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Minimizing matrix effects in the development of a method for the determination of salmeterol in human plasma by LC/MS/MS at low pg/mL concentration levels

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Abstract

Salmeterol is an inhaled bronchodilator drug used for treatment of asthma. Its concentrations in plasma are very low or undetectable by previously developed methods. The present paper describes a method for analysis of salmeterol in human plasma with 2.5 pg/mL lower limit of quantitation. Despite the basic character of the drug the method uses mixed mode anion-exchange solid phase extraction for sample preparation combined with a column switching approach to minimize matrix effects. Samples are separated and detected by LC/MS/MS. The method is easy to use, only requires 0.5 mL of plasma and was validated for use in bioanalytical applications. The method does not suffer from interference from co-administered fluticasone propionate.

Keywords: Salmeterol; Human plasma; Matrix effects; Liquid chromatography; Mass spectrometry; Solid-phase extraction

1. Introduction

Salmeterol is a long-acting β_2-adrenergic receptor agonist drug currently used for treatment of asthma and chronic obstructive pulmonary disease. Currently, the route of administration is by inhalation using both dry-powder inhalers and pressurized metered dose inhalers. Salmeterol is administered on its own or, in cases of moderate-to-severe asthma, in a combination with inhaled corticosteroid fluticasone propionate. The combination formulation (Advair®) treats both the inflammatory and bronchoconstrictive components of asthma [1,2].

Salmeterol is commercially available as the xinafoate (1-hydroxy-2-naphtoic acid; HNA) salt of two enantiomers (Fig. 1). Both *R*- and *S*-enantiomers are long-acting [3] and their metabolism was found to be non-stereoselective in a human hepatic metabolism *in vitro* study which studied microsomal metabolism of salmeterol and its major metabolite α-hydroxysalmeterol [3,4].

HNA is absorbed, distributed, metabolized, and excreted independently from salmeterol and has no apparent pharmacological activity [3].

There is limited information on the pharmacokinetics of salmeterol. There is no data on the extent to which inhaled salmeterol undergoes first-pass metabolism. This is primarily due to the lack of suitable analytical methodology that is able to detect salmeterol at very low plasma concentrations after therapeutic dose administration. Low concentrations of salmeterol in plasma come from the lipophilic character of the drug (log *P* 3.07) and its rapid absorption in tissue. After administration of a therapeutic inhaled dose (50 μg), plasma concentrations could not be detected even 30 min after administration [3]. Larger inhaled doses give approximately proportionately increased blood concentrations. Plasma concentrations from 100 to 200 and 1000 to 2000 pg/mL were detected in healthy volunteers after inhalation of single doses of 50 and 400 μg, respectively [5]. In patients who inhaled 50 μg dose of salmeterol twice daily for 10 months a c_{max} of 70–200 pg/mL was observed in 45–90 min after inhalation, probably due to gastrointestinal absorption of the swallowed drug (most of the dose delivered by a metered-dose inhaler is swallowed) [6]. Following chronic administration of an inhaled dose of 50 μg of salmeterol twice daily, salmeterol

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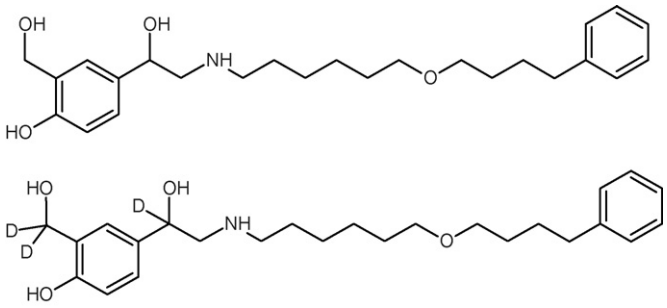

Fig. 1. Chemical structure of salmeterol and salmeterol-D_3 (3-hydroxymethyl-D_2, α-D_1), I.S.

was detected in plasma at mean peak concentrations of 167 pg/mL at 20 min without accumulation after repeated dose [7].

Several methods for determination of salmeterol in biological matrices were reported up to date. Caldwel et al. describe a method based on GC/MS in human plasma with solid-phase extraction (SPE) sample preparation and derivatization with a lower limit of quantitation (LLOQ) of 100 pg/mL [8]. Pleasance et al. described method based on LC/MS/MS and automated SPE down to 69 pg/mL in plasma [9]. Zhou et al. described a method for analysis of salmeterol in human serum with minimal sample preparation and capillary zone electrophoresis at the detection limit of 83 ng/mL [10]. Methods for stereoselective LC determination of salmeterol enantiomers were described and applied to in vitro metabolism studies with LLOQ of 1.04 μg/mL [4,11]. Salmeterol has also been analyzed in animal biological fluids. Lehner et al. described an LC/MS/MS method for analysis of equine urine and serum [12]. This method uses liquid–liquid extraction (LLE) for sample preparation and was used for analysis of high concentration samples up to 14 ng/mL. Another LLE-based LC/MS/MS method for analysis of equine urine after inhaled administration of salmeterol was described by Van Eenoo et al. with the LLOQ of 0.25 ng/mL [13]. A method based on LC with fluorescence detection for analysis of salmeterol in rat and dog plasma was described by Colthup et al. [14,15]. The method had a quantitation limit of 1 ng/mL and used SPE for sample preparation.

