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Validation of a reverse-phase high performance liquid chromatographic method for concurrent assay of a weak base (salmeterol xinafoate) and a pharmacologically active steroid (fluticasone propionate)

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Abstract

The analysis of weakly basic drugs such as salmeterol xinafoate (SX) by reverse-phase liquid chromatography remains a problem, particularly when present in combination with other drugs such as steroids and weak acids. This study describes the validation of an assay for a weakly basic drug, salmeterol (SB), its weakly acidic counter-ion, 1-hydroxy-2-naphthoic acid (XA), and the neutral glucocorticoid, fluticasone propionate (FP) using a second-generation silica stationary phase (Inertsil ODS-2). The assay utilized an Inertsil ODS-2 base-deactivated 250 mm × 4.6 mm, 5 μm HPLC column, with 75:25 methanol:0.6% aqueous ammonium acetate as the mobile phase. Under these near neutral conditions, SB demonstrated a good peak shape (tailing factor = 1.21 ± 0.02, $n = 85$). The method provided a short analysis time: XA, $t_R = 2.96$ min; SB, $t_R = 5.23$ min and FP, $t_R = 7.01$ min. The assay displayed good sensitivity for both XA (LOD for SX = 0.22 μg mL^{-1}) and SB (LOD for SX = 0.26 μg mL^{-1}). The limit of detection for FP was 0.19 μg mL^{-1}. Neither of the drugs was found to interfere in the determination of the other and the assay accuracy (% recovery) was high (the recoveries were: 99.58 ± 1.85% for XA, 99.49 ± 1.88% for SB and 100.24 ± 1.28% for FP). The assay reproducibility was determined with a mean coefficient of variance for the five calibration concentrations of XA = 0.71 ± 0.18%; SB = 1.11 ± 0.64% and FP = 0.92 ± 0.14%. Analysis of a pressurized metered dose inhaler formulation demonstrated recovery of the analytes that are within pharmacopoeial limits. It was shown that RP-HPLC was suitable for the high throughput analysis of the combination of SX and FP.

Keywords: HPLC assay; Salmeterol xinafoate; Fluticasone propionate; 1-Hydroxy-2-naphthoic acid

1. Introduction

Salmeterol is a long-acting and highly selective β2-agonist formulated as its 1-hydroxy-2-napthoate (xinafoate) salt used in the treatment of asthma and chronic obstructive pulmonary disease [1]. Salmeterol xinafoate (SX) dissociates in solution to yield salmeterol base and hydroxynaphthoate, and displays poor aqueous solubility (lower than 62 μg mL^{-1} for the micronized material) [2]. This correlates with its high lipophilicity and poor wettability as indicated by its *n*-octanol/water partition coefficient (log D_{ow} = 1.45 for the charged and log D_{ow} (calc.) = 3.26 for the uncharged molecule [1]). Salmeterol is a weak base (a secondary amine) with an ionizable phenol. Its two pK_a values have been estimated as 8.3 and 10.3, respectively [3], ensuring that the molecule is >99.9% positively charged below neutral pH. Xinafoic acid has a pK_a of approximately 3.8 [4].

Fluticasone propionate (FP) is a glucocorticoid with potent anti-inflammatory activity used in the prevention of asthma [5]. It is very poorly water soluble (<1 μg mL^{-1}) [6] and also displays a high degree of binding to lung tissue [7]. It has activity when delivered topically to the airways in asthma, limiting the side effects associated with the use of systemically administered corticosteroids.

These two drugs are formulated both as dry powder inhalers and pressurized metered dose inhalers, both individually and as a combination formulation. While validated assays have been reported for each drug individually [8], there is no such assay that permits quantification of all the species present: salmeterol, xinafoate and fluticasone propionate. A short analysis time for high-throughput analysis is most desirable and a preliminary review of the literature showed that the currently reported methods were unsuitable for the analysis of the drug combination.

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For example, methods reported for salmeterol indicated long analysis times [2], required high flow rates (e.g. [8,9]) and/or were not validated [10]. Furthermore, the method described by Michael et al. [9] resulted in xinafoate peak which displayed poor peak performance including a split peak, and necessitated the inclusion of an ion-pair reagent in the mobile phase.

The assay of hydrophobic compounds routinely utilizes reversed-phase high performance liquid chromatography (RP-HPLC). RP-HPLC with UV detection was shown to be suitable for the assay of fluticasone propionate in a nasal formulation [11]. Other methods using octadecylsilane (ODS) stationary phases have been reported [12], which show high sensitivity when coupled with mass spectrometric detection techniques.

