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New HPLC assay for urinary salbutamol concentrations in samples collected post-inhalation

S.H.R.A. Mazhar[a], H. Chrystyn[b,*]

[a] School of Pharmacy and Institute of Pharmaceutical Innovation, School of Life Sciences, University of Bradford, Bradford BD7 1DP, UK
[b] Division of Pharmacy and Pharmaceutical Sciences, School of Applied Sciences, University of Huddersfield, Queensgate, Huddersfield HD1 3DH, UK

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ABSTRACT

A new reversed phase high performance liquid chromatography (HPLC) method with florescence detection and two solid phase extraction (SPE) methods have been developed, optimised and validated for determining salbutamol in human urine after an inhalation. SPE methodology for unchanged salbutamol (USAL) and salbutamol plus its metabolites (USALMET) concentrations in urine has been developed using terbutaline as the internal standard. Confirm HCX cartridges were used for USAL and Oasis HLB for USALMET. Calibration lines of salbutamol urine standards were linear over the range 25–300 μg/L with mean (RSD) r^2 values of 0.9983 (0.06%) for USAL and 0.9976 (0.202%) for USALMET. The HPLC method was accurate (mean bias −0.40% for USAL and 0.46% for USALMET) and precise (mean RSD 5.0% for USAL and 2.90% for USALMET). The calculated LOD and LOQ for salbutamol using a 1 mL urine sample were 4.0 and 12.12 μg/L for USAL, and 4.80 and 14.56 μg/L for USALMET, respectively. The mean (RSD) SPE recoveries of salbutamol were 90.82% (2.32%) for USAL and 91.54% (2.96%) for USALMET. Both HPLC and SPE methods were applied to quantify unchanged and metabolised salbutamol excreted in urine after the inhalation of 200 μg salbutamol from metered dose inhalers (MDIs) by 14 healthy volunteers. Charcoal slurries were also ingested to prevent gastro-intestinal absorption. Urine samples were collected at 30 min post-inhalation and then pooled for the next 24 h. All urine concentrations were within the sensitive portion of the assay. The volunteer study revealed that following inhalation from an MDI about 20% of the nominal dose is deposited into the lungs and 46% is delivered to the systemic circulation. The results confirm the application, sensitivity, reliability and robustness of the HPLC and SPE methods for urinary pharmacokinetic studies after salbutamol inhalations using therapeutic doses.

1. Introduction

Salbutamol is a widely prescribed β_2-agonist for relieving bronchospasm in patients with asthma and Chronic Obstructive Pulmonary disease [1]. Salbutamol is first-pass metabolised to an inactive sulphate conjugate in the liver and in the gut wall [2]. It is rapidly absorbed from the gastro-intestinal tract and excreted in the urine as the unchanged drug or as the sulphate ester metabolite. Following inhalation, about 10–30% of the salbutamol dose is reported to be absorbed from the lung and most of the remaining inhaled dose is swallowed and absorbed from the gut [3]. It has been shown that after an inhalation salbutamol is rapidly delivered to the body via the pulmonary route whereas there is a lag time for salbutamol to be absorbed following oral administration [4]. Salbutamol and its metabolite are excreted by the renal route. It has been shown that the amount of salbutamol excreted in the urine in the first 30 min after an inhalation is an index of lung deposition [4]. This index, the relative lung bioavailability, is used as a yardstick in inhalation bioequivalence studies. Also, it has been shown that the amount of salbutamol and its metabolite excreted in the urine over the 24-h period after an inhalation represents the systemic delivery. This index is termed as the relative bioavailability following an inhalation [4].

Although several reversed phase high performance liquid chromatography (HPLC) methods have been reported, for the quantification of salbutamol, only three have used a urine matrix. Morgan et al. [2] worked at low sensitivity because of the presence of interfering peaks in urine while Clark et al. [5] did not hydrolyse urine samples to assay the salbutamol ester metabolite. Both methods do not use an internal standard.

The analysis method of Hindle and Chrystyn [4] has been widely used to identify the relative lung and systemic bioavailability following an inhalation [3]. However, the run time of this assay is long (>50 min) because of the need to split some interferences with the salbutamol and the internal standard (bamethane) peaks which frequently could not be baseline resolved. Also, the solid phase

* Corresponding author. Tel.: +44 1484 472783; fax: +44 1484 272182.
E-mail address: h.chrystyn@hud.ac.uk (H. Chrystyn).

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extraction (SPE) method produced variable recoveries of salbutamol and bamethane. Further, these recoveries from the hydrolysed urine samples were very low (≤30% and ≤40%, respectively). The aim of this study was therefore to overcome these difficulties. Hence a new HPLC method with a reasonably short run time and new SPE methods have been developed, optimised and validated using terbutaline as the internal standard. Two SPE methods have been identified and validated. The first SPE method uses mixed-mode cationic cartridges but can only be used for unchanged salbutamol (USAL). The second method uses polymeric cartridges and can be used for both unchanged and total salbutamol (unchanged plus metabolised) (USALMET). Since many studies only use data for unchanged salbutamol in the first 30 min then the first method is recommended because the extraction cartridges are much cheaper and the sample preparation time is shorter. The application of the assay to quantify salbutamol excreted in urine from participants of an inhaler study is reported.

