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Gas chromatographic–mass spectrometric determination of β_2-agonists in postmortem blood: application in forensic medicine

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Abstract

A solid-phase extraction procedure is described for the simultaneous determination of terbutaline, salbutamol and fenoterol in human postmortem whole blood, using gas chromatography–electron impact mass spectrometry. The limit of quantitation in 1 ml of blood was 1 ng/ml for all analytes. A linear response was observed over the concentration ranges tested, covering both low and high concentrations of each drug. The recoveries in postmortem blood were: terbutaline, 88%; salbutamol, 86%; fenoterol, 92%; orciprenaline (internal standard), 86%. Coefficients of variation for both intra-assay precision and inter-assay reproducibility ranged between 2.2 and 13.0% for all analytes. This method is sensitive and selective, and has been applied successfully to over 60 postmortem blood specimens.

Keywords: Terbutaline; Salbutamol; Fenoterol

1. Introduction

Asthma is one of the most common chronic diseases in Australia, affecting approximately 1 in 5 children and 1 in 10 adults [1]. It also causes substantial morbidity, with over 200 deaths occurring in the state of Victoria, each year [2].

The β_2-adrenoceptor agonists are the most popular first line treatment for the majority of mild to severe asthmatics. They are usually self-administered via metered dose inhalers or nebulisers. Salbutamol and terbutaline are the most widely used β_2-agonists in Australia.

There are concerns regarding the possible contribution of β_2-agonists in asthmatic deaths [3–8]. A proposed mechanism by which this may occur is the excessive use of β_2-agonist inhalants during asthma attacks, leading to cardiac arrhythmia. Over-reliance on inhaled β_2-agonists, together with the under treatment of severe asthma with preventive corticosteroids, may offer further explanations of the possible mechanisms contributing to death.

Measurements of blood concentrations of β_2-agonists are therefore important to establish the possible contribution of drug use to the death.

Numerous LC and GC–MS methods for the determination of β_2-agonists in biological fluids have been published [9–16]. However, they have focused primarily on antemortem plasma, serum or urine specimens. Few have dealt with postmortem specimens [9,10], especially blood which is often haemolysed and degraded from decomposed bodies.

The objective of this study was to develop a sensitive and reliable assay for determining β_2-agon-

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ist concentrations in postmortem and clinical whole blood specimens.

2. Experimental

2.1. Reagents and materials

Acetonitrile and methanol (Mallinckrodt, Australia) were of analytical HPLC grade and ethyl acetate (Ajax Chemicals, Australia) was redistilled prior to use. Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (BSTFA–1% TMCS) was purchased from Pierce (Australia).

Sodium phosphate buffer (pH 7.5) was prepared by adding 50 ml of 0.1 *M* sodium dihydrogen orthophosphate to 150 ml of 0.1 *M* disodium hydrogen orthophosphate (Ajax Chemicals).

Sep-Pak Vac 1-ml C_{18} cartridges were purchased from Waters, Millipore (Australia). Disposable borosilicate culture tubes (Kimble, Biolab Scientific, Australia) and reacti-vials (Pierce, Australia) were silanised with a 5% solution of Surfasil (Pierce, IL, USA) in toluene for 1 h, then rinsed twice in methanol prior to drying.

2.2. Standards and controls

Pure standards of salbutamol hemisulfate and terbutaline hemisulfate were purchased from Sigma (St. Louis, MO, USA). Fenoterol hydrobromide and orciprenaline sulfate were obtained from Boehringer Ingelheim (Melbourne, Australia). Stock solutions for all drugs (1 mg/ml) were prepared daily in deionised water.

Working standards were prepared by diluting stock solutions with deionised water to give concentrations of 10 μg/ml, 1 μg/ml and 0.1 μg/ml. Five-point calibration curves were constructed for each analyte by adding working standards to drug free blood, to give concentrations ranging from 1–15 ng/ml for terbutaline, 1–100 ng/ml for salbutamol and 1–10 ng/ml for fenoterol. For salbutamol concentrations above 100 ng/ml, the blood specimen was diluted and/or the calibration curve extended.

Each assay included in-house controls to provide a measure of quality assurance. These were made from drug-free blood prepared with 5, 15 and 2.5 ng/ml of terbutaline, salbutamol and fenoterol, respectively.

2.3. Specimens

Postmortem blood was obtained from deceased persons autopsied at the Victorian Institute of Forensic Medicine, whose cause of death had been given as due to asthma. Blood specimens were stored at −20°C in tubes containing sodium fluoride and potassium oxalate (1%).

