ELSEVIER

Journal of Chromatography B, 850 (2007) 147–160

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Simultaneous determination of 6 beta-blockers, 3 calcium-channel antagonists, 4 angiotensin-II antagonists and 1 antiarrhytmic drug in post-mortem whole blood by automated solid phase extraction and liquid chromatography mass spectrometry
Method development and robustness testing by experimental design

Lena Kristoffersen [a,*], Elisabeth Leere Øiestad [a], Mimi Stokke Opdal [a], Mette Krogh [a], Elsa Lundanes [b], Asbjørg Solberg Christophersen [a]

[a] *Norwegian Institute of Public Health, Division of Forensic Toxicology and Drug Abuse, P.O. Box 4404 Nydalen, N-0403 Oslo, Norway*
[b] *Department of Chemistry, University of Oslo, P.O. Box 1033 Blindern, N-0315 Oslo, Norway*

Received 6 June 2006; accepted 14 November 2006
Available online 18 December 2006

Abstract

A method for the simultaneous determination of the beta-blockers atenolol, sotalol, metoprolol, bisoprolol, propranolol and carvedilol, the calcium-channel antagonists diltiazem, amlodipine and verapamil, the angiotensin-II antagonists losartan, irbesartan, valsartan and telmisartan, and the antiarrythmic drug flecainide, in whole blood samples from forensic autopsies was developed. Sample clean-up was achieved by precipitation and solid phase extraction (SPE) with a mixed-mode column. Quantification was performed by reversed phase high performance liquid chromatography with positive electrospray ionization mass spectrometric detection (HPLC-MS). The method has been developed and robustness tested by systematically searching for satisfactory conditions using experimental designs including factorial and response surface designs. With the exception of amlodipine, the concentration limit of quantification (cLOQ) covered low therapeutic concentration levels for all the compounds. Within assay precisions and accuracies (bias) were 3.4–21% RSD and from −24 to 21% for the concentration range 1.00–5.00 μM, respectively. Between assay precisions were 4.4–28% RSD for the concentration range from 0.1 to 5 μM and recoveries varied from 9 to 103%. The method is used for determination of cardiovascular drugs in post-mortem whole blood samples from forensic autopsy cases.
© 2006 Elsevier B.V. All rights reserved.

Keywords: Cardiovascular drugs; Beta-blockers; Calcium-channel antagonists; Angiotensin-II antagonists; Antiarrythmic drug; Whole blood; Experimental design; SPE; HPLC-MS

1. Introduction

Cardiovascular diseases are common causes of morbidity and mortality in western industrialised countries. Drugs used in the treatment of cardiovascular disorders are among the most frequently used drugs worldwide as well as in Norway [1]. A combination of several different types of cardiovascular drugs is often used in treatment [2], making drug treatment complex with respect to drug interaction and assessment of clinical effects.

In a study by Buajordet et al. [3], fatal adverse drug events amongst medical department inpatients were found for the whole array of drugs applied for treating heart diseases. In the USA, cardiovascular drug poisonings ranks among the leading agents involved in pharmaceutical poisoning-related deaths [4]. Case reports of deaths in which the beta-blockers sotalol [5,6], metoprolol [7–10] and propranolol [11–13], the calcium-channel antagonists amlodipine [14–16], diltiazem [17–21] and verapamil [22–27] and the antiarrythmic drug flecainide [28–34] were strongly suspected to be causative agents have been published. Deaths related to a combination of cardiovascular drugs and other drugs or alcohol have also been reported [35–38]. However, little is known about the toxicology of the newer

* Corresponding author.
E-mail address: lena.kristoffersen@fhi.no (L. Kristoffersen).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.11.030

cardiovascular drugs, i.e. angiotensin-II antagonists, especially when these drugs are used in combinations with other drugs.

In Norway, when there is an unexpected or suspicious death, a medico-legal autopsy may be ordered by the police. The Norwegian Institute of Public Health, Division of Forensic Toxicology and Drug Abuse (NIPH, Oslo, Norway) performs toxicological analyses in specimens sampled at autopsy. These analyses may be of great importance when cause of death is established.

Several methods have been published for the determination of beta-blockers [39–42], calcium-channel antagonists [43–49], antiarrythmic drugs [50–52] and angiotensin-II antagonists [53–55] in plasma or serum. Validated methods which allow the determination of a single drug or drugs belonging to the same cardiovascular drug group in whole blood have also been published [23,56,57].

The therapeutic treatment of heart disorders often involves several groups of cardiovascular drugs. Furthermore, a common situation in forensic toxicology is the lack of information concerning which drugs have been ingested prior to death. Simultaneous determination of several cardiovascular drug groups is therefore highly desirable, and a few studies have been published [58–62]. With the exception of Gergov et al. [59] and Yawney et al. [62], the ability of these methods to determine the cardiovascular drugs in whole blood samples was not reported. Hence, an automated and sensitive method for simultaneous determination of these drugs in post-mortem whole blood samples is highly in need.

The aim was to develop a quantitative screening method for determination of cardiovascular drugs in forensic autopsy whole blood samples. The chemical properties of compounds, their therapeutic concentration range, possible side-effects and the Norwegian sales statistics [1] were used as selection criteria for inclusion in the method. Based upon these considerations, 14 commonly used cardiovascular drugs in Norway belonging to four different cardiovascular drug classes were selected. In this paper, we present an automated SPE and gradient HPLC method with single MS detection which simultaneously determines the beta-blockers atenolol, sotalol, metoprolol, bisoprolol, propranolol and carvedilol, the calcium-channel antagonists diltiazem, amlodipine and verapamil, the angiotensin-II antagonists losartan, irbesartan, valsartan and telmisartan, and the antiarrythmic drug flecainide in post-mortem whole blood covering both therapeutic and toxic concentrations (Table 1). Experimental design [63,64] has been used in the method development. The method is used for toxicological examination of blood from forensic autopsies as an aid in establishing the cause of death.

2. Experimental

2.1. Reagents

The reference substances were purchased from the following manufacturers: atenolol, metoprolol, sotalol, propranolol and flecainide from Sigma–Aldrich (St. Louis, MO, USA), losartan from Merck (Darmstadt, Germany), bisoprolol, carvedilol and telmisartan from Sequoia Research Products (Oxford, UK), diltiazem from Tanabe Seiyaku Co. (Osaka, Japan), amlodipine from Penn Bio-organics Inc. (Bellefonte, PA, USA), verapamil from Alltech (Lexington, Kentucky USA), irbesartan from Xiangding Chemical International Company (Nanjing, China) and valsartan from Ciba (Basel, Switzerland). Analytical grade potassium dihydrogen phosphate (KH_2PO_4), formic acid (98%) and hydrochloric acid (37%), extra pure ammonia (NH_3, 32%) and HPLC grade methanol were obtained from Merck. AnalaR® ammonium formate was purchased from BDH Laboratory Supplies (Poole, England) and acetonitrile was obtained from Lab-Scan (Dublin, Ireland). Grade 1 water from a Millipore station (Billerica, MA, USA) was used for all procedures.

2.2. Preparation of solutions

For each compound, two separate stock solutions were prepared to a concentration of 2500 μM in methanol, identified as calibration and control, respectively. Aqueous calibration solutions with the concentrations of 0.3, 0.8, 5.0, 12.5, 25.0, 62.5 and 125.0 μM of each compound were prepared from the stock solutions. Aqueous control solutions were prepared from the second set of stock solutions to the concentrations of 1.0, 10.0 and 50.0 μM each. Diazepam-d5 was used as internal standard, a 155 μM stock solution and a 18.5 μM solution were prepared in acetonitrile and water, respectively. The stock and aqueous solutions were stored at −20 and 4 °C, respectively.

2.3. Samples

Spiked calibration and control samples were prepared by adding 50 μL of aqueous calibration or control solutions to 0.45 mL drug free sodium fluoride whole blood from healthy donors (The Blood Centre at Ullevaal University Hospital, Oslo, Norway). Authentic post-mortem whole blood samples were obtained from forensic autopsies received at NIPH.