2. Experimental

2.1. Materials and reagents

All solvents used for chromatography and SPE were of HPLC grade (BDH, UK). Reagent grade orthophosphoric acid (H_3PO_4, 85%, specific gravity 0.85), 7N hydrochloric acid (HCl), ammonia solution (35%, specific gravity 0.88), sodium dodecyl sulphate (Biochemical; SDS), potassium dihydrogen phosphate (KH_2PO_4) and potassium hydroxide pellets (KOH) were also obtained from BDH (UK). Ultra-purified (deionised) water was prepared in-house using a Milli-Q Reagent Water System (Millipore). Salbutamol base, terbutaline hemisulphate and other drugs and compounds tested for method specificity and selectivity were purchased from Sigma–Aldrich (UK).

2.2. Preparation of urine and aqueous standards

Blank urine was obtained from 14 (7 females) volunteers for the preparation of stock and working standard solutions. Quality control (QC) standards were prepared from separate stock solutions. All urine standard solutions and blanks were transferred to polypropylene tubes (25 mL) and frozen at −20 °C. Previous studies have shown that when prepared and frozen at −20 °C, these standards were stable for 12 months [6]. All standard solutions were therefore frozen and used within 12 months. Terbutaline sulphate aqueous solution (500 μg/L) was used as the internal standard *in situ* during the SPE. Salbutamol aqueous standards were prepared in parallel concentrations each containing 500 μg/L terbutaline sulphate. All aqueous standards were stored at 5 °C.

2.3. Solid phase extraction methods

Varian Vac Elute workstations (10 cartridge ports) with on-line laboratory vacuum were used for extraction. Eluates were dried under nitrogen (N_2) in the fume cupboard using a Techne Dri-Block DB-3A sample concentrator, reconstituted with 1 mL mobile phase, thoroughly mixed and transfered to autosampler vials (screw-capped with air-tight seals) for injection onto the HPLC system.

2.3.1. SPE pre-hydrolysis (USAL method)

Solid phase extraction of unchanged salbutamol was carried out using mixed-mode cationic-exchange Isolute Confirm HCX 130 mg cartridges with a 10 mL capacity (IST, UK). To each urine sample (1 mL), internal standard terbutaline (1 mL) was added. Similarly, in the blank urine sample, purified water was added instead of the internal standard. The samples were buffered with 2 mL of 30 mM KH_2PO_4 (pH 7.0) and vortex mixed. The pH of the pre-treated samples (4 mL) was checked. The cartridges were solvated with MeOH (1 mL) and then equilibrated with 1 mL of 15 mM KH_2PO_4 (pH 7.0) followed by loading of the pre-treated samples. Then 2 mL of 15 mM KH_2PO_4 (pH 7.0) was added to the cartridge followed by 2 mL of HCl (0.00001N, pH 5.0). The cartridges were then dried for ∼2 min using a full vacuum. After passing 1 mL HCl (0.005N, pH: 2.50) through the cartridges using a low vacuum they were dried for ∼5 min using a full vacuum. The cartridges were washed with 1.5 mL of MeOH:H_2O (75:25) and then dried again for ∼5 min under full vacuum. The analytes (salbutamol and terbutaline) were eluted with 1 mL of NH_3:MeOH (6:94) into glass test tubes. Low vacuum was applied for ∼2–3 min following complete elution. The eluates were concentrated at 60 °C under a gentle stream of N_2 for ∼15 min.

The samples were allowed to run through under gravity. Care was taken not to let the cartridges dry in any step before sample application.

2.3.2. SPE post-hydrolysis (USALMET method)

Oasis HLB 30 mg in 1 mL (Waters, UK) polymeric cartridges were used for the extraction of total salbutamol (unchanged plus metabolised) and the internal standard. To each cartridge, a 25 mL reservoir (IST, UK) was attached at the top using an adapter (Supelco, UK). The flow rate of each sample application and elution was maintained between 1 and 2 mL/min using a low flow vacuum applied throughout the SPE.

The urine samples were first hydrolysed to convert all metabolised salbutamol back to the free salbutamol. The hydrolysis procedure was 1 mL urine sample and 1 mL terbutaline internal standard in a glass test tube to which 8 mL of 0.1N HCl was added and vortex mixed (10 mL). The test tubes were covered with aluminum foil and placed in a boiling water bath for 60 min. Blank urine was treated similarly except that 1 mL of purified water was added instead of the internal standard. After acid hydrolysis, the samples were left to cool and then 1 mL of 0.5 M KH_2PO_4 (pH 13.0) was added and vortex mixed. The pH of the neutralised hydrolysate (11 mL) was checked.

The cartridges were conditioned with methanol (2 mL) and 2 mL of 45 mM KH_2PO_4 (pH 7.0) before loading the hydrolysed and neutralised samples. After sample elution, 2 mL of 15 mM KH_2PO_4 (pH 7.0) was passed through the cartridges which were then dried under full vacuum for ∼2 min. Thereafter, sequential washing of the cartridges was carried out using 1 mL each of MeOH:H_2O (5:95), MeCN:H_2O (2:98) and THF:H_2O (0.25:99.75) with intermittent drying for ∼1 min (∼2 min for the last step) using a full vacuum. The analytes were eluted and collected in glass test tubes with 2 mL of CH_3COOH:H_2O (2:98) using a low vacuum which was continued for ∼2–3 min after all solutions had eluted. The eluates were concentrated at 120 °C under gentle stream of N_2 for ∼35 min.