As described above, the most sensitive methods that reach the quantitation limits of 69–100 pg/ml were previously reported as various conference abstracts [8,9]. Clearly, more sensitive methodology is needed in order to allow for more extensive pharmacokinetic studies in human plasma or serum. These studies are currently problematic or impossible due to very low plasma concentrations and the inability to detect salmeterol in plasma by existing bioanalytical methods. In fact, due to analytical methodology sensitivity limitations, studies to assess the disposition of salmeterol in therapeutic dose ranges by radionuclide imaging techniques are now mandatory [3].

This paper describes rapid, simple, and sensitive method for determination of salmeterol in human plasma with 2.50 pg/mL limit of quantitation. To the best of our knowledge, this is more than an order of magnitude lower than the existing published methods. We present method development challenges arising from the need for very low quantitation limits and unique, non-intuitive solutions used to overcome them. The method was validated according to current US Food and Drug Administration guidelines for bioanalytical method validations [16] and was successfully used to analyze human plasma samples from a clinical study.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile, methanol, and acetone were HPLC grade and purchased from EMD Chemicals (Gibbstown, NJ, USA) along with formic acid, 98%, ACS grade. Salmeterol xinafoate, >99% and ammonium acetate, reagent grade, ≥98% were from Sigma-Aldrich (St. Louis, MO, USA). Deuterium-labeled salmeterol used as an internal standard, salmeterol-D_3 (3-hydroxymethyl-D_2, α-D_1), >98% chemical purity, 99% isotopic purity, was from CDN Isotopes (Pointe-Claire, Quebec, Canada). Deionized water used for preparation of reagents was Type I, typically 18.2 MΩ cm, generated in-house by Milli-Q water system from Millipore (Billerica, MA, USA). Human plasma (K_3EDTA anticoagulant) was purchased from Bioreclamation (Hicksville, NY, USA).

2.2. Sample extraction

An aliquot of 500 μL of the sample (a standard, a quality control sample, or a subject sample) was spiked with 50.0 μL of working Internal Standard (I.S.) salmeterol-D_3 (3000 pg/mL in methanol) and diluted with 2.50 mL of 100 mM ammonium acetate in water. Sample extraction was performed by SPE using Oasis MAX mixed-mode polymeric anion-exchange sorbent cartridges, 3 mL/60 mg format from Waters (Milford, MA, USA). SPE was carried out using a System 48 positive pressure SPE manifold from SPEware Corp. (San Pedro, CA, USA). After conditioning and equilibrating of SPE columns with 1 mL of acetonitrile and 1 mL of 100 mM ammonium acetate in water, the samples were loaded onto SPE columns. After passing through, SPE columns were washed with 1 mL of 100 mM ammonium acetate in water and dried at maximum pressure (~25 psi, 172 kPa) for approximately 5 min. After drying, samples were eluted slowly with 0.5 mL of 20/80 acetonitrile/water (v/v) and analyzed directly by LC/MS/MS without any further processing.

2.3. LC/MS/MS analysis

2.3.1. LC separation conditions

After extraction, the resulting extracts are injected onto a HPLC system consisting of three Shimadzu LC-10AD high pressure pumps (A and B for high pressure mixing of mobile phase, and C for precolumn backflush, see below), a SCL-10A system controller (Shimadzu, Columbia, MD, USA), a CTC PAL autosampler (Leap Technologies, Chapel Hill, NC, USA) equipped with a 150 μL sample loop and a 100 μL syringe, a Cera LC Column Heater 150 (Baldwin Park, CA, USA), and an electronically actuated six-port high pressure switching

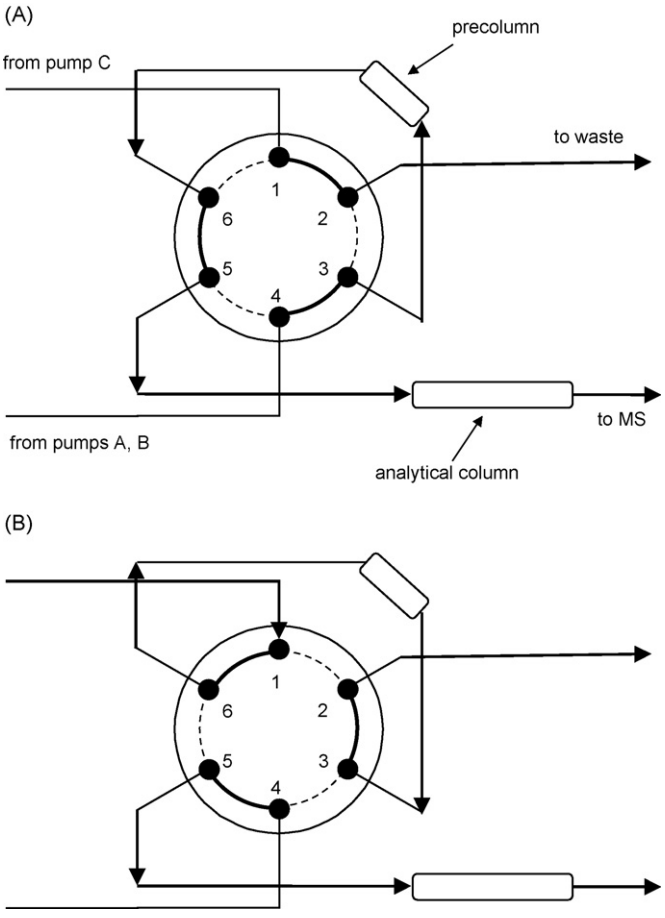

Fig. 2. Schematic diagram of precolumn backflush switching valve setup. Position of the valve: A—at the time of injection and at 4 min, B—at time of 1–4 min.

