Validation of new analytical methodology for determining fenoterolhydrobromide by HPLC: application in pharmaceutical products

Validação de uma nova metodologia analítica para determinação de bromidrato de fenoterol por CLAE: aplicações em produtos farmacêuticos

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RESUMO
Bromidrato de fenoterol é um agente agonista adrenérgico β_2-seletivo utilizado para tratamento de asma e doenças pulmonares crônicas obstrutivas. A metodologia analítica por cromatografia líquida de alta eficiência (CLAE) foi desenvolvida e validada para a determinação quantitativa de bromidrato de fenoterol. A condição analítica empregada incluiu coluna em fase reversa C_{18} (150 mm × 3,9 mm d.i., 5 μm) Thermo®, fase móvel composta de mistura de acetonitrila e água (30:70, v/v) com 0,1% de trietilamina e pH ajustado para 5,0 com ácido fórmico, vazão de 1,0 $mL.min^{-1}$ e detecção em UV a 276 nm. A faixa de linearidade foi de 0,025 a 0,15 $mg.mL^{-1}$; a curva analítica mostrou coeficiente de correlação > 0,999. O limite de detecção (LD) foi de 0,003 $mg.mL^{-1}$ e o limite de quantificação de 0,012 $mg.mL^{-1}$. A repetitibilidade da técnica (desvio padrão relativo) foi ≤ 2,0% e a exatidão revelou média percentual de recuperação de 99,53%. A metodologia proposta é de simples execução, rápida, precisa, exata e sensível. As vantagens sobre as demais técnicas são baixo custo e poucas condições poluentes. Demonstrou, ainda, simplicidade e resultados confiáveis para ser aplicada no controle de qualidade de produtos farmacêuticos contendo bromidrato de fenoterol como ingrediente ativo.
Palavras chave. bromidrato de fenoterol, teste de estresse, cromatografia líquida de alta eficiência, validação analítica.

ABSTRACT
Fenoterol hydrobromide is a β_2-adrenergic agonist agent used for asthma and chronic obstructive pulmonary disease treatment. HPLC methodology was developed and validated for quantitative determination of fenoterol hydrobromide. The methodology was achieved by using a reversed-phase C_{18} column, (150 mm × 3.9 mm i.d., 5 μm) Thermo. The mobile phase was consisted of acetonitrile: water (30:70, v/v) with 0,1% triethylamine, pH adjusted to 5.0 with formic acid and flow rate of 1.0 $mL.min^{-1}$ with UV detection at 276 nm. The concentration range was from 0.025 to 0.15 $mg.mL^{-1}$, and the correlation coefficient of analytical curve was >0.999. The detection limit and the quantifying limit (QL) were 0.003 $mg.mL^{-1}$ and 0.012 $mg.mL^{-1}$, respectively. Intra- and interday relative standard deviations were ≤2.0%. The methodology accuracy showed the percentage mean of 99.53%. The described technique was found to be simple, rapid, precise, accurate and sensitive; the advantages over the others current methodologies are the low-cost and low-polluting conditions. Owing to its simplicity and reliable results, this methodology is suitable to be used in quality control of pharmaceutical drugs containing fenoterol hydrobromide as active component.
Keywords. fenoterol hydrobromide, stress testing, high performance liquid chromatography, pharmaceutical drug validation.

INTRODUCTION

Fenoterol hydrobromide (FEN), 1-(3,5-dihydroxyphenyl)-2-(4-hydroxy-methylphenylamino) ethanol hydrobromide. The molecular formula as bromide is $C_{17}H_{21}NO_4HBr$ and its molecular weight 384.3 g.mol^{-1}. FEN is a direct-acting sympathomimetic with beta-adrenoceptor stimulant activity largely selective for beta$_2$ receptors (a beta$_2$ agonist). It has actions and uses similar to those of salbutamol and is used as a bronchodilator in the management of reversible airways obstruction, as occurs in asthma and in some patients with chronic obstructive pulmonary disease. On inhalation, acts within a few minutes and has duration of action of about three to five hours[1]. The drug product is available as a dry powder inhaler in some countries, may also be given orally for the relief of bronchospasm at a dose of 2.5 to 5 mg three times daily.