The analysis of basic compounds such as salmeterol by RP-HPLC remains problematic due to the occurrence of poor peak shapes and acid-base interaction with unreacted silanol groups on the ODS-coated silica stationary phase [13]. This is usually overcome by the acidification of the mobile phase to suppress the ionization of unreacted silanol groups. Additionally newer generation base-deactivated, high purity silica reverse phases allow improved peak performances at neutral pH [13].

The use of higher flow rates has been reported to be detrimental to the assay of some bases [14]. Acidification by buffering was judged unsuitable as the aim of this work was to assay concomitantly all compounds present: salmeterol base, xinafoic acid and fluticasone propionate. Xinafoic acid is only partially ionized at acidic pH, and its peak shape could be expected to be poor in a mobile phase buffered at lower pH values, to improve the performance of salmeterol base. The use of a higher temperature has been shown to reduce the tailing of the basic compound nortriptyline ($pK_a = 10$) by increasing the rate coefficient of interaction with the stationary phase [14], while the retention times for neutral compounds (FP is a neutral corticosteroid) usually decrease with an increase in temperature. Mobile phases containing the organic modifier methanol have been shown to improve peak performance for basic compounds compared to those based on acetonitrile [15]. The previously reported assays for salmeterol and fluticasone employed acetonitrile (e.g. [8]).

The aim of this work was to develop and validate an assay for salmeterol xinafoate and fluticasone propionate at near-neutral pH using a base-deactivated RP-HPLC column. A further aim was that the assay should have the capacity to quantify both salmeterol base and xinafoic acid with rapid analysis times rendering it suitable for high-throughput analyses.

2. Materials and methods

2.1. Reagents

Salmeterol xinafoate was purchased from Vamsi Labs Ltd. (Solapur, India). Fluticasone propionate was kindly donated by GlaxoSmithKline Research and Development (Ware, UK). Ammonium acetate (HiPerSolv grade) and HPLC grade methanol were purchased from BDH (Poole, UK) and Rathburn Chemicals (Walkerburn, UK) respectively. Deionized water was from the in-house supply. Seretide® (GlaxoSmithKline) pressurized metered dose inhalers were obtained from AAH Hospital Supplies (Coventry, UK).

2.2. HPLC

A HP 1050 modular liquid chromatography system was used for the analysis. The variable wavelength detector of the system was interfaced to a HP Chemstation for data acquisition using a HP 35900 C multichannel interface (Agilent Technologies UK Ltd., Wokingham, UK). Integration was carried out using the HP Chemstation LC data analysis module enhanced integrator (revision A.07.01). Separation was achieved using an Inertsil ODS2 column (5 μm, 200 mm × 4.6 mm) (Capital HPLC Ltd., Broxburn, UK), maintained at 40 °C using a column block heater (Jones Chromatography Ltd., Pontypridd, UK). The mobile phase was a mixture of methanol–0.6% (w/v) aqueous ammonium acetate solution (75:25%, v/v), filtered through a 0.45 μm nylon membrane (Whatman International Ltd., Maidstone, UK) and degassed. The mobile phase flow rate was 1.0 mL min^{-1}. The injection volume was 20 μL and detection was at 228 nm. Data and statistical analysis were performed using Microsoft Excel and Minitab respectively. The choice of 228 nm as the detection wavelength was determined by using a Dionex PDA 100 photodiode array detector, with Chromeleon Client Version 6.60 for analysis (Dionex Corporation, Sunnyvale, CA, USA) following injection of a standard solution of SX and FP.

2.3. Calibration standards

Mixed calibration standard solutions of salmeterol xinafoate and fluticasone propionate were prepared in mobile phase. Approximately 0.005 g of each drug was weighed accurately by difference and made up to 50 mL and dissolved with sonication, before being made up to final volume. The calibration series was prepared by serial dilution of this standard solution with mobile phase, to achieve a concentration range of 2–50 μg mL^{-1} of each drug.

2.4. Linearity

Linearity of the peak area response was determined by replicate injections ($n = 6$) of each of the five calibration standards on day 1. The relative standard deviation (% CV) of the peak height and area of the six injections was used to estimate the instrument precision. Linearity was determined using the LINEST function in Microsoft Excel.