valve from Valco Instruments Co. (Houston, TX, USA). LC separation took place using a Betasil C18 analytical column (100 mm × 2.10 mm, 5 μm particle size, 100 Å pore size) and BetaBasic 8 precolumn (10 mm × 2.1 mm, 5 μm particle size, 100 Å pore size) connected to an electronic high-pressure 6-port switching valve as shown in Fig. 2. The analytical column was temperature-controlled at 30 °C. A 50 μL aliquot of the extracted sample was injected into the HPLC system as described above in the switching valve in position A in Fig. 2 with a mobile phase flow rate of 0.4 mL/min with 65%B isocratic conditions (A: 0.1% formic acid in water, vol.%; B: methanol). Mobile phase C (90/10 acetone/water, v/v) was pumped by an independent LC pump at 0.5 ml/min to waste. At 1 min of the run time, the switching valve was electronically switched to the position B (see Fig. 2). At 4 min of the run time, the valve was switched back to position A. Total run time was 4.5 min. MS acquisition time was 4.0 min. Because of the low concentrations of salmeterol being determined by this method, carryover was of a great concern. To minimize carryover, the autosampler was equipped with two wash stations. Wash 1 was 0.1% formic acid in 90/10 acetonitrile/water (v/v, vol.%) and wash 2 was 50% methanol in water (vol.%). A series of injection syringe washes and injector valve washes were performed after each injection with wash 1 followed by wash 2. The injection syringe was pre-conditioned with wash 2 immediately prior to aspirating a sample.

2.3.2. Mass spectrometric conditions

Salmeterol and salmeterol-D_3, were detected by tandem mass spectrometric detection with Turbo Ion Spray interface in positive ion mode. Data was acquired using Sciex API5000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) in multiple reaction monitoring (MRM) mode and monitored the following precursor $[M+H]^+ \rightarrow$ product ion mass transitions: 416.3 → 232.3, 200-ms dwell time for salmeterol, and 419.3 → 235.3, 100-ms dwell time for salmeterol-D_3. The MS conditions were as follows. Curtain gas: 20 (arbitrary units, a.u.), desolvation gas 1 and 2: 70, 70 a.u., ion spray potential: 2000 V, desolvation temperature: 500 °C, collision gas: 12 a.u., nitrogen, declustering potential: 100 V, entrance potential: 10 V, collision cell exit potential: 22 V, collision energy: 28 eV.

2.4. Method validation

2.4.1. Standard curve and quality control samples

Standard curve was prepared in human plasma at eight nominal concentrations ranging from 2.5 to 1000 pg/mL. Calibration curve standards in matrix were prepared fresh daily for each validation experimental run. QC samples were prepared in bulk at four concentration levels ranging from 2.5 to 800 pg/mL. In addition, one QC level above the upper level of quantitation (ULOQ) of the assay was prepared at 4000 pg/mL for validation of ability to dilute the samples that would be above the ULOQ. All QC samples were stored frozen at −20 °C and were thawed at room temperature before analysis. Each validation experimental run contained the calibration curve in duplicate, blank sample after each ULOQ standard for evaluation of method carryover, a blank with I.S., and each of the four concentration levels of QC samples ($n = 6$).

2.4.2. Validation experiments

The method was evaluated in terms of inter- and intra-assay precision and accuracy, selectivity (matrix effects) in six different sources of human plasma at LLOQ and ULOQ, carryover, ability to dilute, and extraction recovery. Selectivity of detection in terms of cross-channel MS signal contribution was evaluated by extracting samples containing the analyte at the ULOQ concentration without the presence of the I.S. and also blank samples with the I.S. at the working concentration without any analyte added.

In addition, stability of the analyte in matrix short term at room temperature and long term at −20 °C was demonstrated along with short and long-term stability in methanol at room temperature and at 4 °C, respectively. Stability after freeze/thaw cycles was also demonstrated.

Stability during sample collection in whole blood at room temperature was evaluated by spiking the analyte into whole blood at 415 pg/mL and centrifuging the resulting sample at 1 and 2 h intervals. The instrument response results from the resulting plasma samples were then compared to the results

from plasma samples from the blood centrifuged at the time of 0 h.

Extraction recovery was evaluated at three concentration levels (7.5, 400, and 800 pg/mL) by comparing the instrument response (analyte/I.S. area ratio) of plasma QC samples spiked with analyte prior to extraction to those spiked to extracted plasma blank samples after the extraction. The I.S. was added after extraction to both groups.

Reinjection reproducibility was evaluated by re-injecting a previously-extracted validation experiment batch after a period of storage at room temperature.

As mentioned earlier, salmeterol is often co-administered in a formulation with fluticasone propionate. To demonstrate the suitability of the method for analysis of samples containing fluticasone, an interference test was performed. In this test salmeterol plasma QC samples at 7.5 and 800 pg/mL salmeterol concentrations were prepared in the presence of 400 pg/mL fluticasone. The samples were analyzed and the results for salmeterol had to be within 15% of the targeted concentration value for the test to be successful.

Acceptance criteria for the above validation tests were based on current US FDA guidelines to industry for bioanalytical method validations [16]. All validation tests were performed at $n = 6$ unless stated otherwise.

