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Journal of Pharmaceutical and Biomedical Analysis
34 (2004) 45–51
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Validation of a chiral HPLC assay for (*R*)-salbutamol sulfate

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Received 24 March 2003; received in revised form 20 August 2003; accepted 24 August 2003

Abstract

A fast, reliable and specific method for the screening, confirmation, determination and quantitation of salbutamol enantiomers was developed and validated. The described procedure includes a single robust chiral HPLC determination employing a Teicoplanin stationary phase. The method was evaluated for specificity, robustness, linearity, precision and accuracy. Under the chromatographic conditions of the method, known impurities were separated from the active principle.

Keywords: Albuterol; Salbutamol; HPLC validation; Chiral chromatography; Levalbuterol

1. Introduction

(±)-Salbutamol, also known as albuterol, is a β_2-adrenoceptor agonist and currently one of the most prescribed bronchodilators for the treatment of bronchial asthma [1,2].

In view of the regulatory position that argues that racemic drugs should no longer be used because only one enantiomer is active [3], many chiral drugs have to be enantioresolved. This is the case of (±)-salbutamol, in which the *R*-isomer is the most active enantiomer, responsible for all bronchial airway benefits [4] while the *S*-isomer induces hypersensitivity in the airways [5] and is metabolized more slowly than the *R*-isomer [6].

Different analytical methods for quantification of (±)-salbutamol, such as GC–MS [7], HPLC [8] and CE [9], have been previously described. A chiral HPLC method using the Teicoplanin column was validated to determine low levels of each enantiomers in plasma using fluorescence detection [10]. In the case of raw material quality control, a routine HPLC arrange is desired, so this work was conducted in order to validate a chiral HPLC assay method that allows the resolution, detection and quantitation of (*R*)-salbutamol and related substances like its enantiomer and impurities.

2. Experimental

2.1. Equipment

A Waters 2690 HPLC system was used with a quaternary pump and autosampler, with a Waters 996 photodiode array detector. For data acquisition, Millenium 3.20 software was used.

All separations were achieved using a 250 mm × 4.6 mm Chirobiotic T column (amphoteric glycopep-

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doi:10.1016/j.japna.2003.08.020

tide Teicoplanin bonded to a 5 μm silica gel) from ASTEC.

All samples and standard solutions were chromatographed at ambient temperature (25 ± 2 °C) using an acetonitrile/methanol/acetic acid/triethylamine mixture (60:40:0.3:0.2, v/v/v/v) as the mobile phase (flow rate of 1.5 ml min^{-1}), with detection at 276 nm and an injection volume of 10 μl, unless otherwise indicated.

2.2. Chemicals

HPLC grade acetonitrile (ACN) and methanol (MeOH) from Fisher (Fair Lawn, NJ, USA) were used to prepare the mobile phase, together with acetic acid (AA) from J.T. Baker (Phillipsburg, NJ, USA) and triethylamine (TEA) from Merck (Schuchardt, Hohenbrunn, Germany) of reagent grade quality.

(±)-Salbutamol sulfate was kindly provided by Laboratorio Pablo Cassará S.R.L. (Buenos Aires, Argentina).

(*R*)-Salbutamol sulfate (*R*-SS) and (*S*)-salbutamol sulfate (*S*-SS) were prepared according to a previous report [11]. The purity of these compounds was 99.57 and 99.35%, respectively against albuterol sulfate USP standard.

Pure water was produced by a Millipore Milli-Q Plus System (Molsheim, France).

The usual impurity of (±)-salbutamol [12] as (±)-1-(4-hydroxy-3-methoxymethyl-phenyl)-(2-(*tert*-butylamino)-ethanol (hereafter abbreviated as Imp) was isolated from commercial (±)-salbutamol sulfate by preparative HPLC chromatography and spectroscopically characterized.

^{1}H NMR (D_2O): δ (ppm) 1.56 (s, 9H), 3.47 (m, 2H), 3.58 (s, 3H), 4.66 (s, 2H), 6.98 (d, 1H, $J = 8.4$ Hz), 7.37 (dd, 1H, $J = 8.4$, 2.2 Hz), 7.49 (d, 1H, $J = 2.2$ Hz).

