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Validation of an HPLC method for the quantification of ambroxol hydrochloride and benzoic acid in a syrup as pharmaceutical form stress test for stability evaluation

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Abstract

A method is described for ambroxol, *trans*-4-(2-amino-3,5-dibromobenzylamino) cyclohexanol hydrochloride, and benzoic acid separation by HPLC with UV detection at 247 nm in a syrup as pharmaceutical presentation. Optimal conditions were: Column Symmetry Shield RPC8, 5 μm 250 × 4.6 mm, and methanol/(H_3PO_4 8.5 mM/triethylamine pH = 2.8) 40:60 v/v. Validation was performed using standards and the pharmaceutical preparation which contains the compounds described above. Results from both standards and samples show suitable validation parameters. The pharmaceutical grade substances were tested by factors that could influence the chemical stability. These reaction mixtures were analysed to evaluate the capability of the method to separate degradation products. Degradation products did not interfere with the determination of the substances tested by the assay.

Keywords: Ambroxol; Benzoic acid; HPLC; Syrup; Pharmaceutical

1. Introduction

Ambroxol, *trans*-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol hydrochloride, is a compound with potent mucolytic activity, for which it is used as an expectorant and bronchosecretolytic in therapeutics [1,2]. It is a pharmacologically active metabolite of bromhexine, *N*-cyclohexyl-*N*-methyl-(2-amino-3,5-dibromobenzyl)amine hydrochloride. Ambroxol stimulates the transportation of the viscous secretion in the respiratory organs and reduces the standstillness of the secretion. Benzoic acid is an antimicrobial preservative frequently included in liquid pharmaceutical forms. Fig. 1 shows the chemical structure of ambroxol and benzoic acid with their pk_a.

Ambroxol hydrochloride can be found in pharmaceutical preparations such as drops, granules, injections, syrups and tablets. Several different

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methods have been used for the individual determination of ambroxol hydrochloride in pharmaceutical solutions and tablets including TLC [3,4], spectrophotometry [2], HPLC [5], flow injection [6,7] and capillary electrophoresis [8]. More complex methods have been reported for ambroxol determination in biological fluids [1,2,8,9].

None of them combine the analysis of one acid and basic compound and only one of them [8] has been applied to a syrup, which has the problems due to the presence of excipients, stabilizers and flavouring substances. Furthermore, capillary electrophoresis is not yet a routine technique in pharmaceutical quality control and full validation parameters are not presented in this paper.

Owing to acid–base characteristics of the compounds herein analysed, separation is highly dependent of pH, so the effect of this variable has been studied. Two different types of stationary phases were assayed as well as different strategies to avoid tailing in peaks: changes in pH of mobile phase or addition of modifiers.

After optimizing the separation, the method was validated for standards and syrup of a pharmaceutical preparation containing these substances by determining linearity, precision and accuracy. Simultaneously, as the method could be used for stability testing, standards and samples were subjected to degradation conditions to prove that no interferences appeared.

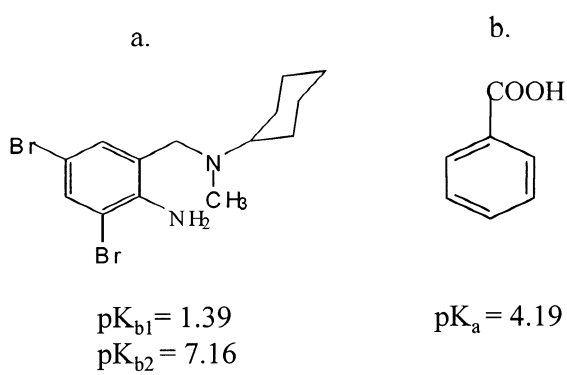

Fig. 1. Chemical structures of the two analytes: (a) ambroxol hydrochloride; (b) benzoic acid.

2. Experimental

2.1. Instrumentation and chromatographic analysis

A Beckman (Palo Alto, USA) HPLC system provided with a 126 pump, an automatic injector (507e), a 168 Diode Array detector and a Gold System data processor were used. The chromatographic analysis were performed on a 5 μm Symmetry Shield RP8 (Waters) column (25 × 0.46 cm) kept in a Biorad column oven at 35°C.