2.5. Intra-day precision

The same series of standards was re-analyzed and the % CV at each of the concentration levels was calculated from the pooled data ($n = 12$) for each concentration level.

2.6. Inter-day precision

Two further calibration curves were analyzed by the preparation of a fresh series of calibration standards on days 2 and

3. The concentration varied slightly from day-to-day, due to the method employed, which involved preparing the standard solutions by weight difference. Therefore the % CVs were calculated by normalizing the area and height responses to unit concentration prior to analysis. Additionally linearity of the combined data from the three calibration curves was determined.

2.7. Limits of detection and quantification (LOD and LOQ)

These were calculated each day using Eqs. (1) and (2) [16]:

$$\text{LOD} = \text{intercept} + 3\,\text{Sy} \quad (1)$$

$$\text{LOQ} = \text{intercept} + 10\,\text{Sy} \quad (2)$$

where the intercept was determined by linear regression and Sy is the standard deviation of the y estimate from the linear regression.

2.8. Interference of cosolutes with the determination of SX and FP

The percentage recovery of SX from standard solutions ($n = 3$) and from a standard cosolution of SX and FP ($n = 4$) was determined by external standardization using a standard SX calibration curve. The percentage recovery of FP was determined in the same fashion using a standard FP calibration series. The approximate concentration of SX and FP in the standards was 50 μg mL^{-1}.

2.9. Accuracy

The percentage recovery of SX and FP from standard solutions ($n = 11$) was determined by external standardization (with an approximate concentration of SX and FP in the standard solutions of 50 μg mL^{-1}).

2.10. System suitability

System suitability data were determined for SX and FP using the data analysis output from the HP Chemstation. The parameters calculated using this revision of the enhanced integrator function were the tailing factor, tangent theoretical plates, tangent resolution and tangent peak width according to the formulae of the USP XX [17].

2.11. Analysis of a formulation matrix

A commercial combination product of salmeterol xinafoate and fluticasone propionate (Seretide® 50 Evohaler) containing 25 μg of salmeterol base (as xinafoate salt) and 50 μg fluticasone propionate in hydrofluoroalkane 134a propellant was tested using the dosage uniformity sampling apparatus (DUSA) of the British Pharmacopoeia [18]. Briefly five doses were fired to waste to prime the valve, then six doses were collected at a flow rate of 28 L min^{-1} using a GF/A glass fibre pre-filter (Whatman International Ltd., Maidstone, UK). The device was washed with mobile phase, and the filter was sonicated for 5 min in the washings, before being made up to final volume. This procedure was repeated a further three times, and the drug recovery was assayed by external standardization using the above chromatographic method.

3. Results and discussion

3.1. Resolution and system suitability

The wavelength used in the study was found to be suitable for the assay of all three compounds (see Fig. 1(a)–(c)). The UV maximum (240 nm) for fluticasone propionate (FP) was at a minimum in the UV spectrum for salmeterol base (SB). The choice of 228 nm was shown to be close to the absorption maxima of FP, SB and xinafoic acid (XA). This is, to the best of our knowledge, the first time a UV spectrum for salmeterol base has been published and shows that the UV spectrum for salmeterol reported by Michael et al. [9] is actually the resultant spectrum of the twin absorbances of XA and SB in solution.

The chromatographic system provided good resolution of the three compounds of interest SB, its counter-salt XA and FP. Fig. 2 shows a chromatograph of the lowest concentration standard from day 1, while Table 1 shows the system suitability data. XA eluted first followed by SB and FP with retention times of $t_R = 2.96 \pm 0.02$ min, $t_R = 5.23 \pm 0.11$ min and $t_R = 7.01 \pm 0.17$ min, respectively ($n = 85$ injections). This rapid analysis time is furthermore achieved using a lower flow rate than any of the previously reported studies for SX [2,8,9].

The low degree of tailing with salmeterol base, and its high degree of resolution at neutral pH even without ion-pair reagents was noted, despite conventional recommendation that such bases should be routinely analyzed using mobile phases buffered at low pH [19]. The parameters derived from the calibration series which were analyzed using different batches of mobile phase, were highly reproducible. All peaks showed efficient separation, with narrow peak widths and low peak dispersion (the number of theoretical plates was never below 5000, and a low variance was obtained over 85 injections).

