spectrum (upper spectrum), the structure and the hit list found by computer library search (lower part).

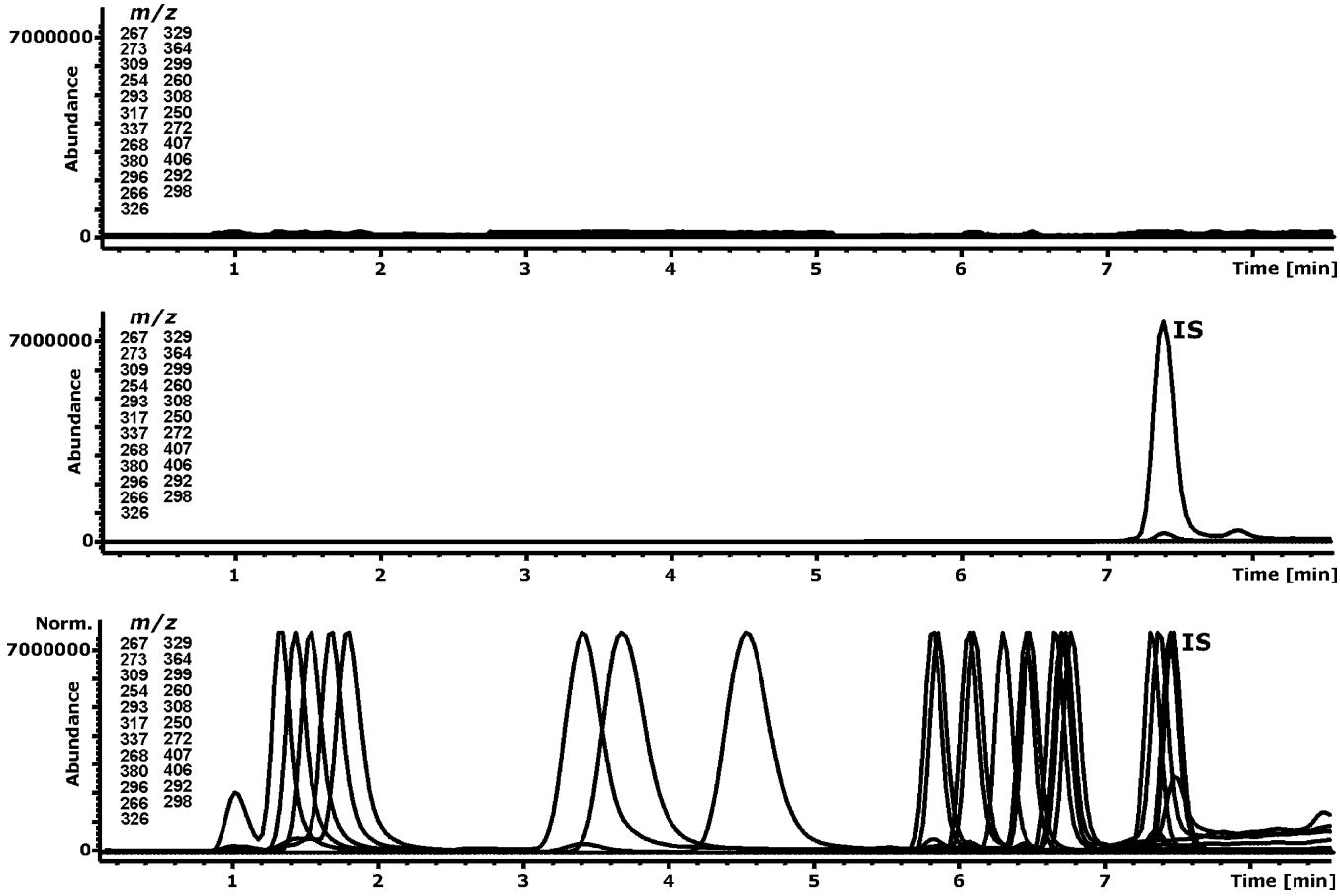

Fig. 4. Smoothed and merged mass fragmentograms (SIM mode) of extracts of a blank plasma sample (top), of a blank plasma sample spiked with 1.0 mg/l trimipramine-d_3 (middle), and of a MEDIUM QC sample (bottom, same extract as used in Fig. 2).

the full scan spectra were recorded at 100 and 200 V with a cycle time of 1.52 s. It should be kept in mind that the same fragmentor voltage selected in different apparatus may result in different abundances of the formed fragments [37–39]. Therefore, each user has to select that fragmentor voltage of his specific apparatus which produces mass spectra similar to those shown in Fig. 1. In the authors' experience with three different LC–MSD apparatus, this allowed the successful use of the presented screening procedure.

In Fig. 2, smoothed, normalized and merged mass chromatograms (scan mode) of the given ions of a MEDIUM QC sample extract are shown. All drugs were sufficiently separated within only 7.5 min regarding the high selectivity of MS detection.

For illustration of the screening and identification procedure, smoothed and merged mass chromatograms of the ions given above of an authentic plasma extract indicating beta-blockers are shown in the upper part of Fig. 3. The mass spectrum underlying the marked peak (lower spectrum), the reference spectrum (upper spectrum), the structure and the hit list found by computer library search [22] are shown in the lower part of Fig. 3.

