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Clean-up, detection and determination of salbutamol in human urine and serum

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Salbutamol {2-(*tert*-butylamino)-1-[4-hydroxy-3-(hydroxymethyl)phenyl]ethanol}, also known as albuterol, is clinically the most widely used β_2-adrenoceptor agonist in the treatment of bronchial asthma. During this study, we evaluated liquid–liquid extraction (LLE) and solid-phase extraction (SPE) in order to develop a reliable extraction method followed by analysis using liquid chromatography and gas chromatography. An assay is described which involves SPE as the clean-up method followed by gas chromatography-mass spectrometry to determine salbutamol levels in human serum after oral administration. The SPE method requires the use of a hyper-cross-linked styrene–divinylbenzene bonded phase (ENV+) without involving any sample pre-treatment to obtain 60–65% recoveries for salbutamol and terbutaline as the internal standard. Distilled water and 1% trifluoroacetic acid in methanol were found to be the most suitable washing solvent and eluting solvent, respectively. A detection limit of 2 ng mL^{-1} was achieved by derivatization with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide to form trimethylsilyl (TMS)-salbutamol (*m/z* 369) and TMS-terbutaline (*m/z* 356). The relationship between the ratio of the peak area of salbutamol to that of the internal standard and concentration was linear for the range tested (2–200 ng mL^{-1}) and the correlation of coefficient was 0.9999 with a *y*-intercept not significantly different from zero. The inter-day relative standard deviation (RSD) was $< 10\%$ for all three concentrations. The intra-day RSD was 14% for 2 ng mL^{-1}. This assay was then successfully applied to human serum samples obtained from clinical trials after oral administration of salbutamol.

Introduction

Salbutamol {2-(*tert*-butylamino)-1-(4-hydroxy-3-(hydroxymethyl)phenylethanol}, also known as albuterol, is clinically the most widely used β_2-adrenoceptor agonist in the treatment of bronchial asthma (Fig. 1). The metabolism of salbutamol in the human body proceeds mainly *via* the formation of the sulfate conjugate.[1] This process occurs principally in the liver, *via* the action of the liver microsomal enzymes. *In vivo* studies have shown that sulfate formation also occurs in the small intestine. Salbutamol is rapidly absorbed after oral administration and after inhalation. About 60–90% of a dose is excreted in the urine in 24 h, of which approximately 50% is unconjugated salbutamol and 50% is the 4′-*O*-sulfate derivative. Up to about 12% is eliminated in the feces.[2]

In addition to its antiasthmatic effects, β_2-agonists have been pharmacologically proven to be able to improve nitrogen retention, reduce body fat and promote muscle growth. As a result, there is much interest in the determination of these substances in body fluids. However, the hydrophilicity of the β_2-agonists coupled with the low concentrations found in urine and plasma makes the analysis relatively difficult.

In recent years, various assays have been developed to determine salbutamol in biological matrices. Black and Hansson reported a sensitive and quantitative method for the determination of salbutamol and other β_2-agonists in *post-mortem* human whole blood and urine using GC-MS in the selected ion monitoring (SIM) mode.[3] Ramos *et al*. described a derivatization procedure for confirmatory residue analysis of urine for β_2-agonists using methyl-(MBA) and butyl boronic acid (BBA)[4] and a simple and sensitive method for the determination of salbutamol in rat tissues using liquid chromatography with electrochemical detection.[5] Gupta *et al*. claimed to achieve a quantification limit of 2 ng ml^{-1} by reversed phase column liquid chromatography with fluorescence detection for the determination of salbutamol levels in plasma.[6] In this paper, we propose a GC-MS assay for salbutamol using a solid-phase extraction (SPE) procedure that is fast, reliable and meets the sensitivity requirements for both drug residue analysis and bioequivalence purposes.

Experimental

Chemicals, buffer and instruments

Salbutamol, terbutaline and bamethan were obtained from Sigma (St. Louis, MO, USA). Trifluoroacetic acid (TFA) of HPLC grade and triethylamine (TEA) were purchased from Fluka (Buchs, Switzerland). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and sodium 1-octanesulfonate were supplied by Sigma. Phosphoric acid, sodium dihydrogenphosphate, potassium hydroxide, sodium chloride and ammonium acetate were purchased from R & M Chemicals (Essex, UK). Dimethyldichlorosilane (5% in toluene) for glassware silanization was purchased from Supelco (Bellefonte, PA, USA). The SPE cartridges ENV+, HCX, C_{18}, CN-silica (ISOLUTE International Technologies, UK) and OASIS HLB (Waters, Milford, MA, USA) were used in the extraction evaluation. All solvents were analytical-reagent and HPLC grade.