2.4. Extraction details

Aliquots (1 ml) of blood standards, controls and unknowns were added to 10-ml poly-propylene plastic extraction tubes (Biolab Scientific, Australia). Orciprenaline (10 μl of 10 μg/ml solution) was added to each tube as the internal standard. A 2-ml volume of 0.1 *M* sodium phosphate buffer, pH 7.5 was added. The tubes were mixed, then sonicated briefly using an ultrasonic processor (Extech Equipment, Australia). The samples were centrifuged at 3500 rpm for 10 min and the supernatant transferred to clean plastic extraction tubes. The pellet was discarded.

The samples were applied to 1-ml C_{18} Sep-Pak Vac cartridges which had been pre-conditioned with 3 ml methanol–acetonitrile (50:50), 2 ml of deionised water and 2 ml of 0.1 *M* sodium phosphate buffer pH 7.5. The samples were allowed to run through under gravity. The cartridges were left to dry briefly before being washed thoroughly with 5–10 ml of deionised water. After thorough drying of the cartridges under full vacuum for 20 min, the analytes of interest were eluted with 2 ml of methanol–acetonitrile (50:50) into culture tubes.

The 2-ml eluate was transferred to reacti-vials and evaporated under nitrogen at 70°C. A 15-μl volume of BSTFA–1% TMCS and 60 μl of ethyl acetate were added and the solution was allowed to derivatise for 15 min at 70°C. After cooling to room temperature, the derivatised samples were transferred to autosampler vials.

2.5. Chromatography

A 2-μl aliquot was injected into a gas chromatograph (Hewlett- Packard 5890A) attached to a mass-selective detector (HP 5970A). Data was recorded on a HP-UNIX 98785A Chem Station. The capillary column was a 25 m Ultra-2, crosslinked 5% phenyl

methyl silicone column, 0.2 mm I.D, 0.33 μm film thickness, using helium as the carrier gas. The injector and detector temperatures were maintained at 250°C and 310°C, respectively. The temperature program was initially set at 90°C for 2 min, increasing at 15°C/min until 310°C, then held for 15 min.

Analysis was accomplished by selected ion monitoring of ions between 18 and 21 min at *m/z* 356 and 72 for orciprenaline, *m/z* 356 and 86 for terbutaline, *m/z* 369 and 86 for salbutamol and between 25 and 27 min for ions *m/z* 322 and 412 for fenoterol. The choice of base and qualifier ions was determined from the mass spectra of the derivatised compounds (Fig. 1). β_2-Agonist drugs were identified based on a comparison of peak retention time to drug standards and the relative abundance and ratio of the base and qualifier ions (±20%).

2.6. Method validation

Coefficients of variation for intra-assay precision and inter-assay reproducibility were calculated at 3 levels for each analyte. These quality control specimens were prepared by spiking blank blood with the following concentrations: 1 ng/ml of terbutaline, salbutamol and fenoterol, in the low control; 5, 15 and 2.5 ng/ml of terbutaline, salbutamol and fenoterol, respectively, in the mid-range control; and 10, 100 and 5 ng/ml of terbutaline, salbutamol and fenoterol, respectively, in the high control.

Recoveries of the various analytes were calculated by comparing the peak area response of an extracted and derivatised blood standard, spiked with known drug concentrations, to the peak area of known amounts of unextracted derivatised standards.

3. Results and discussion

Fig. 1 shows the EI mass spectrum of the TMS derivatives. The 3 prominent ions for orciprenaline, terbutaline and salbutamol were similar, whilst the spectrum of fenoterol showed a greater degree of fragmentation. Table 1 shows the characteristic ions and the relative abundances for each of the β_2-agonists.