Various analytical methods have been applied for the determination of FEN in raw material, pharmaceuticals and biological fluids. These methods include liquid chromatography[2-7], gas chromatography[8], voltammetry[9,10], fluoro-immunoassay[11], coulometry[12], colorimetric-flow injection[13], electrophoresis[14-16] and spectrophotometry[17].

The aim of this work was to develop and validate an efficient method using high performance liquid chromatography and applied to pharmaceutical preparations marketed in Brazil.

MATERIALS AND METHODS

Chemicals and samples

FEN (100% on a dry basis) was kindly supplied by Prati-Donaduzzi and was used as a reference standard without further purification. Acetonitrile (HPLC grade), triethylamine (analytical grade) and formic acid (analytical grade) were obtained from Merck. Ultrapure water was obtained from a Milli-Q Plus apparatus. The oral solutions (samples A, B and C) containing 5 mg.mL^{-1} FEN were obtained from three different pharmaceutical companies. Placebos were prepared using EDTA, sodium chloride, hydrochloride acid (0.01 mole.L^{-1}) and benzalkonium chloride (pharmaceutical grade).

Equipment and analytical conditions

The method was performed on a chromatographic system, consisted of a solvent delivery pump model 305 and 306 (Gilson), an UV–VIS detector model 118 (Gilson), an auto injector fitted with 10 µL loop. A reversed-phase C_{18} column, (150 mm × 3.9 mm i.d., 5 µm) Thermo, was used for the separation. The mobile phase was acetonitrile:water (30:70, v/v) with 0.1% triethylamine and pH adjusted to 5.0 with formic acid and was filtered through a 0.45-µm filter Millipore (Millex HV) hydrophilic membrane. The flow rate was 1.0 mL.min^{-1}. The injection volume was fixed at 10 µL, and UV detection was at 276 nm. All analyses were at room temperature and the mobile phase was prepared at the beginning of the day.

Method validation

The method was validated according to the International Conference on Harmonization[18] and AOAC International[19] guidelines for validation of analytical methods.

Selectivity and Specificity

The selectivity was assessed by comparing the chromatograms obtained from excipients (placebo). An amount of placebo equivalent to sample containing 12.5 mg FEN was used. Ingredients to prepare the placebo were similar to those presented in the commercial formulations and in the same ratio. The systems responses were examined in triplicate for the presence of interference or overlaps with FEN responses.

Forced degradation studies were performed in order to provide an indication of specificity of the method. The chemical oxidation was made with 3% of hydrogen peroxide, the acid hydrolysis with 1 mole.L^{-1} of hydrochloric acid, and the alkaline hydrolysis with 1 mole.L^{-1} of sodium hydroxide. All the solutions were heated at 80ºC for four hours. After the degradation treatments, the samples were allowed to equilibrate to room temperature, neutralized with acid or base (when necessary), and diluted with mobile phase (0.075 mg.mL^{-1} of FEN). The samples were analyzed against a freshly prepared control standard solution (with no degradation treatment).

Linearity

A series of solutions containing FEN from 0.025 to 0.15 mg.mL^{-1} was obtained by appropriate dilutions of the standard stock solution. Water was used as the solvent for dilution. The calibration graph was obtained by plotting mean peak area versus FEN concentrations. All analyses were performed in triplicate.

Precision

A stock solution was prepared and stored refrigerated until use. Standard solutions of FEN were obtained by dilutions at 0.05 $mg.mL^{-1}$ in water. For quantitative determination in pharmaceuticals, an amount of sample equivalent to 10 mg of FEN was used to obtain final sample solutions of 0.06 $mg.mL^{-1}$ in water. On three consecutive days, three and ten separate aliquots of stock standard and stock sample solutions were diluted and analyzed. Duplicate determinations were made with each solution in each day and a mean response was calculated.