3. Results and discussion

3.1. Method development

The goal in method development for analysis of salmeterol in human plasma was to achieve the LLOQ of 2.5 pg/mL in plasma that is required for support of pharmacokinetic and bioequivalence studies with salmeterol and its mixed formulation with fluticasone propionate.

The choice of instrumental technique used for detection and analysis of the resulting extracts is typically limited to the state-of-the-art instrumentation such as tandem mass spectrometry (MS/MS). The use of MS/MS still poses significant challenges considering the extreme nature of the concentration ratio of the analyte to endogenous compounds in the matrix. Even though mass spectrometry is a highly selective technique in MRM mode and in most cases only detects the desired analyte signal, it can suffer from ionization effects that cause problems with reproducibility and sensitivity. Ionization effects (ion suppression, ion enhancement) take place in the ionization source of the mass spectrometer and result from ionization competition between the analyte of interest and a competitor (i.e. co-extracted matrix) [17–19]. These are usually irreproducible between samples but can be compensated for by a proper choice of an I.S. For this work, stable isotope-labeled I.S. was used. This I.S. compensates for ionization effects by being influenced in the ionization source to the equivalent extent as the analyte (i.e. the I.S. does not eliminate ionization effects, only masks them). Therefore, any ion suppression occurring would still have adverse effect on the method sensitivity. Matrix effects, on the other hand, are any undesirable effects caused by a sample matrix. Matrix effects include not only the ionization effects, but also effects from physiological matrix variability and the resulting sample preparation irreproducibility, effects of matrix on chromatographic separation, etc. [17,18].

3.1.1. Sample preparation

In general, because of extremely low concentration detection levels required, the extraction of a large aliquot volume of the samples is typically required. These can be as high as 1–2 mL of plasma being extracted and typically concentrated into a small volume that is analyzed by a suitable analytical methodology. As a result, there is increased demand on elimination of possibly interfering compounds during sample preparation for the purpose of minimizing or eliminating matrix effects.

Initially, we considered common sample preparation methods based on protein precipitation and LLE. Protein precipitation does not clean the sample of unwanted material and, due to large extraction volumes, it was deemed not suitable for our application. LLE was optimized using solvents with varying polarity and pH values. Due to the low selectivity of LLE and resulting matrix effects from extraction of large volumes (minimum of 1 mL of plasma extracted) the technique was also deemed unsuitable (data not shown).

SPE was chosen as the method for sample preparation because of its higher selectivity, wide choice of separation mechanisms, greater potential for minimizing matrix effects, ability to automate, and lower solvent consumption. Salmeterol is a basic drug (see Fig. 1). It is pK_a is ~9.3 (–NH– group, calculated by ACD/PhysChem History software, v. 9.2, ACDLabs, Toronto, ON, Canada), so under physiological and most laboratory conditions, the nitrogen in the molecule is protonated. Based on the structure and pK_a alone, one would expect a cation-exchange or reverse phase extraction mechanism as the most appropriate for the drug extraction from a biological matrix. We screened multiple SPE materials based on different retention chemistries (mixed mode cation and anion exchange, reverse phase, weak reverse phase C_2) under multiple loading and elution pH conditions. As expected, Waters Oasis MCX mixed-mode cation exchange and HLB reverse phase provided high recoveries of salmeterol. However, using these materials, we were unable to reach the desired limit of quantitation. The likely reason for this is a high amount of co-extracted matrix by these sorbents due to low selectivity of the HLB sorbent and the requirement for highly organic and basic elution conditions with the MCX sorbent. Increasing the sample volume to greater than 1 mL was impractical due to problematic sample processing (i.e. SPE bed clogging). In addition to these two sorbents, high recovery was also achieved using Oasis MAX mixed-mode anion exchange sorbent under neutral sample loading and elution conditions (pH ~6.9). Under these conditions, the secondary amine group in the salmeterol molecule is protonated and the molecule is retained by weak reverse-phase interactions in the packing material due to highly lipophilic nature of the compound (log P 3.07, calculated by ACD/PhysChem History software, v. 9.2, ACDLabs, Toronto, ON, Canada). Optimization of the wash and elution steps in the SPE procedure was performed as follows: 0.5 mL of salmeterol human plasma samples were extracted by SPE as outlined in Section 2 and, in the elution step of the SPE, the replicate samples

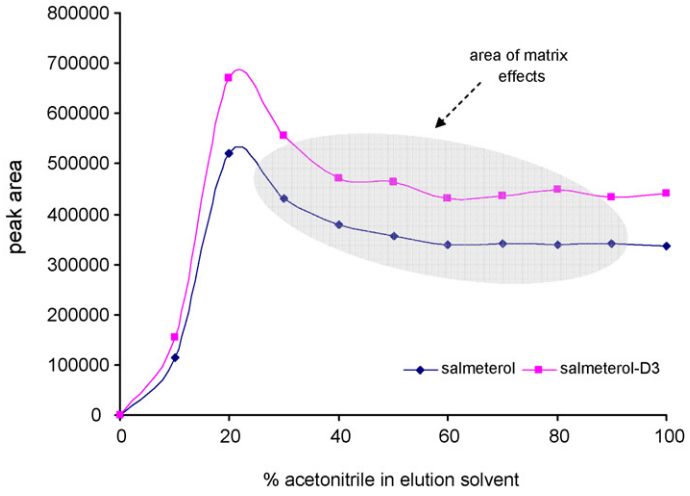

Fig. 3. Optimization of mixed mode anion exchange conditions for the SPE of salmeterol from human plasma.