^{13}C NMR (D_2O): δ (ppm) 25.3, 30.3, 57.6, 58.1, 70.2, 115.8, 125.4, 127.3, 127.9, 132.6, 156.4.

When Imp was chromatographed on the Chirobiotic T column, two peaks were obtained and were attributed to its two enantiomers, Imp1 and Imp2.

2.3. Standards

Albuterol Sulfate USP was used as the (±)-salbutamol sulfate standard.

In-house *R*-SS and *S*-SS were used as the standard drugs for the assays, prepared by methanol crystallization of the corresponding drug, vacuum-drying (40 °C) for 24 h, and its titer obtained by assaying these compounds by pharmacopoeial methods [13] against albuterol sulfate USP standard. Then the *R*-SS, used as standard, was 99.57% of chemical purity and >99.99 of optical purity.

2.4. Preparation of mobile phase

Mobile phase solution A (MPA) was prepared by carefully adding AA (3 ml) and TEA (2 ml) to 400 ml methanol and mixing well. This solution and ACN 100% were filtrated through HV (durapore) membrane in PVDF 0.45 μm pore diameter and degassed by sonication. The HPLC was programmed to pump and mix pure ACN and MPA in a 60:40 ratio, which means ACN/MeOH/AA/TEA mobile phase.

2.5. Preparation of standard solutions and samples

Approximately 25 mg of the standard *R*-SS were weighed precisely and dissolved in 25 ml of filtrated Milli-Q water.

All working samples were prepared by mixing *R*-SS and *S*-SS solutions, in order to obtain the required *R*/*S* ratio, except for the working sample of the specificity test where (±)-salbutamol sulfate was used.

2.5.1. Linearity

Working samples were prepared in duplicate from *R*-SS solutions of 2 mg ml^{-1} and *S*-SS solutions of 1 mg ml^{-1}. Aliquots of each solution were diluted in Milli-Q water to obtain solutions of the required concentrations. First, solutions of *R*-SS were prepared in concentrations of 0.125; 0.250; 0.375; 0.500; 0.625; 0.750; 0.875; 1.000; 1.125; 1.250; 1.375; 1.500 mg ml^{-1}. Then, mixture solutions of *R*-SS and *S*-SS of 1 mg ml^{-1} were prepared in *R*/*S* ratio of 0.2/0.8; 0.4/0.6; 0.6/0.4; 0.8/0.2 and 0.995/0.005.

2.5.2. Accuracy and precision

Mixture samples were prepared by dissolving the corresponding amounts of each compound of *R*-SS and *S*-SS in 25 ml of water to reach solutions of 1 mg ml^{-1} and the required *R*/*S* (w/w) ratio. For the addition of 0.5% of *S*-SS, a 0.2 mg ml^{-1} solution was previously

prepared and an aliquot of this solution was added to a 25 ml volumetric flask to obtain the required mixture.

2.5.3. Specificity

Working samples were prepared by subjecting 1 mg ml^{-1} (±)-salbutamol sulfate aqueous solutions to acid (0.05 M H_2SO_4), base (0.1 M NaOH) and UV light (254 nm, pH 5.48). After the degradation treatments were completed (120 h), all samples were neutralized with acid/base, if needed.

2.5.4. Robustness

Assays were performed on mixtures of *R*-SS and *S*-SS in a *R*/*S* ratio of 50/50 and 99.5/0.5 aqueous solutions of 1 mg ml^{-1}.

2.5.5. Stability

An assay was performed on *R*-SS standard solution, which was prepared by weighing the corresponding amount to obtain solutions of 1 mg ml^{-1}.

3. Results

Prior to performing the validation assays, chromatographic conditions for the HPLC method were studied in order to achieve appropriate system suitability parameters, such as resolution (commonly abbreviated as *R*, but to avoid misunderstandings we will use Res.), tailing (*T*), number of theoretical plates (*N*), capacity factor (k') and retention time (t_R).