Accuracy

Known amounts of FEN were added to the samples and analyzed by the proposed method. Three aliquots of the standard solutions were used to fortify 2.0 mL sample solutions, in three separate volumetric flasks. The final FEN concentration of these fortified solutions was 0.100, 0.125 and 0.150 $mg.mL^{-1}$. All solutions were prepared in triplicate and analyzed.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ for the proposed analytical method were determined based on standard error and slope of the analytical curve[18].

Robustness

This parameter was evaluated by variations on the established chromatography conditions. The mobile phase composition was increasing and decreasing 1%, the flow rate was changed in 0.1 $mL.min^{-1}$ and the UV detection was analyzed in 275 an 277 nm. Triplicate measurements were made for each variation and the RSD value was calculated.

RESULTS AND DISCUSSION

To achieve this percentage of mobile phase it was tested different concentrations of organic and aqueous phase such as (50:50, v/v, 40:60, v/v and 80:20, v/v, respectively). A satisfactory separation and peak symmetry were obtained with mobile phase consisting of acetonitrile and water (30:70, v/v) with 0.1% triethylamine and pH adjusted to 5.0 with formic acid. The pH of the mobile phase was adjusted with formic acid after addition of triethylamine. pH lower than 3.5 and greater than 7.0 showed poor efficiency and symmetry. Quantitation was achieved with UV detection at 276 nm on peak area.

Selectivity and specificity

Specificity is the ability of the method to accurately measure analyte response in the presence of potential sample components (excipients and degradation product). All related excipients were used to prepare placebo sample solutions, which were analyzed by using the proposed method (Figures 1 and 2). The results were compared with those obtained for the analysis of the standard FEN solutions at the same concentration level. No interference from excipients was observed.

Linearity

The linearity was evaluated by analyzing standard solutions at six different concentration levels of FEN ranging from 0.025 to 0.15 $mg.mL^{-1}$. The correlation coefficient was found to be >0.999 indicating excellent correlation[18]. Relevant data on the linearity of the proposed methods is presented in Table 1.

Table 1. Linear regression and LOD and LOQ data

Statistical parameter	HPLC
Concentration range, $mg.mL^{-1}$	0.025 - 0.15
Regression equation	$y = 7^{E+7}x - 63726$
Correlation coefficient (r)	0.9996
LOD, $mg.mL^{-1}$	0.003
LOQ, $mg.mL^{-1}$	0.012

LOD = Limit of detection; LOQ = Limit of quantification

Precision

The method precision was evaluated by measuring inter-and intraday repeatability. The intraday repeatability was evaluated by analyzing a single concentration of FEN in replicate (ten times) and is expressed in terms of RSD with the respective confidence interval. The RSD values were found to be <2.0%, indicating good intraday repeatability[18] (Table 2). The interday repeatability (reproducibility) was determined by analyzing sample solutions prepared from the same stock solution on 3 consecutive days (in triplicate), at the same concentration level. Interday repeatability is expressed in terms of RSD values with the respective confidence intervals. The RSD values were also below 2.0% indicating good reproducibility for the method (Table 2).

