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Validation of high-performance liquid chromatography assay for quantification of formoterol in urine samples after inhalation using UV detection technique

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Abstract

A novel high-performance liquid chromatography (HPLC) assay for the estimation of formoterol in urine samples was developed and validated. A solid phase extraction (SPE) using Oasis HLB was optimised to isolate formoterol from a urine matrix followed by HPLC with UV detection. This extraction procedure concentrated the final analyte forty times so that UV detection can be used to determine even a low concentration of formoterol in urine samples. The urinary assay was performed in accordance with FDA and ICH regulations for the validation of bioanalytical samples. The samples were injected onto a C18 Spherisorb® (250 mm × 4.6 mm × 5 μm) analytical column maintained at 30 °C. The mobile phase consisted of 5 mM of potassium dihydrogen orthophosphate buffer (adjusted to pH 3 with ortho phosphoric acid):acetonitrile (ACN) (70:30, v/v), and the formoterol peak was detected at wavelength 214 nm. The extraction recovery of formoterol from the urine sample was >95%. The calibration curve was linear ($r^2 = 0.99$) over formoterol concentrations ranging from 1.5 to 25 ng/mL ($n = 6$). The method had an accuracy of >92% and intra and inter-day precision CV% of <3.9% and <2.2%, respectively, at three different concentrations low, medium and high (10, 15, 20 ng/mL). The limit of quantification (LOQ) for formoterol was found to be 1.50 ng/mL. The accuracy and precision at the LOQ level were 95% and %CV <3.7% ($n = 10$), respectively. The method reported is simple, reliable, precise, and accurate and has the capacity to be used for determination of formoterol in urine samples.
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Keywords: Formoterol; HPLC; Assay; Validation

1. Introduction

Chemically, formoterol is ((RR)-(±)-*N*-[2-hydroxy-5-[1-hydroxy-2-[[2-(4-methoxypheny)-1-methylethyl]amino]ethyl] phenyl] formamide) [1] (Fig. 1). It is a phenylethylamine derivative with one phenolic hydroxyl and one secondary amino group, and is widely marketed as a racemate of the enantiomers, which have the RR + SS configuration [2]. Formoterol is marketed as Oxis® (AstraZeneca) Turbuhaler and Foradil Aerolizer® (Novartis AG). Each delivered dose of Oxis Turbohaler 12 contains 12 μg of formoterol fumarate dihydrate.

Formoterol is a long-acting beta2-adrenoceptor agonist with a rapid onset of action [3]. Maximum bronchodilation is achieved within 2 h, with effects persisting for approximately 12 h. This is significantly longer than the bronchodilatory effects of equivalent doses of salbutamol, fenoterol or terbuataline [4]. It has been reported to be more effective than a shorter-acting beta-2 agonist in the treatment of nocturnal and exercise-induced asthma [3,5,6]. Formoterol has been recommended by the British Thoracic Society (BTS) as an add on therapy with corticosteroids to improve lung function and prevent exacerbations [7]. Literature also suggests that the addition of formoterol to inhaled corticosteroids does not increase significant undesirable cardiac effects [8].

The pharmacological effects of formoterol are similar to beta-2 adrenoceptor agonist drugs. They stimulate the intracellular adenyl cyclase, the enzyme that catalyzes the conversion of adenosine triphosphate (ATP) to cyclic-3′,5′-adenosine monophosphate (cyclic AMP). The increased cyclic AMP levels cause relaxation of bronchial smooth muscles and inhibit

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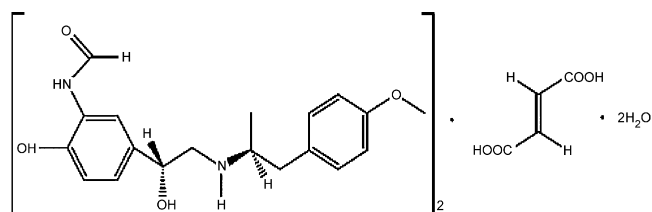

Fig. 1. Molecular structure of R,R formoterol fumarate.