2.4. HPLC method

The mobile phase was pumped through the system at a flow rate of 1 mL/min using a Gilson 307 pump to which an on-line membrane degasser (Thermal Separation Products) was attached. Hundred microliter samples were injected into the system with a 200 μL loop using a SIL-9A Autosampler (Shimadzu, Japan). The stationary phase was a Zorbax column (ODS 5 μm, 25 cm × 0.46 mm ID; Phenomenex) with a Security Guard cartridge (ODS 4 mm × 3 mm ID; Phenomenex). Columns were maintained at a temperature of 30 °C using a Column Chiller Model 7950 (Jones Chromatography, UK). Florescence detection was made using a Spectroflourometric Detector RF-551 (Ver. 2.4, 12 μL flow cell; Shimadzu, Japan) set at an excitation of 269 nm and emission

at 312 nm. Detection of the chromatograms was made using a Shimadzu (Japan) Chromatopac CR-6A. The mobile phase was 10:8:14:68% (v/v) MeCN:THF:MeOH:buffer. The buffer was 5 mM KH_2PO_4 adjusted to pH 2.5 (with H_3PO_4) that contained 25 mM of the ion-pairing agent SDS. The mobile phase was filtered (0.45 μm, Millipore) and degassed by sonication (Decon FS200 B) under vacuum for 10 min. The working pressure was between 145 and 150 bar.

2.5. Validation

The efficiency of chromatographic separation and solid phase extraction was evaluated and validated according to criteria described in the literature [7–12].

2.5.1. Validation of HPLC

Selectivity was determined by injecting: (a) blank mobile phase, Milli-Q water and blank urine collected from volunteers 0.5 h before a salbutamol inhalation, (b) aqueous and urine standards, and (c) volunteers' urine samples collected 0.5 and 0.5–24 h after salbutamol inhalation. Both un-hydrolysed and hydrolysed urine specimens were extracted and assayed.

Intra-day accuracy and repeatability (RSD of peak height ratio) were determined by injecting three salbutamol concentrations (50, 100 and 200 μg/L) in triplicate. The accuracy and precision of the calibration curves to measure the unknown concentrations or QC samples were determined by drawing calibration curves excluding these concentration points and expressed as mean measured concentrations (with their respective biases) and RSD.

The inter-day accuracy and precision were determined for all concentrations of the standard calibration curve. Inter-day accuracy ($n = 6$) was determined as the mean measured concentration of each calibration point, which was obtained using a linear regression equation of each calibration curve and reported as bias. Inter-day precision ($n = 6$) was expressed as the RSD of the peak height ratios of individual calibration points.

Limits of detection (LOD) and quantitation (LOQ) were calculated using the data obtained from the linear regression equations ($n = 6$) [12]. LOD and LOQ calculated by linear regression were subsequently validated by the repeated analysis of three salbutamol aqueous and urine standards prepared at concentrations near the LOD (5, 10 and 15 μg/L) and the RSD was calculated [12].

The ruggedness of the assay was estimated by the day-to-day variability of the chromatographic response of three salbutamol urine standards and one unknown sample (volunteer KA24) evaluated in duplicate on 5 different days. Robustness was estimated by varying various HPLC conditions such as the mobile phase constituents, temperature, buffer strength and molarity of the ion-pairing agent.

2.5.2. Validation of extraction recovery, precision and accuracy

Intra-day recovery of salbutamol was determined by repeated SPE ($n = 3$) of three urinary salbutamol QC samples selected at high, mid and low points of the calibration range (50, 100 and 200 μg/L). The inter-day extraction recovery ($n = 6$) was determined using the extraction and assays of the whole calibration curve standards. The peak heights of salbutamol urine extracts were compared to the peak heights obtained with the direct injections of salbutamol aqueous standards assuming 100% recovery in order to provide an estimate of the extraction recovery. The intra- and inter-day accuracy and precision were determined as the percent relative recovery and RSD, respectively.

The study to optimise the conditions for the SPE when more than 1 mL of urine sample was used, consisted of extracting and injecting 8 replicates of hydrolysed salbutamol urine standards (50 μg/L) and a volunteer's 0.5–24 h urine sample (NK24) for each test volume (1–5 mL) after the volunteer had inhaled five puffs of salbutamol from an MDI (Ventolin Evohaler™; GlaxoSmithKline, UK). The results were expressed as the recovered amount, bias and RSD.

2.5.3. Stability studies

Two stability studies were carried out, one for establishing the stability of salbutamol and terbutaline urine concentrates in the mobile phase post-reconstitution and post-SPE, and the other for concentrated urine extracts frozen at −20 °C for up to 40 days for later reconstitution.

The first study involved determining the stability of urine concentrates in the mobile phase over 0–38 h after reconstitution at ambient temperature (25–27 °C). This was assessed by repeated ($n = 5$) HPLC determinations of three urine and aqueous salbutamol QC samples (50, 100 and 200 μg/L), a volunteer's urine sample containing no salbutamol (blank) and a 0.5–24 h post-salbutamol inhalation urine sample.

The second stability study was carried out to determine the recovery of extracted salbutamol and terbutaline in frozen urine concentrates for a period of 10–40 days post-SPE using the same samples and the frequency of SPE as mentioned in the first study. The concentrated urine extracts collected as SPE eluates in glass test tubes were sealed with parafilm, further enveloped in polythene bags and frozen at −20 °C till defrosted and assayed. The first set of samples was considered as reference day-1 with no freezing.

The urine salbutamol QC samples, volunteer's 0.5–24 h post-inhalation samples and blanks were all extracted the same day in duplicate using the SPE USAL method. Accuracy (recovery) and precision were determined against aqueous standards to establish the stability at each test point.