Eluent had a flow rate of 1 ml/min and the mobile phase which gave the best results was methanol/(H_3PO_4 8.5 mM/triethylamine pH = 2.8) 40:60 v/v. Detection was performed at 247 nm and peaks were identified with retention times as compared with standards and confirmed with characteristic spectra using the photodiode array detector.

2.2. Reagents

All solvents were HPLC grade quality purchased from Scharlau (Barcelona, Spain). General reagents were from Merck (Darmstadt, Germany) and standards of ambroxol hydrochloride, benzoic acid, syrup containing these active compounds, ambroxol cinfa 15 mg, and all the components of the placebo were supplied by Laboratorios CINFA S.A. (Pamplona, Spain).

2.3. Methods

Mobile phase consisted of aqueous buffer–methanol at proportion of 60:40. Buffers were prepared with 8.5 mM H_3PO_4 and pH adjusted by adding triethylamine. The assayed pH value was in the recommended working range for the column (from 2.5 to 7.5) and it was measured in all instances before the addition of methanol.

For optimization, the sample solutions were prepared dissolving the analytes in the mobile phase to give concentrations of 0.3 mg/ml ambroxol hydrochloride and 0.2 mg/ml benzoic acid. Injection volumes were 50 μl to produce adequate UV responses to detect degradation products when existing.

2.4. Validation

Once chromatographic conditions were established, method validation was performed following ICH specifications [10,11]. Standard and sample linearity was verified by analysis in triplicates of five points in the range of 0.15–0.45 mg/ml for ambroxol hydrochloride and 0.10–0.30 mg/ml for benzoic acid which corresponded to 50–150% of the expected sample values.

Recoveries were evaluated with the same method by comparing the calculated concentrations and measured concentrations.

Instrumental repeatability and intermediate precision were determined by processing two series of injections of the same standard, corresponding to the mid point in linearity range, on 2 different days. Reatability and intermediate precision of the method applied to final product were determined by processing two series of 10 samples, prepared as explained below, on 2 different days and with the corresponding standards for quantification.

Sample treatment: 2.9081 g of sample were weighted in a volumetric flask of 25 ml and completed to volume with mobile phase. The samples were filtered through nylon membranes of 0.45 μm of pore size before passing to injection vials.

2.5. Accelerated degradation

Stability testing [12] is performed to ensure that drug products do not degrade until the sell-by date has expired. Analytical methods to be employed for stability testing should be able to separate degradation products from the active substances.

Degradation conditions included the following: acidic media with phosphate buffer 0.2 M, pH = 1.0; water; basic media with Na_2HPO_4 0.4 M pH = 8.9 and oxidating media with 0.3% H_2O_2. In all cases 1% (w/v) of standard or sample were heated in autoclave for 1 h at 120°C. Dilutions were done for reaching the same concentration as in the valuation.

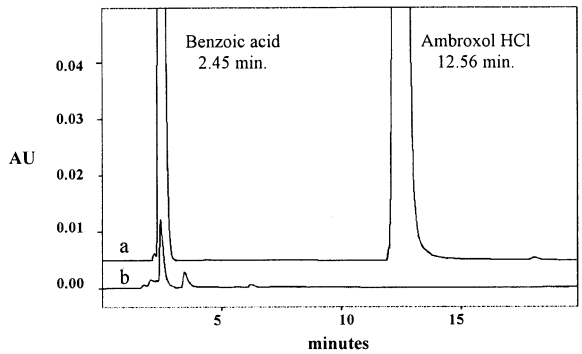

Fig. 2. Superimposed chromatograms of standards: (a) benzoic acid 2.45 min and ambroxol hydrochloride $t = 12.56$ min, and placebo; (b) Conditions: Symmetry Shield RP8 column, acetonitrile/(H_3PO_4 8.5 mM/tryethylamine pH = 2.8) 40:60 v/v, flow rate of 1 ml/min and UV detection at 247 nm.