2.4. Instrumentation and quantification

Aspec XL robot from Gilson Inc. (Middleton, WI, USA) was used in the SPE procedure. The HPLC-MS system was an Agilent 1100 series system (Agilent Technologies, CA, USA) consisting of an online vacuum degasser, a quaternary pump, well-plate autosampler, a thermostatted column compartment and a LC/MSD SL detector. LC/MSD ChemStation Rev. A. 09.03 (Agilent) was used for instrument control and data collection. Peak heights ratios (compound versus diazepam-d5) were used in the quantification. For quantification weighted ($1/x$) quadratic calibration curves were used in which the peak height ratios of the analyte versus internal standard were plotted versus the analyte concentrations. The weighting factor was normalised to the smallest concentration. The normalisation was done by multiplying the weight ($1/x$) with the lowest calibration concentration level a, giving a weight factor $1/x \times a$. The calibration curve concentration ranges in whole blood were from 0.03 to 12.5 μM for irbesartan and telmisartan, 0.08 to 12.5 μM for amlodipine and losartan, 0.50 to 12.5 μM for valsartan and 0.03 to 6.25 μM for the remaining nine compounds. The target ions used for quantification are listed in Table 1.

Table 1
Structures, drug classification, compound MS group for quantification, target ions (*m/z*), fragmentor voltages (V) and therapeutic concentration range (μM and μg/mL) in plasma or serum and conversion factor (CF)[a]

Structures	Type MS group	$[M+H]^+$ V	Fragment ion V	Therapeutic range and CF[a]
Atenolol	Beta-blocker	267.4	225.3	0.38–3.76 μM
	1	120 V	180 V	CF 3.76
				0.1–1 μg/mL [72]
Sotalol	Beta-blocker	273.3	134.3	1.84–11 μM
	1	100 V	180 V	CF 3.67
				0.5–3 μg/mL [72]
Metoprolol	Beta-blocker	268.4	165.3	0.07–2.24 μM
	1	140 V	190 V	CF 3.74
				0.02–0.6 μg/mL [72]
Bisoprolol	Beta-blocker	326.4	308.4	0.03–0.31 μM
	2	140 V	210 V	CF 3.07
				0.01–0.1 μg/mL [72]
Propranolol	Beta-blocker	260.3	183.3	0.08–3.47 μM
	2	120 V	190 V	CF 3.86
				0.02–0.9 μg/mL [72]
Carvedilol	Beta-blocker	407.4	283.3	0.05–0.37 μM
	2	150 V	210 V	CF 2.46
				0.02–0.15 μg/mL [73]
Diltiazem	Calcium-channel antagonist	415.3[b]	178.2[b]	0.24–0.96 μM
	2	150 V	220 V	CF 2.41
				0.1–0.4 μg/mL [72]
Amlodipine	Calcium-channel antagonist	409.3[b]	238.2[b]	0.014–0.04 μM
	2	110 V	110 V	CF 2.45 0.006–0.018 μg/mL [69]

Table 1 (*Continued*)

Structures	Type MS group	$[M+H]^+$ V	Fragment ion V	Therapeutic range and CF[a]
Verapamil	Calcium-channel antagonist	455.4	165.3	0.11–1.1 μM
	2	190 V	250 V	CF 2.20 0.05–0.5 μg/mL [72]
Losartan	Ang-II-antagonist	423.3	207.3	0.2–3.3 μM
	2	100 V	220 V	CF 2.36 0.08–1.4 μg/mL [69]
Irbesartan	Ang-II-antagonist	429.4	207.3	2.2–12.3 μM
	2	140 V	220 V	CF 2.33 0.9–5.3 μg/mL [69]
Telmisartan	Ang-II-antagonist	515.3	276.3	0.98 μM[c]
	2	230 V	350 V	CF 1.94 0.51 μg/mL [55]
Valsartan	Ang-II-antagonist	436.3	207.3	3.8–13.6 μM
	2	90 V	220 V	CF 2.30 1.6–5.9 μg/mL [69]
Flecainide	Antiarrhythmic	415.3[b]	301.3[b]	0.43–2.1 μM
	2	150 V	260 V	CF 2.41 0.18–0.87 μg/mL [74]
Diazepam-d5	Benzodiazepine	290.3	198.3	CF 3.45
	2	160 V	270 V	

[a] CF = 1000/molecular weight of compound. To convert from μg/mL to μM: μg/mL × CF. To convert from μM to μg/mL: μM/CF.
[b] Quantified using fragment ion.
[c] Cmax after a single oral dose of 80 mg (Micardis).

2.5. Chromatographic conditions

HPLC separation was performed on an Atlantis® dC18 column (2.1 mm × 150 mm, 3.0 μm) from Waters Corporation (Milford, MA, USA). The mobile phase consisted of 10 mM ammonium formate adjusted to pH 3.1 with formic acid (A) and acetonitrile (B). A linear gradient from 90% A and 10% B was run over 10 min up to 10% A and 90% B, which was held for 3 min. Re-equilibration of the HPLC column was achieved as the start conditions were held for 5 min before the next injection. The mobile phase flow rate was 0.3 mL/min and the column temperature was 25 ± 0.8 °C. The selected ion monitoring (SIM) mode (Table 1) electrospray MS conditions (positive mode) were: nitrogen drying gas (12 L/min, 350 °C), nitrogen nebulizer gas (241 kPa, 35 psi), and a capillary voltage of 2000 V. The injection volume was 5 μL and the autosampler temperature was 5 °C to avoid stability problems.

2.6. Sample precipitation and SPE procedure

To 0.5 mL whole blood, 50 μL 18.5 μM internal standard was added. The mixed sample was precipitated with 1 mL ice cold acetonitrile:methanol solution (85:15, v:v, stored at −20 °C) and immediately mixed on a whirli mixer. The samples were frozen (−20 °C) for 30 min before centrifugation (2260 × *g*) at 4 °C for 10 min. The supernatant was decanted and mixed with 0.2 mL 4.5 M HCl before dilution with 3.3 mL water. The diluted sample was applied to an Oasis® MCX (mixed-mode sorbent, reversed phase and cation exchanger, 30 mg, 1 mL) extraction column obtained from Waters. The SPE procedure was performed on an Aspec robot (Gilson) (Table 2). The eluate was evaporated to dryness under a stream of N_2 at 50 °C (Turbovap, Zymark Corporation, Hopkinton, MA, USA). The residue was dissolved in 150 μL acetonitrile:10 mM ammonium formate (pH 3.1) (20:80, v/v) before HPLC-MS analysis.

In the SPE Box-Behnken design [63,64], each supernatant was added 0.2 mL 4.5 M HCl and diluted with water to a final volume of 5 mL before application to the SPE column. The elution and SPE washing solution volume were 2 mL, the SPE method parameters were otherwise held constant as described in Table 2.

Table 2
Automated SPE procedure on Aspec XL robot for the determination of 14 cardiovascular drugs in post-mortem whole blood

Process	Reagent	Flow rate (mL/min)
Condition	1 mL methanol	10.0
Condition	1 mL H_2O	10.0
Load	5 mL (precipitated, pH adjusted and diluted blood)	1.5
Rinse syringe	2 mL H_2O	12
Wash	1 mL KH_2PO_4 pH 3.4	10.0
Rinse syringe	0.5 mL H_2O	12
Wash	1.5 mL methanol:H_2O (60:40)	10.0
Drying	3 mL air	10
Elute	1.5 mL acetonitrile:NH_3 (95:5)	1.5
Rinse syringe	0.5 mL H_2O	12

2.7. Experimental design

Full factorial, reduced factorial and response surface Box-Behnken designs [63,64] were used in the development and robustness testing of the LC separation and/or the SPE method. In the SPE Box-Behnken design a summarized normalised response was found by normalising the results obtained in each experiment by the largest peak height observed for that compound in the data table. The summarized normalised response, including all the compounds and internal standard ($n = 15$) was calculated for each experiment number in the data table, giving the highest obtainable response to be 15. Descriptive statistics was used to get a summary of the distribution of the response. All the experiments in the design table were included in the descriptive statistics. A 95% confidence level was used for evaluation of statistical significance. Only significant effects were included in the multiple linear regression models (MLR). Two replicates were used in the SPE experiments. Three center point samples were analysed. Unscrambler 9.1 software (Camo Process AS, Oslo, Norway) was used for modelling.

2.8. Method validation

The matrix effect (ME%) was determined by analysing two sample sets as described by Matuszewski et al. [65]. The first set (set 1) consisted of six mobile phase samples each prepared by mixing 100 μL mobile phase, 30 μL spiked aqueous calibration solution and 30 μL diazepam, giving a concentration of 0.9 μM of each drug and 2.5 μM diazepam in the sample. The second set (set 2), originating from six different persons post-mortem whole blood extracts, was spiked after sample preparation and evaporation as described for set 1. The mean peak heights and their RSDs were calculated for set 1 and set 2. The matrix effect (ME%) was determined by equation 1 (Eq. (1)). ME% values of 100% indicates absence of any matrix effects, whereas <100% indicate ion suppression and values >100% indicate ion enhancement.

$$\mathrm{ME\%} = \frac{\text{Mean peak height Lot}_{1-6}\text{ Set2}}{\text{Mean peak height Lot}_{1-6}\text{ Set1}} \times 100 \quad (1)$$

Analyses of cardiovascular drug free post-mortem whole blood samples from eight different persons were carried out to study the assay selectivity with regard to endogenous interferences. To evaluate possible drug interferences, whole blood spiked with a number of drugs and narcotics (Table 5) was extracted.