2.6. Volunteer study

Fourteen healthy volunteers (7 females) participated in a cross-over study after giving informed written consent. The study was approved by the Local Research Ethics Committee. This volunteer study consisted of two parts (Parts 1 and 2), each part involving four different commercially available salbutamol metered dose inhalers (MDIs) that delivered 100 μg per dose and inhaled on separate study days. The MDIs were Ventolin™ (GlaxoSmithKline, UK) which contained CFC propellant and the Ventolin Evohaler™ (GlaxoSmithKline, UK), Airomir (TEVA UK Ltd.) and Salbulin™ (Meda, UK) which are all formulated with the HFA143a propellant. All volunteers were trained to inhale from each MDI using the recommended optimal inhalation technique [13].

In Part 1, on separate study days (one week apart), each volunteer inhaled two puffs (200 μg) from one of the four salbutamol MDIs. The MDI was randomly selected. In Part 2, each volunteer repeated this study and swallowed 100 mL of a charcoal slurry immediately before and after inhalation (activated charcoal 25 g in 200 mL of water; Carbomix™, Penn Pharmaceuticals, UK). The oral charcoal was administered to prevent the oral absorption of the swallowed fraction of the MDI dose [14–16].

On all occasions urine samples were collected 0.5 h before and after inhalation and thereafter volunteers pooled their urine for 24 h. The volume and pH of all urine samples were recorded. Aliquots of each urine samples were stored at −20 °C till extracted and assayed. Urine samples collected at 0.0–0.5 h post-inhalation were assayed for unchanged salbutamol (USAL0.5) using the USAL method. Pooled urine samples collected during 0.5–24 h were assayed for their unchanged salbutamol (USAL24) using the USAL method and for their salbutamol plus metabolite concentration (USALMET24) using the USALMET method.

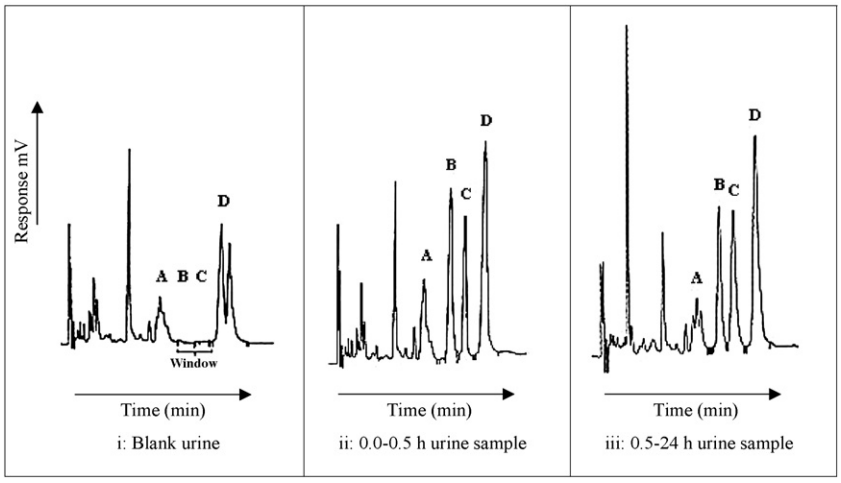

Fig. 1. Un-hydrolysed (i) blank, (ii) 0.0–0.5 h and (iii) 0.5–24 h urine samples of a male volunteer extracted using Isolute Confirm HCX cartridges—USAL method (B = salbutamol, C = terbutaline, A and D = unknown peaks).

3. Results and discussion

3.1. Validation of HPLC method

3.1.1. Representative chromatograms

Representative HPLC chromatograms of un-hydrolysed and hydrolysed blank and a 0.5–24 h volunteer's urine samples are shown in Figs. 1 and 2. Salbutamol eluted after ~24 min (RSD 0.31%; range 24.2–24.5 min) and terbutaline in ~27 min (RSD 0.26%; range 26.9–27.1 min) ($n = 50$) with baseline resolution ($R_s = 1.8$, RSD = 3.3%, $n = 5$).

3.1.2. Specificity and selectivity

For specificity and selectivity, a total of 45 drugs commonly used as adjuvant therapy, including β_2-agonists, β-antagonists, sympathomimetics, analgesics and steroids, were used. Of the tested compounds none interfered with salbutamol. Terbutaline and bamethane were also fully resolved and were the only candidates for the internal standard. However, the retention time of bamethane was very long (>45 min).

3.1.3. Linearity and range

The mean ($n = 6$) regression equations using peak height ratios of salbutamol to terbutaline for aqueous standards (SAS), un-hydrolysed (USAL) and hydrolysed (USALMET) urine stan-

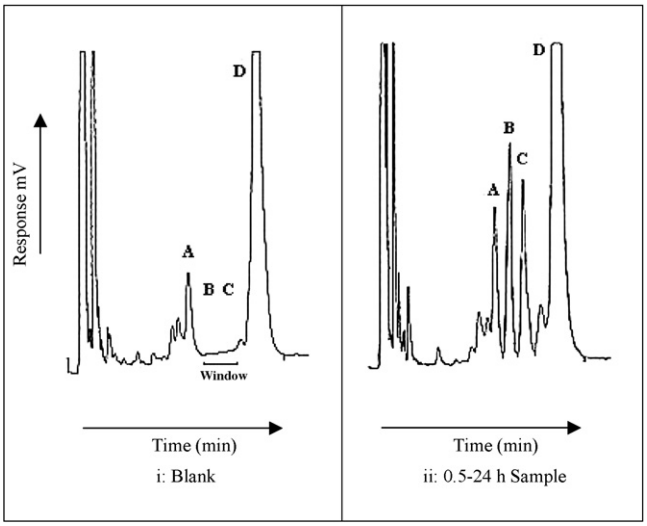

Fig. 2. Hydrolysed (i) blank and (ii) 0.5–24 h urine sample of a female volunteer extracted using Oasis HLB cartridges—USALMET method (B = salbutamol, C = terbutaline, A and D = unknown peaks).