In this study, flow rate was tested between 1.0 and 2.0 ml min^{-1}, injection volume between 10 and 20 μl and mobile phase composition ACN/MeOH/AA/TEA between 60:40:0.3:0.2 and 70:30:0.5:0.2 (v/v/v/v). The final experimental conditions were set as described in Section 2.1. An example chromatogram obtained by using those conditions is shown in Fig. 1.

The Imp1 and Imp2 were reported to be frequent impurities in commercial (±)-salbutamol sulfate [12]. Note that these impurities were well resolved under the chromatographic conditions as is confirmed by the values $\text{Res.}_{R\text{-SS}-\text{Imp2}} > 2$ and $\text{Res.}_{\text{Imp2}-\text{Imp1}} > 3$ (see also t_R in Fig. 1).

3.1. Linearity

The linearity of the method was studied over a concentration range of 0.125–1.500 mg ml^{-1}. To evaluate the influence of one enantiomer on the other, linearity was studied over a *R*/*S* range between 20/80 and 99.5/0.5. Triplicate injections of each sample were performed. The correlation of the instrumental peaks area response versus compound concentration of *R*-SS, singly and in the presence of its enantiomer, showed excellent linearity over the range studied, with $r^2 \geq 0.99$ (Table 1).

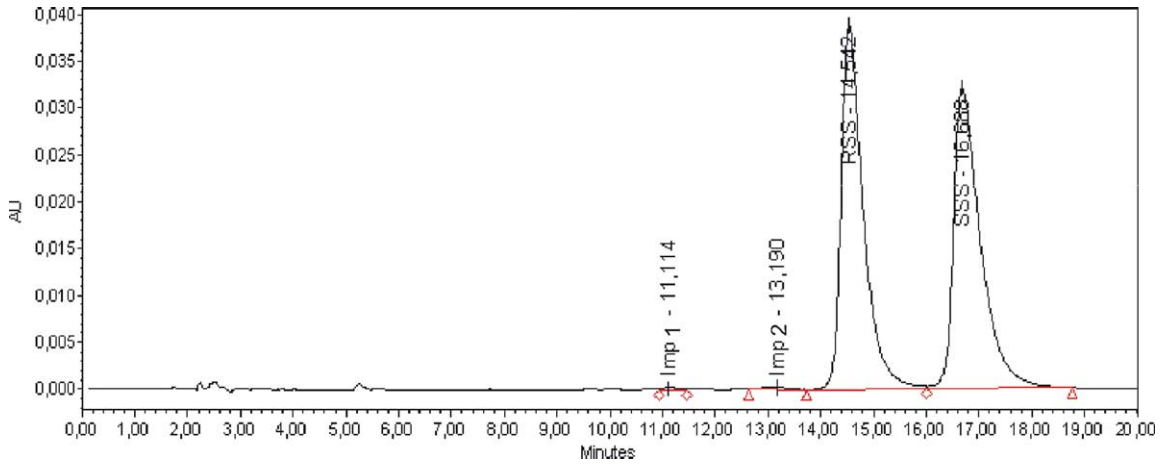

Fig. 1. Chromatogram of (±)-salbutamol sulfate sample on Teicoplanin column. Imp1 and Imp2: both enantiomers of 1-(4-hydroxy-3-methoxy methyl-phenyl)-2-(*tert*-butylamino)-ethanol, *R*-SS: (*R*)-salbutamol sulfate and *S*-SS: (*S*)-salbutamol sulfate. For chromatographic conditions, see Section 2.1.