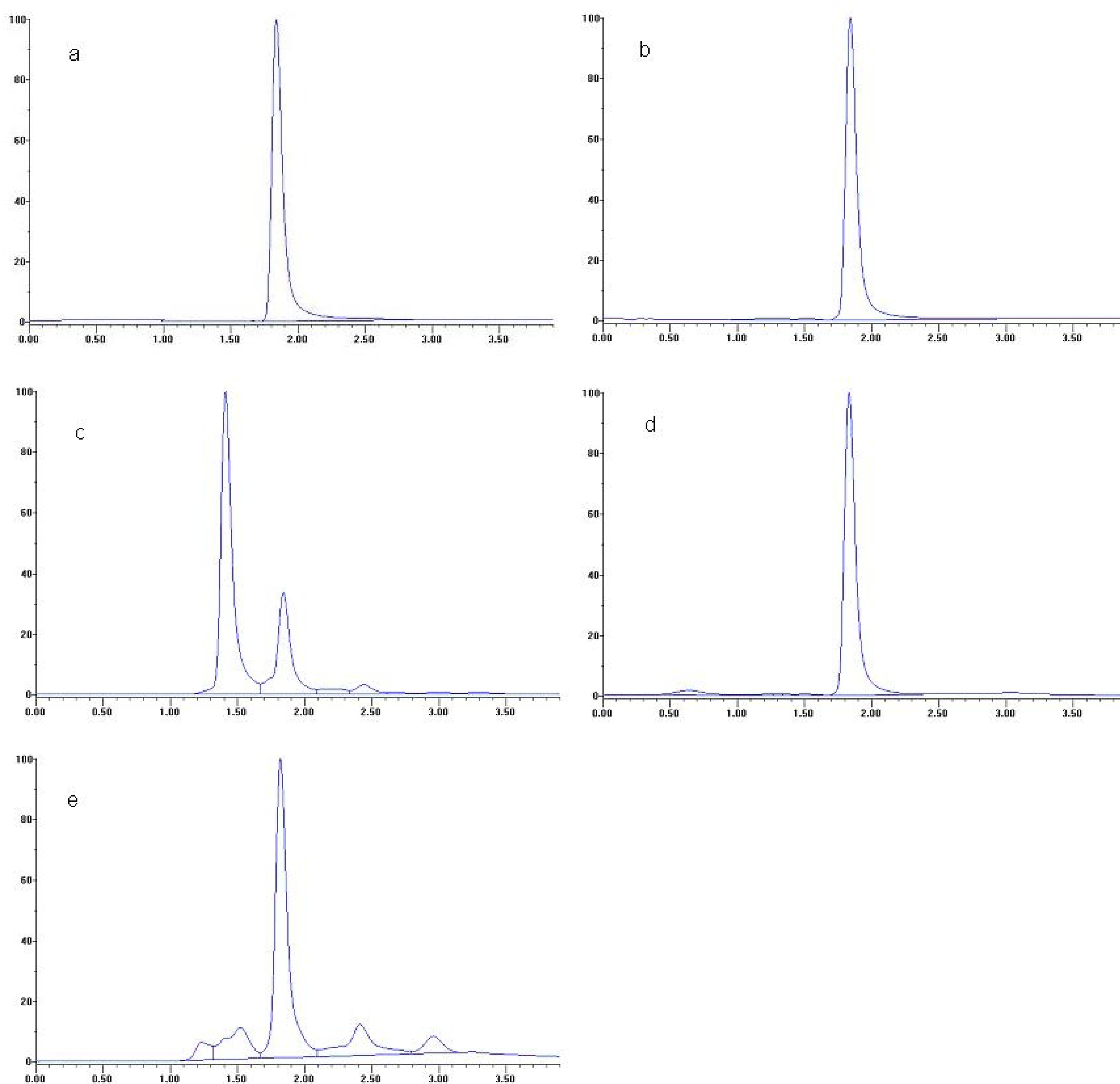

Figure 1. Representative chromatograms of FEN standard (a), neutral hydrolysis (b), chemical oxidation (c) acid hydrolysis (d) and alkaline hydrolysis (e). Concentration: 0.075 mg.mL^{-1}. Chromatographic conditions: Thermo C_{18} column (150 x 3.9 mm, 5 µm); mobile phase: acetonitrile:water (30:70, v/v) with 0,1% triethylamine and pH adjusted to 5.0 with formic acid; flow rate, 1.0 mL.min^{-1}, UV detection at 276 nm and temperature of 20 ± 1 °C

Accuracy

Standard additions for fortification and recovery experiments were used to determine the accuracy of the proposed method. The accuracy of the method was checked at 3 concentration levels, 0.1, 0.125 and 0.15 mg.mL^{-1}. Triplicate analyses were performed with the proposed method and the measurements were made at each concentration. Accuracy is expressed as the percentage of standard recovered from the sample matrix with the corresponding RSD and confidence interval. The mean recovery of FEN was excellent ranged from of 97.2% to 101.4%[19], this data are showed in Table 3.

