Journal of Chromatography B, 876 (2008) 163–169

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Investigation of interaction between salmeterol and fluticasone propionate and its effect on quantitative accuracy of an LC/MS/MS assay in human plasma at low pg/mL concentrations

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ARTICLE INFO

Article history:
Received 3 July 2008
Accepted 19 October 2008
Available online 25 October 2008

Keywords:
Fluticasone
Salmeterol
Interaction
Human plasma
Liquid chromatography
Mass spectrometry

ABSTRACT

This paper reports an LC/MS/MS method for analysis of salemeterol and fluticasone propionate in human plasma based on combined SPE-based extraction and separate LC/MS/MS conditions. Previously reported interaction between analytes was confirmed and eliminated by their separation in the sample preparation step to ensure no negative impact on their quantitation. The method was validated per FDA guidelines in the range of 2.5–500 pg/mL for salmeterol and 5–500 pg/mL for fluticasone propionate. The method is suitable for plasma analysis of combined salmeterol/fluticasone formulation without adverse effects of inter-analyte interactions on quantitation.

1. Introduction

Advair® is used for the treatment of airflow obstruction in patients with chronic obstructive pulmonary disease associated with chronic bronchitis. It is an inhalation powder that includes both salmeterol xinafoate and fluticasone propionate [1–3]. Salmeterol is a selective, long-acting beta-2-adrenergic agonist and fluticasone propionate is a synthetic corticosteroid with anti-inflammatory activity (see Fig. 1 for structures). Both compounds have been also marketed separately.

Michael et al. reported that an interaction between salmeterol and fluticasone propionate takes place in methanol/water [4,5]. In their studies, they report an intermolecular association occurring between salmeterol and fluticasone propionate in solution and in the ionization source of a mass spectrometer. In addition, in solvent solution, there are various compositions of monomers and dimers of both salmeterol and fluticasone propionate. To our knowledge, no information has been reported to date regarding whether the same interaction exists when both compounds are contained in human plasma. Such an interaction could have adverse effect on quantitation of either compound at low pg/mL concentrations in human plasma.

Analytical methods for the determination of salmeterol in biological matrices have been published [6–11]. These methods have limitations because lower limits of quantitation (LLOQ) are not low enough to analyze plasma concentrations of salmeterol in inhaled therapeutic dose ranges. We have recently reported a salmeterol method with a LLOQ of 2.50 pg/mL, which is more than an order of magnitude lower than any other published salmeterol method [12]. There are several methods for the determination of fluticasone propionate [13–17]. To our knowledge, there are currently no published methods for the simultaneous analysis of salmeterol and fluticasone propionate in human plasma at physiologically relevant concentrations. One publication for the simultaneous determination of both compounds has a limit of detection of 0.26 μg/mL for salmeterol and 0.19 μg/mL for fluticasone propionate [18]. In addition, we are not aware that the salmeterol–fluticasone propionate interaction and its implications in quantitative analytical applications have been addressed. From an analytical perspective, significant analytical issues may be associated with such an interaction and ways to overcome them and their impact on quantitative determination of salmeterol and fluticasone propionate should be explored.

Here we report a method for the determination of salmeterol and fluticasone propionate in human plasma based on a combined extraction at 2.50 pg/mL (salmeterol) and 5.00 pg/mL

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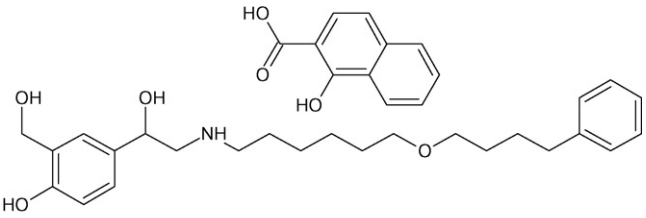
doi:10.1016/j.jchromb.2008.10.034

Structure 1a. Structure of Salmeterol Xinafoate.



Structure 1b. Structure of Fluticasone Propionate.