Table 1
Linearity: linear regression parameters

Sample	Range (mg ml^{-1})	Slope × 10^4	Intercept × 10^4	r^2
R-SS	0.125–1.500	25.4 ± 0.6	−3 ± 2	0.9991 ± 0.0001
R-SS (in presence of *S*-SS)	0.200–0.995	23 ± 3	9 ± 5	0.99 ± 0.01
S-SS (in presence of *R*-SS)	0.005–0.800	24.6 ± 0.4	0.5 ± 0.2	0.99993 ± 0.00002

Table 2
Accuracy: recovery results for total area and *R*/*S* ratio

Sample levels	*R*/*S* ratio	Measured *R*/*S* ratio	Mean ± R.S.D. (%) for *R*/*S* ratio recovery (%) ($n = 10$)	Mean ± R.S.D. (%) for mass recovery ($n = 10$)
R/*S* 22.2/77.8	0.285 ± 0.003	0.282 ± 0.008 (22.0/78.0)	99 ± 1	101.3 ± 0.6
R/*S* 52.4/47.6	1.10 ± 0.03	1.11 ± 0.06 (52.6/47.4)	101.7 ± 0.5	101.7 ± 0.6
R/*S* 99.5/0.5	199	169 ± 4 (99.4/0.6)	85 ± 1	99 ± 2

3.2. Accuracy/recovery studies

Two kinds of recovery were analyzed.**(a) Mass recovery:** Triplicate solutions of *R*-SS and *S*-SS were prepared as stated in point 2.5 for *R*/*S* ratios of 20/80, 50/50 and 99.5/0.5 and chromatographed versus *R*-SS standard solutions. Recovery was calculated for ten runs of each solution. Results are summarized in Table 2. The results obtained indicated a good percentage (99–101%) of recovery for both enantiomers.

***R*/*S* ratio recovery (%):** The *R*/*S* ratio recovery (%) (ratio between peak areas of *R*-SS and *S*-SS against the theoretical ratio in solution, per 100) was studied, in experiments performed at different *R*/*S* ratios. Results were calculated for 10 runs. As shown in Table 2, excellent results for the recovered *R*/*S* ratios were obtained even when *S*-SS was present in low concentration (for $R/S = 99.5/0.5$ the recovered *R*/*S* was 99.4/0.6). The *R*/*S* ratio recovery (%) was 99 ± 1 and 101.7 ± 0.5% for *R*/*S* ratios of 22.2/77.8 and 52.4/47.6, respectively. When *S*-SS was present in a <1% concentration the ratio recovery (%) was 85 ± 1, due to the small variation in the *S*-SS recovered of, from 0.50 to 0.6.

3.3. Precision

The study was performed with the same samples as those prepared for the accuracy assay, making four injections for the levels *R*/*S* 20/80 and 50/50 and 10 injections for *R*/*S* 99.5/0.5. The relative standard deviation (R.S.D. (%)) of the area of both *R*-SS and *S*-SS peaks and of the *R*/*S* ratio were calculated. Intermediate precision was determined by the assay of the samples at days 1, 2, 4 and 7 and by two different analysts on day 1. Tables 3 and 4 summarize the chromatographic results.

Table 3
Precision: results for day 1

R/*S* ratio of sample	R.S.D. (%)			*n*
	R-SS	*S*-SS	*R*/*S* ratio	
20/80	0.1	0.2	0.2	4
50/50	0.1	0.4	0.3	4
99.5/0.5	0.2	5	5	10

Table 4
Precision: intermediate precision

R/*S* sample	Peak or ratio under analysis	R.S.D. (%) ($n = 4$)			
		Day 2	Day 4	Day 7	Analyst 2
20/80	*R*-SS	0.2	0.6	1	1
	S-SS	0.3	0.7	1	2
	R/*S* ratio	0.1	0.1	0.2	0.2
50/50	*R*-SS	0.3	0.5	0.8	0.5
	S-SS	0.6	0.6	0.7	0.6
	R/*S* ratio	0.4	0.2	0.2	0.2
99.5/0.5[a]	*R*-SS	0.7	0.6	1	0.1
	S-SS	8	8	8	6
	R/*S* ratio	7	8	8	6

[a] $n = 10$.

Table 5
Specificity: results of degraded (±)-salbutamol sulfate samples (area (%))

Condition	HPLC peak area (%)			
	R-SS	*S*-SS	Imp1[a]	Imp2[a]
0.05 M H_2SO_4				
Initial	49.59	49.85	0.16	0.39
120 h	49.59	49.88	0.15	0.38
0.1 M NaOH				
Initial	49.70	49.98	0.15	0.17
120 h	49.73	49.75	0.16	0.35
Light				
Initial	49.59	49.85	0.16	0.39
120 h	49.48	49.92	0.22	0.38

[a] Imp1 and Imp2: both enantiomers of 1-(4-hydroxy-3-methoxymethyl-phenyl)-2-(*tert*-butylamino)-ethanol.