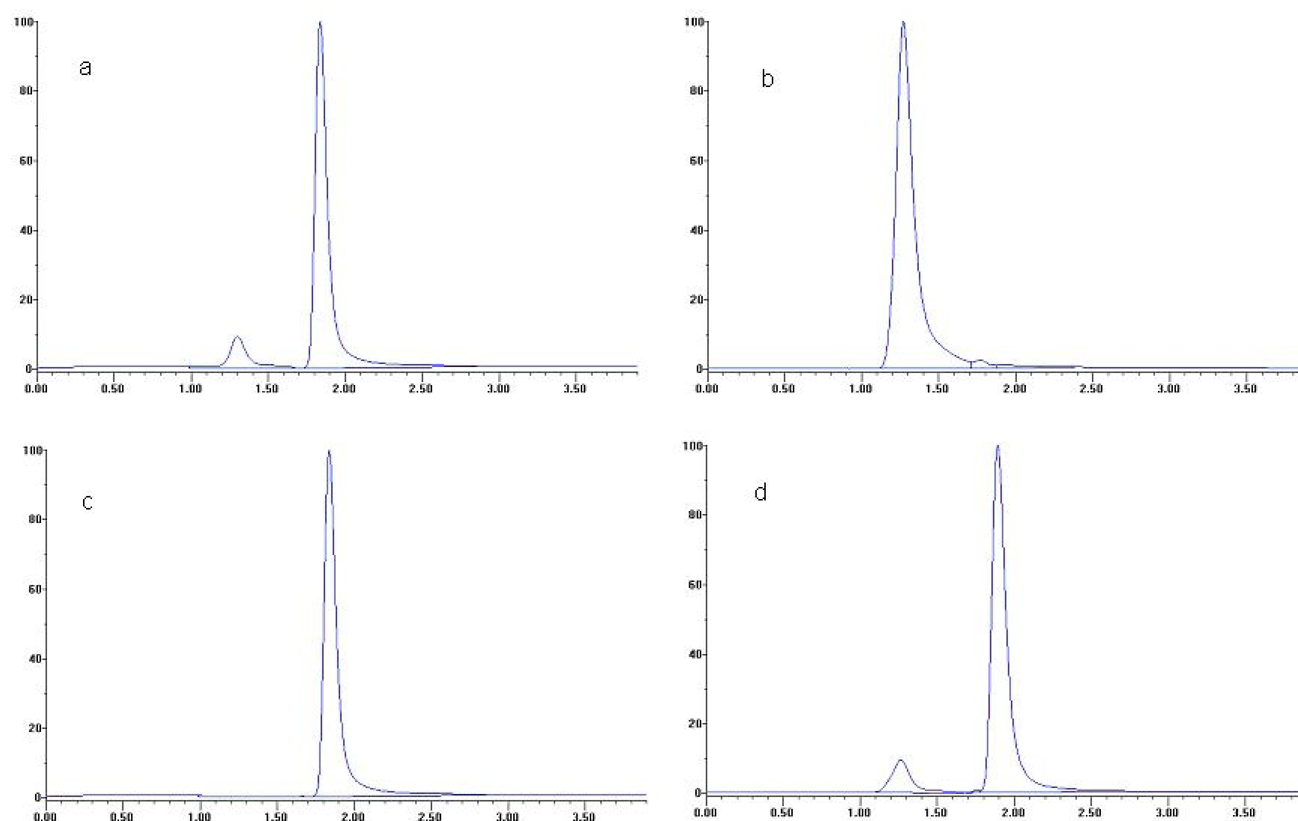

Figure 2. Representative chromatograms of sample A (a), placebo (b), FEN standard (c) and placebo with FEN standard added (d). Concentration: 0.1 $mg.mL^{-1}$ of FEN. Chromatographic conditions: Thermo C_{18} column (150 x 3.9 mm, 5 µm); mobile phase: acetonitrile:water (30:70, v/v) with 0,1% triethylamine and pH adjusted to 5.0 with formic acid; flow rate, 1.0 $mL.min^{-1}$, UV detection at 276 nm and temperature of 20 ± 1 °C