the release of mediators of immediate hypersensitivity from cells, especially from mast cells [9]. The assay of formoterol in biological fluids is limited by the low systemic concentrations; the lack of a chromophore for detection and it has not been successfully derivatised to improve assay sensitivity. There have been limited reports on analytical methods for the determination of formoterol from biological fluids. Various techniques include capillary electrophoresis (CEC) [10], high-performance liquid chromatography (HPLC) [11,12] and hyphenated techniques like HPLC coupled with mass spectrophotometer (LC–MS–MS) [13] and gas chromatography/mass spectrophotometer (GC–MS) [14,15]. However, these latter methods are not readily available to all laboratories. A HPLC method to measure formoterol in urine samples has been reported by Hennion et al. [1999] for the bioequivalence of formoterol fumarate in urine samples using LC–MS–MS interfaced with atmospheric pressure chemical ionisation (APCI) [16]. The method uses a liquid–liquid extraction (LLE) for the isolation of formoterol fumarate from urine. The formation of emulsions with biological samples and poor reproducibility affects the reliability of this method [16]. The use of GC–MS has been reported for the detection of derivatised formoterol (bispentafluoropropionyl-methyl derivative) in urine samples [14]. However, the operating temperature was very high thereby causing the formoterol to degrade [14]. Another method has reported the use of electrochemical detection [11], which has a very high sensitivity and selectivity but has low reproducibility.

The aim of the present work was therefore to develop a sensitive and easy to perform method for the quantitation of formoterol in urine samples collected after inhalation of the drug. The proposed method uses a SPE to isolate and concentrate formoterol from urine samples and to enhance the sensitivity of the method. Separation was achieved using a C18 reversed-phase column and UV detection at 214 nm.

2. Experimental

2.1. Chemicals

A primary reference standard of formoterol fumarate dihydrate was supplied from by Sigma-Aldrich (Dorset, UK). HPLC grades solvent were from Fisher Scientific Ltd. (Leicestershire, UK). Buffer salts were obtained from Sigma (Dorset, UK) and B.D.H chemicals (Poole, UK). Ultrahigh purified water was obtained from a Milli-Q water dispensing system (Massachusettes, USA).

2.2. Equipment and analytical method

Waters Oasis® (1 cc/30 mg) HLB (hydrophilic–lipophilic balance) reversed-phase sorbent solid phase extraction cartridge (Milford, USA) was used to isolate formoterol from urine samples. A Hewlett Packard 1050 series HPLC system with a multiple solvent delivery system containing an auto sampler with a variable injection loop, variable wavelength UV/visible detector and variable temperature column heater was used. Integration software Prime Mutichannel Data Station (Ver 4.2.0) was supplied by HPLC technologies (Herts, UK).

The chromatographic separations were carried out at 30 °C on a C18 Spherisorb® (250 mm × 4.6 mm × 5 μm) analytical column (Waters; Milford, USA). The analytical column was protected with a C18 (4 × 3 mm i.d.) security cartridge system (Phenomenex; Torrance, USA). The mobile phase was 5 mM potassium dihydrogen orthophosphate buffer (adjusted to pH 3 with ortho phosphoric acid):acetonitrile (ACN) (70:30, v/v). The mobile phase was filtered prior to use using a membrane filter (47 mm diameter, pore size 0.25 μm) and sonicated under vacuum for 10 min. The mobile phase was delivered at a flow rate of 1.0 mL/min and the injection volume was 100 μL.

2.3. Preparation of urine standard calibration solutions

A stock solution containing 100 μg/mL of formoterol was prepared using water: methanol (60:40, v/v). Blank urine samples were obtained from 12 healthy volunteers (6 males and 6 females). This was further used to prepare urine standards for the validation.

Urine standards were prepared by mixing 50 mL of the 100 μg/mL stock, with 5000 mL of blank urine to obtain a concentration of 1000 ng/mL. Ten microliter of 1000 ng/mL urine standard was diluted to 100 mL to obtain a concentration of 100 ng/mL (sub-stock). Calibration standards in the concentration range of 2.5, 5, 10, 15, 20, 25 ng/mL, and were prepared in 50 mL volumetric flasks. All urine standards/samples were filtered through a 0.45 μm filter to eliminate any solid impurity.