Table 1
Intra- and inter-day HPLC accuracy and precision (repeatability and reproducibility).

Nominal concentration (μg/L)	USAL method		USALMET method		USAL method	USALMET method
	Mean measured concn[a] (RSD%)	Mean bias (%)	Mean measured concn[a] (RSD%)	Mean bias (%)		
(a) Intra-day accuracy ($n = 3$)					(a) Intra-day repeatability ($n = 3$) RSD (%)	
50	50.11 (5.72)	0.23	52.70 (0.92)	5.40	6.69	3.13
100	105.16 (4.62)	5.16	100.04 (3.24)	0.04	5.30	1.03
200	201.46 (3.96)	0.73	207.04 (1.66)	3.52	4.90	1.70
(b) Inter-day accuracy ($n = 6$)					(b) Inter-day reproducibility ($n = 6$) RSD (%)	
25	24.30 (15.03)	−2.81	24.81 (11.04)	−0.78	5.68	5.59
50	49.03 (5.36)	−1.94	52.12 (3.31)	4.25	4.77	2.80
75	73.92 (5.43)	−1.44	74.57 (2.47)	−0.57	6.42	2.37
100	104.10 (4.12)	4.10	99.28 (2.80)	−0.73	6.28	2.01
150	147.64 (3.62)	−1.58	150.03 (3.05)	0.02	4.21	3.47
200	200.36 (3.05)	0.18	205.87 (1.50)	2.94	5.84	1.98
250	252.88 (2.07)	1.15	244.66 (2.99)	−2.14	3.41	2.11
300	297.47 (1.73)	−0.84	302.05 (1.80)	0.68	3.41	2.83

[a] Concn = concentration.

dards were $y = 0.00488x + 0.00372$, $y = 0.00482x + 0.0000166$ and $y = 0.00448x + 0.01455$, respectively. The corresponding RSD of their slopes were 2.72, 1.56 and 1.68% with respective standard deviation (SD) of the intercepts at 0.00299, 0.0058 and 0.0065. These slopes were not different from each other indicating that the urine matrix had a minimal effect on the method [17]. This could be due to the effective clean-up of the urine samples during extraction. The mean intercept (SD; 95% confidence interval) of USAL and USALMET at the lower limit of quantitation (LLOQ, 25 μg/L) was 0.05% (4.8; −0.022, 0.019) and 11.9% (5.8; −0.010, 0.039), respectively.

The regression lines were linear over the range 25–300 μg/L. The mean r^2 (RSD) values for SAS, USAL and USALMET were 0.9992 (0.1002%), 0.9983 (0.06%) and 0.9976 (0.202%), respectively. The calibrations were also found linear over the range 5–1000 μg/L. Since a smaller range is reported to tolerate larger deviations from linearity and make the method more rugged to non-linearity [18], a small concentration range with more close points spanned over the intended use of the method was selected.

3.1.4. Accuracy and precision

The intra- and inter-day accuracy and precision (Table 1) for both USAL and USALMET urine standards were within acceptable limits of ±15% [10]. The results also depict the accuracy of the HPLC assay in precisely measuring unknown concentrations.

3.1.5. Limit of detection and quantitation (LOD and LOQ)

The calculated LOD of salbutamol from SAS, USAL (1 mL sample) and USALMET (1 mL sample) standards was 2.0, 4.0 and 4.8 μg/L, respectively, and the LOQ was 6.1, 12.1 and 14.6 μg/L. Repeated assays of three salbutamol concentrations, 5, 10 and 15 μg/L using the SAS method produced RSD values ($n = 10$) of 4.1, 2.7 and 1.3%, respectively while the same concentrations using the USALMET method gave RSD values ($n = 7$) of 10.6, 3.9 and 3.9%. The HPLC method is therefore highly sensitive for quantifying salbutamol extracted in urine after inhalation.

3.1.6. Robustness

The influence of different chromatographic parameters upon separation was evaluated by systematically varying the chromatographic conditions [12]. Only one condition was changed while the others were kept constant. Slight variations in mobile phase constituents may change the width of the window for salbutamol and terbutaline which, however, remained fully resolved with respect to each other. The change in operating temperature by ±5 °C did not affect resolution except back-pressure. An increase in phosphate buffer molarity of up to 10 mM and of the ion-pair agent sodium dodecyl sulphate in the mobile phase up to 30 mM decreased the retention time with sharp peaks. However, this reduced resolution of salbutamol and terbutaline in hydrolysed urine samples and the width of the window squeezed by the unknown matrix peaks "A" and "D" (Figs. 1 and 2). Increasing molarity of the ion-pair agent also increased back-pressure.

3.2. SPE recovery, accuracy and precision

The pooled mean (RSD) intra-day percentage relative recoveries (%RR) of salbutamol using USAL and USALMET samples was 91.3 (0.05%) and 92.5 (2%) and the pooled mean precision RSD of %RR was 4.4 and 2.6%, respectively. The pooled mean (RSD) inter-day %RR of salbutamol using USAL and USALMET samples was 90.8 (2.3%) and 91.5 (3.0%) and the pooled mean precision RSD of %RR was 2.9 and 3.3%, respectively. The parallel %RR of terbutaline, added as the internal standard, was 90.2 (2.9%) and 96.4 (1.8%) with precision RSD of 4.9 and 3.9%.