Concentration limit of detection (cLOD) and quantification (cLOQ) were determined by analysing a minimum of seven different cardiovascular drug free post-mortem whole blood samples ($n = 1$ or 2) in 10 successive assays, one replicate in each assay. A whole blood sample spiked to a concentration near the presumed cLOQ (0.03 μM of atenolol, sotalol, metoprolol, propranolol, diltiazem, flecainide, carvedilol, verapamil, irbesartan and telmisartan, and 0.08 μM of amlodipine and losartan and 1.00 μM valsartan) was analysed in 10 different assays, one replicate in each assay. cLOD and cLOQ were defined as the analyte concentration equal to the measured mean concentra-

tion of the post-mortem drug free whole blood samples plus 3 and 10 SD of the cLOQ sample, respectively.

Within assay precisions were determined by analysing spiked whole blood samples ($n = 10$; 1.0 and 5.0 μM) in one assay. Between assay precisions were determined at three concentration levels (0.10, 1.0 and 5.0 μM) of spiked whole blood samples in ten different assays, one replicate in each assay. Three persons performed the assays during 1 month. Accuracy was calculated as the percent deviation of the measured mean from the theoretical concentration.

Recoveries were determined at two concentration levels (1.0 and 5.0 μM, $n = 5$). Total recovery was determined the following way: To 0.45 mL whole blood sample, 50 μL of a 10 or 50 μM aqueous control solution was added. The sample was precipitated and extracted (Table 2). To the SPE eluate, 50 μL of 18.5 μM diazepam-d5 was added, and the eluate was evaporated and the residue dissolved according to the procedure described previously. The SPE recovery was determined as follows: To the supernatant of the precipitated whole blood sample, 50 μL of 10 or 50 μM working control solution was added and the sample was extracted. To the SPE eluate, 50 μL of 18.5 μM diazepam-d5 was added. As controls, corresponding to 100% recovery, a whole blood sample ($n = 3$) was precipitated and extracted. To the corresponding SPE eluate, 50 μL of the 10 or 50 μM working control solution and 50 μL of 18.5 μM diazepam-d5 were added, and the sample was evaporated and dissolved. Recoveries were calculated by comparing peak height ratios (compound versus diazepam-d5) of spiked whole blood samples and controls.

3. Results and discussion

3.1. Optimisation of LC separation

Adequate resolution is needed to obtain satisfactory quantification of compounds. However, modelling of the resolution of successive pairs of compounds could not be performed because the retention order of two consecutive compounds can change (e.g. A-B can become B-A) as well as the selectivity (e.g. A-B-C can become C-A-B). Therefore, retention time was studied in the experimental designs.

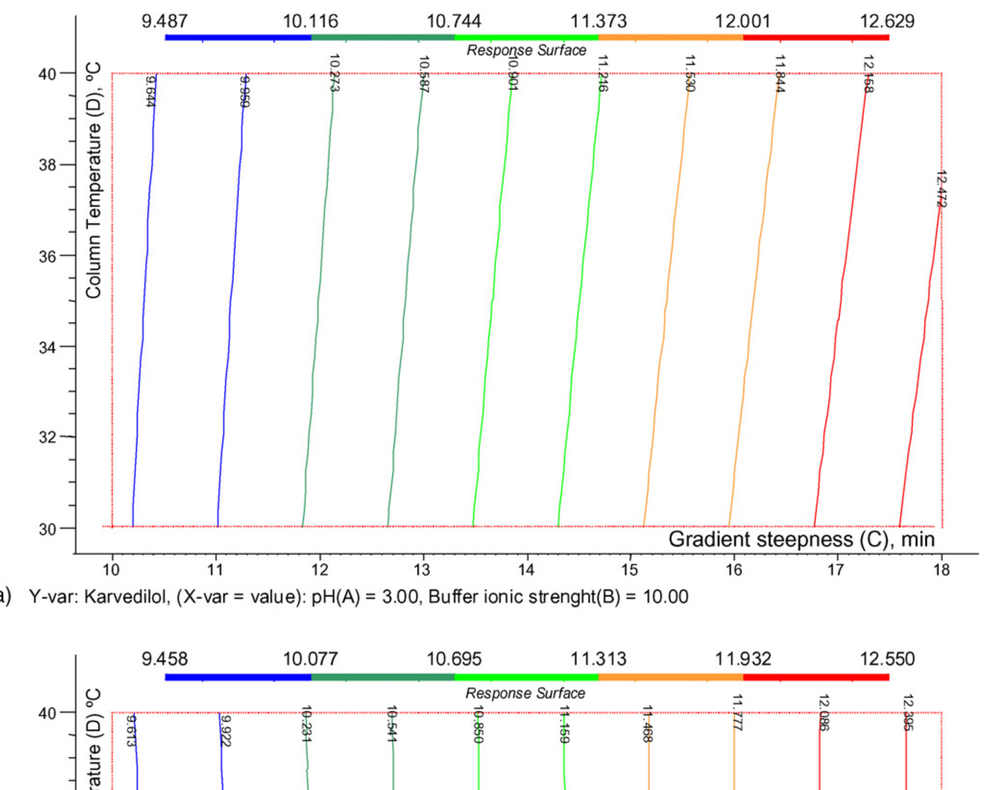
Based on preliminary experiments with an Atlantis C18 column and a mobile phase with acetonitrile and ammonium formate buffer (pH 3) or ammonium acetate buffer (pH 5), the most important factors were selected and studied in a full factorial design 2^4 (16 experiments). The impact of the factors pH (3–5), buffer ionic strength (5–10 mM), gradient steepness (10–18 min, 10–90% acetonitrile) and column temperature (30–40 °C) on the retention time of the compounds (except flecainide which was added to the method at a later step) were studied. None of the investigated combinations resulted in separation of all the compounds. However, at two factor level combinations, amlodipine and carvedilol was the only pair not separated. The retention of these compounds differed regarding the interaction between column temperature and gradient steepness, seen in the contour plot as different directions of the contour lines (Fig. 1). With an 18 min gradient, the difference in retention between amlodipine and carvedilol was negligible. However, at the steepest gradient the retention of carvedilol increased with decreasing column temperature, whereas for amlopidine the retention decreased with decreasing column temperature (Fig. 1). The factorial design results therefore indicated that, when using the steepest gradient, lowering of the column temperature could result in a separation of the compounds. This was confirmed in the experiments with a column temperature of 25 °C, the steepest gradient (10 min) and a mobile phase with acetonitrile and 10 mM ammonium formate pH 3.1. At these conditions all the compounds were completely or partly separated (Fig. 2).

A response surface Box-Behnken design, including the factors buffer pH (2.7, 3.0, 3.3), buffer ionic strength (8, 10, 12 mM) and column temperature (23, 25, 27 °C), was performed in order to study the properties and robustness of the LC separation at small deviations from the separation conditions (pH 3.1, 10 mM and 25 °C). Studying the retention time, one or several significant effects were found for all the compounds. The Box-Behnken design showed satisfactory descriptive statistics regarding the retention time with RSD < 0.8% for all the compounds with the exception of telmisartan (2.9%) and irbesartan (1.5%). The pH was found to be critical for the separation, showing an alteration in retention order and/or coelution involving irbesartan, telmisartan and/or valsartan when the pH was 2.7 and 3.3 (buffer ionic strength and column temperature were varied as described above). At pH 3.0 neither shift in retention order nor coelution was observed when altering the buffer ionic strength and column temperature from low to high level. Based on these findings it was stated that the HPLC method was robust with regard to consistency of retention order and no coelution when the pH was in the range 3.0–3.1, buffer ionic strength 10 ± 2 mM and column temperature 25 ± 2 °C.

3.2. MS detection

The target ions and optimal individual fragmentor voltages (Table 1) were found by flow injection analysis in both positive and negative mode. Positive ionization mode gave the overall best responses. The capillary voltages 2000 and 4000 V (recommended by the manufacturer) were evaluated. With the exception of valsartan, the highest responses, measured as peak heights, were obtained using 2000 V. Positive ionization mode and a capillary voltage of 2000 V were therefore chosen for all the compounds.