2.4. Extraction of formoterol from urine

To 20 mL of each urine standard/sample, 2 mL of Borate buffer (100 mM) was added to maintain the pH of the urine standard/sample at around 8.5–9.5. This pH range was used to retain the formoterol in the cartridge because below this range the formoterol is protonated and therefore the extraction recovery for formoterol was low. The urine standards/samples were filtered using 0.45 μm filter to eliminate any solid impurities. Thirty milliliter sample reservoirs were connected to the solid-phase extraction cartridges and each were conditioned using 2 mL of methanol. Twenty milliliter of pre-treated urine sample/standard were then applied to the cartridge and allowed to elute through the bed at a flow rate of 1–1.2 mL/min. After elution of urine sample a full vacuum was applied for 0.5 min and the cartridge was then washed with 2 mL of 2.5 mM borate buffer (prepared from sodium tetraborate decahydrate, the pH was not adjusted and was around ∼9.4). The cartridge was then dried for

1 min using a full vacuum followed by the addition 2 mL of 1% NH_4OH (30% NH_4OH in 10% methanol; 1:99, v/v) and then dried for 0.5 min using a half pressure vacuum. Sample tubes for collection were placed into the extraction station for the collection of the final aliquot. The analyte was eluted with 2 mL of 2% glacial acetic acid solution (glacial acetic acid in 70% methanol; 2:98, v/v). It was dried under a stream of nitrogen and the residue was reconstituted in 0.5 mL mobile phase.

2.5. Clinical study

Approval was obtained from the local ethics committee and all the volunteers gave their written informed consent to take part in the study. Four healthy volunteers (two males and two females) provided urine samples before inhalation (time zero) and at 0.5, 1, 2, 4, 6, 9, 12, 18, 24 h post inhalation of 48 μg (12 μg × 4) formoterol on two separate study days. One of the two study doses involved the inhalation of 12 μg × 4 doses of formoterol from a turbohaler (Oxis, AstraZenica; UK). The other study dose involved repetition of the inhalation with the co-administration of 12.5 g of charcoal in 100 mL of water before the inhalation study dose and 12.5 g of charcoal in 100 ml of water after the inhalation.

2.6. Biological assay validation

The optimised method was validated to determine the formoterol in the urine samples post inhalation. The validation procedure was based on FDA and ICH guidelines for bioanalytical method validation for human studies [17–19].

2.6.1. Specificity

Blank urine samples collected from 12 volunteers were individually spiked with formoterol. Both the blank and the spiked urine were assayed. A photodiode array detector was used to check for the presence of coeluting peaks of formoterol metabolite.

2.6.2. Method linearity

Six calibration standards (1.5–25 ng/mL) were prepared covering the expected range, including the limit of quantification (LOQ). The standards were prepared in urine matrix. Blank sample were also analysed (without the formoterol) along with the calibration standards. ANOVA in conjunction with linear regression should be used.

2.6.3. Sensitivity

The sensitivity of the analytical technique was expressed as the limit of quantification, which is the minimum plasma concentration of formoterol that can be quantitatively determined with a peak height to base line ratio of at least 10:1, and the limit of detection (LOD) as peak height to base line ratio of 3:1. The LOQ is accepted if the analyte peak response is identifiable, discrete and reproducible with a precision of 20% and accuracy of 80–120% [17]. Formoterol in urine at a concentration of 1.5 ng/ml was extracted and injected on five separate days (inter-day and intra-day) to determine the precision of the formoterol peak.

2.6.4. Accuracy/recovery

Both accuracy and recovery were studied from replicate sets of

i. formoterol standard spiked in an extracted blank urine matrix; and
ii. extracted formoterol urine standard at known concentrations levels corresponding to low (5 ng/mL), medium (10 ng/mL) and high (20 ng/mL). The measurement was performed by using five determinations per concentration.