Preparation of standard solutions

Stock standard solutions (100 μg mL^{-1}) of salbutamol, terbutaline and bamethan were prepared in both UHQ water and HPLC grade methanol and were stored at 4 °C until used. All the stock standard solutions were further diluted to obtain 10, 1 and 0.1 ng μL^{-1} working standard solutions for the preparation of spiked salbutamol in human serum and urine.

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Preparation of spiked human serum and urine samples

Human serum. Accurately measured aliquots of the diluted salbutamol standard solutions were each placed in silanized tapered tubes followed by the addition of 100 μL of 1 ng μL^{-1} terbutaline standard solution. A 0.5 mL volume of blank serum was added to volume to give standard solutions containing 2, 4, 10, 20, 100 and 200 ng mL^{-1} of salbutamol.

Human urine. Accurately measured aliquots of the diluted salbutamol standard solutions were each placed in silanized tapered tubes followed by the addition of 100 μL of 1 μg μL^{-1} bamethan standard solution. A 3 mL volume of blank urine was added to volume to give standard solutions containing 1, 2, 5, 10, 50 and 100 μg mL^{-1} of salbutamol.

Instrumentation and chromatographic conditions

GC-MS analyses were performed using an HP system (Hewlett-Packard, Palo Alto, CA, USA) consisting of an HP 6890 gas chromatograph connected to an HP 5972 quadrupole mass spectrometer. The gas chromatograph was equipped with an HP-5 cross-linked 5% phenylmethyl siloxane fused silica capillary column (25 m × 0.2 mm id, 0.11 μm film thickness). Helium was used as the carrier gas with a head pressure of 10.4 psi. The injector and transfer line was maintained at 230 °C. Several column temperature programs were studied in order to achieve optimal separation. The mass spectrometer conditions were as follows: electron ionization, ion source temperature 110 °C and ionization voltage 70 eV for both full scan mode and SIM mode. In the SIM mode, the ions at *m/z* 369 for salbutamol and 356 for terbutaline were detected.

Development of extraction methods

Extraction procedure. The 1 mL ISOLUTE ENV+ cartridges containing 10 mg of sorbent were positioned in the respective Luer fittings of the vacuum manifold. No vacuum pressure was applied for the extraction. The cartridges were preconditioned with 1 mL of methanol and 1 mL of distilled water. An aliquot of 0.5 mL of serum was loaded in each cartridge and the cartridges were subsequently washed with 1 mL of washing solvent. Several types of washing solvent were studied, namely distilled water, 5, 10 and 20% methanol in distilled water, 10% acetonitrile in distilled water and 10% acetic acid in distilled water. A vacuum of 10–20 cmHg was applied for 5 min to dry the cartridges. Elution of the analytes was performed with two 500 μL portions of either methanol or 1% TFA in methanol. The eluates were evaporated to dryness under a gentle stream of nitrogen at 37 °C. The dried residue was tightly capped with a stopper after derivatization with 50 μL of MSTFA at 60 °C for 30 min. The derivatized sample was transferred into a 100 μL autosampler vial. The derivative (1 μL) was then injected into the GC column by splitless injection using the autosampler.

Extraction recovery. In order to determine the efficiency of the extraction procedure, an identical set of methanolic standard solutions were prepared with concentrations equal to those in the spiked plasma and urine standards. These methanolic standards were evaporated to dryness under a gentle stream of nitrogen at 37 °C. The dried standard was derivatized with 50 μL of MSTFA at 60 °C for 30 min, then capped tightly with a stopper. The derivatized standard was transferred into a 100 μL autosampler vial. The derivative (1 μL) was then injected into the GC column by splitless injection using the autosampler. The peak area obtained was compared with that obtained from the spiked standard.