were eluted with 1 mL of solvent with progressively increasing elution strength (0% acetonitrile to 100% acetonitrile in water in 10% increments). The extract was then evaporated and reconstituted in 0.5 mL of mobile phase and analyzed by LC/MS/MS. Fig. 3 shows the results of this optimization. The optimized SPE wash solvent composition is 100% aqueous, whereas maximum signal was achieved using 20% acetonitrile as an elution solvent. Analyte signal decreased at compositions of greater than 20% acetonitrile due to an increase in co-extraction of endogenous matrix components, consequently causing matrix effects and ion suppression in MS detector. Fig. 3 indicates that a relatively weak elution solvent can be used to elute salmeterol from the MAX sorbent. This may be because the elution is aided by electrostatic repulsion of the molecule from the anion-exchange functionality embedded in the sorbent because the analyte is protonated at these neutral pH conditions. As a result, the wash and elution steps were selected as stated in Section 2. The weak elution strength of the elution solvent and the use of anion-exchange SPE sorbent may also help to retain negatively charged phospholipids which would be unlikely eluted with 20% acetonitrile and are known to cause matrix effects in LC/MS/MS applications. Another advantage of the current SPE procedure is direct injection of eluted extracts into the LC/MS/MS without time-consuming evaporation and re-constitution in a suitable solvent. This is due to the low elution strength of the SPE elution solvent compared to the elution strength of the LC mobile phase. Consequently, this leads to an increased sensitivity in the reverse-phase LC because of peak focusing resulting from the difference in elution strength of the extract solvent and the mobile phase used for separation. This elution strength difference also allowed for the possibility of larger injection volumes in LC separation which further increased sensitivity. Injection volumes of 50 μL or more could be used without adverse effect on chromatographic performance. At these injection volumes the extract volume was sufficient for repeated analysis if necessary. The SPE procedure developed provided seamless compatibility with the LC separation due to the strong retention of matrix contaminants and weak retention of analyte on the SPE sorbent. The SPE elution volume was further optimized and it was determined that the equivalent recovery is achieved at 0.5 mL elution volume. A decrease in the elution volume further minimized the extraction-based matrix effects by allowing for more matrix retention on the SPE sorbent and more selective elution of salmeterol. As a result of this SPE sample preparation design, it was possible to decrease the sample volume to 0.5 mL of plasma while maintaining the acceptable S/N ratio at the desired LLOQ of 2.5 pg/mL.

3.1.2. LC/MS/MS optimization

Electrospray ionization (ESI) was used in MS detection of salmeterol. Atmospheric pressure chemical ionization (APCI), although less prone to ionization effects, did not provide adequate sensitivity. Ionization conditions were optimized by infusion of salmeterol (200 ng/mL, 5 μL/min) into a stream of mobile phase at the chromatographic flow rate and composition.

In most current bioanalytical applications, relatively short LC columns are used (i.e. length of 50 mm or less). In most applications, short columns provide adequate combination of speed and separation capacity due to high selectivity of MS/MS detection. In the current application, because of the difference in elution strength of the extract solvent and the mobile phase required for separation, and a high injection volume, there was a concentration of co-extracted matrix on a typical short reverse-phase column which caused ion suppression and deterioration of chromatographic performance over time. This was mainly due to limited separation capacity of the column. Consequently, we decided to use a longer column with higher separation capacity (Betasil C18 100 mm × 2.10 mm, see Section 2) which allowed us to separate the co-extracted matrix from the analyte. As mentioned earlier, the SPE procedure developed results in very clean extracts and the amount of co-extracted matrix components is minimal but still exists in significant quantities compared to low pg/mL levels of salmeterol. Co-extracted matrix contaminants can accumulate on the analytical column over time, especially with the large injection volumes used in our application. Initially, this caused gradual shift of salmeterol peak retention time. Over the analysis of approximately 70 samples, the retention time decreased by more than 1 min. To eliminate this problem, a column switching approach was used, which is shown in Fig. 2. The sample is injected by the autosampler to the HPLC system with the initial position of the switching valve (position A in Fig. 2) and carried to a pre-column. The pre-column contains a less retentive stationary phase (C_8) than the analytical column (C_{18}). The less retentive pre-column was intentionally used for the purpose of rapid transfer of analyte to the analytical column since the analyte would not be retained strongly on this stationary phase. However, many matrix-effect causing contaminants (such as phospholipids) are well retained on this stationary phase using our mobile phase conditions. Complete transfer of salmeterol to the analytical column occurs within 1 min into the analysis time. At this time, the switching valve is switched to the position B (see Fig. 2). The separation on the analytical column continues unaffected since the analyte is already transferred at the time of switching, but the precolumn is now back-flushed with strong mobile phase C (90/10 acetone/water, v/v), which