Selected ion monitoring mode was used in the identification of the compounds. As described by The Society of Forensic Toxicologists (SOFT) and the American Academy of Forensic Sciences Toxicology Section (AAFS) Forensic Laboratory Guidelines [66] one qualifying ion for each compound and internal standard, in addition to a primary ion for each, was used in the identification. Furthermore, for forensic toxicology applications, the detection of a compound at a concentration above the cLOQ, should be confirmed in a different sample extract with a different method. Hence, an unambiguous identification of compound is achieved.



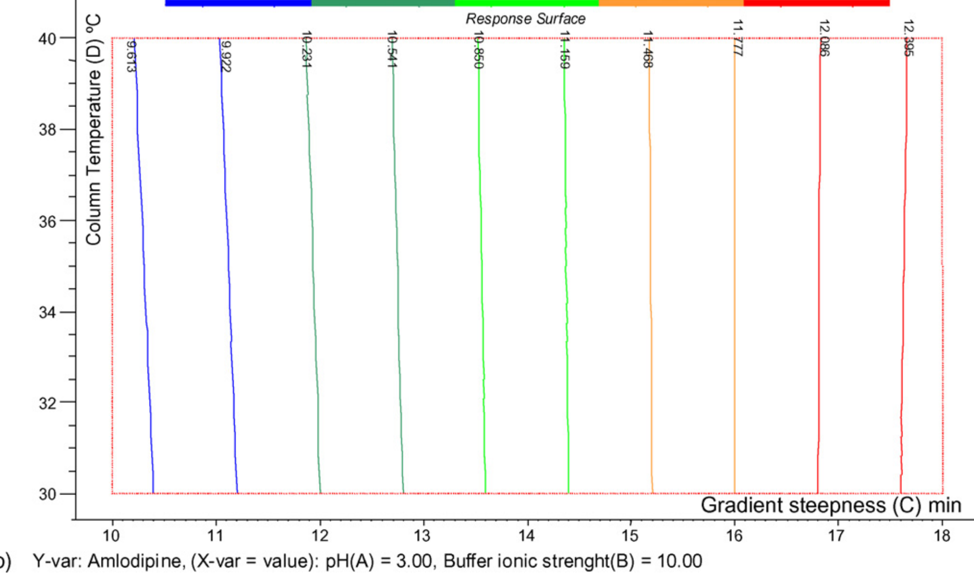


Fig. 1. Contour plot of the interaction effect between gradient steepness (C) (*x*-axis) and column temperature (D) (*y*-axis) on the responses retention time of (a) carvedilol and (b) amlodipine. The remaining variables were kept constant using 10 mM ammonium formate pH 3.0. The retention times were displayed as contour lines, i.e. lines which show where the response has the same predicted value.

3.3. *Optimisation of sample treatment*

Since the method included basic, acidic and amphoteric compounds (Table 1), there was no pH value at which all the compounds were simultaneously uncharged. Liquid–liquid extraction was considered inconvenient as a two steps extraction, one basic and one acidic, would be time consuming. A simple precipitation before HPLC-MS analysis was considered insufficient partly due to the dilution of the sample, but also due to the increased risk of ion suppression or ion enhancement resulting from the presence of other compounds and salts [67]. Previous studies have shown that satisfactory results were obtained by SPE for cardiovascular drugs [41,44,46,50,57,58,68–70]. Based on these considerations, precipitation and dilution of the sample followed by SPE was chosen.

Whole blood from forensic autopsies can be of very variable quality (e.g. viscosity, state of decay, blood clots). Precipitation of the sample before SPE was therefore necessary in order to prevent clogging of the SPE column. Blanchard [71] studied different precipitation agents and their effectiveness. Based on his results, we chose to evaluate the precipitation agents acetonitrile, methanol, perchloric acid and combinations of these. Perchloric acid gave low recoveries due to coprecipitation of the compounds. Methanol was found to be a less effective precipitation agent than acetonitrile and a larger volume of methanol was required to precipitate the same volume of whole blood.

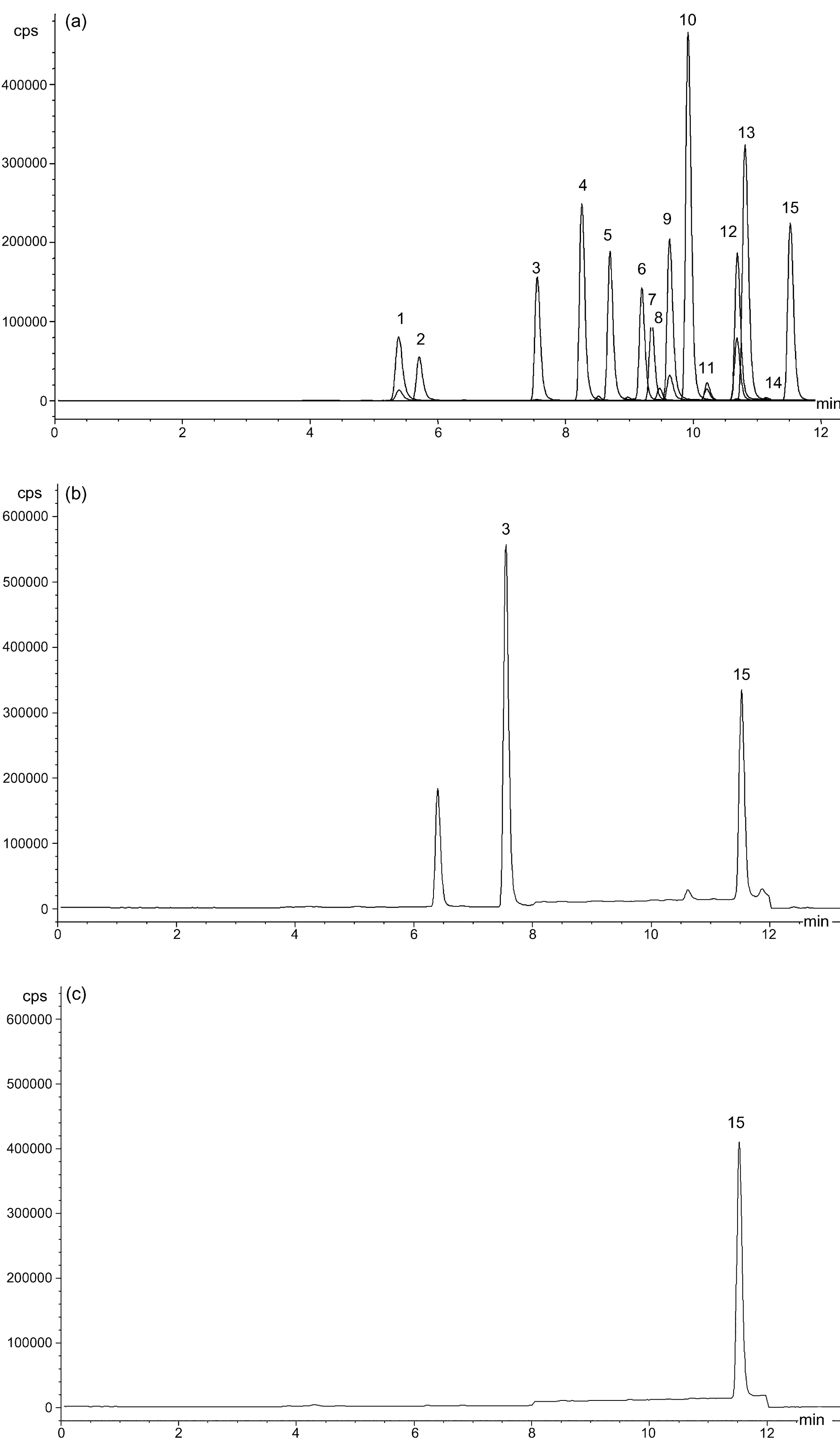


Fig. 2. (a) Selected ion chromatogram of a spiked whole blood sample (1.25 μM) of the studied compounds as well as the internal standard diazepam-d_5. (b) Total ion chromatogram of a autopsy sample where 3 μM metoprolol where found as well as the internal standard diazepam-d5 and (c) total ion chromatogram of a cardiovascular drug negative autopsy sample. (1) Atenolol 5.4 min, (2) sotalol 5.7 min, (3) metoprolol 7.6 min, (4) bisoprolol 8.3 min, (5) propranolol 8.7 min, (6) diltiazem 9.2 min, (7) flecainide 9.3 min, (8) amlodipine 9.5 min, (9) carvedilol 9.6 min, (10) verapamil 9.9 min, (11) losartan 10.2 min, (12) irbesartan 10.7 min, (13) telmisartan 10.8 min, (14) valsartan 11.1 and (15) internal standard Diazepam-d5 11.5 min.