Table 2. Precision results and statistical data obtained in the proposed method

	HPLC		
Parameter	**Sample A**	**Sample B**	**Sample C**
Intraday repeatability[a]			
Day 1, $mg.mL^{-1}$	5.21 ± 0.08	5.25 ± 0.06	5.01 ± 0.06
RSD, %	2.00	1.72	1.75
Day 2, $mg.mL^{-1}$	5.12 ± 0.07	5.13 ± 0.07	4.89 ± 0.06
RSD, %	1.98	1.93	1.78
Day 3, $mg.mL^{-1}$	5.19 ±0.07	5.17 ± 0.07	4.94 ± 0.06
RSD, %	1.76	1.80	1.59
Interday repeatability[b]			
Days 1-3, $mg.mL^{-1}$	5.18 ± 0.04	5.19 ± 0.04	4.49 ± 0.04
RSD, %	2.00	1.99	1.96

[a]n = 10; calculated after statistical treatment by analysis of variance.
[b] n = 30; calculation based on arithmetic mean.

Table 3. Results obtained for recovery of FEN added to sample A, B and C

	HPLC		
Sample	**Added ($\mu g.mL^{-1}$)**	**Found ($\mu g.mL^{-1}$)**	**Recovery, %** [a]
	50.00	49.93	99.87%
A	75.00	75.16	100.17%
	100.00	97.24	97.24%
	50.00	50.37	100.5%
B	75.00	74.69	99.58%
	100.00	97.92	97.92%
	50.00	50.69	101.40%
C	75.00	74.51	99.36%
	100.00	99.73	99.73%

a = mean of three determinations

Limits of Detection and Quantitation

The limit of detection (LOD) and quantitation (LOQ) were determined for the proposed method. The limits were based on the standard deviation of the response and slope of the curve at the lowest concentrations[18]. The theoretical values obtained for LOQ were experimentally prepared and cross-checked by theoretical value. The LOD and LOQ were 0.003 and 0.012 $mg.mL^{-1}$, respectively (Table 1).

Stability

The standard of FEN was exposed to extreme conditions as acid and alkali solutions, oxidizing agents and temperature. After stress conditions, FEN was mainly degraded when was exposed to basic hydrolysis, the peak area was reduced in 45.4% to the standard not stressed and small unknown peaks were observed before and after of the FEN. In the exposure to chemical oxidation, the absorbance also revealed a decrease in 24.1% of the peak area, and for acid hydrolysis it was reduced in 5.8% in relation to the FEN standard.

Robustness

Robustness of analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage[18]. This search evaluated the robustness under variations on the mobile phase, UV detection and the flow rate. Triplicate analyses were made for each variation and the values obtained showed that the changes with the mobile phase and UV detection was practically invariable, ranged from 98 to 102%. For the flow rate the recovery showed a variation slightly higher, but the method is still considered robust.

CONCLUSIONS

The proposed method for the determination of FEN in pharmaceutical formulations showed to be efficient and sensitive. Chromatographic parameters such as mobile phase, pH and flow rate can be modified to control FEN retention time on column. The excipients of the commercial samples assayed did not interference in the analysis and the absence of interference demonstrated the specificity of the method. The proposed method was found to be simple, rapid, precise, accurate, and sensitive. Once water was used as the solvent for dilution most of the time it is a low-cost and low-polluting method. The method can be used for routine quality control of FEN in commercial samples. Factor such as mobile phase pH and acetonitrile concentrations should be controlled to obtain adequate resolution and separation.

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