The USAL method reported here is the mixed-mode SPE which is based on a control of the pH and thereby ionisation of the analyte(s)

Table 2
Sample preparation and pre-treatment before SPE.

Total urine output (mL) in the sampling period (h)		Volume of urine sample to be taken	Volume of internal standard to be used	Pre-treatment of un-hydrolysed urine sample (USAL method)				Pre-treatment of urine samples for hydrolysis and of hydrolysed urine (USALMET method)				
0–0.5	0.5–24			Molarity of buffer KH_2PO_4 pH 7.0	Volume of buffer to add	Total volume of treated sample	Final molarity of treated sample	Normality of HCl to be added to sample	Volume of HCl to be taken	Final normality of treated sample	Volume of KH_2PO_4, pH 13.0 to be used	Total volume of treated sample
Upto 75	Upto 750	1 mL	1 mL	30 mM	2.0 mL	4.0 mL	15.00 mM	0.10N	8 mL	0.01N	1 mL	11 mL
75–150	750–1500	2 mL		60 mM	2.0 mL	4.0 mL	15.00 mM	0.11N				12 mL
150–225	1500–2250	3 mL		100 mM	0.7 mL	4.7 mL	14.89 mM	0.12N				13 mL
225–300	2250–3000	4 mL		100 mM	0.9 mL	5.9 mL	15.25 mM	0.13N				14 mL
300–400	3000–4000	5 mL		100 mM	1.1 mL	7.1 mL	15.49 mM	0.14N				15 mL

[19,20]. The previously reported SPE method [4] lacked this control. The mean intra- and inter-day recoveries were within the accepted value of ±15% [10] indicating that the USAL method was efficient, accurate and precise.

Extracting salbutamol from hydrolysed urine posed difficulties in controlling the pH, osmolarity and ionic strength of the analytes in the urine sample optimally with consequent variable and decreased recoveries of salbutamol and terbutaline. Since polymeric cartridges are reported to possess a higher retaining capacity of analytes [17,20,21], Oasis HLB cartridges were used for extracting hydrolysed urine samples using the USALMET method. Oasis HLB were chosen instead of Oasis MCX (polymeric cationic) cartridges as the latter did not produce clean extracts. The USALMET method gave reproducible recoveries of both salbutamol and terbutaline which were comparable to the USAL method. This also indicates that the drying of eluates at 120 °C under a gentle stream of nitrogen in USALMET method did not affect the integrity of the analytes.

Acid hydrolysis of salbutamol has been used to free it from its sulphate ester conjugate [4,22,23]. This is the first work where an internal standard has been used during acid hydrolysis to study the effect of any degradation of salbutamol itself in addition to freeing it from its conjugate. Terbutaline, being a structural analogue, possesses very similar physico-chemical properties as that of salbutamol [24,25]. It was therefore added to the samples to reflect the stressful conditions salbutamol undergoes during acid hydrolysis. Forsdahl and Gmeiner [22] have reported decomposition of salbutamol at 60 °C for 1 h and could recover only 63% of intact salbutamol. This may be because they used 6 M HCl with the final concentration of the acidified urine at 2 M. Evans et al. [23] used 1 M HCl and reported that salbutamol remained unaffected by hydrolysis in a boiling water bath for 1 h. The results of this work are in agreement with the findings of Evans et al. [23]. The samples were acidified to give a final concentration of 0.01N HCl. The similarities and consistencies in recoveries of salbutamol and terbutaline indicate that both remained stable during acid hydrolysis for 1 h at boiling temperature.

Although Oasis HLB cartridges could also be used for unchanged salbutamol when the samples were not hydrolysed they are more expensive than Confirm HCX cartridges. Also the preparation time when using Oasis HLB cartridges is longer because more steps are involved with the extraction. Thus on grounds of economy it is recommended that the Confirm HCX cartridges are used for unchanged salbutamol and when urine samples are hydrolysed for their salbutamol plus metabolite amounts then Oasis HLB cartridges are used.

3.3. Use of increased/multiple sample volume

The urine output of individuals is difficult to control over a set collection period. This is particularly important when patients are unable to inhale salbutamol dose correctly and completely from an MDI with resultant low levels excreted in urine. In such circumstances, a large output of urinary volume may necessitate the use of more than the usual 1 mL of urine sample for the SPE to ensure consistent chromatographic response. The optimised and validated SPE conditions (based on the inhalation of two puffs of salbutamol (200 μg) by volunteers from an MDI) using 1–5 mL of the urine sample are shown in Table 2. Table 3 shows the results of the volunteer study using 1–5 mL of sample volume for the SPE. The individual and mean RSD values were within the acceptable limits [10] for both salbutamol and terbutaline which indicates that the extraction remained accurate and reproducible with the use of different sample volumes (1–5 mL). It was found that with a higher urine volume in a given period the inherent urinary interferences were diluted which presented little concern for the resolution of salbutamol or terbutaline when more than 1 mL of sample was used for SPE to concentrate the sample.