The studied SPE columns were Oasis HLB (Hydrophilic-Lipophilic Balance Sorbent) and Oasis MCX (cation exchange and reversed phase sorbent). The HLB columns showed recoveries >50% for the angiotensin-II antagonists, calcium-channel antagonists and several of the beta-blockers, however the beta-blockers atenolol and sotalol were not retained when using acetonitrile as precipitation agent. The loss of atenolol and sotalol were due to lack of retention in sample load and the organic solvent washing. The MCX columns showed low recovery of valsartan, but satisfactory recoveries of the other compounds (Table 6), allowing both acetonitrile-methanol precipitation of sample and washing with higher concentration of organic solvent. Since the therapeutic concentration range (Table 1) was high for valsartan, low recovery was tolerated. The MCX column was therefore chosen.

A response surface Box-Behnken design was applied with focus on the steps in the sample preparation which had been identified as critical in the preliminary investigations. The impact of precipitation agent (100:0, 92.5:7.5, 85:15 acetonitrile:methanol), organic solvent SPE wash (60, 80, 100% methanol) and volume of precipitation agent (0.5, 1.0, 1.5 mL) on the summarized normalised response was investigated. The precipitation agent and %methanol in the SPE washing solution showed significant effects on the summarized normalised response (Fig. 3). Since the compounds losartan and valsartan were only retained by hydrophobic interactions on the MCX column, the organic wash step was critical for these. This can be seen as a minimum point at high levels of methanol in the wash solution. In the contour plot the predicted highest response, representing the overall highest recovery for all the compounds, was a combination of 60% methanol in the wash solution and precipitation with acetonitrile or 15% methanol added to acetonitrile. Traces of precipitate were observed when decanting the pure acetonitrile supernatant, but not when methanol-acetonitrile was used as precipitation agent, using 1 mL precipitation solvent. The precipitation agent volume was found not to be significant for the summarized normalised response. However, the precipitation volume was highly significant for the two beta-blockers atenolol and sotalol, showing a significant decrease in recovery when increasing the precipitation volume from 0.5 to 1.5 mL. The loss of atenolol and sotalol were presumed to occur in the SPE sample load due to the polar character of these compounds. Supernatants with traces of precipitate were observed when using 0.5 mL of the precipitation agents. Based on these observations a 1.0 mL acetonitrile:methanol (85:15 v/v) solution was used for precipitation of the samples, and 60% methanol was used in the SPE wash. These factor levels were used in the center samples in the robustness experiment (Table 3).

Table 3
Low (−1), center (0) and high (+1) level of the variables studied in the fractional factorial design $2^{III,5-3}$ for robustness testing of the precipitation and SPE procedure

Variables	Units	−1 ($n = 2$)	0 ($n = 3$)	+1 ($n = 2$)
Precipitation reagens (methanol:acetonitrile)	% Methanol	12	15	18
Flow rate methanol:H_2O wash	mL	1	1.5	2
Wash solution composition (methanol:H_2O)	% Methanol	55	60	65
Flow rate elution	mL	1	1.5	2
Elute solution composition (acetonitrile:NH_3)	% NH_3	3	5	7

In order to investigate the robustness of the sample clean-up, the precipitation and SPE procedure was tested at modest deviations from the conditions specified in the method (Table 2). A fractional factorial design $2^{III,5-3}$ was performed (Table 3). The response was the peak height of the compounds. The design

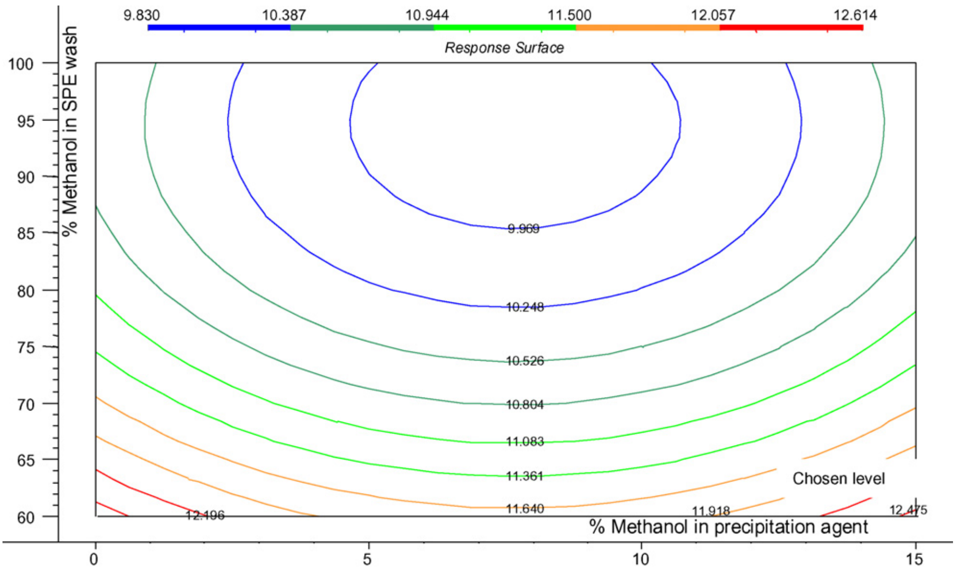


Fig. 3. Contour plot with the variables precipitation agent (A; %methanol) (*x*-axis) and %methanol in SPE wash (B; %methanol) (*y*-axis) on the summarized normalised response. The volume of the precipitation agent (C; mL) was 1.0 mL. The response was displayed as contour lines, i.e. lines which show where the response has the same predicted value. The maximum obtainable response value was 15.

showed non-significant effects ($p > 0.05$) and descriptive statistics for all the experiments with RSD < 14% for the compounds metoprolol, bisoprolol, propranolol, flecainide, amlodipine and irbesartan. Some compounds showed significant effects, but acceptable RSDs < 9%, these were atenolol, sotalol, diltiazem, carvedilol, verapamil, telmisartan and diazepam-d5. For these compounds the SPE method showed satisfactory robustness to small changes in the procedure. However, as expected from the SPE Box-Behnken design, recovery of losartan (RSD 31%) and valsartan (RSD 55%) was not robust to changes in the methanol content in the washing step, and reduced recovery when increasing the methanol content from 55 to 65% was observed. This variable was therefore critical in the sense of maximising the recovery of these compounds.

3.4. Method validation

The matrix effects (ME%) ranged from 94–114% (Table 4) and RSDs for the peak heights of drugs in set 1 were from 0.4 to 2.1% and for set 2 from 4.2 to 12%, respectively. The higher variability in set 2 compared to set 1 might indicate a matrix effect. The RSDs for set 2 using the peak height ratio of drug and internal standard showed improved RSDs for all the drugs when compared to no correction with internal standard, and ranged from 3.3 to 11%. This indicates that the internal standard had a compensating effect both on the precision and reliability of the quantification of the drugs. However, an examination of the matrix effects for each of the post-mortem whole blood lots indicated that the ME% value from one blood lot was higher for almost all the drugs when compared to the other blood lots, leading to the higher RSDs values of set 2. The same matrix effect was not observed for the internal standard diazepam indicating a matrix effect in this particular post-mortem whole blood which was not completely corrected for by the internal standard. However, the highest ME% value for this particular blood lot was 120%, i.e. maximum 20% ion enhancement was observed in this post-mortem whole blood sample when compared to a biological matrix free mobile phase sample. The observed matrix effects were therefore considered to be acceptable. However, as with all forensic toxicology determinations [66], a confirmation should always be carried out by a different method and possible matrix effects differences between this and the confirmation method should be taken into consideration when interpreting the analytical results.

Endogenous peaks above cLOD were not detected in any of the post-mortem whole bloods analysed in the selectivity experiments. No interferences were observed from any of the investigated drugs and narcotics which are shown in Table 5.