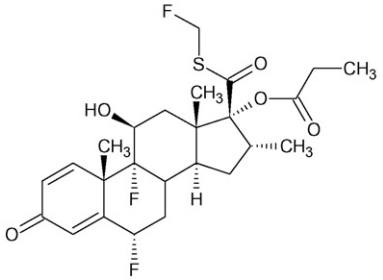


Fig. 1. Structures of salmeterol xinafoate and fluticasone propionate.

(fluticasone) that overcomes the analytical challenges associated with salmeterol–fluticasone propionate interaction. The limits of quantitation in our assay are several orders of magnitude lower than the only other reported combination assay [18]. To the best of our knowledge, this is the only method that addresses quantitation of each analyte in the presence of the other one via detailed interference evaluation and consideration for inter-analyte interactions that may be present in reconstitution solution after the sample preparation. Essentially, the method separates salmeterol from fluticasone propionate in human plasma during sample extraction and both compounds are injected onto a HPLC system under separate, optimized conditions for MS detection.

2. Experimental

2.1. Chemicals and reagents

Fluticasone propionate, >98% and fluticasone-D_3 propionate, >98% chemical purity, were obtained from Toronto Research Chemicals, Inc. (Toronto, Ontario, Canada). Salmeterol xinafoate, >99% and ammonium acetate, reagent grade, ≥98% were obtained from Sigma–Aldrich (St. Louis, MO, USA). Salmeterol-D_3 (3-hydroxymethyl-D_2, α-D_1), >98% chemical purity, 99% isotopic purity, was obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). Acetonitrile, methanol, acetone (HPLC-grade) and formic acid, 98% (ACS grade) were obtained from EMD Chemicals (Gibbstown, NJ, USA). *N*,*N*-Dimethylformamide (HPLC-grade) was obtained from Burdick and Jackson (Muskegon, MI, USA). 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 obtained from Bioreclamation (Hicksville, NY, USA).

2.2. Standard and QC preparation

Stock solutions were prepared in methanol for salmeterol and *N*,*N*-dimethylformamide for fluticasone propionate. All dilutions of stock solutions for both compounds were prepared in methanol. Standards were prepared by spiking 5% methanolic solution into human plasma. QCs which contained one analyte only were prepared by spiking 5% methanolic solution containing the compound into plasma. QCs which contained salmeterol and fluticasone propionate were prepared by first spiking 2.5% methanolic solution of one analyte into plasma, and then spiking the other analyte with 2.5% methanolic solution.

2.3. Sample extraction

An aliquot of 500 μL of the plasma sample (calibrator, quality control sample, or a subject sample) was spiked with 50.0 μL of working Internal Standard (I.S.): 3000 pg/mL salmeterol-D_3 and 4000 pg/mL fluticasone propionate-D_3 in methanol. The aliquot was then diluted with 2.50 mL of 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 water, the samples were loaded onto SPE columns. After passing through, SPE columns were washed with 1 mL of water and dried at maximum pressure (~25 psi, 172 kPa) for approximately 5 min. After drying, samples were eluted slowly for salmeterol with 0.5 mL of 20/80 acetonitrile/water (v/v) and analyzed for salmeterol directly by LC/MS/MS without any further processing as described elsewhere [12]. This elution for salmeterol simultaneously served as an additional SPE wash for fluticasone propionate. Following the wash (salmeterol elution), fluticasone propionate samples were eluted from the column with 1 mL of 60/40 acetonitrile/water (v/v). Fluticasone propionate extracts were evaporated to dryness at 50 °C and reconstituted in 0.200 mL of 60/40 acetonitrile/water (v/v). Reconstituted extracts were analyzed by LC/MS/MS for fluticasone propionate.

2.4. LC/MS/MS analysis

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 pre-column backflush, see below for more details), 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 valve from Valco Instruments Co. (Houston, TX, USA).

Data was acquired using Sciex API 5000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) in multiple reaction monitoring (MRM) mode.