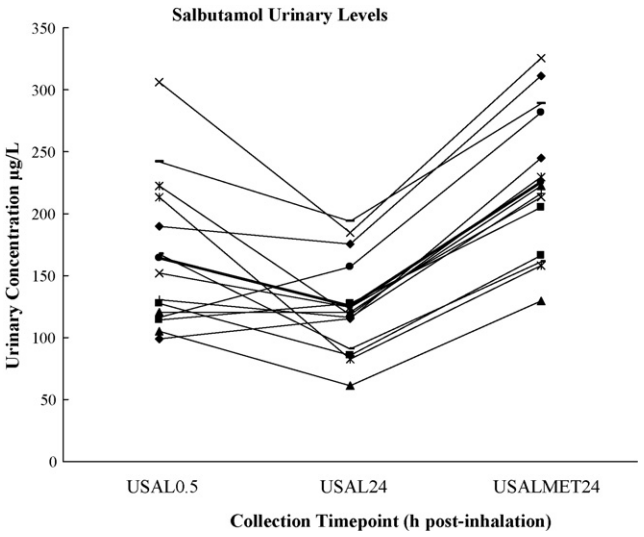

Fig. 3. Mean urinary salbutamol concentration of each of the 14 individuals following MDI inhalation (Part 1). The bold line indicates the mean of all the volunteers' samples ($n = 56$).

3.4. Stability study

Analysis of a large number of samples over a prolonged period of time (overnight) makes it necessary that stability of the analyte(s) and internal standard in the carrier solvent for injection onto the automatable HPLC system be assured [10,12]. The mean ($n = 5$) salbutamol and terbutaline recovered from the urine standards and the volunteer's sample (dissolved in the mobile phase) left at room temperature for up to 36 h were consistent (>91% and ≥94%) and precise (RSD ≤2% for both), respectively. The mean ($n = 10$) salbutamol and terbutaline recovered from the urine standards and the volunteer's sample extracted concentrates frozen at −20 °C for up to 40 days were also consistent (>88% for both) and precise (RSD ≤4% and ≤3%), respectively. The mean (RSD) recovered amount of salbutamol over 0–36 h ($n = 5$) and over 40 days ($n = 10$) from the 0.5–24 h urine sample of the volunteer (KA24) was 210.5 μg (2.0%) and 187.6 μg (8.1%), respectively. The deviations in recovery over the specified period were within the acceptable limits of ±15% [10]. The mean (SD) percent change in measured concentration of salbutamol and terbutaline with subsequent injections ($n = 4$) as compared to the 1st injection was ≤3% (3.1) and ≤2% (2.7), respectively. The mean (SD) percent change in measured concentration of salbutamol and terbutaline after freezing and defrosting ($n = 8$) was ≤−2% (1.2) and ≤−3% (3.3), respectively. Also, the chromatograms of urine standards and the volunteer's sample did not show the appearance of any interfering or additional peaks over the test time-frame and no changes in chromatography were observed. These stability studies indicate that salbutamol and terbutaline left at room temperature for up to 36 h (dissolved in the mobile phase) and their extracted concentrates frozen at −20 °C for up to 40 days did not show any significant variation of the measured concentration and recovery. The results of the two stability studies also demonstrate that the HPLC method is ruggedly robust.

3.5. Volunteer study

The applicability of the method was demonstrated by determining urinary salbutamol concentrations post-inhalation using doses (two) equivalent to normal clinical practice [26]. In the past larger doses have been used to overcome assay sensitivity issues [4,5].

Fig. 3 shows the average urinary concentrations for each of the 14 volunteers and the overall mean values ($n = 56$) following the

Table 3
Recovery and reproducibility of SPE (USALMET method) with increasing volume of hydrolysed urine standard (50 μg/L) and a volunteer's 0.5–24 h urine sample (USALMET24).

Sample volume (mL)	Accuracy and precision with increasing volume of hydrolysed urine standards and samples (n = 8)						
	Salbutamol urine standard (50 μg/L)				Volunteer's 0.5–24 h urine sample (NK24)		
	Concn[a] found (μg/L)	Recovered amount (μg)	Bias (%)	RSD (%)	Concn[a] found (μg/L)	Recovered amount (μg)	RSD (%)
1	43.9	43.87	−12	5.93	114.6	189.55	6.63
2	88.2	44.09	−12	2.03	220.71	182.53	3.51
3	134	44.69	−11	3.36	326.44	179.98	3.10
4	183	45.68	−8.6	2.64	419.38	173.41	7.19
5	217	43.32	−13	2.89	492.68	162.98	5.23
Mean		44.33	−11.3	3.37		177.69	5.13
SD		0.90	1.8	1.51		10.05	1.82
RSD		2.03				5.66	

[a] Concn = concentration.

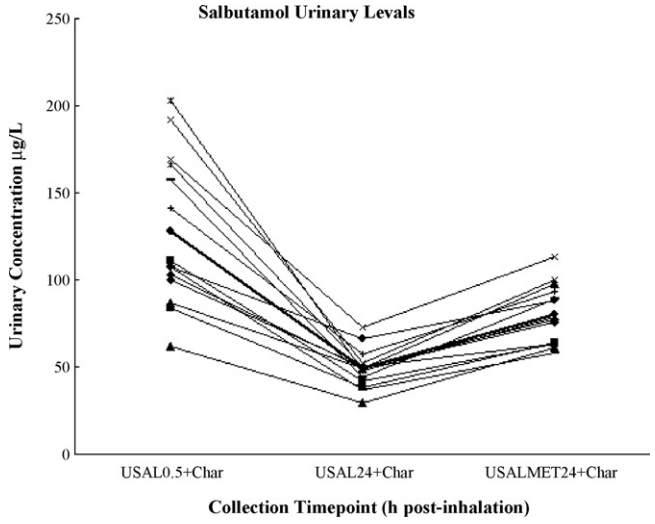

Fig. 4. Mean urinary salbutamol concentration of each of the 14 individuals following MDI inhalation with the co-administration of oral charcoal (Part 2). The bold line indicates the mean of all the volunteers' samples (n = 56) (char = charcoal).