Quantification and validation

In order to obtain calibration curves for the assay of human serum, three stock standard solutions of salbutamol in distilled water were independently prepared and appropriate volumes were added to 0.5 mL aliquots of drug-free serum to give three

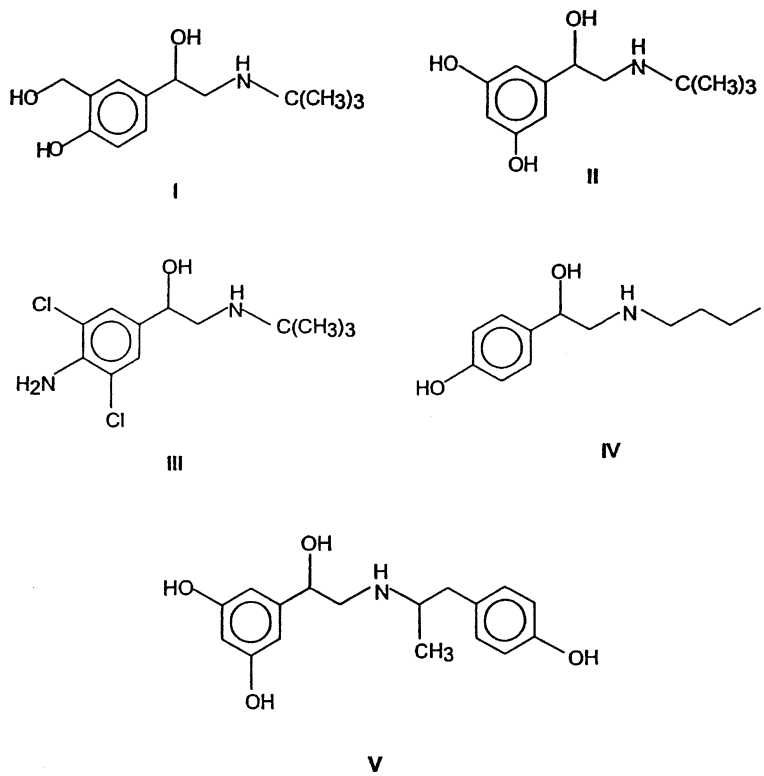

Fig. 1 Structure of some clinically important β-adrenoceptor agonists. I = Salbutamol; II = terbutaline; III = clenbuterol; IV = bamethan; V = fenoterol.

replicate spiked standards of 2, 4, 10, 20, 100 and 200 ng mL^{-1}. The serum standards were subjected to SPE (extraction B) and analyzed as described above.

The intra-day variation of the assay at 2, 20 and 200 ng mL^{-1} was assessed using aqueous standards by extracting and analyzing six replicates of each independently prepared spiked standard on the same day. The inter-day variation was determined at 2, 20 and 200 ng mL^{-1} by extracting and analyzing the spiked standard solutions on six consecutive days.

Pharmacokinetic study and clinical trials

In order to demonstrate the applicability of the present method to analyze biological matrices from human subjects, the concentration of salbutamol in serum samples after 12 human volunteers had received one CR tablet (8 mg) as a single dose without food (standard breakfast). All subjects were healthy males aged 22–36 years, weighing 64–86 kg. Each subject provided a medical history, results of blood analysis and urine analysis and had a physical examination including ECG as a screening process. Blood samples from each subject were obtained at 0 (prior to dosing), 1, 2, 3, 4, 6, 7, 8, 10, 12, 16, 20, 24 and 30 h after administration. Serum was separated from the blood samples and frozen at −20 °C until analysis.

Results and discussion

Evaluation of different extraction methods

In the process of developing a reliable extraction method, we evaluated liquid–liquid extraction (LLE) and SPE using CN-silica, C_{18}, OASIS HLB, ISOLUTE HCX and ISOLUTE ENV+ cartridges. For LLE, we studied four solvent systems: chloroform, ethyl acetate, chloroform-butan-1-ol (4 + 1) and ethyl acetate–propan-2-ol (4 + 1). This method was generally found to give poorer recoveries and proved to be more tedious for β_2-agonists.

Although different bonded phases have been described for extracting salbutamol and/or its metabolite(s) from biological matrices,[3–13] we obtained poor recoveries (30–40%) for salbutamol using CN-silica and ISOLUTE C_{18}. However, high recoveries (80–90%) were achieved using a mixed-phase hydrophobic–cation exchanger (HCX) and 5% TEA in methanol as the eluent. A small amount of TEA should be added to methanol in order to enhance the efficiency of ion exchange as TEA itself behaves as a cation. We also obtained 60–65% recoveries for salbutamol using the polymeric bonded phase ISOLUTE ENV+ and OASIS HLB with distilled water as the washing solvent and 1% TFA in methanol as the eluent.