2.6.5. Precision

Precision was studied using five determinations at known concentration levels corresponding to low (5 ng/mL), medium (10 ng/mL) and high (20 ng/mL) levels in the calibration range. This study was repeated for five days to determine the precision between days.

2.6.5.1. Intermediate precision. A second analyst conducted intermediate precision on different days with different instruments.

2.6.6. Robustness

Deliberate variations in mobile phase pH and composition, temperature, and flow rates were introduced. As well, different columns from different suppliers were studied.

2.6.7. Stability

The stability studies evaluate the stability of the analyte during sample collection, handling, after short-term storage and after going through freeze-and-thaw cycles and the analytical process [17–20]. The stability of aqueous standard solutions was also evaluated.

3. Results and discussion

3.1. Optimisation of method

Initially the urinary extraction was performed using Waters Bond elute LRC C-8, Bond elute CBA and Sep-Pak C-18 cartridges. Upon analysis, it was found that these cartridges were non-specific and gave poor recovery. Extraction procedure was further performed using a Waters Oasis HLB (hydrophilic–lipophilic balance) reversed-phase sorbent cartridge. The HLB cartridge is a hydrophilic–lipophilic water-wettable reversed-phase sorbent with a balanced ratio of two monomers, the hydrophilic N-vinyl pyrrolidine and lipophilic divinylbenzene [21,22]. This cartridge was finally selected for the study because it gave the most efficient sample clean up with a recovery >96% for formoterol in urine samples.

The presence of residual silanol groups on the silica surface caused peak tailing problems and an extended run time when operating at a high level (pH > 5). A number of approaches have

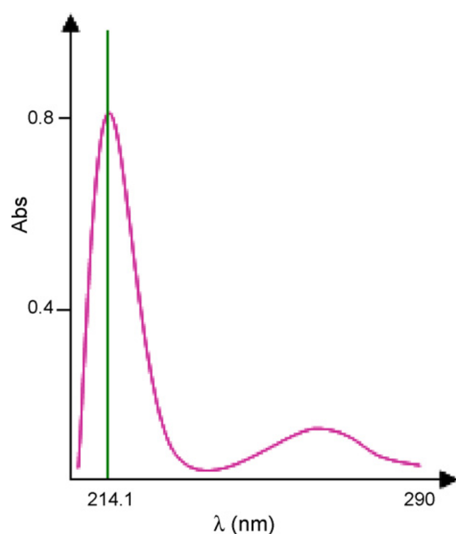

Fig. 2. UV spectrum of formoterol.

been adopted to overcome this problem such as ion suppression [23] and ion pair chromatography [24]. In this study the pH of the mobile phase was lowered to pH 3 to avoid these problems. When the pH is lowered, the silanols become protonated [25], thus eliminating the attractions between the ionised silanol groups and the NH_2 groups of the solutes.

Formoterol peak was monitored at different wavelengths and wavelength 214 nm was optimised due to enhanced sensitivity [26]. Even though, this wavelength is non-specific for bioanalysis, a study was performed to prove the specificity of the method beyond any reasonable doubt. Also, a UV spectrum was applied for formoterol samples (see Fig. 2). Different lots of urine samples from the different donors were analysed. No interfering peaks were observed at elution time of formoterol. A number of potential internal standards were evaluated, initially using their chromatographic behaviour under the conditions optimised for the separation of formoterol. Compounds that had retention time of less than 10 min were excluded from further investigation, as they would elute together with the urine matrix. Pindalol yielded a peak closer to a formoterol peak. On solid phase extraction pindalol gave inconsistent recovery (average CV% was 42.1% (n = 10)). Frequent calibration standards and quality-control standards prepared in a urine matrix, were extracted along with the samples to make up for any variability that might have existed in the extraction procedure. These standards and samples were then injected into the HPLC and the mean regression equation was compared with the sample data.

3.2. Assay validation

3.2.1. Specificity

The volunteer samples were analysed using the optimised HPLC parameters. Chromatograms of extracted urine consisting of both blank and spiked urine samples are shown in Fig. 3(a) and (b). The figures show that formoterol was eluted at 20.1 min and was separated from the co-extracted endogenous urine components.