Within assay precisions and accuracies were in the range 3.4–21% and from −24 to 21%, respectively (Table 6). Between assay precisions were in the range 4.4–28% (Table 7). The cLOQ for atenolol and sotalol was 0.05 and 0.07 μM, however, for both compounds the between assay accuracy at 0.10 μM was not satisfactory (atenolol: +31% bias, sotalol: +43% bias, Table 7). When introducing this method to the forensic toxicology routine determination, the cLOQs for both compounds were set to 0.5 μM, resulting in a between assay precision (0.6 μM, $n = 14$) of 13 and 15% RSD and +21 and 24% bias for atenolol and sotalol, respectively. The therapeutic concentrations of atenolol and sotalol ranged from 0.4 and 1.8 μM, respectively (Table 1), and hence the method was still able to monitor therapeutic concentration ranges of these compounds. With the exception of amlodipine (cLOQ 0.135 μM), the cLOQs (Table 7) covered low therapeutic concentration levels for the compounds (Table 1). The calibration curve ranges were up to 6 or 12.5 μM, the method was therefore suitable for determination of therapeutic and toxic

Table 4
Mean peak heights of compounds, mean peak heights ratios of compound and internal standard (IS), RSD for sets 1 and 2 and matrix effect (%ME) in six different lots of post-mortem whole blood

Compound	Set 1[a]		Set 2[b]		Set 2[b]		
	Mean peak height[c]	RSD %	Mean peak height[c]	RSD %	Mean peak ratio Compound/IS	RSD %	ME[d] %
Atenolol	5.59	0.5	5.86	4.2	0.25	3.3	105
Sotalol	3.92	0.4	4.01	4.4	0.17	3.7	103
Metoprolol	9.62	0.6	9.90	7.1	0.43	6.1	103
Bisoprolol	12.63	0.6	12.97	7.6	0.56	6.4	103
Propranolol	10.63	0.9	10.83	6.8	0.47	5.7	102
Carvedilol	7.38	1.9	7.27	11	0.31	9.1	99
Diltiazem	6.94	0.8	7.13	7.6	0.31	6.4	103
Amlodipine	0.51	1.7	0.50	12	0.02	11	97
Verapamil	20.56	1.2	21.25	8.3	0.91	6.9	103
Losartan	1.55	0.4	1.51	8.8	0.07	7.2	98
Irbesartan	9.09	0.9	8.54	6.2	0.37	4.0	94
Telmisartan	12.26	2.1	12.72	8.9	0.55	7.4	104
Valsartan	0.67	2.1	0.77	5.1	0.03	4.9	114
Flecainide	5.22	1.1	5.31	7.2	0.23	6.0	102
Diazepam (IS)	25.05	0.8	23.25	3.1			93

[a] Compound standards in mobile phase.
[b] Compounds spiked to extracts from six different post-mortem whole blood lots.
[c] In arbitrary units, $\times 10^4$.
[d] Matrix effect expressed as the ratio of the mean peak height of an compound spiked postextraction (set 2) to the mean peak height of the same compound standards (set 1) multiplied by 100. A value > 100% indicates ionization enhancement, and a value < 100% indicates ionization suppression.

Table 5
Assay selectivity of drug interferences, 0.5 mL whole blood was spiked before sample preparation with antidepressants, analgesics, cardiovascular drugs, antiepileptica, antipsychotics, hypnotica and sedatives, narcotic substances and others

Compound
Antidepressants:
Moclobemide (20)
Venlafaxine (5)
Mirtazapine (2)
Citalopram (4)
Reboxetine (2)
Doxepine (5)
Paroxetine (2)
Fluvoxamine (2.5)
Nortriptyline (4)
Amitriptyline (5)
Mianserin (2.5)
Trimipramine (4)
Fluoxetine (5)
Sertraline (2)
Clomipramine (5)
Nefazodone (5)
Analgesics:
Paracetamol (1000)
Salicylic acid (200)
Oxycodone (2.5)
Ketobemidone (2.5)
Tramadol (10)
Pethidine (10)
Dextropropoxyphene (5)
Fentanyl (0.05)
Morphine (2.6)
Methadone (2.5)
Codeine (2.5)
Buprenorphine (1.3)
Cardiovascular drugs:
Isradipine (13)
Nifedipine (13)
Felodipine (13)
Amiodaron (13)
Antiepileptica:
Lamotrigine (20)
Phenobarbital (200)
Carbamazepine (100)
Phenytoin (200)
Clonazepam (0.2)
7-Aminoclonazepam (0.6)
Valproic acid (2000)
Antipsychotics:
Amisulpride (5)
Olanzapine (2)
Risperidone (2)
Haloperidol (0.4)
Clozapine (8)
Levomepromazine (4)
Chlorpromazine (4)
Dixyrazine (4)
Perfenazine (0.4)
Chlorprothixene (4)
Zuclopenthixol (1)
Flupenthixol (0.8)
Quetiapin (4)
Hypnotica and sedatives:
Alprazolam (0.2)
Diazepam (2.4)
n-Desmethyldiazepam (2.4)
Flunitrazepam (0.06)
7-Aminoflunitrazepam (0.1)
Nitrazepam (0.6)
7-Aminonitrazepam (0.3)
Midazolam (0.8)
Oxazepam (6.4)
Zopiclone (0.6)
Zolpidem (0.6)
Phenazepam (0.1)
Narcotics:
6-Monoacatylmorphine (6-MAM) (1)
Cocaine (6)
Benzoylecgonine (13)
Amphetamine (25)
Methamphetamine (10)
3,4-Methylenedioxymethamphetamine (MDMA) (10)
3,4-Methylenedioxyamphetamine (MDA) (10)
3,4-Methylenedioxyethylamphetamine (MDEA) (10)
Tetrahydrocannabinol (THC) (27)
Lysergic acid diethylamide (LSD) (0.3)
Others:
Theophyllin (1000)
Promethazine (5)
Alimemazine (2)
Carisoprodol (10)
Meprobamat (10)
Ketamine (25)

The compounds concentrations (μmol/L) in whole blood are marked in brackets.

levels of several of the compounds. Although low therapeutic concentrations of amlodipine could not be determined, the compound was included in the method in order to determine high therapeutic and toxic concentrations.

The total recoveries ranged from 9 to 103% for all the studied compounds (Table 6). The total recovery of valsartan and the SPE recoveries were both approximately the same, indicating that the loss of valsartan was due to the SPE method, and not the precipitation. The therapeutic concentrations of valsartan was reported to be high, and ranged from 3.8 μM [72] (Table 1). Analyses of whole blood spiked with 1.0 μM (cLOQ) valsartan showed an acceptable precision (Tables 6 and 7), however the recovery and accuracies were not satisfactory ($\geq -20\%$ bias). Despite its low recovery, valsartan was included in the method, however, the method was only regarded as semi-quantitative for valsartan.

3.5. Application

Fig. 2(b) and (c) show TIC chromatograms of two autopsy samples, the first shows a sample where a concentration of 3 μM metoprolol was found and the second a negative sample. The ingested metoprolol dose was unknown. The method has been used for the analyses of post-mortem whole blood samples from forensic autopsies in cases where sudden cardiac death with known use of cardiovascular drugs were indicated, or in cases with unknown cause of death. During the first months of routine analysis, it was found that in about 10% of the samples cardiovascular drugs were determined at concentrations above

Table 6
Within assay precisions ($n = 10$), accuracies expressed as bias and total and SPE recoveries ($n = 5$) of cardiovascular drugs in whole blood

Compound	Concentration theoretical (μM)	Concentration measured Mean ± 1 SD (μM)	RSD[a] (%)	Bias[a] (%)	Total recovery[a] ±1 SD (%)	SPE recovery[a] ±1 SD (%)
Atenolol	1.00	1.12 ± 0.05	4.5	12	46 ± 0.6	62 ± 1.2
	5.00	5.66 ± 0.66	12	13	54 ± 1.6	65 ± 3.3
Sotalol	1.00	1.21 ± 0.08	6.6	21	46 ± 2.3	62 ± 3.1
	5.00	5.66 ± 0.57	10	13	56 ± 1.4	62 ± 3.0
Metoprolol	1.00	0.99 ± 0.05	5.1	−1.4	85 ± 1.5	99 ± 2.5
	5.00	4.73 ± 0.47	10	−5.5	85 ± 1.3	97 ± 2.4
Bisoprolol	1.00	0.95 ± 0.04	4.3	−5.9	84 ± 0.8	95 ± 2.1
	5.00	3.81 ± 0.32	8.4	−24	85 ± 1.8	80 ± 0.9
Propranolol	1.00	1.09 ± 0.08	7.3	8.7	80 ± 1.5	95 ± 1.7
	5.00	4.41 ± 0.50	11	−12	83 ± 1.3	95 ± 2.5
Carvedilol	1.00	0.98 ± 0.04	4.2	−1.5	91 ± 2.9	96 ± 2.3
	5.00	4.20 ± 0.30	7.1	−16	85 ± 1.7	94 ± 4.5
Diltiazem	1.00	1.00 ± 0.03	3.4	0.5	84 ± 1.5	93 ± 1.6
	5.00	3.93 ± 0.25	6.4	−21	86 ± 1.2	91 ± 3.5
Amlodipine	1.00	1.10 ± 0.11	9.6	10	96 ± 3.2	102 ± 3.6
	5.00	4.14 ± 0.33	7.9	−17	76 ± 3.5	86 ± 1.9
Verapamil	1.00	1.00 ± 0.04	3.5	0.5	88 ± 1.9	96 ± 2.1
	5.10	4.58 ± 0.39	8.4	−10	88 ± 1.7	97 ± 3.1
Losartan	1.00	0.76 ± 0.06	7.8	−24	50 ± 5.7	52 ± 11
	5.00	4.96 ± 0.77	16	−0.8	41 ± 3.7	52 ± 8.8
Irbesartan	1.00	0.91 ± 0.07	7.8	−8.9	80 ± 5.4	85 ± 6.1
	5.00	5.13 ± 0.28	5.5	2.6	83 ± 2.4	92 ± 4.5
Telmisartan	1.00	0.97 ± 0.04	4.4	−3.2	103 ± 4.2	107 ± 3.1
	4.90	4.20 ± 0.35	8.2	−14	87 ± 1.2	97 ± 4.5
Valsartan	1.00	0.78 ± 0.03	4.0	−22	18 ± 1.7	21 ± 2.6
	5.00	4.63 ± 0.96	21	−7.3	9 ± 1.6	13 ± 2.0
Flecainide	1.00	0.94 ± 0.03	3.5	−5.8	83 ± 1.3	95 ± 1.5
	5.00	4.59 ± 0.37	8.1	−8.3	88 ± 1.8	97 ± 3.0