Distilled water was considered a better washing solvent than other solvents for both extraction methods as the least fraction of analyte was washed out. The choice was made in order to effect a compromise with the elution efficiency in the eluting step. Acidified methanol usually elutes a larger fraction of basic analytes than methanol from a hydrophobic solid phase.

The decision to utilize the ISOLUTE ENV+ as the preferred extraction cartridges was based on the following reasons. SPE using mixed-phase cartridges (HCX) gave better extraction recoveries but involved more steps, *e.g.*, pH adjustment prior to loading, extra washing step and pH adjustment after elution to avoid losses during drying. SPE using HCX cartridges involved larger conditioning, washing and elution volumes because of the larger sorbent mass involved. SPE using OASIS HLB cartridges suffered from poor flow characteristics although producing satisfactory recoveries. SPE using ISOLUTE ENV+ required only 10 mg of sorbent, gave satisfactory extraction recoveries, produced cleaner extracts and had superior flow characteristics. These features led to a robust method with a high throughput when large amounts of sample are involved. Lastly, SPE using ISOLUTE ENV+ is able to extract efficiently many polar compounds that cannot be retained from an aqueous matrix by C_8 or C_{18} silica based sorbents.

Quantification and validation

The relationship between the ratio of the peak area of salbutamol to that of the internal standard and concentration was linear for the range tested (2–200 ng mL^{-1}) and the correlation of coefficient was 0.9999 with a *y*-intercept not significantly different from zero (Fig. 2). The low noise baseline, good extraction recovery and relatively high detector response allow the quantification of salbutamol to levels as low as 2 ng mL^{-1}.

The intra-day and inter-day precision and accuracy were determined by analyzing replicates of plasma samples (n = 6) spiked with salbutamol at 2, 20 and 200 ng mL^{-1} and the internal standard at 100 ng mL^{-1}. As shown in Table 1, the intra-day relative standard deviation (RSD) was > 10% for 2 ng mL^{-1}, indicating that the assay was nearing its limit of quantification. The inter-day RSD was < 10% for all three concentrations.

Chromatographic conditions

The conditions for chromatographic separation using GC-MS-SIM were selected to meet the objectives of achieving high selectivity and sensitivity. At the required sensitivity, it was not possible to eliminate all the serum extraneous peaks by a one-step extraction procedure. The separation of salbutamol and the internal standard from these extraneous peaks has to be achieved by adopting the optimal chromatographic conditions. The column temperature was maintained at 150 °C for 1 min followed by a gradient of 15 °C min^{-1} to 280 °C, which was held for 1 min.

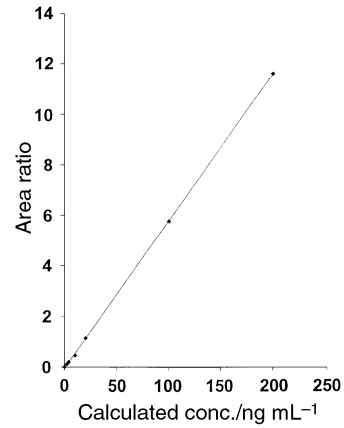

Fig. 2 Calibration curve for the quantification of salbutamol in human serum.

Table 1 Intra- and inter-day variation, accuracy and extraction recoveries of salbutamol in spiked serum samples (n = 6)

Concentration/ng mL^{-1}	RSD (%)		Standard deviation (%)		Recovery (%)	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
2	14.19	8.19	0.1892	0.2838	61.16	60.85
20	6.79	7.83	0.7126	0.9027	60.61	62.51
200	7.43	8.37	8.9859	8.6746	65.72	66.18

