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Determination of bromhexine and ambroxol in pharmaceutical dosage forms, urine and blood serum

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Abstract

Data presented in this paper show that bromhexine and its pharmacologically active metabolite can easily be determined by capillary zone electrophoresis. The composition of the running buffer had a significant effect on the reproducibility of the migration time for which a carrier solution containing 30 mM phosphate buffer (pH 3.0), 5 M urea and 10% (v/v) acetonitrile was used. The method was validated with respect to its response linearity and reproducibility. The method is suitable for the determination of bromhexine and ambroxol in several samples such as pharmaceuticals, urine and serum. Photodiode-array detection permitted the rapid identification of both drugs in the sample analyzed.

Keywords: Bromhexine; Ambroxol

1. Introduction

Bromhexine (BHX: 2-amino-3,5-dibromo-*N*-cyclohexyl-*N*-methylenzenemethan amine) and its major metabolite, ambroxol (ABX: trans-4-[(2-amino-3,5-dibromophenyl-methyl)amino] cyclohexanol) are used as bronchosecretolytic and expectorant drugs. They are administered as hydrochloric forms in daily doses of 30–120 mg using oral, rectal, inhalation and intravenous routes, producing good results in the treatment of chronic bronchitis and alveolar proteinosis. BHX and ABX hydrochlorides have been formulated alone and with other drugs, and are available commercially as drops, tablets, granules and injections.

Several different methods have been used for the individual determination of BHX and ABX, includ-

ing ultraviolet spectrophotometry [1] and visible spectrophotometry of the compound formed by the coupling of the diazoted BHX derivative with *N*-(1-naphthyl)ethylenediamine [2]. Gas-liquid chromatography in conjunction with electron capture, mass spectrometry or flame ionization [3–5] and high-performance liquid chromatography using UV and amperometric detection has also been used [1,6–8]. Flow-injection methods using a continuous liquid-liquid extractor coupled on-line to a spectrophotometer or an atomic absorption spectrometer have been described for the determination of BHX and ABX using orange IV and Bromothymol Blue dyes, the inorganic complexes BiI_4^- , $\text{Co}(\text{SCN})_4^{2-}$ and Reinecke's salt to form the ion-pairs with these drugs which are extracted in organic solvents [9–12].

Gas liquid chromatography (GC) [3,13] and high-performance liquid chromatography (HPLC) [1,6,8,14] have been used for the individual de-

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termination of BHX or ABX in human urine and plasma.

Capillary electrophoresis (CE) is a highly efficient and fast technique, the application of which in the biological and pharmaceutical fields has developed rapidly in recent years [15–17]. CE combines high resolution and ease of automation with modest sample requirements and low solvent consumption. However, the use of CE for the analysis of BHX and ABX has not hitherto been reported.

Prior to developing a system for the simultaneous determination of BHX and ABX it was noticed that only one GC method for the analysis of both drugs in equine urine has been published. The GC method described makes use of mass spectrometry detection [18]. The aim of this work was the separation of BHX and ABX by capillary zone electrophoresis (CZE) in order to develop a procedure for the determination of both drugs in pharmaceutical preparations and biological fluids. This work also showed that CZE combined with photodiode-array detection permits the rapid identification of these analytes in the samples.

2. Experimental

2.1. Apparatus and running conditions

Electrophoresis was carried out on a P/ACE Series 5000 instrument fitted with a diode-array detector (Beckman, Palo Alto, CA, USA). Separations were performed in a 570 mm × 0.075 mm I.D. fused-silica capillary tube (Beckman). Integration of the electropherograms was achieved by the Chromatography Software System Gold V.810 (Beckman). The sample was introduced by using electrokinetic (10 kV for 10 s) and hydrodynamic (0.5 psi for 2 s) injection modes. To perform field-amplified sample injection with a water plug, water was injected hydrodynamically (0.5 psi for 5 s) to achieve a plug length of about 6.5 mm before the sample was introduced electrokinetically (10 kV for 10 s). A constant voltage of 25 kV (current, 30 μ A) was applied and the temperature of the cartridge was automatically maintained at 20°C. Fresh capillaries were pretreated by rinsing with 0.1 M sodium hydroxide for 5 min, applying 12 kV for 15 min, rinsing with buffer, and

equilibrating for 30 min under the subsequent running conditions. Between experiments the capillary was rinsed with buffer for 5 min. If the species or the concentration of the additive was changed, the capillary was first rinsed with 0.1 M sodium hydroxide followed by the new buffer. If not stated otherwise, a 30 mM phosphate buffer of about pH 3.0 containing urea (5 M) and acetonitrile (10% v/v) was used as the running buffer and the detection of the analytes was performed at 210 nm.