3. Results and discussion

There are many possible ways of suppressing the interaction of residual silanols in the silica-gel surface with basic analytes, which frequently leads to inferior separations due to the tailing of the peaks. The reduction of ionisation of acidic SiOH sites by employing mobile phases of low pH (pH < 4) or, in contrast, the decrease of ionisation of the basic sample by increasing the pH of the mobile phase are the easiest methods. Other approaches take advantage of the addition of 'silanol blockers' [13], e.g. triethylamine to the mobile phase and this has proved to be interesting to permit ambroxol elution in the present work.

The optimisation was started with mobile phase of phosphate buffer 8.5 mM pH 6.5/ACN 60:40 (v/v), pH 6.5 achieved with triethylamine. This value was selected because it was considered to be a compromise for acid and basic compounds. However, in these conditions the superimposed chromatograms showed that the placebo interfered with the standard of benzoic acid as can be seen in Fig. 2. The optimisation was continued by changing the amount of acetonitrilo and the pH of the buffer as well as adding tetrahydrofuran. This last compounds could affect to selectivity of esters that appear in the placebo. Nevertheless, none of these changes were good enough to get adequate resolution between the different components.

Another subject to consider is the organic solvent employed in the mobile phase. The presence of methanol in the mixture has a strong influence in the dissociation of both the buffer and the basic solute. The methanol content in the mobile phase has also effect on the retention characteristics of basic solutes, [13]. So, the next step was to change acetonitrilo to methanol and analyse the standards and placebo again with different kinds of mobile phases, changes in pH of the phosphate buffer and in the amount of MeOH. The best results, Fig. 3, were obtained with the following mobile phase: H_3PO_4 8.5 mM pH 2.8 (with triethylamine)/MeOH = 60:40.

Elution order of ambroxol hydrochloride and benzoic acid changed with pH in the mobile phase. A basic compound, as ambroxol hydrochloride, is more retained in RP HPLC in its molecular or unionized form, that is with higher pH values in the mobile phase. The opposite happens with benzoic acid and that is the cause of the elution order changes observed between pH = 6.5 and 2.8.

Although preliminary development assays were performed with a column described as having an intermediate hydrophobicity and silanol activity, as NovaPak C18, Symmetry Shield RP8 column was then assayed because it has one of the lowest hydrophobicity and silanol activity, and it had proved to have better behaviour with ionizable compounds as seen in previous works in the laboratory [14].

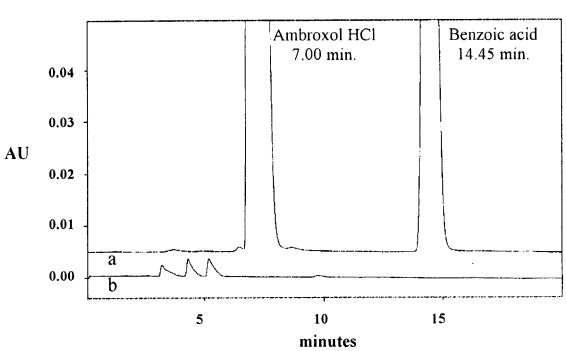

Fig. 3. Superimposed chromatograms of standard: (a) ambroxol hydrochloride 7 min and benzoic acid 14.45 min, and placebo; (b) Conditions: Symmetry Shield RP8 column, methanol/(H_3PO_4 8.5 mM/tryethylamine pH = 2.8) 40:60 v/v, flow rate of 1 ml/min and UV detection at 247 nm.

A stationary phase with lower hydrophobicity and silanol activity (Symmetry Shield RP8) was chosen to reduce the secondary interactions leading to tailing in peaks and higher capacity factors for basic compounds.

Dorsey and Cooper [15] showed the roll of stationary phases in reversed-phase chromatography, as a function of chain density and that the predominant driving force in the retention changes from an enthalpic to an entropic mechanism. Symmetry Shield with a 15.0% carbon load and C8 is expected to have a higher density of chains than Nova Pak with a 7.3% carbon and C18.