2.4.1. Salmeterol LC/MS/MS conditions

The separation conditions used for salmeterol have been described in detail elsewhere [12]. Briefly, LC separation for salmeterol took place using a Betasil C18 analytical column and C8 precolumn. Salmeterol elutes from the precolumn to the analytical column but endogenous matrix compounds are retained and backflushed with each injection. Isocratic conditions are used with the analytical column using a mobile phase of methanol:0.1% formic acid in water (65:35) at 0.4 mL/min for 4.5 min.

Salmeterol and salmeterol-D_3, were detected with Turbo Ion Spray interface in positive ion mode. The following precursor $[M+H]^+ \rightarrow$ product ion mass transitions were monitored: 416.3 → 232.3, 200-ms dwell time for salmeterol, and 419.3 → 235.3, 100-ms dwell time for salmeterol-D_3. The source desolvation temperature was 500 °C. Fig. 2 is a chromatogram of salmeterol at 2.50 pg/mL.

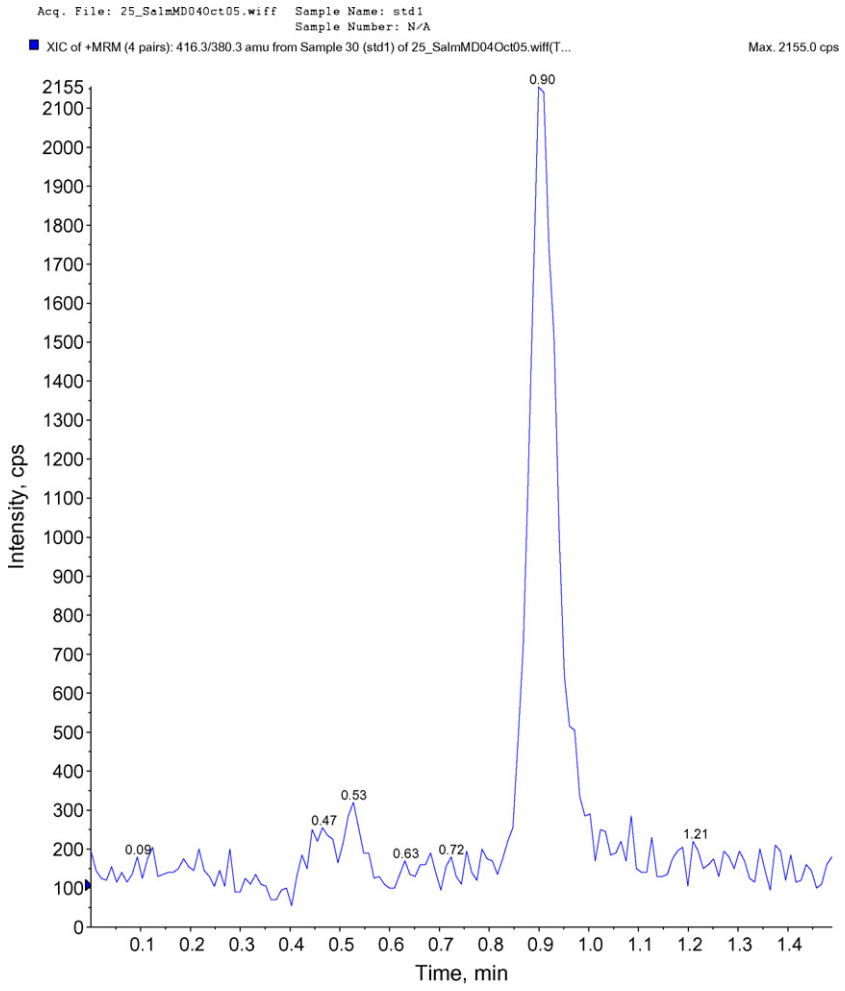


Fig. 2. Chromatogram of salmeterol at 2.50 pg/mL.