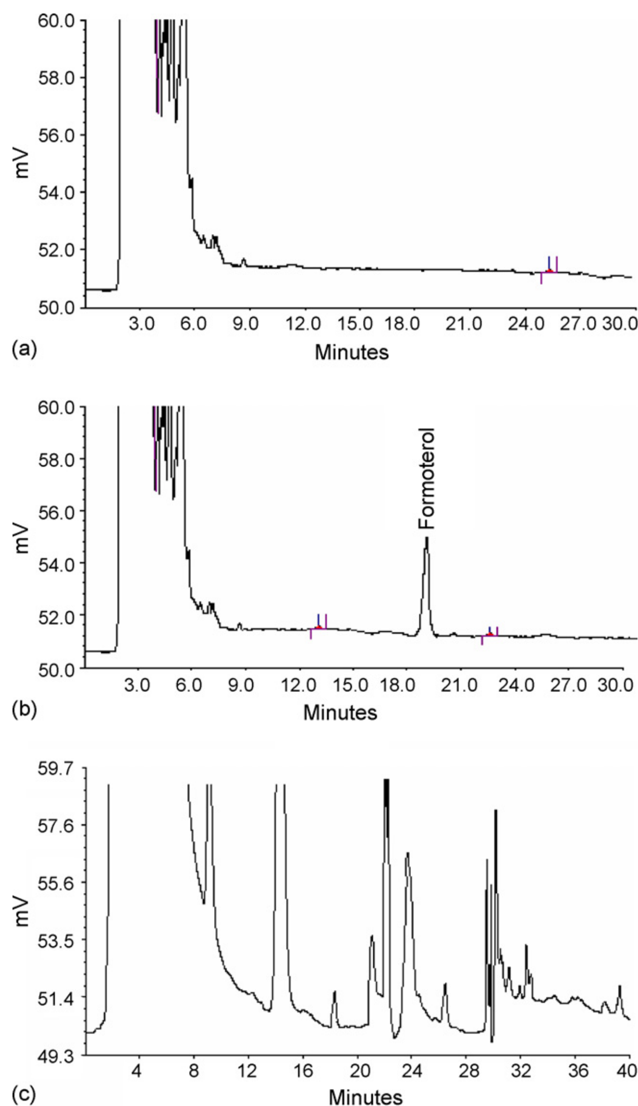

Fig. 3. (a) Chromatogram of a pooled blank urine sample; Column C18 waters spherisorb (250 mm × 4.6 mm × 5 μm), mobile phase 5 mM KH_2PO_4 (adjusted to pH 3 with OPA): ACN (70:30, v/v), flow rate 1 mL/min, column temperature 30 °C, injection volume 100 μl, wavelength of 214 nm. (b) Chromatogram of spiked urine sample where the formoterol is 5 ng/mL; for chromatographic conditions see (a). (c) Chromatogram of a direct injection of unextracted urine of formoterol; for chromatographic conditions see (a).

On separate occasions the extracted blank urine samples were spiked with formoterol and some commonly used over the counter drugs (paracetamol, aspirin and ibuprofen). The chromatogram in Fig. 4 evaluates the absence of any interference caused by the common over the counter drugs. The formoterol peak in the extracted urine sample was monitored in the UV range from 200 to 400 nm with a DAD-detector. The peak purity test showed peak homogeneity, thus indicating that there were no coeluting metabolites or impurities present with the formoterol peak.

3.2.2. Method linearity

The calibration curve was linear in the range from 1.5 to 25 ng/mL. The samples were extracted and injected in duplicate.