2.2. Reagents and solutions

The chemicals used for preparation of the solutions were purchased at the highest grade possible. Demineralized water from a Milli-Q system (Millipore Iberica, Madrid, Spain) was used for the preparation of the solutions. Phosphoric acid, acetic acid, sodium dihydrogen phosphate, sodium acetate, sodium citrate and sodium hydroxide were of analytical-reagent grade, and are available from Merck (Darmstadt, Germany). Methanol, ethanol and acetonitrile (HPLC grade) were obtained from Romil (Loughborough, UK). Bromhexine, ambroxol and imipramine hydrochlorides, *N*-(2-acetamido)-2-iminodiacetic acid (ADA) and 2-(*N*-morpholino) ethanesulphonic acid (MES) were purchased from Sigma (St. Louis, MO, USA).

Stock standard solution of BHX (376.1 μ g/ml) and ABX (378.1 μ g/ml) were prepared by dissolving appropriate amounts of their corresponding hydrochlorides in water. Working standard solutions of lower concentrations were freshly prepared by appropriate dilution of the stock standard solution.

For the studies where a modifier was added to the electrophoretic solution, the buffer solution was mixed with the required amount of the modifier and then the pH was readjusted with 20 mM hydrochloric acid or sodium hydroxide when necessary.

All solutions were filtered through a 0.45 μ m filter (Millisolve Kit, Millipore), and then degassed by sonication and evacuation.

2.3. Sample preparation

2.3.1. Pharmaceutical preparations

An accurately weighed portion of finely powdered tablets, formulated granules, or liquid formulation,

each containing approx. 5.0–7.0 mg of BHX or ABX hydrochlorides, were transferred into a 50 ml volumetric flask and the contents were diluted with 20 ml of 20 mM hydrochloric acid. The contents of the flasks were shaken vigorously on a mechanical shaker for 10 min, sonicated for 15 min and diluted to volume with demineralized water. A 5 ml portion of the liquid was filtered through a 0.45 μm filter and transferred into a 15 ml volumetric flask, to which 1.0 ml of internal standard solution (imipramine hydrochloride, 30 mg per 100 ml) was added. The volume was adjusted to 15.0 ml with the electrophoretic buffer.

2.3.2. Urine

The samples were spiked with BHX and ABX hydrochlorides and diluted in the running buffer (1:5). After filtering with a 0.45 μm pore size filter, the solution was injected directly into the capillary.

When the injection of raw urine gave a high background or the urine compounds caused poor capillary performance, a liquid–liquid extraction of the endogenous compounds was carried out. The sample (5 ml), which had been adjusted to pH 4.5 and syringe filtered, was extracted with ethyl acetate (10 ml), followed by extraction with petroleum ether (10 ml). The resulting solution was degassed and injected into the capillary.

2.3.3. Blood serum

The sample of blood serum (3.0 ml) was collected from a healthy volunteer and BHX and ABX were added at similar concentrations to those used in clinical applications [19]. The serum was treated with perchloric acid to separate the proteins. After centrifugation, the liquid supernatant was adjusted to pH 3.0 with 2 M sodium hydroxide and filtered through a 0.45 μm filter; it was then transferred into a 10 ml volumetric flask, to which the internal

standard was added. The volume was adjusted to 10 ml with the electrophoretic buffer.

3. Results and discussion

BHX and ABX are weak bases which can protonate in the benzylamino group to give the corresponding cations. Fig. 1 shows the structures of BHX and its pharmacologically active metabolite.

From some preliminary experiments, it was observed that BHX precipitates under basic conditions ($\text{pH} \geq 7.5$); therefore, measurements were always carried out at pH values lower than 7.0. Various buffer systems were used in this study to observe the influence of buffer pH on electrophoretic behaviour of the drugs in terms of relative separation and changes in migration time.

The assay was started with 50 mM phosphate buffer as electrolyte. In the pH range studied (3.0–6.5) both BHX and ABX migrate as cations. Changing the pH from 3.0 to 6.5 decreased the migration time of both drugs and worsened the resolution. The effect of electrolyte concentration on the mobility of BHX and ABX was studied by increasing the phosphate buffer (pH 3.0) concentration from 20 to 100 mM at 15 kV and 20°C. An increase in the ionic strength of the buffer improved the resolution and increased the migration time of both compounds. To limit the Joule heating effect inside the capillary, the maximum voltages were chosen from the plot of current vs. voltage and were 12, 15 and 18 kV for 100, 50 and 25 mM solutions, respectively. It is worth noting that the repeatability of the BHX and ABX migration times were poor although the relative values remained constant.