Table 1
Retention time and peak performance parameters for XA, SB and FP when analyzed by RP-HPLC ($n = 85$)

Compound	Retention time (min)	USP peak width	USP tailing factor	USP theoretical plates	USP resolution
Xinafoic acid	2.96 ± 0.02	0.19 ± 0.02	1.26 ± 0.01	5700 ± 208	1.00 ± 0.00
Salmeterol base	5.23 ± 0.11	0.33 ± 0.04	1.21 ± 0.02	5655 ± 110	10.394 ± 0.35
Fluticasone propionate	7.01 ± 0.17	0.37 ± 0.04	1.09 ± 0.03	7787 ± 414	5.97 ± 0.12

All values are mean ± S.D.

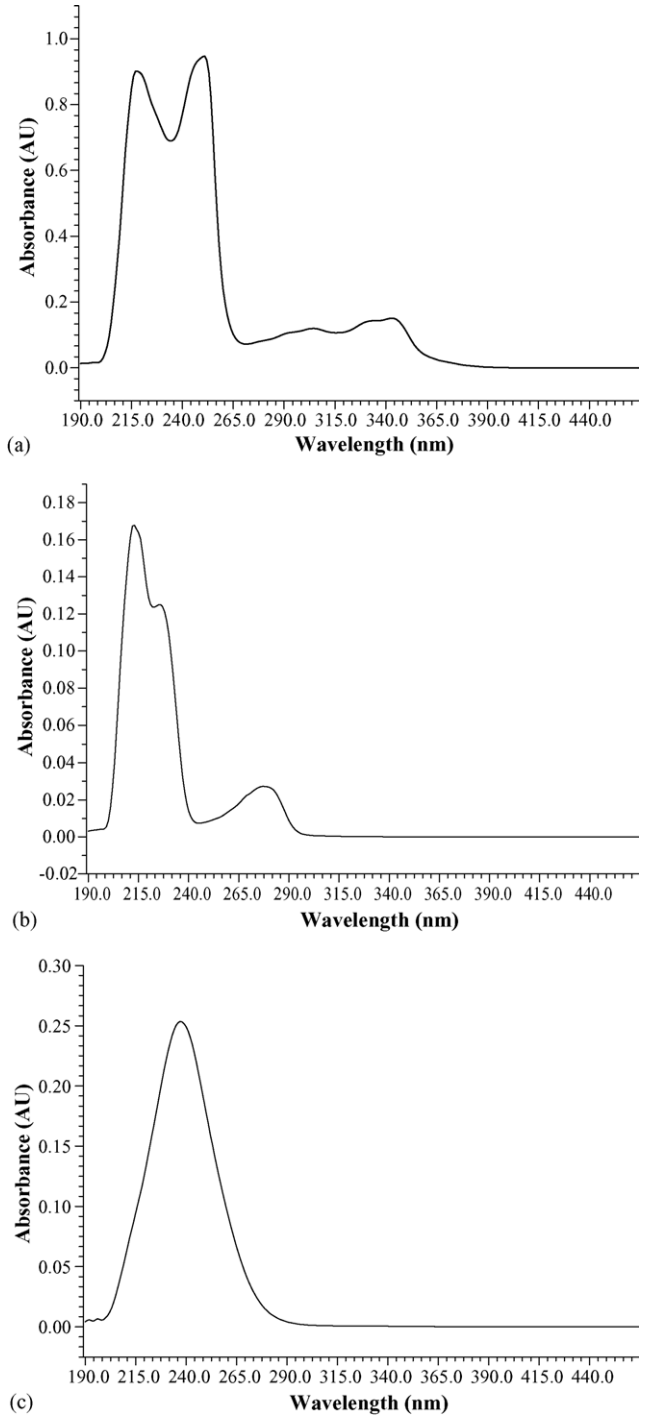

Fig. 1. UV spectra of xinafoic acid (a), salmeterol base (b) and fluticasone propionate (c) as determined by photodiode array detection.