It can be observed that the method was repeatable and precise within 7 days and when performed by two different analysts.

3.4. Specificity

Forced degradation studies were performed on (±)-salbutamol sulfate to provide an indication of the specificity of the method. Intentional degradation was attempted using acid and base and UV light, in order to verify if any degradation product co-eluted or altered the analytical method. The degraded solutions were analyzed at 1, 6, 24, 48, 72, 96 and 120 h and compared to a standard solution of (±)-salbutamol sulfate. The results for the initial and final (120 h) assays are shown in Table 5.

In general, solutions were quite stable. Diode-array spectra of the samples, taken at several points of the peaks and compared to the apex spectra, did not reveal any co-eluting degradation product or impurity. In addition, resolution between *R*-SS and the closest peak, Imp2, was greater than 2.

3.5. Robustness

The capability to remain unaffected by small but deliberate variations in the method parameters was studied in order to anticipate the problems which may arise during the application of the method, and allow the setting of method parameter limits.

Table 6
Robustness: Plackett–Burman design

Experiment	Factor[a]			Result
	A	*B*	*C*	
1	+	+	+	ρ_1
2	−	+	−	ρ_2
3	+	−	−	ρ_3
4	−	−	+	ρ_4
	E_A	E_B	E_C	

[a] Factor *A*: mobile phase acidity; factor *B*: flow rate; factor *C*: column temperature.

The effect of variation in the mobile phase acidity, flow rate and column temperature on the resolution (Res.), reproducibility (R.S.D. (%)), and *R*/*S* ratio were studied following a Plackett–Burman design [14,15]. The effect of each factor (E_F) was evaluated in four experiments according to the combinations expressed in the matrix of Table 6.where "+" and "−" express the upper and lower levels respectively, and $\rho_{1–4}$ represent the results in the following parameters:

(a) resolution (Res.),
(b) reproducibility (R.S.D. (%)),
(c) *R*/*S* ratio.

The effect of each factor ($E_F = E_A, E_B$ or E_C) was calculated as

$$E_F = \frac{1}{2}\sum \rho(+) - \frac{1}{2}\sum \rho(-)$$

The effect is considered important if

$$|E_F| > [s(2)^{1/2}]$$

where s is the standard deviation obtained in the precision study for each parameter.

Two levels of the critical factors previously mentioned were tested as follows:

Factor *A*: mobile phase acidity. Variations of the AA amounts in the mobile phase. Upper level: 3.5 ml AA in 400 ml MeOH; lower level: 2.5 ml AA in 400 ml MeOH (nominal level: 3.0 ml AA in 400 ml MeOH).

Factor *B*: flow rate. Upper level: 1.6 ml min^{-1}; lower level: 1.4 ml min^{-1} (nominal level: 1.5 ml min^{-1}).

Factor *C*: column temperature. Upper level: 30 °C; lower level: 22 ± 2 °C (nominal level: 25 °C).

Assays were performed on mixtures of *R*-SS and *S*-SS in a *R*/*S* ratio of 50/50 and 99.5/0.5, aqueous solutions of 1 mg ml^{-1}. Results are shown in Table 7.

Table 7
Robustness assays

Experiment	*R*-SS/*S*-SS = 50/50			*R*-SS/*S*-SS = 99.5/0.5		
	Res.[a]	R.S.D. (%) of *R*-SS ($n = 10$)	*R*-SS/*S*-SS	Res.[a]	R.S.D. (%) of *R*-SS ($n = 10$)	*R*-SS/*S*-SS
1	2.64	0.2	1.000	5.37	0.3	158
2	2.15	0.3	1.002	4.97	0.4	189
3	2.24	0.2	1.006	5.23	0.1	191
4	2.73	0.4	0.999	5.58	0.2	148
E_A[b]	4.4×10^{-16}	0.2	0.003	0.03	0.1	6.0
E_B[b]	0.09	0.05	0.002	0.24	0.2	4.0
E_C[b]	0.49	0.05	0.004	0.38	0.1	32.0
s[c]	9.80	0.1	0.004	0.09	0.2	12.6
$s(2)^{1/2}$	13.86	0.1	0.006	0.13	0.3	17.8

[a] Res.: resolution.
[b] *E*: effect of each factor.
[c] *s*: standard deviation in the precision study.