2.4.2. Fluticasone propionate LC/MS/MS conditions

LC separation for fluticasone propionate took place using a Phenomenex Prodigy ODS(3) analytical column (100 mm × 2.10 mm, 5 μm particle size). The analytical column was temperature-controlled at 30 °C. Extracted sample was injected into the HPLC system with a mobile phase flow rate of 0.5 mL/min with 70%B isocratic conditions (A: water and B: acetonitrile). Mobile phase C (90/10 acetone/water, v/v) was backflushed into the column by an independent LC pump at 0.8 mL/min between 2.0 and 3.0 min to clean the column from endogenous compounds injected from the sample extract. The retention time of the fluticasone propionate fluticasone propionate-D3 propionate was 1.45 min. Total run time was 4.5 min. The autosampler was equipped with two wash stations. Wash 1 was 5/95 dimethyl sulfoxide/water (v/v) and wash 2 was 80/20 acetonitrile/water (v/v). A series of injection syringe washes and injector valve washes were performed after each injection with wash 1 followed by wash 2.

Fluticasone propionate and fluticasone propionate-D_3 propionate were detected with APCI (Heated Nebulizer) interface in positive ion mode. The following precursor $[M+H]^+ \rightarrow$ product ion mass transitions were monitored: 501.2 → 313.2, 250-ms dwell time for fluticasone propionate, and 505.2 → 314.2, 100-ms dwell time for fluticasone propionate-D3 propionate. The ^{13}C isotopic peak of fluticasone propionate-D_3 propionate is monitored because fluticasone propionate contributes signal to the ^{12}C peak transition at 504.2 → 313.2 or 504.2 → 314.2. The MS conditions were as follows. Curtain gas: 15 (arbitrary units, a.u.), desolvation gas 1 and 2: 90 and 40 a.u., nebulizer current: 5 V, desolvation temperature: 300 °C, collision gas: 12 a.u., nitrogen, declustering potential: 110 V, entrance potential: 5 V, collision cell exit potential: 18 V, collision energy: 17 eV. Fig. 3 is a chromatogram of fluticasone propionate at 5.00 pg/mL.

2.5. Method validation

A salmeterol-only assay was fully validated and the data for the validation has been published [12]. Included in that validation was an interference test in which QCs at two salmeterol concentration levels were prepared in plasma: 7.50 and 800 pg/mL. Both of these QCs were also prepared with 400 pg/mL fluticasone propionate.

A fluticasone propionate-only assay was also fully validated and data is not shown here. A similar interference test was conducted for fluticasone propionate in the presence of salmeterol. Interference QCs were created to evaluate the effect on fluticasone concentrations while in the presence of 200 pg/mL salmeterol. 500 μL low (15.0 pg/mL) and high (800 pg/mL) fluticasone propionate QCs ($n = 6$) were spiked with 20.0 μL of 5000 pg/mL salmeterol solution in methanol.

To demonstrate acceptable results for the interference experiments: (1) four of six QCs for each level must be within ±15% of nominal concentration and (2) the %Theoretical and %CV for the mean of the test QC samples must be within ±15%. Acceptance criteria for the above validations were based on current US FDA guidelines to industry for bioanalytical method validations [19].