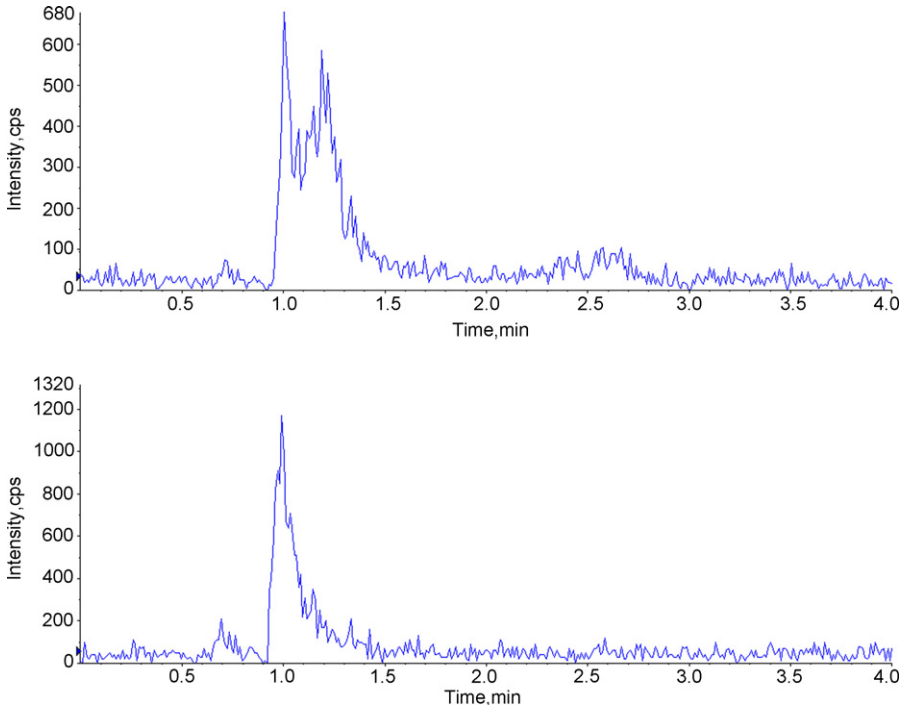

Fig. 4. Chromatogram of extracted blank human plasma. Top trace—salmeterol, bottom trace—salmeterol-D_3 I.S.

elutes the accumulated matrix contaminants to waste. After the salmeterol peak is detected by the MS detector, the precolumn is switched back in-line with the analytical column and allowed to equilibrate for 0.5 min. Using this column switching approach, we were able to achieve stable retention times of salmeterol. By using the less retentive stationary phase in the pre-column than the analytical column, the run time and sample throughput was not compromised significantly while the matrix effects resulting from irreversible accumulation of co-extracted matrix contaminants on the LC column were eliminated. Representative chromatograms of extracted blank plasma and a LLOQ sample at 2.5 pg/mL are shown in Figs. 4 and 5.

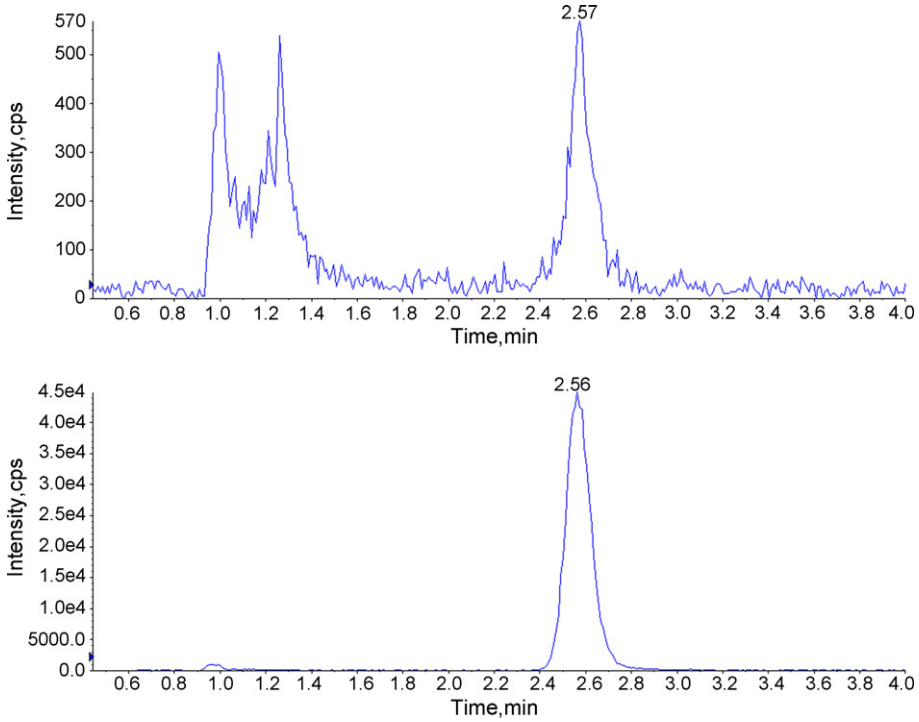

Fig. 5. Chromatogram of salmeterol at lower limit of quantitation of 2.5 pg/mL in human plasma. Top trace—salmeterol, bottom trace—salmeterol-D_3 I.S. (300 pg/mL). Salmeterol elutes at ~2.5 min.