For orciprenaline, the 2 main ions were *m/z* 72, formed by β cleavage of the isopropyl amino portion, and *m/z* 356 [M−72]. The spectrum for terbutaline gave ions at *m/z* 86, formed by β-cleavage of *tert.*-butyl aminomethylene, and *m/z* 356 [M−86], whilst *m/z* 86 and *m/z* 369 [M−86] were the main ions for salbutamol. Similar β-cleavage also occurred for fenoterol, producing ions at *m/z* 356 and *m/z* 236. The ion *m/z* 412 was produced by cleavage of $CH_2 \cdot C_6H_4$-O-TMS (i.e. [M−179]), and the *m/z* 322 ion was a product of [M−179] with an additional loss of the O-TMS from the side chain and formation of a double bond adjacent to the phenyl group. A similar, minor fragmentation pathway was also observed with the other silylated β_2-agonists. The loss of O-TMS from the [M−15] ions and the formation of a double bond, gave ions *m/z* 322, *m/z* 336 and *m/z* 350 for orciprenaline, terbutaline and salbutamol, respectively. The *m/z* 73 ion, arising from TMS [$(CH_3)_3Si$], was common to all of the silylated β_2-agonists, as was the *m/z* 147 ion $[(CH_3)_2Si{=}O{-}Si(CH_3)_3]^+$, which is common in polyhydroxy TMS compounds containing 2 or more TMS groups [17].

Based on the percentage of the total signal produced, 2 ions were usually chosen for selected ion monitoring of each of the silylated β_2-agonists. Other ions can be chosen for confirmation purposes, however, selected ion monitoring of these ions would increase the detection limit.

The accuracy, precision and reproducibility of the described extraction method was investigated at three levels for each analyte and the results are shown in Table 2. The accuracy of all analytes for both intra- and inter-assay ranged between 92–113%, which was within ±20% of the target concentrations. The coefficients of variation for all analytes were less than 9.7% (intra-assay) and 13.0% (inter-assay) for the low control, and less than 5.8% (intra-assay) and 8.7% (inter-assay) for the mid-range control. For the high control, the coefficients of variation were less than 5.0% and 6.7% for intra-assay and inter-assay, respectively.

Calibration curves were constructed by calculating the peak-area ratios of all drugs relative to the internal standard at each known concentration. Linear regression was performed and the standard curves were found to be linear over the concentration ranges tested. All three analytes gave regression fits of $r^2 \geq 0.99$. Typical equations were: terbutaline $y = 8.0247e{-}3 + 1.1776e{-}2x$, salbutamol $y = 1.2525e{-}2 + 1.8880e{-}2x$ and fenoterol $y = 5.8143e{-}3 +$