Quantification of the studied analytes was performed in the SIM mode to improve sensitivity and precision. The protonated molecular ion was chosen as target ion for all analytes with exception of nadolol. The protonated molecular ion of this drug is *m/z* 310, which is identical with the ^{13}C isotope peak of the protonated molecular of diacetolol (*m/z* 309). Because nadolol and diacetolol were chromatographically not fully separated, the more selective fragment ion *m/z* 254 at fragmentor voltage 200 V was chosen as target ion for nadolol to avoid interference with diacetolol. The compounds were divided up into four groups which were monitored in four different traces, each with a different gain value (Table 1) in order to account for the large differences of the therapeutic concentration ranges of the various beta-blockers. Furthermore, the target ions of compounds eluting close to the end of a time window were monitored also in the following time windows (*m/z* 260, 308, 250 in time windows 1.3 and 1.4; *m/z* 268 in time windows 2.2 and 2.3). This allowed quantification of the respective analytes even if the separation line of the time windows was not situated exactly between the peaks. This procedure allowed a reliable, selective and sensitive quantification of the analytes. The use of only the target ion (without qualifiers) for quantification was acceptable, because the drugs had already been identified in the full scan mode [25,28,31].

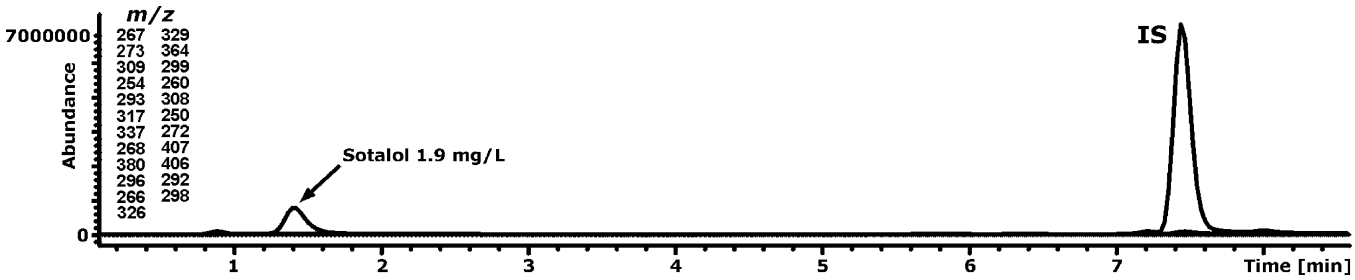

Fig. 5. Smoothed and merged mass fragmentograms (SIM mode) of an authentic plasma extract (same extract as used in Fig. 3) indicating 1.9 mg/l of sotalol.

In Fig. 4, smoothed and merged mass fragmentograms (SIM mode) are shown of extracts of a blank plasma sample extract (top), of a blank plasma sample spiked with the IS (1.0 mg/l trimipramine-d_3, middle), and of a MEDIUM QC sample (bottom). The extract of the spiked plasma was the same as used in Fig. 2.

3.3. Assay validation

The described procedure was validated according to internationally accepted recommendations [23,40–43]. The validation data are summarized in Table 2.

The assay was found to be selective for all tested compounds. As exemplified in Fig. 4 (top), no interfering peaks were observed in the extracts of the different blank plasma samples. Interferences with common drugs typically taken in combination were tested and could be excluded due to different retention time and/or mass spectra.

The assay was linear from sub-therapeutic to overdose concentrations of all compounds. Reference plasma concentrations of the studied analytes are included in Table 2. A weighted least squares model was used for calculation of calibration curves to account for unequal variances (heteroscedasticity) across the calibration range. The low and high level recoveries ranged from 46.9 to 94.8% for all studied analytes. Because screening and identification were carried out in the full scan mode, this mode was also used for determination of the LODs. All LODs were lower or at least equal to the corresponding LOQs in the SIM mode. The LOQs corresponded to the lowest concentrations used for the calibration curves with a signal-to-noise ratio of at least 10. The complete validation data concerning LOD, linearity, accuracy, precision and recovery are shown in Table 2. Within-day (repeatability) and total precision (combination of within- and between-day effects) were determined and lay within the required limits of ≤15% R.S.D. (≤20% R.S.D. at LOQ). Accuracy data (in terms of bias) were also determined and all lay within the acceptance interval of ±15% (±20% at the LOQ) of the nominal values [40,41].

In processed samples, the analytes were stable for a period of more than 24 h at room temperature. No instability of analytes in spiked samples was observed over three freeze/thaw cycles or during storage at −20 °C for a one-month period.

The procedure has proven to be applicable in the analysis of authentic plasma samples during routine work. For example, Fig. 5 shows smoothed and merged mass fragmentograms (SIM mode) of such an authentic plasma sample (same extract as used for Fig. 3) indicating 1.9 mg/l of sotalol. As these fragmentograms were recorded in the SIM mode they differ from Fig. 3.

The presented assay is the first fully validated procedure for the simultaneous determination of a large number of beta-blockers in plasma. It has proved to be selective, sensitive, linear, accurate and precise. Furthermore, the *y*-intercepts of the calibration curves were either not significantly different from zero ($P \leq 0.05$) or less than 5% in comparison to the response at high therapeutic concentrations. Therefore, in emergency toxicology, it should be acceptable to confine to a one-point calibration using a HIGH control sample of the various analytes, respectively.

4. Conclusions

The LC–APCI–MS assay presented here allowed fast and reliable screening and identification as well as accurate, precise and sensitive quantification of 21 beta-blocker drugs and one of their relevant metabolites in plasma. It has proven to be appropriate for isolation, separation, screening, identification and validated quantification of the studied analytes in plasma for clinical toxicology and compliance monitoring.

In addition, despite some differences in sample preparation, this assay is part of a general LC–MS procedure for screening and identification (full-scan mode) as well as for quantification (SIM mode) of other toxicologically relevant compounds in plasma. The variety of different substances covers oral antidiabetics [25], benzodiazepines [28], neuroleptics [31] and substances relevant in the diagnosis of brain death [44].

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