Final conditions for which the method was validated were: Symmetry Shield RP8 column, and methanol/(H_3PO_4 8.5 mM/triethylamine pH = 2.8) 40:60 v/v.

Validation results appear in Table 1. Both standards and samples show a good linearity for the two analytes with correlation coefficients over 0.999; RSD of slopes ranged between 0.6 and 0.7 for the two compounds in standards and samples showing the good fit of individual points to the regression line. Ambroxol hydrochloride, which has a smaller experimental t value than tabulated ($P = 95\%$) does not present a significant bias. On the other hand, benzoic acid has an experimental t value superior to the tabulated one (2.160 for $n - 2 = 13$ and $P = 95\%$). It presents statistically significant bias which do not influence much to the extreme concentrations, it is mainly due to the good fit of the points in the regression line. In the smallest concentration the error is only 1.7%. Recoveries are 100% $\pm$0.93 for ambroxol and 100% $\pm$1.38 for benzoic acid in raw material and 101% $\pm$1.42 and 100% $\pm$1.76 in the pharmaceutical form. Maximum RSD in precision was 0.9 for intermediate precision of ambroxol.

In relation to the accelerated degradation, the results showed that, when each of the assayed substances were decomposed with the stress test, the decomposition products did not interfere with the determination of the primary substances, except for a small peak in the placebo in neutral media ($t_r = 13.8$ and area 0.12631). This time corresponds with the retention time of benzoic acid, ($t_r = 13.9$ and area 10.29446) in the same condi-

Table 1
Summary of method validation parameters[a]

	r	a ± C.L.	b ± C.L.	Range
Linearity and range raw material				
Ambroxol	0.9997	0.7 ± 5.5	1117 ± 17	0.15–0.45 mg/ml
Benzoic acid	0.9995	24 ± 7	1752 ± 35	0.1–0.3 mg/ml
Linearity and range final product				
Ambroxol	0.9992	2 ± 9	1126 ± 27	50–150%
Benzoic acid	0.9988	17 ± 11	1798 ± 52	50–150%
Precision final product RSD (%)				
	Repeatability	Intermediate precision		
	Day 1	Day 2	Day 1+2	
Instrumental				
Ambroxol	0.44	0.43	0.46	
Benzoic acid	0.30	0.44	0.80	
Method				
Ambroxol	0.80	0.86	0.90	
Benzoic acid	0.54	0.71	0.59	
	Mean recovery (%)	RSD (%)		
Accuracy raw material				
Ambroxol	100	0.93		
Benzoic acid	100	1.38		
Accuracy final product				
Ambroxol	101	1.43		
Benzoic acid	100	1.76		

[a] a, intercept; b, slope; C.L., confidence limits.

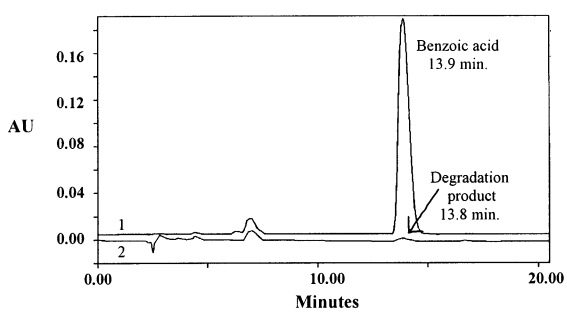

Fig. 4. Chromatograms of: (a) benzoic acid; and (b) placebo (after stress conditions in the media neutral). Conditions: Symmetry Shield RP8 column, methanol/(H_3PO_4 8.5 mM/tryethylamine pH = 2.8) 40:60 v/v, flow rate of 1 ml/min and UV detection at 247 nm.

tions. Nevertheless, the area of the peak in the placebo is only 1.2% of the peak of benzoic acid. Fig. 4 includes the chromatograms of these two substances.

In conclusion, the studied method is suitable for separation, quantification and stability testing of ambroxol and benzoic acid in the pharmaceutical preparation.

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