In a second trial, different buffer compositions were used in order to improve the separation and reproducibility of the migration time. Citrate and

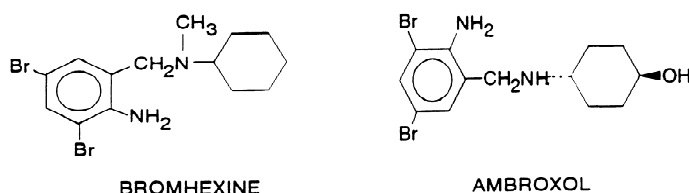


Fig. 1. Structures of bromhexine and ambroxol.

acetate buffer and the biological buffers ADA and MES showed no advantages over the use of phosphate buffer. Taking into account that a poor precision of the migration time indicates adsorption problems on the silica wall when the temperature control of the capillary is satisfactory, another approach was tried, which involved the addition of a wide variety of buffer additives.

The effect of adding an organic solvent to the electrophoretic buffer is hard to predict because it affects several variables, including viscosity, dielectric constant and zeta potential. The presence of acetonitrile, methanol or ethanol in the buffer slightly improved the separation and reproducibility at the expense of a longer analysis time. It is of interest to stress that acetonitrile yielded a base line of great stability.

To resolve the reproducibility problems in the separation of BHX and ABX, micellar electrokinetic capillary chromatography (MEKC) was also used, in which anionic, cationic and zwitterionic surfactants were studied. In addition, the use of modifiers such as cyclodextrins was also considered. However, it was not possible to develop a suitable MEKC method for the determination of both drugs with good resolution and reproducibility.

3.1. Capillary zone electrophoresis method

The simultaneous presence of acetonitrile and urea in the phosphate buffer (pH 3.0) gave the most reproducible values in the migration time of BHX and ABX together with good resolution and a very stable base line. The optimised composition of the electrophoretic buffer was: 30 mM phosphate buffer (pH 3.0), 5 M urea and 10% (v/v) acetonitrile, and the applied voltage was 25 kV.

As the temperature was raised (18–35°C), the decrease in viscosity of the buffer solution and the increasing current inside the capillary shortened the migration time. To obtain reproducible results it is very important to keep the temperature constant during the analysis by using capillary cooling. The resolution of BHX and ABX was better at low temperatures, so 20°C was chosen for further studies.

Hydrodynamic (by pressurizing the sample vial) and electrokinetic injection modes were studied.

Higher sensitivities were found using the electrokinetic injection mode provided with stacking conditions. For that, the samples were dissolved in pure water or phosphate buffer (pH 3.0) of lower specific conductivity than the electrophoretic buffer. Further enhancement can be obtained by introducing a short plug of water into the capillary column before sample injection to establish an enhanced electric field [20].

A study was performed to determine the degree of trace enrichment at various injection times and any sacrifice in resolution that occurred. It was possible to perform pressure injections up to 20 s without substantial loss in resolution and with fair linearity of peak area and height with injection time (analyte quantity).

To measure the reproducibility, eleven replicates of standard solutions of each compound were injected under the optimum conditions. The BHX and ABX peaks were completely separate from each other (Fig. 2), and the migration times remained constant.

The response of BHX and ABX were constant for eleven consecutive runs with an R.S.D. of 1.8% and 2.4%, respectively, for each peak area. When the same sample was run 20 times over a seven day period, the response varied widely with an R.S.D. of 11% for each peak area. However, the relative response of BHX and ABX remained constant with an R.S.D. of 3.1%. Thus, an internal standard was

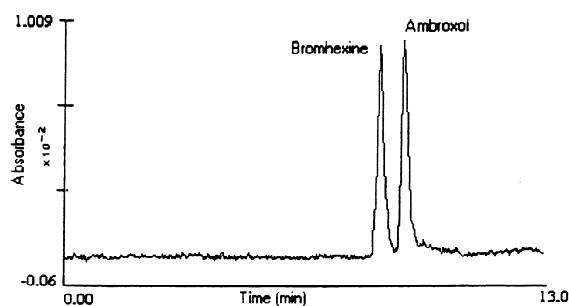


Fig. 2. Electrophoretic separation of a standard solution of bromhexine (20 μM) and ambroxol (20 μM). The separation was driven by an electrolyte solution containing 30 mM phosphate buffer (pH 3.0), 5 M urea and 10% (v/v) acetonitrile. Voltage applied=25 kV. Hydrodynamic injection (2 s).

required to normalize the responses of BHX and ABX. After surveying a number of compounds, imipramine was chosen since its peak was near these of the drugs but well resolved from them, and it migrates from the capillary first, which saves time.