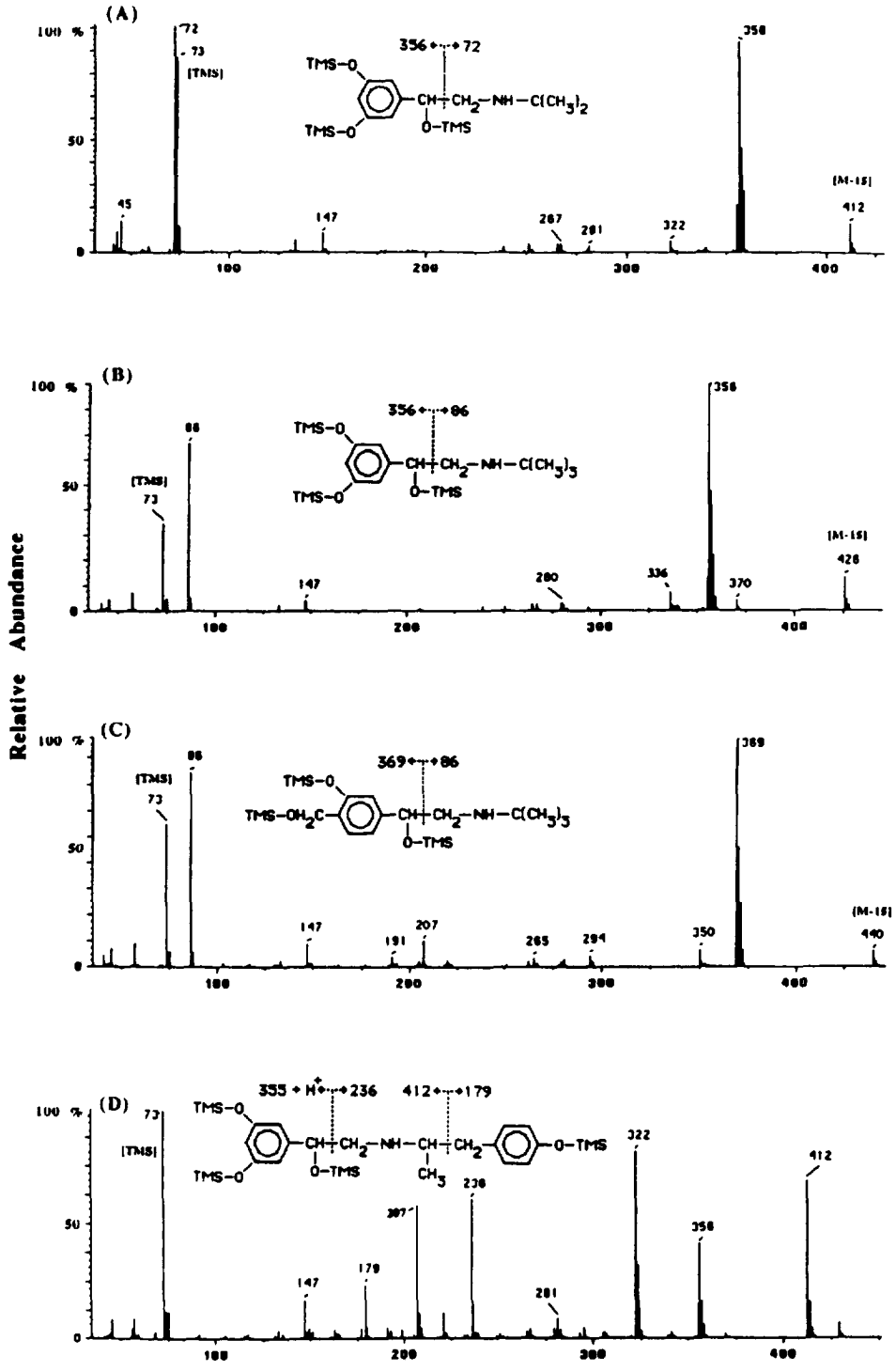

Fig. 1. Mass spectra of the TMS-derivatives of (A) orciprenaline [MW 428], (B) terbutaline [MW 442], (C) salbutamol [MW 455] and (D) fenoterol [MW 591].

Table 1
Fragment ions of the TMS-derivatives of β_2-agonists

Derivative	[M]	*m/z* values of characteristic fragment ions[a]
TMS-orciprenaline	428	72(100) 356(95) 73(87) 357(47) 358(27) 356(22) 45(15) 412(13) 147(9)
TMS-terbutaline	442	356(100) 86(74) 357(51) 73(38) 358(24) 426(13) 355(12) 336(7) 147(4)
TMS-salbutamol	455	369(100) 86(83) 73(62) 370(52) 371(28) 207(11) 147(10) 350(7) 440(6)
TMS-fenoterol	591	73(100) 322(86) 412(70) 236(61) 207(58) 356(45) 323(33) 179(23) 147(17)

[a] Relative abundances are given in parentheses.

$2.5927e-2x$. The average extraction recoveries for the described method in postmortem blood were terbutaline 88%, salbutamol 86%, fenoterol 92% and the internal standard 86%.

Previous studies on the analysis of terbutaline and salbutamol in postmortem serum and blood have not provided coefficients of variation and recovery data [9,10]. The recoveries, precision and reproducibility of our method for the analysis of β_2-agonists in postmortem whole blood are comparable to those obtained by others using antemortem serum or plasma specimens [11,15,16].

Table 2
Accuracy, precision and reproducibility data for β_2-agonists in postmortem blood

β_2-Agonist	Concentration added (ng/ml)	Concentration detected[a] (ng/ml)	Accuracy (%)	C.V. (%)
Intra-assay (n=6–8)				
Terbutaline	1.0	1.03±0.10	103	9.7
	5.0	5.08±0.21	102	4.1
	10.0	10.07±0.24	101	2.4
Salbutamol	1.0	1.01±0.09	101	8.9
	15.0	14.98±0.54	100	3.4
	100	99.43±2.22	99	2.2
Fenoterol	1.0	0.92±0.07	92	7.6
	2.5	2.59±0.15	104	5.8
	5.0	5.04±0.25	101	5.0
Inter-assay (n=6)				
Terbutaline	1.0	1.13±0.13	113	11.5
	5.0	5.06±0.30	101	5.9
	10.0	10.33±0.57	103	5.5
Salbutamol	1.0	1.08±0.14	108	13.0
	15.0	15.19±0.86	101	5.6
	100	102.8±4.52	103	4.4
Fenoterol	1.0	0.97±0.11	97	11.3
	2.5	2.44±0.21	98	8.7
	5.0	4.97±0.33	99	6.7

[a] Mean±S.D.