MDI inhalations (Part 1). Similar values following inhalation from the MDIs with the co-administration of oral charcoal (Part 2) are shown in Fig. 4. For each individual the USAL and USALMET data from the four study doses have been averaged. Following inhalation from the MDIs (Part 1) the range of salbutamol concentrations was 22.1–501.3, 36.7–315.4 and 49.3–512.4 μg/L, respectively, for USAL0.5, USAL24 and USALMET24. Similar ranges for the MDI inhalations with the co-administration of oral charcoal (Part 2) were 33.5–302.4, 12.1–94.8 and 37.0–195.7 μg/L.

The range of urine volumes for all the 0–0.5 and 0.5–24 h collection periods was 15–580 and 370–2805 mL, respectively and the pH of all samples ranged from 4.7 to 8.

The mean (n = 56) amount of salbutamol (unchanged and metabolite fraction) recovered in the urine samples after the inhalation of two puffs (200 μg) from four different MDIs (Part 1) and with the co-administration of oral charcoal (Part 2), by the 14 volunteers, is shown in Table 4.

The percentage of salbutamol recovered in the urine in the first 0.5 h (USAL0.5) post-inhalation from an MDI, without and with the co-administration of oral charcoal, is consistent with that reported by other researchers [4,27–31]. The amount of salbutamol excreted unchanged in urine in the first 0.5 h after inhalation is believed to be mainly derived from the lung and is used as an index of relative bioavailability [3,4,32]. The recovery of salbutamol in the first 30 min post-inhalation is considered to elicit the rapid bronchodilaton and hence clinical effectiveness of an MDI as measured by spirometry. This index of relative bioavailabity of salbutamol is used to identify the correlation of its pharmacokinetics and pharmacodynamics. The total amount of salbutamol and its metabolite excreted in the urine in the 24 h post-inhalation reflects the systemic delivery and is considered an indicator of the relative bioavailability of salbutamol to the body following an inhalation [3,4,32]. In Part 1, this amount was similar to that previously reported [4,33]. Hence approximately 46% of the nominal inhaled dose was delivered to the body via the pulmonary and gastro-intestinal routes and excreted in the urine. As a large proportion of the inhaled dose is swallowed [34], the salbutamol dose recovered in urine from 0.5 to 24 h contains both unchanged and metabolised fractions. Since only unchanged salbutamol is effective in relieving bronchospasm, it is therefore necessary to ascertain the proportions of these fractions.

Charcoal blockage is used to separate absorption via the pulmonary and oral routes [14–16] and to identify the total effective lung dose after inhalation [3]. Hence there was a difference between the amounts excreted in the urine in the 24 h collections between the MDI inhalation with and without the co-administration of oral charcoal. The amount excreted over 24 h with the co-administration of charcoal represents the amount that was deposited into the lungs

Table 4
Mean (SD) amount of salbutamol dose recovered in the urine samples of 14 (7 female) healthy volunteers after inhaling two puffs (200 μg) of salbutamol from four MDIs without and with charcoal ingestion.

	Urinary recovery of salbutamol dose (μg) in the given period (h)				Total recovered dose (μg) 0.0–24 h
	USAL0.5[a]	USAL24[a]	Metabolite fraction 0.5–24[b]	USALMET24[c]	
MDI alone (Part 1)					
Mean (SD)	6.44 (3.36)	48.09 (17.06)	37.42 (15.89)	85.51 (21.7)	91.94 (22.43)
% of nominal dose (SD)	3.22 (1.68)	24.04 (8.53)	18.71 (7.94)	42.75 (10.85)	45.97 (11.21)
MDI + charcoal (Part 2)					
Mean (SD)	6.57 (3.23)	19.99 (8.01)	12.82 (6.81)	32.81 (11.04)	39.38 (11.72)
% of nominal dose (SD)	3.28 (1.61)	10.00 (4.00)	6.41 (3.41)	16.41 (5.52)	19.69 (5.86)

[a] Assayed using the USAL method.
[b] Obtained from USALMET24 minus USAL24.
[c] Assayed using the USALMET method.

and delivered to the systemic circulation. This was found to be 20% of the nominal dose. This value compares well with Olsson et al. [35] and Chrystyn et al. [36].

4. Conclusion

The HPLC method was found to be linear (over the range tested), precise, accurate and sensitive for determining salbutamol concentrations in human urine following the inhalation of normal doses. Two SPE methods for extracting salbutamol from unhydrolysed and hydrolysed urine were efficient, reproducible and robust. A method using Confirm HCX cartridges is recommended for unchanged salbutamol and a different method using Oasis HLB is recommended for total salbutamol (salbutamol plus its metabolite). These methods were reliably applied to urinary pharmacokinetic studies using 14 volunteers after the inhalation of two 100 μg doses of salbutamol. The concentrations of unchanged and total salbutamol were all within the sensitive range of the assay. This volunteer study revealed that about 20% of the nominal dose is delivered to the lungs and 46% to the systemic circulation following inhalations from a metered dose inhaler.

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