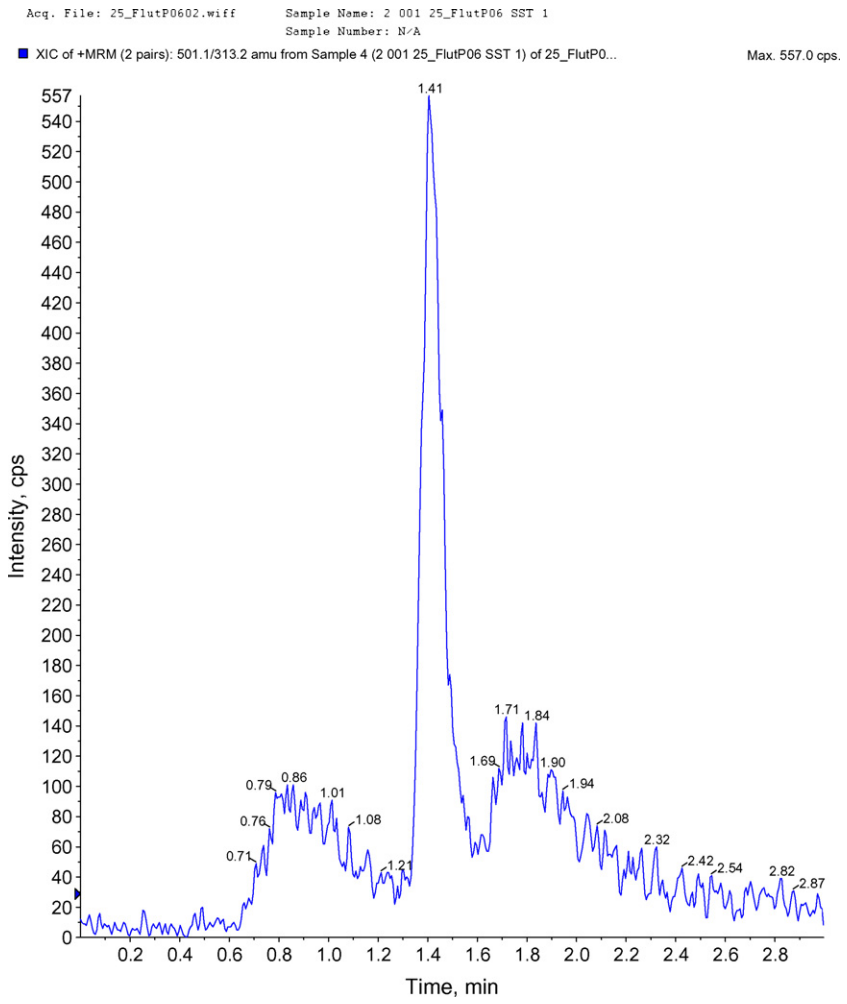


Fig. 3. Chromatogram of fluticasone at 5.00 pg/mL.

These guidelines do not provide clarity on the topic of interference testing in terms of suggested experimental design and implications on assay accuracy.

Subsequent to the full validations of salmeterol and fluticasone propionate, a partial validation was performed in which standards with salmeterol-only and fluticasone propionate-only were used to quantitate QCs prepared with salmeterol-only, fluticasone propionate-only, and QCs with both salmeterol and fluticasone propionate combined. The extraction method that was used in the partial validation was essentially the same as those in the full validations (see Section 2 for details), with the exception that the fluticasone propionate extracts received an additional wash of 100% water prior to the acetonitrile/water (20/80, v/v) wash. Salmeterol and fluticasone propionate were collected in separate SPE elutions and were injected under separate LC/MS/MS conditions (see Section 2 for details). Repeating this same design for the partial validation later on provided stability data for salmeterol and fluticasone propionate in human plasma when present together in the same samples.

2.5.1. Standard curve and quality control samples

Two separate standard curves were prepared—one with salmeterol-only and the other with fluticasone propionate-only. Standard curve was prepared in human plasma at eight nominal concentrations for each ranging from 2.50 to 500 pg/mL for salmeterol and 5.00 to 500 pg/mL for fluticasone propionate. Calibration curve standards in matrix were prepared fresh. QC samples were prepared in bulk—with salmeterol-only, fluticasone propionate-only, and a combination of salmeterol and fluticasone propionate. The QCs with both analytes in them were prepared by spiking solvent solution of each analyte separately into plasma. The concentrations of salmeterol-only QCs were 2.50, 200, and 400 pg/mL. The concentrations of fluticasone propionate-only QCs were 5.00, 200, and 400 pg/mL. The concentrations of the combined analyte QCs were 7.50 pg/mL salmeterol with 400 pg/mL fluticasone propionate and 200 pg/mL salmeterol with 15.0 pg/mL fluticasone propionate. All QC samples were stored frozen at −20 °C and were thawed at room temperature before analysis. Each run contained the calibration curve in duplicate, blank sample after each ULOQ standard for evaluation of method carryover, a blank with I.S., and QC samples ($n = 6$ for each level).