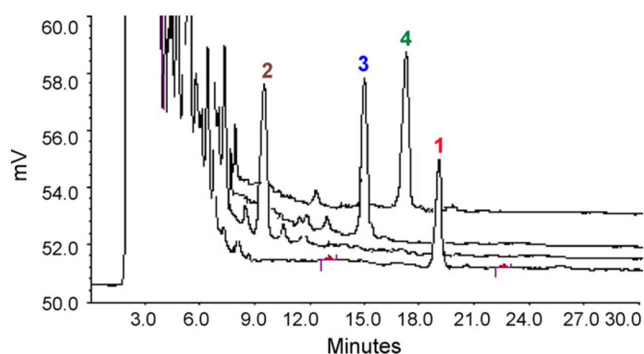

Fig. 4. Overlay of over the counter drugs with formoterol. Peak 1: formoterol; Peak 2: paracetamol; Peak 3: aspirin; Peak 4: ibuprofen.

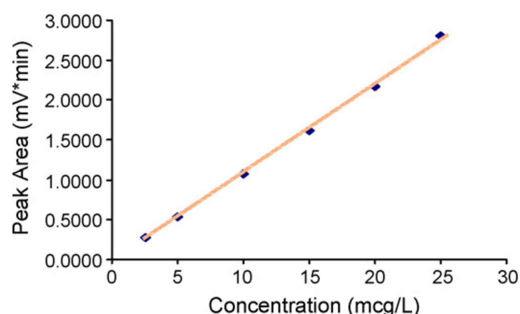

Fig. 5. Linearity curve.

The linear response (Fig. 5) for formoterol in urine gave an average correlation (r^2) of 0.9991 ($n = 6$).

ANOVA associated with linear regression was conducted and it was found that $F_{\text{statistics}} > F_{\text{critical}}$ and accordingly, the null hypothesis was rejected. Therefore, it may be concluded that the assumption of a linear relationship between the detector response and concentration of formoterol is valid.

3.2.3. Sensitivity

The limit of quantification and limit of detection were 1.5 and 0.7 ng/mL, respectively. The precision study revealed a relative standard deviation of <3.7% ($n = 10$) for the formoterol peak and an accuracy of 95%.

3.2.4. Accuracy/recovery

Accuracy was determined by comparing the calculated concentration of the extracted formoterol urine standard with the true concentration of formoterol. The accuracy pertains to the extraction efficiency within the limit of variability. The accuracy of the method ranged from 92 to 96% (Table 1).

Table 1
Accuracy data for formoterol

Actual concentration (ng/mL)	Observed concentration (mean±S.D., ng/mL)	%Accuracy
5	4.81 ± 0.43	96.28
10	9.51 ± 0.15	95.17
20	18.48 ± 0.32	92.42

Table 2
Recovery data following extraction of formoterol from urine matrix using SPE

Concentration (ng/mL)	S-1*	S-2**	%Recovery
5	0.59	0.58	98.31
	0.58	0.56	96.50
	0.58	0.59	100.76
	0.54	0.53	97.18
	0.59	0.54	92.14
Mean			96.98
10	1.21	1.14	94.54
	1.21	1.15	95.38
	1.21	1.17	96.70
	1.21	1.14	94.88
	1.22	1.15	94.64
Mean			95.23
20	2.16	1.95	90.38
	2.14	2.03	94.81
	2.14	2.10	97.86
	2.14	2.13	99.52
	2.14	1.98	92.70
Mean			95.06

* Formoterol standard spiked in extracted blank urine matrix.
** Extracted formoterol urine standard.

The analytical recovery was assessed by comparing the peak area of the extracted formoterol urine standard with the peak area of the formoterol standard externally spiked with extracted blank urine matrix. The recovery of formoterol was found to be consistent, precise and reproducible. The mean recovery of formoterol for low-to-high concentrations is >95.0% (Table 2).

3.2.5. Precision

The precision expressed as the intra-day coefficient of variation (CV%) ranged from 1.3 to 4.7% and as the inter-day CV% (Tables 3a and 3b), it ranged from 0.6 to 2.8%.

3.2.5.1. Intermediate precision.

The inter-day CV% ranged from 1.4 to 3.5% and intra-day CV% ranged from 1.3 to 3.4%.

3.2.6. Robustness

Minor variations in the method parameters did not have any affect on experimental results.