Low limits of detection are required for the analysis of β_2-agonists in body fluids due to the low concentrations obtained after inhalation. The limit of detection was determined by estimating the minimum concentration equivalent to or greater than three times the background noise whilst still allowing detection of the qualifier ion ratio. This gave detection limits of 1 ng/ml for all analytes for a 1 ml blood specimen. At 1 ng/ml the coefficient of variation following repetitive analyses was ≤20% for all analytes. This was used as the practical limit of quantitation. Lower limits of detection could be obtained if the base ion only is measured.

Extracts of postmortem blood showed chromatograms free of significant interferences from endogenous peaks and displayed good chromatographic separation of each of the analytes of interest. Fig. 2 shows typical chromatograms for an extracted postmortem blank blood, an extracted spiked blood standard and three extracted postmortem blood specimens. Orciprenaline, terbutaline, salbutamol and fenoterol eluted at approximately 19.29, 19.56, 20.57 and 26.12 min, respectively. An unidentified endogenous peak regularly appeared at 20.67 min, in both blank and postmortem bloods. An extracted postmortem blood specimen from an asthmatic death (Fig. 2C) shows the presence of salbutamol at a concentration of 36 ng/ml, and Fig. 2D shows an extracted postmortem blood specimen with a terbutaline and salbutamol concentration of 4.1 ng/ml and 33 ng/ml, respectively. Fig. 2E shows the presence of salbutamol at a concentration of 663 ng/ml, using 200 μl of postmortem blood obtained from an asthmatic death.

In a preliminary study, postmortem blood specimens were obtained from 24 asthmatic deaths and analysed for the presence of β_2-agonists (Table 3). 19 cases were positive for salbutamol, with concentrations ranging from 2.3–1690 ng/ml. The me-