2.5.2. Stability

The stability of salmeterol and fluticasone propionate when combined together in human plasma was tested. This was performed by storing 7.50 pg/mL salmeterol with 400 pg/mL fluticasone propionate in human plasma and 200 pg/mL salmeterol with 15.0 pg/mL fluticasone propionate in human plasma for 56 days at −20 °C and then analyzing them against freshly prepared standards and QCs.

3. Results and discussion

3.1. Solvent solution experiment

Our experiment confirmed that there is a likely interaction between salmeterol and fluticasone propionate in solvent solution. This interaction was previously reported by Michael et al. [4,5]. Five different solutions that contained both salmeterol and fluticasone propionate were prepared in 50/50 methanol/water (v/v). The concentration of salmeterol was 100 ng/mL in each solution. The concentration of fluticasone propionate in each solution was 1.00, 10.0, 100, 1000, or 10,000 ng/mL. As the concentration of fluticasone propionate increased, the peak area of salmeterol decreased (see Fig. 4). There are several possible implications on quantitation using an assay that contains salmeterol and fluticasone propionate concurrently in solution, in matrix, or in extracts. Most importantly, inaccurate sample concentrations could result for just one analyte, or the other, or both analytes. Such inaccurate results would be difficult to notice, since a run would still likely meet acceptance criteria as QCs would quantitate against standards as expected. Standards and QCs would be prepared the same way—but not the same as actual clinical study samples. Also, curve linearity may be altered as a result of an increased or decreased interaction at different concentrations and thus sample concentrations could be biased.

3.2. Method development

Initial efforts to develop the method focused on having one extraction and one instrument method. However, our approach changed to a one extraction/two separate injection method for the following reasons:

(1) Due to the potential problem of the interaction of salmeterol and fluticasone propionate in solvent solution, we ensured that both compounds would not exist at any time in solution, including reconstituted extracts. If a single HPLC column was used for separation, the interaction and the impact may have occurred already in reconstitution solvent and any impact on quantitation accuracy would be unknown.
(2) Salmeterol and fluticasone propionate have different optimal mass spectrometer conditions. Electrospray ionization worked for salmeterol while APCI worked for fluticasone propionate. When electrospray ionization was used for fluticasone propionate, chromatographic performance deteriorated after multiple samples were injected, perhaps due to the large injection volume being used. The method was intended for analysis of large number of samples unattended in bioequivalence studies in regulated environment where method consistency was required. Suppression could be a potential issue with electrospray ionization and therefore, APCI is more suitable for fluticasone propionate.
(3) The separation mechanism used here with SPE would not necessarily correspond to the same separation mechanism using a single reverse-phase HPLC column for both compounds. The wash and elution solvents with an anion-exchange SPE column are neutral pH meaning the compounds are retained using reverse-phase like conditions. However, because the column is an anion-exchange, endogenous compounds may be retained differently on SPE than there are on a reverse-phase HPLC column. In addition, at neutral pH, salmeterol is protonated and it's retention on the SPE column is further weakened, allowing us to use weak elution solvent that can be directly injected into LC/MS and serving as a charge-driven separation tool from fluticasone. Therefore, a one SPE separation/one injection method may not provide optimal separation from endogenous compounds.

An interference experiment was performed using both the fluticasone propionate and salmeterol methods to determine whether the other compound would impact quantitation. The result was that neither compound interfered with the other. Extracts from evaluation of fluticasone propionate interference on salmeterol quantitation in salmeterol method validation were analyzed by fluticasone propionate LC/MS/MS method. Fluticasone propionate was not detected in these extracts, thus leading to the conclusion that salmeterol and fluticasone propionate are separated during the SPE process. This conclusion was the basis for combining the methods and eliminating the interaction in sample extracts of both validated methods (salmeterol and fluticasone propionate).