3.2. Linearity and sensitivity

The peak area response displayed excellent linearity for the three agents in the range 2.1–52.3 μg mL^{-1} for SX and 2.0–49.3 μg mL^{-1} for FP ($r^2 > 0.999$), for all three calibration curves. Linear regression analysis of peak area response as a function of concentration (day 1) are shown in Table 2, and the combined data obtained for the regression analyses carried out on the 3 days' calibration curves are shown in Table 3. When the

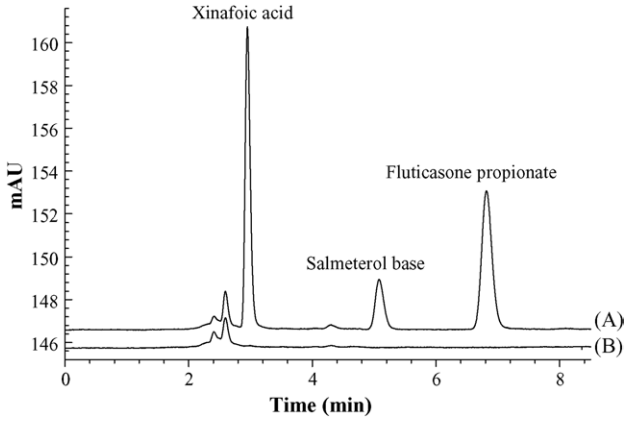

Fig. 2. Chromatogram of (A) standard mixture of SX and FP overlaid with a chromatogram of (B) solvent blank.

data for all 3 days were combined, the calibration curves were shown to be linear ($r^2 > 0.999$). The limits of detection and quantification calculated using Eqs. (1) and (2) were in the ranges 0.19–0.26 and 0.63–0.86 μg mL^{-1}, respectively (Table 4). The peak height response as a function of concentration displayed poorer linearity and a higher limit of detection (data not shown) than the corresponding plot of peak area response, thus peak area was used in all studies. Significantly the linearity and similar sensitivity when either XA or SB peak area responses were plotted as a function of salmeterol xinafoate concentration show that, on the basis of linearity, either compound may be used for the assay of total salmeterol xinafoate content.

3.3. Precision

Inter- and intra-day variation (Table 5) was determined at five concentration levels ($n = 6$). Xinafoic acid and salmeterol base peak responses did not display any dependence of repeatability on solute (salmeterol xinafoate) concentration, while fluticasone propionate exhibited lower repeatability at lower concentration levels (Table 5). Nevertheless, the repeatability had a % CV < 2% at all concentration levels. The reproducibility of the assay, as indicated by the data derived for the three calibration curves was also excellent, with % CV < 2% for all analytes. Both reproducibility and repeatability was better for the xinafoic acid peak

Table 2
Linear regression analysis of peak area response plotted as a function of concentration (day 1)

	Xinafoic acid	Salmeterol base	Fluticasone propionate
Range (μg mL^{-1})	2.1–52.3	2.1–52.3	2.0–49.3
Intercept	−0.344	−0.009	−0.607
Standard deviation of the intercept	±0.737	±0.251	±0.546
Standard deviation of *y*-estimate	2.686	0.915	1.989
Slope	37.523	10.606	31.373
Standard deviation of the slope	0.027	0.009	0.021
Correlation coefficient	0.99999	0.99998	0.99999

Table 3
Linear regression analysis of the calibration curves on different days, for peak area response plotted as a function of concentration

Compound	Day	Intercept	Standard deviation of the intercept	Slope	Standard deviation of the slope	Correlation coefficient
Xinafoic acid	1	−0.344	0.737	37.523	0.027	0.99999
	2	−1.403	1.084	37.581	0.040	0.99997
	3	2.897	1.120	37.949	0.040	0.99997
	Pooled	−1.431	1.349	37.749	0.049	0.99986
Salmeterol base	1	−0.009	0.251	10.606	0.009	0.99998
	2	−0.183	0.326	10.598	0.012	0.99996
	3	−1.601	0.357	10.633	0.012	0.99997
	Pooled	−0.551	0.214	10.629	0.008	0.99995
Fluticasone propionate	1	−0.607	0.546	31.373	0.021	0.99999
	2	−2.645	0.928	31.314	0.029	0.99998
	3	0.028	0.783	31.102	−0.649	0.99998
	Pooled	−0.724	0.981	31.118	0.037	0.99988

Table 4
Limits of detection (LOD) and quantitation (LOQ) for the HPLC determination of salmeterol xinafoate and fluticasone propionate

Analyte	Calibrant	LOD ($\mu g\,mL^{-1}$)	LOQ ($\mu g\,mL^{-1}$)
SX	XA	0.22	0.72
SX	SB	0.26	0.86
FP	FP	0.19	0.63

than salmeterol base, indicating that although both peaks show excellent linearity, the XA peak may prove more suitable for the determination of salmeterol xinafoate.