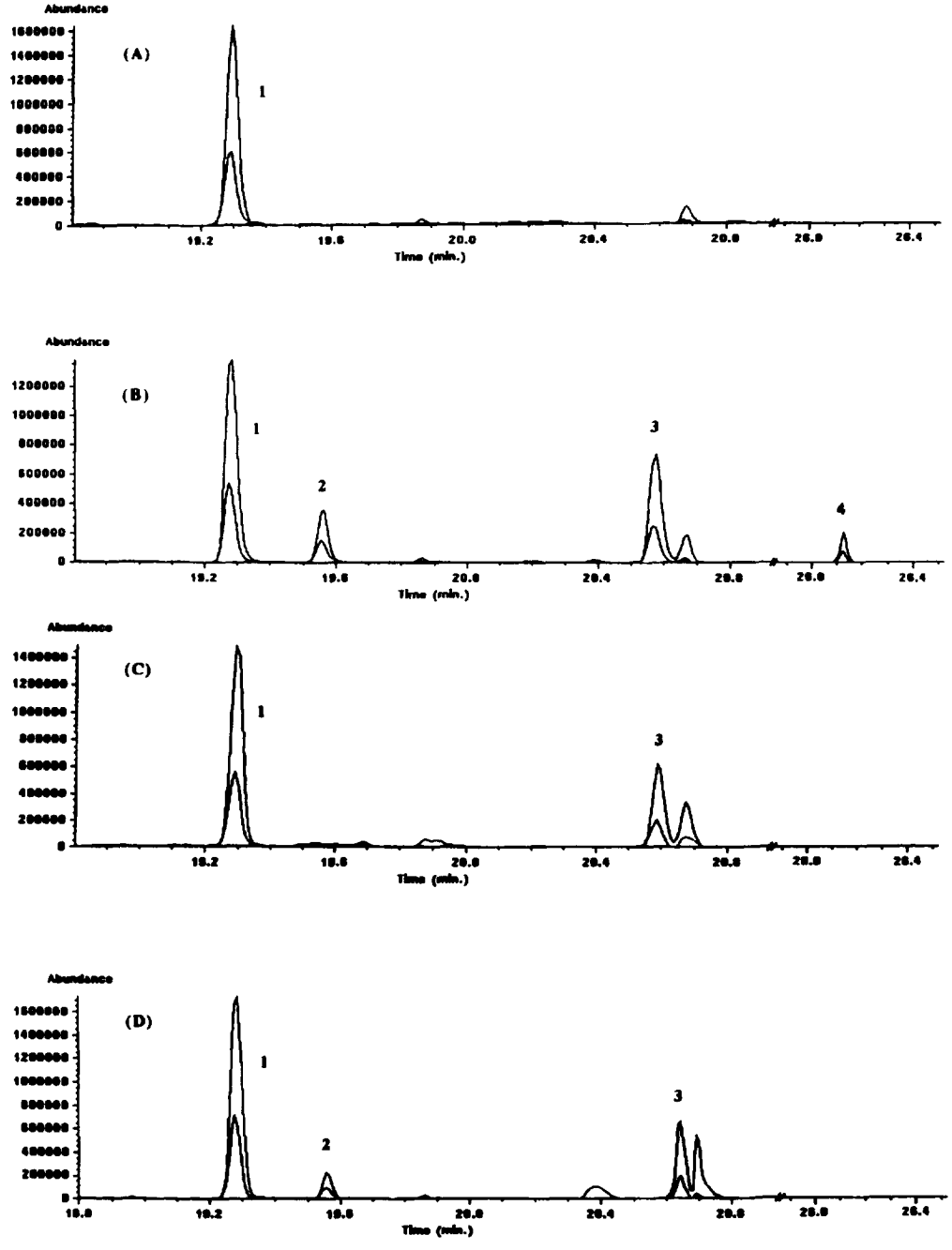

Fig. 2. GC–TIC-MS chromatograms showing the response to (A) an extracted postmortem blank blood containing 100 ng/ml of orciprenaline (1) as internal standard; (B) an extracted blood standard containing 100 ng/ml of orciprenaline, 15 ng/ml of terbutaline (2), 50 ng/ml of salbutamol (3) and 10 ng/ml of fenoterol (4); (C) an extracted postmortem blood specimen from an asthmatic, containing 36 ng/ml of salbutamol; (D) an extracted postmortem blood specimen containing 4.1 and 33 ng/ml of terbutaline and salbutamol, respectively; and (E) an extracted postmortem blood specimen (200 μl) from an asthmatic, containing 663 ng/ml of salbutamol. Conditions defined in Section 2.6.

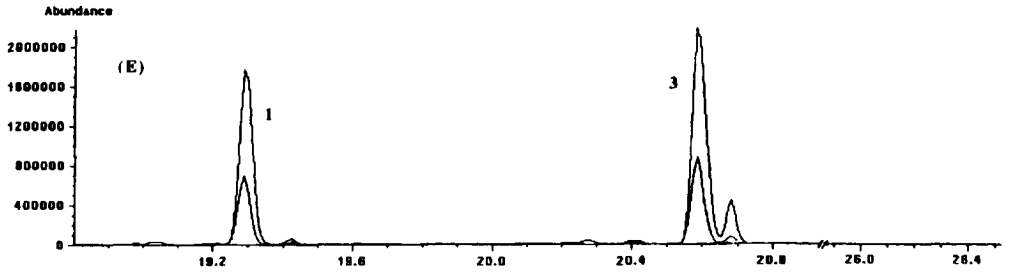

Fig. 2. (*continued*)

dian concentration was 33.6 ng/ml. Over 50% of the bloods analysed were higher than the reported therapeutic range for salbutamol of up to 20 ng/ml [12]. In a previous study, salbutamol concentrations measured in 5 patients admitted with salbutamol self-poisoning ranged from 50–76 ng/ml [13]. The 3 highest salbutamol concentrations in our study were 663, 1494 and 1690 ng/ml, which may represent potentially toxic concentrations of salbutamol in blood.

Four asthmatic cases were positive for terbutaline, with concentrations ranging from 2.8–4.9 ng/ml, and the median concentration was 4.0 ng/ml. These correspond to reported therapeutic concentrations of up to 10 ng/ml [18]. Fenoterol was not detected in any of the 24 cases and 3 cases were negative for all β_2-agonists.

A number of cases ($n=6$) were also analysed in which β_2-agonists were known not to be involved. Terbutaline, salbutamol and fenoterol were not detected in these cases. In addition, the β-agonist adrenaline does not interfere with the chromatography of the above analytes. This method is not only applicable to blood but has been found to be applicable for plasma and urine.

In conclusion, a sensitive and specific GC–MS procedure is described, suitable for the simultaneous determination of terbutaline, salbutamol and fenoterol in postmortem whole blood using solid-phase extraction.

Table 3
Prevalence and blood concentrations of β_2-agonists in 24 postmortem asthmatic deaths

β_2-Agonist	Number of positive cases	Concentration[a] (ng/ml)
Salbutamol	19	2.3–1690 (33.6)
Terbutaline	3	2.8–4.9 (4.0)
Fenoterol	0	-

[a] Median concentrations are given in parentheses.

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