Table 3a
Intra-day precision

Extraction in a day	%CV		
	5 ng/mL	10 ng/mL	20 ng/mL
1	3.86	3.14	2.64
2	4.29	1.39	1.39
3	4.29	2.61	1.49
4	2.57	1.64	1.67
5	4.70	2.77	1.99
Mean	3.94	1.98	1.84

Table 3b
Inter-day precision

Days	%CV		
	5 ng/mL	10 ng/mL	20 ng/mL
1	1.44	2.10	0.60
2	2.18	2.83	0.81
3	0.40	1.21	1.22
4	0.05	2.54	1.27
5	1.55	2.48	1.21
Mean	1.12	2.23	1.02

3.2.7. Stability

i. Short-term stability
   Three aliquots each of low-and-high concentration test samples were thawed at room temperature and kept at this temperature for 24 h and analysed. The accuracy from samples ranged from 97 to 101% after short term stability testing.

ii. Freeze and thaw cycles
   Three aliquots of each level of concentration near the low (5 ng/mL) and high (20 ng/mL) range of the calibration curve were prepared and frozen at −20 °C for 24 h. The samples were thawed unassisted at room temperature and analysed. The samples were refrozen for 24 h under the same conditions. The freeze–thaw cycle was repeated two more times, and then analysed on the third cycle. The accuracy for samples ranged from 95 to 99% after freeze thaw stability testing.

iii. Standard aqueous solutions
   The standard solutions were found to be stable for five months when refrigerated at +4 °C. The samples were analysed every two weeks and were compared with freshly prepared standards. The concentration on comparison with freshly prepared standard after the storage was 99.9% (w/w).

iv. Processed samples stability
   The samples reconstituted with mobile phase were found to be stable for at least 48 h at 22 °C on the sample tray of the autosampler. The accuracy following its storage period was 94–98% of the nominal values of 5 and 20 ng/mL of formoterol.

4. Application of the method

The mean (S.D.) urinary excretion rate of formoterol following inhalation with (F + CH) and without (F) charcoal are shown in Fig. 6. The mean (S.D.) amounts excreted in the urine during the 24 h post inhalation with and without charcoal were 3.66 μg (0.42) and 4.80 (g (0.36) (Table 4a). This represents 7.6% (0.89) and 10.0% (0.75) (Table 4a) of the nominal dose, respectively. Individual amounts of formoterol excreted in the urine over the first 30 min post inhalation with and without charcoal have a mean (S.D.) of 1.46 μg (0.42) and 1.83 μg (0.73), respectively (Table 4b).

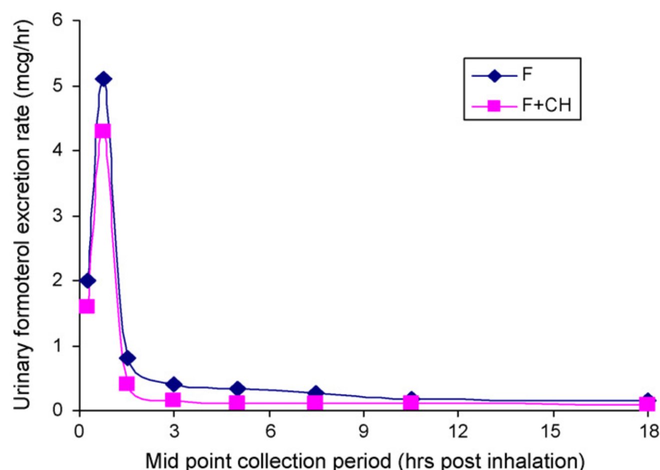

Fig. 6. Mean (S.D.) urinary formoterol excretion rates following the inhalation of 48 μg with (F + CH) and without (F) the co-administration of 25 g oral charcoal.