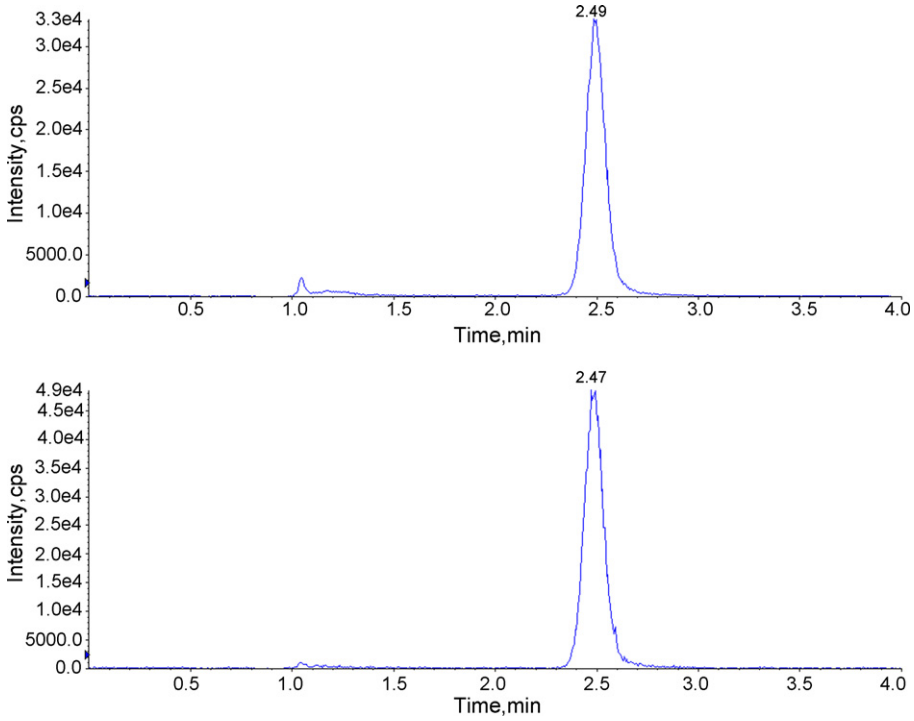

Fig. 6. Chromatogram of a human subject sample after inhaled administration of a therapeutic dose (50 μg) of salmeterol. Determined salmeterol concentration 150 pg/mL. Top trace—salmeterol, bottom trace—salmeterol-D_3 I.S. (300 pg/mL). Salmeterol elutes at ~2.5 min.

The usefulness of the method was demonstrated by analysis of plasma samples from a human subject after inhalation of therapeutic dose (50 μg) of salmeterol. Representative chromatogram from a human subject sample and a time-concentration plasma profile are shown in Figs. 6 and 7.

3.2. Method validation

Validation of method for determination of salmeterol in human plasma (K_3EDTA) by LC/MS/MS in the range of 2.50–1000 pg/mL was performed according to current US FDA guidelines [16].

Accuracy and precision were evaluated in three separate validation runs on three different days at four concentration levels (including LLOQ) at $n = 6$. Intra- and inter-day accuracy and precision were evaluated. Back-calculated calibration data over three accuracy/precision runs are shown in Table 1. Intra- and Inter-day accuracy and precision data are shown in Table 2. Method carryover was evaluated in the three accuracy/precision validation runs. No carryover was detected in any of the carryover evaluation blank samples immediately following the ULOQ samples. A blank sample after a ULOQ sample is presented in Fig. 4.

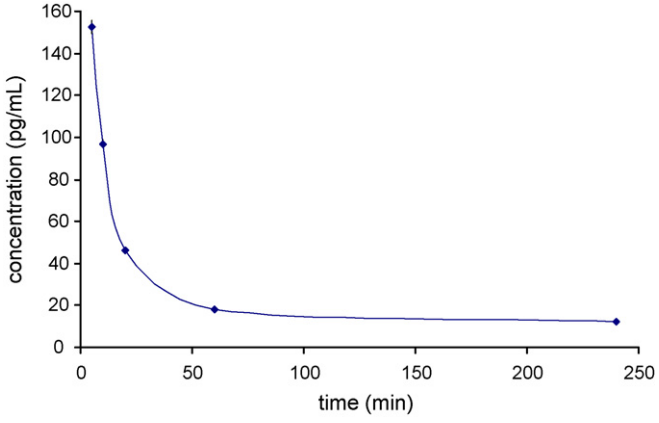

Fig. 7. Plasma concentration profile of salmeterol after inhaled administration of a therapeutic dose (50 μg) of salmeterol to a human subject.

Ability to dilute the samples was validated by diluting plasma samples at 4000 pg/mL by a dilution factor of 10. Accuracy and precision were 104.8% and 2.4%, respectively.

Method selectivity has been evaluated by analysis of blank extracted human plasma samples from six different sources of human serum. In addition, QC samples at LLOQ and ULOQ of the assay were prepared in the six plasma sources tested. No peaks were detected at the retention time of salmeterol and the I.S. in any of the tested plasma sources. The results of the selectivity test at LLOQ and ULOQ concentration levels are in Table 3. Calibration curve slope can be influenced by matrix effects and vary in different sources of human plasma [18,19]. As a result, the selectivity test was performed not only at the LLOQ level but also at the ULOQ level. Agreement of LLOQ and ULOQ selectivity samples with their respective targeted concentrations confirm lack of slope variation and matrix effects. Selectivity of the MS detection was evaluated by analyzing samples of the analyte at the ULOQ level without the I.S. added and also samples with the I.S. without the analyte added. No cross-channel contribution was detected.

Extraction recovery was evaluated at three concentration levels (7.5, 400, and 800 pg/mL, $n = 6$ each) as described in

Table 1
Back-calculated concentrations of calibration standards for salmeterol over three validation accuracy/precision runs

	Standard concentration (pg/mL)							
	2.50	5.00	25.0	100	250	500	850	1000
Mean	2.53	4.86	25.4	105	246	519	825	961
S.D.	0.0846	0.200	0.628	2.14	5.22	5.19	10.1	19.4
%CV	3.3	4.1	2.5	2.0	2.1	1.0	1.2	2.0
%Bias	1.2	−2.8	1.6	5.0	−1.6	3.8	−2.9	−3.9
n[a]	6	6	6	6	6	6	6	6

[a] *n* value over three different days.