3.3. Sample preparation

Optimization of the wash and elution steps in the SPE procedure was performed as follows: 0.5 mL of human plasma with salmeterol or fluticasone propionate were extracted as outlined in Section 2 and in the elution step of the SPE, the replicate samples were eluted with 1 mL of solvent with progressively increasing elution strength (0–100% 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. 5 shows the results of this optimization. For salmeterol, the strongest (highest organic content without loss of analyte) wash conditions were 100% aqueous. The optimal elution condition was achieved using 20% acetonitrile. This elution condition for salmeterol is also the optimal wash condition for fluticasone propionate. It is the optimal wash condition for fluticasone

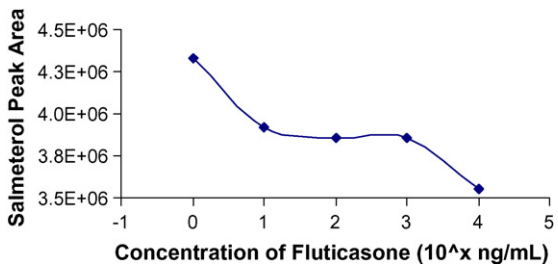


Fig. 4. Effect of varying fluticasone concentrations in the presence of 100 ng/mL salmeterol in neat solution.

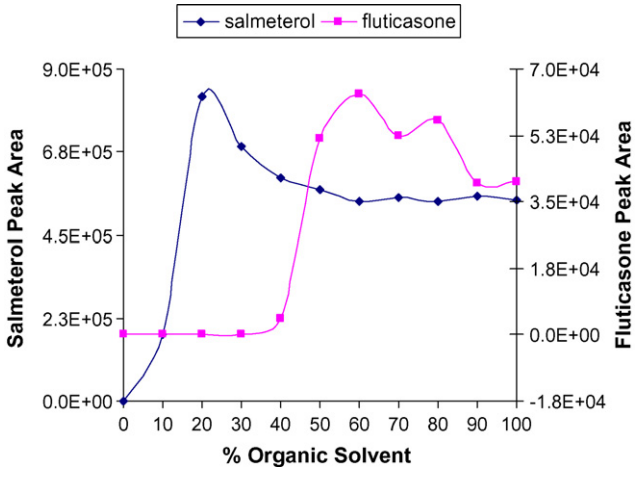


Fig. 5. Fluticasone and salmeterol elution profiles using an ion-exchange SPE column.

Table 1
Effect of salmterol on fluticasone propionate (FP) at varying concentrations.

%Theoretical	FP	
	15 pg/mL QCs	800 pg/mL QCs
Run 1		
Without salmeterol	105	104
With 1000 pg/mL salmeterol	142	99
Run 2		
Without salmeterol	108	103
With 200 pg/mL salmeterol	111	105

propionate not only because it provides the highest recovery (see Fig. 5), but because salmeterol is separated and any potential interaction is eliminated. The optimal elution condition for fluticasone propionate is 60% acetonitrile. Analyte signal decreases at compositions of a higher percent of organic beyond optimal conditions due to an increase in co-extraction of endogenous matrix components. Using an elution of greater than 20% acetonitrile for salmeterol and 60% for fluticasone propionate would needlessly increase matrix effects [12]. As a result of this experiment, it was observed that the elution conditions used for salmeterol simultaneously served as optimal wash conditions for fluticasone propionate. That is, while salmeterol is eluted from the SPE column, fluticasone propionate is retained on the column. This is the key step in sample preparation that separates salmeterol from fluticasone propionate and eliminates inter-analyte interactions in resulting extracts.

3.4. Analyte interactions

The interference tests conducted for the validations of salmeterol and fluticasone propionate met acceptance criteria.