Table 4a
Represents the urinary excretion data for 24 h following inhalation without charcoal (F) and with charcoal (F + C)

Volunteers	Without charcoal		With charcoal	
	Amount excreted in 24 h (μg)	% Dose excreted in 24 h	Amount excreted in 24 h (μg)	% Dose excreted in 24 h
1	3.94	8.21	4.95	10.32
2	3.12	6.50	4.26	8.87
3	4.06	8.46	5.01	10.44
4	3.51	7.31	4.98	10.39
Mean	3.66	7.62	4.80	10.00
S.D.	0.42	0.89	0.36	0.75

A previous urinary pharmacokinetic method, using mass spectrometry detection, has shown that the urinary excretion following inhalation of a 12 μg dose in a capsule was about 7% [13]. Another study (in abstract form) has reported that the urinary excretion of formoterol after inhalation from this capsule formulation was approximately 4.4%, and when inhaling 54 μg from a formoterol Turbuhaler (AstraZeneca), it was 8.8% [27]. The study used concurrent oral administration of charcoal. The latter value is similar to the 7.7% we have found in this study following inhalation using the Turbuhaler with the co-

Table 4b
Represents the urinary excretion data for 30 min following inhalation without charcoal (F) and with charcoal (F + C)

Volunteers	Without charcoal	With charcoal
	Amount excreted in 30 min (μg)	Amount excreted in 30 min (μg)
1	1.01	1.09
2	1.78	2.41
3	1.19	1.31
4	1.86	2.51
Mean	1.46	1.83
S.D.	0.42	0.73

administration of oral charcoal. The comparison of the 30 min urinary excretion data indicates that significant oral absorption may be occurring immediately after inhalation, and so it is likely that co-administration of charcoal will be required. Also, the suggestion that higher concentrations were found following acid hydrolysis of the urine samples suggests that metabolites are present as previously suggested [28]. The lung deposition could thus be higher than the 7.7% suggested from the co-administration of charcoal. The above data highlights the potential to extend the urinary pharmacokinetic methods developed in-house [29] to analyse formoterol.

5. Conclusion

Previous pharmacokinetic studies of formoterol presence following inhalation have used plasma samples [13]. Due to the small doses used and the volume of distribution, all plasma formoterol concentrations are very low. Thus expensive assay detection techniques (LC–MS) are required to determine the concentration of formoterol in blood plasma. This method uses SPE to concentrate the final analyte (40 times) so that UV detection can be used to determine the low concentration of formoterol in urine samples. Therefore, this method can be used routinely in all laboratories which are not equipped with LC–MS. The method utilises simple solid phase extraction procedure, which gave efficient recoveries without using internal standard. We have demonstrated how urinary pharmacokinetic methods are used to identify the relative lung deposition following inhalation [30]. In order for our pharmacokinetic method to be applied to formoterol, we have developed a robust and reliable assay that does not need sophisticated detection methods.

The validation procedure was carried out based on FDA and ICH regulations [17–19] for validation of bioanalytical samples. Acceptable assay precision %CV <2.8 inter-day and %CV <4.7 intra-day was achieved. The SPE method for extraction of fomoterol from urine samples had a recovery of >95.0%. Excellent linearity was achieved over a range of 1.5–25 ng/mL with an average correlation co-efficient of 0.9991 ($n = 6$). All the potential internal standards failed to give consistent recoveries using the developed SPE procedure; to reduce the systemic variability, frequent calibration standards and quality control standards were injected between batches. As well, the developed method has proven to have consistent recoveries and inter and intra-day precision values, and thus the use of internal standard was deemed unnecessary [31].

In addition to its high sensitivity and robustness, the proposed method has proved reliable for the routine determination of formoterol in urine samples collected post inhalation.

As well, the robustness of the method makes it easy for an operator to learn the technique quickly and to generate reproducible results. The method is also very economical, with an approximate cost per sample of less than one pound sterling for the supply and material. In fact, a single analytical column under the assay condition has lasted for the entire period of method validation and clinical study.

In conclusion, a simple, robust, precise and reproducible method was developed for determination of formoterol in urine. The method was then validated according to the FDA and ICH guidelines set forth for bioanalytical method validation for human studies [17–19] and was successfully used to quantify formoterol in urine samples. This method has an adequate degree of robustness and simplicity to be used in determination of formoterol in clinical studies.

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