Table 2
Accuracy and precision data for salmeterol over three validation runs from analysis of variance (ANOVA)

	Nominal QC concentration			
	2.50 (pg/mL)	7.50 (pg/mL)	400 (pg/mL)	800 (pg/mL)
Mean observed concentration	2.47	7.70	410	803
% Bias	−1.2	2.7	2.5	0.4
Between run precision (%CV)	1.4	1.9	0.8	1.3
Within run precision (%CV)	6.8	2.7	2.9	2.0
Total variation (%CV)	7.0	3.3	3.0	2.4
n[a]	18	18	18	18
Number of runs	3	3	3	3

[a] *n* value over three validation runs.

Section 2. The mean extraction recovery was 73.6% with mean %CV of 5.8%. No concentration dependency was observed.

Reinjection reproducibility was evaluated at room temperature where a previously extracted validation experiment batch was re-injected after 115 h of storage at ambient temperature (data not shown).

As mentioned earlier, salmeterol is often co-administered with fluticasone propionate in a combined inhaled formulation. Maximum peak plasma concentrations of fluticasone after inhalation of therapeutic doses were reported to be approximately 100 pg/mL [20–23] but concentrations of up to 245 pg/mL were also reported [24]. In addition, Michael et al. reported possible formation of inter-molecular adducts of salmeterol and fluticasone in solution and in the ionization source of MS detector [25,26], which may complicate the determination of salmeterol in the presence of fluticasone. In fact, due to these interactions, we encountered significant difficulties when trying to develop a combined bioanalytical method for salmeterol and fluticasone when they are concurrently present in the same extract (data not shown, to be discussed in future publication). To the best of our knowledge, the salmeterol-fluticasone interference testing has not been addressed in any of the previously established methods for determination of salmeterol. As a result, an interference test was conducted with the current method where the plasma samples containing salmeterol at 7.5 and 800 pg/mL were prepared in the presence of 400 pg/mL fluticasone, which is approximately 4× higher than the physiologically-relevant maximum observed fluticasone concentrations in human plasma. Interference test results are in Table 4. The results indicate that the presence of fluticasone up to 400 pg/mL in salmeterol samples does not affect the quantitation of salmeterol. Interference in the presence of metabolites of fluticasone and salmeterol (primarily α-hydroxysalmeterol) was not investigated. The risk of interference from metabolites of fluticasone and salmeterol in the current method is very low considering that fluticasone metabolites would be separated from salmeterol in the SPE step and due to extremely low concentrations of salmeterol metabolites.

Table 3
Selectivity at the lower and upper limit of quantitation of salmeterol in six different sources of human plasma

Run date	LLOQ 2.50 (pg/mL)	ULOQ 1000 (pg/mL)
Mean	2.37	949
S.D.	0.180	22.6
% CV	7.6	2.4
% Nominal	94.8	94.9
% Bias	−5.2	−5.1
n	6	6

In addition to the tests above, stability of salmeterol in human plasma was demonstrated for up to 6 h at room temperature, 100 days at −20 °C, and four freeze/thaw cycles. Stability in

Table 4
Interference testing for salmeterol in the presence of 400 pg/mL fluticasone in human plasma

Salmeterol concentration	7.50 (pg/mL)	800 (pg/mL)
Mean	7.97	795
S.D.	0.279	13.9
% CV	3.5	1.7
% Nominal	106.3	99.4
% Bias	6.3	−0.6
n	6	6

human blood (K_3EDTA) during sample collection for up to 2 h at room temperature was also demonstrated. Salmeterol was found stable in methanol at 4 °C for up to 182 days and 6 h at room temperature.

4. Conclusions

A method for determination of salmeterol in human plasma has been developed and validated with the lower limit of quantitation of 2.5 pg/mL, which is more than an order of magnitude lower than previously available methods. The method uses simple and effective SPE for sample preparation. Because of the LLOQ required for the method, combined approach to minimizing matrix effects in the assay was employed. This included "reverse" SPE; that is, basic salmeterol molecule was retained by a weak reverse-phase mechanism on a mixed-mode anion-exchange SPE sorbent. These conditions provided required orthogonality with the LC/MS/MS separation and detection. This strategy is applicable to other drugs and is being used extensively in our laboratory. This unique approach allowed for easy interface with LC separation where the sample was further cleaned by a column switching approach. Differential stationary phase retention ability between precolumn and the analytical column further aided in minimizing matrix effects and allowed us to reach the LLOQ of 2.5 pg/mL with only 0.5 mL of plasma sample size requirement. Due to its high sensitivity, the method is suitable for analysis of salmeterol in human plasma after inhaled administration of salmeterol. Since salmeterol is commonly co-administered with fluticasone propionate, interference testing was performed to demonstrate that fluticasone does not interfere with the determination of salmeterol. Fluticasone is present in a combined formulation with salmeterol in Advair® Diskus. It was demonstrated that the method is suitable for analysis of salmeterol in human plasma containing up to 400 pg/mL fluticasone. The method was successfully used in analysis of plasma samples from a clinical trial.

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