[a] The within assay precision and recovery experiments for the two concentration levels were carried out more than 6 months apart.

Table 7
cLOD, cLOQ, between assay precisions and bias for cardiovascular drugs in whole blood determined on ten assays performed during 1 month

Compound	cLOD (μM)	cLOQ (μM)	Concentration theoretical (μM)	Concentration measured Mean ± 1 SD (μM)	RSD (%)	Bias (%)
Atenolol	0.014	0.046	0.10	0.13 ± 0.02	18	31
			1.00	1.13 ± 0.16	14	13
			5.00[a]	5.50 ± 0.65	12	10
Sotalol	0.021	0.069	0.10	0.14 ± 0.02	14	43
			1.00	1.21 ± 0.19	8.9	21
			5.00[b]	5.66 ± 0.55	9.7	13
Metoprolol	0.020	0.067	0.10	0.12 ± 0.01	9.5	16
			1.00	1.07 ± 0.10	8.9	6.6
			5.00	5.19 ± 0.54	10	3.8
Bisoprolol	0.014	0.047	0.10	0.11 ± 0.01	7.5	4.4
			1.00	0.97 ± 0.05	5.0	−3.5
			5.00	4.80 ± 0.29	6.0	−4.8
Propranolol	0.014	0.046	0.10	0.12 ± 0.01	8.1	22
			1.00	1.12 ± 0.06	5.7	12
			5.00	5.58 ± 0.55	9.8	12
Carvedilol	0.017	0.055	0.10	0.12 ± 0.01	8.2	18

Table 7 (*Continued*)

Compound	cLOD (μM)	cLOQ (μM)	Concentration theoretical (μM)	Concentration measured Mean ± 1 SD (μM)	RSD (%)	Bias (%)
			1.00	1.00 ± 0.11	11	0.1
			5.00	4.24 ± 0.34	7.9	−15
Diltiazem	0.005	0.015	0.10	0.11 ± 0.01	6.4	7.0
			1.00	1.01 ± 0.07	6.4	0.5
			5.00	4.93 ± 0.34	6.8	−1.6
Amlodipine	0.041	0.135	0.10	0.11 ± 0.01	8.9	5.3
			1.00	0.86 ± 0.08	9.6	−14
			5.00	4.65 ± 0.30	6.5	−7.1
Verapamil	0.005	0.017	0.10	0.11 ± 0.01	4.4	8.6
			1.00	1.02 ± 0.06	5.4	1.6
			5.00	4.92 ± 0.26	5.2	−1.3
Losartan	0.080	0.265	0.10[c]			
			1.00	0.86 ± 0.13	16	−14
			5.00	4.42 ± 0.27	6.1	−12
Irbesartan	0.023	0.075	0.10	0.09 ± 0.01	7.9	−8.4
			1.00	0.93 ± 0.09	10	−7.5
			5.00	4.47 ± 0.34	7.6	−11
Telmisartan	0.018	0.057	0.10	0.10 ± 0.01	8.2	−3.7
			1.00	0.86 ± 0.14	16	−14
			5.00	4.24 ± 0.45	11	−15
Valsartan	0.323	1.030	0.10[c]			
			1.00	0.80 ± 0.10	13	−20
			5.00	3.95 ± 1.11	28	−21
Flecainide	0.008	0.027	0.10	0.10 ± 0.01	6.7	5.0
			1.00	0.97 ± 0.05	5.1	−3.5
			5.00	4.66 ± 0.24	5.1	−6.7

[a] $n = 7$.
[b] $n = 6$.
[c] Not analysed because theoretical concentration was <LOQ.

the therapeutic concentration range. However, further studies on the post-mortem redistribution as well as plasma (serum)/whole blood concentration ratios should be performed in order to evaluate these findings.

Published, validated methods have mainly been developed for therapeutic drug monitoring (TDM) and pharmacokinetic studies of cardiovascular drugs in plasma or serum. Today, TDM is mainly used for the antiarrythmic drugs and digitalis [73]. TDM involving other cardiovascular drugs could however play a role in the future with increased focus on individualized drug therapy [74]. The presented method is supposed to, with minor changes, to be applicable for other matrices, such as plasma, serum, autopsy tissues and vitreous humour, and hence may be used in TDM and other forensic toxicology applications.

4. Conclusion

The developed forensic analysis method is intended for determination of cardiovascular drugs in whole blood samples and allows automated, simultaneous determination of 14 cardiovascular drugs including the beta-blockers, calcium-channel antagonists, angiotensin-II antagonists and antiarrythmic drug at therapeutic and toxic levels. To our knowledge, this is the only published method that allows the simultaneous determination of several drugs covering four different cardiovascular drug groups in post-mortem whole blood. Furthermore, response surface and factorial designs were successfully used to optimise and test the robustness of the SPE and HPLC procedures.

References

[1] The department of pharmacoepidemiology Norwegian Institute of Public Health, Drug Consumption in Norway 2000–2004, Norwegian Institute of Public Health, Oslo, 2005.
[2] T.M. Speight, N.H.G. Holford, Avery’s Drug Treatment, Adis International, 2006.
[3] I. Buajordet, J. Ebbesen, J. Erikssen, O. Brors, T. Hilberg, J. Intern. Med. 250 (2001) 327.
[4] W.A. Watson, T.L. Litovitz, G.C. Rodgers Jr., W. Klein-Schwartz, N. Reid, J. Youniss, A. Flanagan, K.M. Wruk, Am. J. Emerg. Med. 23 (2005) 589.
[5] M. Montagna, A. Groppi, Arch. Toxicol. 43 (1980) 221.
[6] D. Perrot, B. Bui-Xuan, J. Lang, Y. Bouffard, B. Delafosse, G. Faucon, J. Motin, J. Toxicol. Clin. Toxicol. 26 (1988) 389.
[7] A. Mozayani, P. Singer, G. Jones, J. Anal. Toxicol. 19 (1995) 519.
[8] T.P. Rohrig, D.A. Rundle, W.N. Leifer, J. Anal. Toxicol. 11 (1987) 231.
[9] E.T. Shore, D. Cepin, M.J. Davidson, Ann. Emerg. Med. 10 (1981) 524.
[10] M. Stajic, R.H. Granger, J.C. Beyer, J. Anal. Toxicol. 8 (1984) 228.
[11] N. Fucci, C. Offidani, Am. J. Forensic Med. Pathol. 21 (2000) 56.
[12] J.W. Jones, M.A. Clark, B.L. Mullen, J. Forensic Sci. 27 (1982) 213.
[13] J. Kristinsson, T. Johannesson, Acta Pharmacol. Toxicol. (Copenh) 41 (1977) 190.