Even with the separation conditions of salmeterol and fluticasone propionate during SPE, evidence still exists of a salmeterol–fluticasone propionate interaction at high concentrations. An additional experiment was performed in which relative concentration levels of salmeterol and fluticasone propionate varied to determine if there was any effect on quantitation. Table 1 shows the results of this experiment. At 1000 pg/mL concentrations of salmeterol, the quantitation of 15.0 pg/mL fluticasone propionate is adversely affected, which is a clear indication that an interaction is occurring. Salmeterol concentrations of 1000 pg/mL are physiologically improbable. In one study, the mean peak plasma concentrations in 14 healthy subjects dosed with Advair Diskus 500/50 (500 μg fluticasone propionate powder and 50 μg salmeterol powder) was 200 pg/mL [20]. In our experiment, fluticasone propionate at 15.0 pg/mL is unaffected when 200 pg/mL salmeterol is added. Also, salmeterol at 7.50 pg/mL is unaffected when 400 pg/mL fluticasone propionate is added [12].

Table 2
Stability of salmeterol and fluticasone propionate (FP) when combined together at −20 C for 56 days.

Analysis of salmeterol					
Salmeterol concentration (pg/mL)	7.50	7.50	200	200	400
FP concentration (pg/mL)	0	400	0	15.0	0
Mean	7.08	7.90	199	194	385
S.D.	0.239	0.153	3.44	2.23	5.01
%CV	3.4	1.9	1.7	1.1	1.3
%Theoretical	94.4	105.3	99.5	97	96.3
n	6	6	6	6	6
Analysis of FP					
FP concentration (pg/mL)	15.0	15.0	200	400	450
Salmeterol concentration (pg/mL)	0	200	0	7.50	0
Mean	15.3	15.1	194	410	440
S.D.	1.79	1.07	6.5	9.82	24.3
%CV	11.7	7.1	3.4	2.4	5.5
%Theoretical	102	100.7	97	102.5	97.8
n	6	6	6	6	6

3.5. Stability

Stability of salmeterol and fluticasone when present together in human plasma was demonstrated at −20 °C for 56 days. See Table 2 for results.

4. Conclusions

We have validated a method for the simultaneous analysis of salmeterol and fluticasone propionate in human plasma. The LLOQ for salmeterol is 2.50 pg/mL and for fluticasone propionate it is 5.00 pg/mL. Because an interaction occurs between salmeterol and fluticasone propionate in solvent solution, the method ensures that neither compound exists together at anytime in solvent solution. Solvent solutions used to prepare standards and QCs do not have salmeterol and fluticasone propionate combined together, but instead contain only one analyte. Standards and QCs prepared in matrix are thus spiked two times (once for each analyte). In human plasma, there does not appear to be a significant interaction between the compounds at physiologically relevant concentrations (≤500 pg/mL for each). However, there is an adverse interaction in matrix with higher (1000 pg/mL) concentrations of salmeterol when fluticasone propionate has lower (15.0 pg/mL) concentrations. With the current dosages of Advair® available, salmeterol concentrations above 500 pg/mL are improbable and even more unlikely with fluticasone propionate concentrations around 15 pg/mL. The potential for an interaction between compounds in solvent solution exists with reconstituted extracts containing both salmeterol and fluticasone propionate. Although there are no known published methods for the simultaneous analysis of salmeterol and fluticasone propionate, one should interpret data with caution from results using a method that include both compounds contained in reconstituted extracts and other solutions used in the method procedure. In our method, salmeterol is separated from fluticasone propionate using a Waters MAX SPE column that is optimized to elute salmeterol at 20% acetonitrile conditions. This elution is simultaneously the optimized wash used for fluticasone propionate prior to elution at 60% acetonitrile conditions. Thus, both compounds are separated during extraction and each compound is injected under separate, optimized LC/MS/MS conditions. Such optimized LC/MS/MS conditions further provide maximum sensitivity and thus the stated LLOQ for each compound was achieved in validation while interference-free from the other analyte.

Salmeterol and fluticasone propionate are administered as a combined formulation. The issue of inter-analyte interaction is important from an analytical perspective since accuracy of quantitation can be influenced from such an interaction. Other drug formulations in the same class of compounds (bronchodilators in combination with corticosteroids) exist and potential interactions with associated analytical challenges may need to be investigated to ensure quantitation accuracy. The presented approach can serve as an example of such investigation.

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