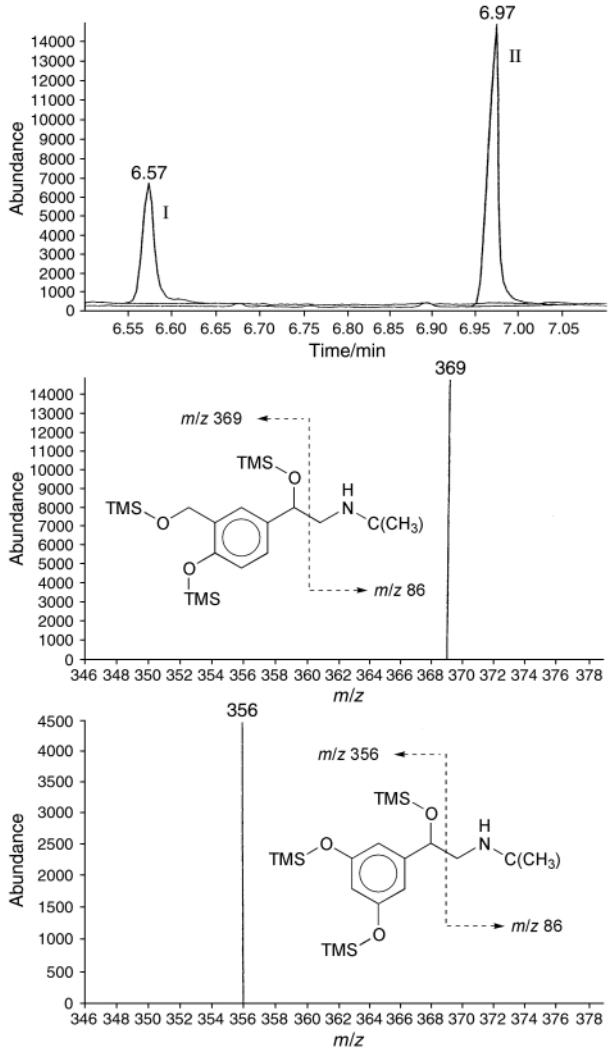

Fig. 3 Chromatogram and mass spectra of a serum sample 2 h after oral administration of salbutamol. I = terbutaline (*m/z* 356); II = salbutamol (*m/z* 369).

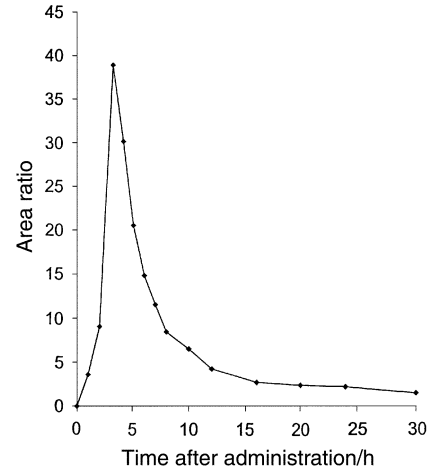

Fig. 4 Pharmacokinetic profile of salbutamol in human serum for 30 h after oral administration of an 8 mg salbutamol sustained-release preparation.

Application to assay of clinical samples

The developed assay was successfully applied to serum samples from 12 volunteers. Fig. 3 shows the chromatogram of a serum sample 2 h after oral administration of salbutamol and the mass spectra of the trimethylsilyl (TMS) ethers of salbutamol (*m/z* 369) and the internal standard (*m/z* 356) obtained by GC-MS-SIM. The retention times for salbutamol and the internal standard are 6.97 ± 0.02 and 6.57 ± 0.02 min, respectively. In this region, there is no significant interference from blank serum. At 2 ng mL^{-1}, salbutamol produced a peak that could be quantified with satisfactory precision and accuracy. Fig. 4 shows the pharmacokinetic profile of salbutamol in all volunteers after oral administration of 8 mg salbutamol in a sustained-release preparation for 30 h. The time of maximum serum salbutamol concentration, T_{max}, was 3–4 h and the serum salbutamol concentration at T_{max}, C_{max}, was calculated as 35–40 ng mL^{-1}.

Conclusion

We have developed an assay that is rapid, sensitive and reproducible to determine serum levels of 2 ng mL^{-1} of salbutamol. This assay involves SPE using a hyper-cross-linked styrene–divinylbenzene bonded phase (ENV+) without any sample pre-treatment to obtain 60–65% recoveries (n = 6) followed by GC-MS. This assay utilizes terbutaline as the internal standard. In this assay, salbutamol and the internal standard were recovered from 0.5 mL of human serum. The samples were then derivatized to TMS ethers using MSTFA followed by analysis by GC-MS with SIM for the ions of *m/z* 369 of TMS-salbutamol and *m/z* 356 of TMS-terbutaline. The correlation coefficient was 0.9999 when the assay was quantified in the concentration range 2–200 ng mL^{-1} in serum. The method was also validated for 2, 20 and 200 ng mL^{-1} of salbutamol in serum. The inter-day RSD < 10% for all three concentrations. The intra-day RSD was 14% for 2 ng mL^{-1}. The method was successfully applied to clinical samples from human volunteers.

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