Resolution, R.S.D. (%) of the *R*-SS peak area and *R*/*S* ratio were almost unaffected by the factors *A*, *B* and *C*, in both mixtures, under the conditions studied. Although factors *B* and *C* show some influence on the chromatographic parameters, this test allows us to set the method limits for system suitability.

3.6. Stability of the analytical solutions

The stability of the *R*-SS analytical solutions was monitored by analyzing standard solutions aged at room temperature, with and without protection from light, at 4 °C (refrigerator) and at −18 °C (freezer) against freshly prepared standards. In all conditions, the recovery was between 100 ± 1 and $102 \pm 2\%$ indicating that *R*-SS in solution was stable for at least 10 days. During the stability studies no additional peaks were developed and no changes in the *R*-SS peak slope were observed.

3.7. Limits of detection (LOD) and quantitation (LOQ)

The limits of detection and quantitation were determined by serial dilutions of *R*-SS solutions in order to obtain signal/noise ratios of ≈3:1 for LOD and ≈10:1 for LOQ. The LOD and LOQ values were found to $\approx 5 \times 10^{-5}$ and $\approx 3 \times 10^{-3}$ mg ml^{-1}, respectively.

4. Conclusion

An enantioselective HPLC method to evaluate Salbutamol enantiomeric purity was developed and validated. Experimental design techniques were successfully employed to the evaluation of robustness as part of the method characterization. The method was found to be fast, accurate, highly sensitive and precise for quantification purposes or e.e. (%) determination. The simplicity of the method means that it is well suited to routine quality control of *R*-SS.

Acknowledgements

The authors thank Laboratorio Pablo Cassará (Buenos Aires, Argentina) for supplying the (±)-salbutamol sulfate.

References

[1] R.B. Penn, Clin. Rev. Allerg. Immunol. 14 (1996) 37–45.
[2] D. Handley, Exp. Opin. Invest. Drugs 7 (1998) 2027–2041.
[3] FDA, Chirality 4 (1992) 338–340.
[4] D. Hartley, D.J. Middlemis, J. Med. Chem. 14 (1971) 895–896.
[5] (a) D. Handley, Chirality 10 (1998) 262;
(b) S. Mitra, Mol. Pharmacol. 53 (1998) 347–354.
[6] D. Boulton, J.P. Fawcett, Clin. Pharmacol. Ther. 62 (1997) 138–144.

[7] C. Gabiola, M.A. García-Calonge, M.P. Portillo, J.A. Martínez, A.S. del Barrio, J. Microcolumn Sep. 8 (1996) 361–364.
[8] Y. Tan, S. Soldín, J. Chromatogr. B 422 (1987) 187–195.
[9] R. Gotti, S. Furlanetto, V. Andrisano, V. Cavrini, S. Pinzauti, J. Chromatogr. A 875 (2000) 411–422.
[10] K. Fried, P. Koch, I. Wainer, Chirality 10 (1998) 484–491.
[11] C. Ferrayoli, M. Palacio, M.F. Bresina, S. Palacios, Enantiomer 5 (2000) 289–291.
[12] E. Redenti, M. Fiaschi, M. Zanol, P. Ventura, G. Moneti, Eur. Mass Spectrom. 3 (1997) 89–91.
[13] USP 26, 2003, p. 56; EP 4, 2001, p. 1878.
[14] ICH guideline Q2B, Validation of Analytical Procedures: Methodology, November 1996.
[15] H. Fabre, J. Pharm. Biomed. Anal. 14 (1996) 1125–1132.