[14] A.R. Koch, D.P. Vogelaers, J.M. Decruyenaere, B. Callens, A. Verstraete, W.A. Buylaert, J. Toxicol. Clin. Toxicol. 33 (1995) 253.
[15] S.H. Cosbey, D.J. Carson, J. Anal. Toxicol. 21 (1997) 221.
[16] S.S. Johansen, J. Genner, J. Clin. Forensic Med. 10 (2003) 169.
[17] F.L. Cantrell, S.R. Williams, Clin. Toxicol. 43 (2005) 587.
[18] J.R. Kalin, K.M. Wood, A.J. Lee, J. Anal. Toxicol. 18 (1994) 180.
[19] G. Romano, N. Barbera, C. Rossitto, G. Spadaro, J. Anal. Toxicol. 26 (2002) 374.
[20] T.A. Roper, R. Sykes, C. Gray, Postgrad. Med. J. 69 (1993) 474.
[21] H.A. Kaliciak, S.N. Huckin, W.S. Cave, J. Anal. Toxicol. 16 (1992) 102.
[22] L.F. Chan, L.H. Chhuy, R.J. Crowley, J. Anal. Toxicol. 11 (1987) 171.
[23] L.T. Chan, L.H. Chhuy, R.J. Crowley, J. Chromatogr. 402 (1987) 361.
[24] H.P. Gelbke, H.J. Schlicht, G. Schmidt, Arch. Toxicol. 37 (1977) 89.
[25] E.J. Janowska, E.M. Chudzikiewicz, Vet. Hum. Toxicol. 38 (1996) 210.
[26] U. Kuhlmann, H. Schoenemann, T.F. Muller, M. Keuchel, H. Lange, Intensive Care Med. 25 (1999) 1473.
[27] A. Tracqui, C. Tournoud, P. Kintz, M. Villain, C. Kummerlen, P. Sauder, B. Ludes, Hum. Exp. Toxicol. 22 (2003) 515.
[28] T. Benijts, D. Borrey, W.E. Lambert, E.A. De Letter, M.H.A. Piette, C.V. Peteghem, A.P. De Leenheer, J. Anal. Toxicol. 27 (2003) 47.
[29] E. Brazil, G.G. Bodiwala, D.C. Bouch, J. Accid. Emerg. Med. 15 (1998) 423.
[30] A.R. Forrest, I. Marsh, J.H. Galloway, P.B. Gray, J. Anal. Toxicol. 15 (1991) 41.
[31] B. Levine, D. Chute, Y.H. Caplan, J. Anal. Toxicol. 14 (1990) 335.
[32] M.J. Lynch, J. Gerostamoulos, Leg. Med. 3 (2001) 233.
[33] C. Rogers, D.T. Anderson, J.K. Ribe, L. Sathyavagiswaran, J. Anal. Toxicol. 17 (1993) 434.
[34] N. Romain, C. Giroud, K. Michaud, M. Augsburger, P. Mangin, Forensic Sci. Int. 106 (1999) 115.
[35] M. Holzbecher, R.A. Perry, H.A. Ellenberger, J. Forensic Sci. 27 (1982) 715.
[36] H. Kinoshita, T. Taniguchi, M. Nishiguchi, H. Ouchi, T. Minami, T. Utsumi, H. Motomura, T. Tsuda, T. Ohta, S. Aoki, Forensic Sci. Int. 133 (2003) 107.
[37] G.W. Kunsman, C.M. Kunsman, C.L. Presses, J.C. Garavaglia, N.J. Farley, J. Forensic Sci. 45 (2000) 926.
[38] D.W. Sadler, C. Quigley, J. Forensic Sci. 40 (1995) 903.
[39] M. Delamoye, C. Duverneuil, F. Paraire, P.d. Mazancourt, J.C. Alvarez, Forensic Sci. Int. 141 (2004) 23.
[40] H. Kataoka, S. Narimatsu, H.L. Lord, J. Pawliszyn, Anal. Chem. 71 (1999) 4237.
[41] H.H. Maurer, O. Tenberken, C. Kratzsch, A.A. Weber, F.T. Peters, J. Chromatogr. A 1058 (2004) 169.
[42] P. Ptacek, J. Macek, J. Klima, J. Chromatogr. B 789 (2003) 405.
[43] G. Bahrami, S. Mirzaeei, J. Pharm. Biomed. Anal. 36 (2004) 163.
[44] A.B. Baranda, C.A. Mueller, R.M. Alonso, R.M. Jimenez, W. Weinmann, Ther. Drug Monit. 27 (2005) 44.
[45] E. Molden, P.W. Johansen, G.H. Boe, S. Bergan, H. Christensen, H.E. Rugstad, H. Rootwelt, L. Reubsaet, G. Lehne, Clin. Pharmacol. Ther. 72 (2002) 333.
[46] C.A. Mueller, A.B.B. Gonzalez, W. Weinmann, J. Mass Spectrom. 39 (2004) 639.
[47] W.M. Mullett, M. Walles, K. Levsen, J. Borlak, J. Pawliszyn, J. Chromatogr. B 801 (2004) 297.
[48] R.V.S. Nirogi, V.N. Kandikere, K. Mudigonda, M. Shukla, S. Maurya, Biomed. Chromatogr. 20 (2006) 833.
[49] T. Yasuda, M. Tanaka, K. Iba, J. Mass Spectrom. 31 (1996) 879.
[50] T. Breindahl, J. Chromatogr. B Biomed. Sci. App. 746 (2000) 249.
[51] K. Doki, M. Homma, K. Kuga, S. Watanabe, I. Yamaguchi, Y. Kohda, J. Pharm. Biomed. Anal. 35 (2004) 1307.
[52] K. Katori, M. Homma, K. Kuga, I. Yamaguchi, K. Sugibayashi, Y. Kohda, J. Pharm. Biomed. Anal. 32 (2003) 375.
[53] L. Gonzalez, J.A. Lopez, R.M. Alonso, R.M. Jimenez, J. Chromatogr. A 949 (2002) 49.
[54] C. Hempen, L. Glasle-Schwarz, U. Kunz, U. Karst, Anal. Chim. Acta 560 (2006) 41.
[55] P. Li, Y. Wang, Y. Wang, Y. Tang, J.P. Fawcett, Y. Cui, J. Gu, J. Chromatogr. B 828 (2005) 126.
[56] C. Dupuis, J.M. Gaulier, A.L. Pelissier-Alicot, P. Marquet, G. Lachatre, J. Anal. Toxicol. 28 (2004) 674.
[57] R.D. Johnson, R.J. Lewis, Forensic Sci. Int. 156 (2006) 106.
[58] E. Caudron, S. Laurent, E.M. Billaud, P. Prognon, J. Chromatogr. B 801 (2004) 339.
[59] M. Gergov, I. Ojanpera, E. Vuori, J. Chromatogr. B 795 (2003) 41.
[60] F. Mangani, G. Luck, C. Fraudeau, E. Verette, J. Chromatogr. A 762 (1997) 235.
[61] S. Ojanpera, A. Pelander, M. Pelzing, I. Krebs, E. Vuori, I. Ojanpera, Rapid Commun. Mass Spectrom. 20 (2006) 1161.
[62] J. Yawney, S. Treacy, K.W. Hindmarsh, F.J. Burczynski, J. Anal. Toxicol. 26 (2002) 325.
[63] K.H. Esbensen, Multivariate Data Analysis – in practice. An introduction to multivariate data analysis and experimental design, CAMO ASA/CAMO Process AS, 2001.
[64] G.E.P. Box, S.J. Hunter, W.G. Hunter, Statistics for Experimenters: Design, Innovation, and Discovery, John Wiley and Sons Inc., New York, 2005.
[65] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[66] The Society of Forensic Toxicologist and The Toxicology Section of the American Academy of Forensic Sciences. SOFT/AAFS Forensic Laboratory Guidelines – Version 2006. http://www.soft-tox.org. 20-10-2006.
[67] H.H. Maurer, Anal. Bioanal. Chem. 381 (2005) 110.
[68] M.K. Angier, R.J. Lewis, A.K. Chaturvedi, D.V. Canfield, J. Anal. Toxicol. 29 (2005) 517.
[69] S.B. Black, A.M. Stenhouse, R.C. Hansson, J. Chromatogr. B Biomed. Sci. App. 685 (1996) 67.
[70] O.v. Richter, M. Eichelbaum, F. Schonberger, U. Hofmann, J. Chromatogr. B Biomed. Sci. App. 738 (2000) 137.
[71] J. Blanchard, J. Chromatogr. B Biomed. Sci. App. 226 (1981) 455.
[72] A.C. Moffat, M.D. Osselton, B. Widdop, L.Y. Galichet, Clarke's Analysis of Drugs and Poisons in Pharmatceuticals, Body Fluids and Postmortem Material, Pharmaceutical Press, 2004.
[73] G. Jürgens, N.A. Graudal, J.P. Kampmann, Clin. Pharmacokinet. 42 (2003) 647.
[74] R. Donnelly, Br. J. Clin. Pharmacol. 57 (2004) 535.