3.4. Assay accuracy

3.4.1. Recovery of salmeterol xinafoate from a cosolution

A two-sided *t*-test showed no significant difference on the recovery of SX whether analyzed on its own ($n = 3$) or in cosolution with FP ($n = 4$), calculated on the basis of salmeterol base ($p = 0.511$) or xinafoic acid peak ($p = 0.536$) responses. The equivalence of SB and XA peak area responses in the assay for SX content was determined by a paired *t*-test on the recovery of SX calculated on the basis of the SB or XA peak areas, when SX was in cosolution with FP. There was no significant difference ($p = 0.063$) between the calculated recoveries (98.89 ± 2.57 and $98.84 \pm 2.61\%$, respectively, $n = 4$).

Table 5
Repeatability and reproducibility values of the calibration standards used for the quantitative determination of salmeterol xinafoate and fluticasone propionate

Concentration ($\mu g\,mL^{-1}$)	% CV intra-day ($n = 12$)			% CV of inter-day ($n = 17$)		
	XA	SB	FP	XA	SB	FP
50	0.09	0.36	0.24	0.75	0.35	0.85
25	0.57	1.73	0.22	0.73	1.45	0.82
15	0.06	0.54	0.23	0.85	0.56	1.16
5	0.21	0.72	0.22	0.39	1.33	0.96
2	0.57	1.52	0.85	0.83	1.87	0.82
Mean	0.30	0.97	0.35	0.71	1.11	0.92

3.4.2. Recovery of fluticasone propionate from a cosolution

There was no statistical significance in the recovery of FP when the analyte was in cosolution ($n = 4$) with SX, or analyzed on its own ($n = 4$; two-sided *t*-test $p = 0.141$).

3.4.3. Accuracy determination

Accuracy was calculated by pooling the data from the following four separate experiments and is shown in Table 6:

- SX analyzed using SB or XA calibration curves ($n = 3$).
- FP analyzed using FP calibration curve ($n = 4$).
- SX/FP cosolution analyzed using SB or XA and FP calibration curves ($n = 4$).
- SX/FP cosolution analyzed using XA or SB with FP cosolution calibration curves ($n = 4$).

A paired *t*-test showed there was a significant difference in the recovery of SX judged on the basis of xinafoic acid or salmeterol base from the pooled recoveries ($n = 11$; $p = 0.001$). However, neither the recovery of SX nor that of FP was shown to be significantly different from 100% (one-sample *t*-test, $p = 0.593$ for FP, $p = 0.389$ for SX calculated on the basis of the salmeterol base peak, and $p = 0.466$ for SX calculated on the basis the of xinafoic acid peak).

3.5. Analysis of a formulation matrix

The percentage recovered dose per shot (total recovered dose from DUSA analysis divided by 6) was within the general BP limits for pressurized inhalations of 75–125% of the stated dose

Table 6
Accuracy (expressed as percentage recovery) for the quantitative determination of salmeterol xinafoate and fluticasone propionate

Analyte	Calibrant	Accuracy ($n = 11$)[a]	95% Confidence interval	Range
SX	XA	99.58	98.34–100.82	5.57
SX	SB	99.49	98.23–100.75	5.65
FP	FP	100.24	99.43–101.05	3.59

[a] $n = 12$ for FP.

Table 7
Analysis of a combination salmeterol xinafoate 25 μg and fluticasone propionate 50 μg pressurized metered dose inhaler

Analyte	Quantified	% Recovery ($n = 4$)	±S.D.
SX	XA	87.59	2.73
SX	SB	86.65	3.36
FP	FP	93.89	2.85

content. The recovery of SX and FP is shown in Table 7. A paired *t*-test showed no significant difference between the recovery of SX calculated on the basis of xinafoic acid or salmeterol base ($p = 0.389$). Although the recovery was significantly different from 100%, it was well within the allowable limits for pressurized inhalers tested by this method, taking into account normal emitted dosage variability, and difficulties in sample recovery.

4. Conclusions

Using the chromatographic system presented here, the separation of weakly basic, acidic and neutral compounds has been possible. This was attributed to the careful selection of a new generation base-deactivated column, which decreased the negative interactions between the stationary phase and the charged basic species, that can lead to peak broadening. It has proved possible to determine salmeterol xinafoate on the basis of either the xinafoic acid or the salmeterol base peak, both of which show good peak performance. The chromatographic system can be used to determine salmeterol xinafoate and fluticasone propionate, without either interfering in the detection or quantitation of the other. Finally the assay method has been shown to be suitable to evaluate product performance of a combined pressurized metered dose inhaler.

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