The detection limits for BHX and ABX were found to be over the range 12–26 nM at a signal-to-noise ratio of 2.

Peak area is better than peak height, because height increases are non-linear at high concentrations. The relative responses of BHX and ABX to the internal standard using hydrodynamic (2 s), electrokinetic (10 s, 10 kV) and field amplification with water plug injection modes were linear in the ranges 1.0×10^{-5} – 5×10^{-4} M, 1.0×10^{-6} – 2×10^{-5} M and 4.0×10^{-8} – 2.0×10^{-6} M, respectively. The correlation coefficients for the calibration graphs were 0.999, 0.990 and 0.995, respectively.

3.2. Applications

The CZE assay described here is characterized by long-term stability and reproducibility. More than 3000 analyses could be performed without replacement of the capillary. As a consequence of excessive sample dilution, the sample requirement is only a few μ l, making it possible to perform studies even though only minimal amounts of stored samples were available.

To demonstrate the usefulness of the procedure for the determination of BHX and/or ABX, pharmaceutical formulations and human serum and urine were analyzed for the presence of these drugs.

3.3. Determination of BHX or ABX in pharmaceuticals

Several commercial pharmaceutical preparations in different physical forms were analyzed. The results are included in Table 1. All values are in good statistical agreement (93.3–101.6%) with the declared contents.

3.4. Determination of BHX and ABX in urine and blood serum

In order to establish that the extraction procedure described in Section 2 did not remove BHX and ABX in urine, the following experiment was performed. Samples of BHX or ABX (5 ml of 1.0×10^{-3} mol l⁻¹) were added into each of three 25 ml volumetric flasks. The first and second flasks were made to volume with phosphate buffer (pH 4.5), and the third with urine which had been adjusted to pH 4.5 and syringe-filtered. An aliquot (5 ml) of flask 2 was extracted with 10 ml ethyl acetate, and with 10 ml petroleum ether. An aliquot (5 ml) of flask 3 received similar treatment. When these solutions were mixed with 1 ml of imipramine 2×10^{-4} mol l⁻¹ and injected, the peak area ratios of BHX (or ABX) to imipramine were similar from all three solutions.

A urine sample spiked with BHX and ABX and the internal standard and then subjected to the above cleanup sequence gave the electropherogram shown in Fig. 3.

Fig. 4 is an electropherogram of a serum sample

Table 1
Determination of bromhexine and ambroxol in real pharmaceutical formulations

Preparation	Formulation	Drug	Label claim	Amount found ^a	% of label claimed ^a
Bisolvon (Fher)	Tablet	Bromhexine	4 mg	4.0±0.05	100.8±1.4
Alongamicina (Alonga)	Ampoule	Bromhexine	4 mg	3.9±0.11	99.2±2.8
Clamoxil (Smit)	Capsule	Bromhexine	16 mg	16.2±0.23	101.6±1.4
Mucosan (Fher)	Syrup	Ambroxol	3 mg/ml	2.8±0.17	93.3±5.7
Mucibron (Hosbon)	Syrup	Ambroxol	3 mg/ml	2.9±0.05	97.7±1.9
Naxpa (Norag)	Syrup	Ambroxol	3 mg/ml	2.9±0.10	96.6±3.3
Motosol (Europharma)	Bag	Ambroxol	60 mg	60.9±1.6	101.5±2.6

^aMeans of three samples±standard deviation.

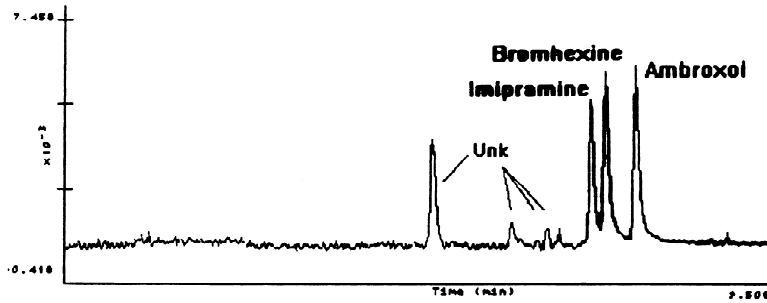


Fig. 3. Electropherogram of urine-1 sample following cleanup. Hydrodynamic injection (2 s). Other conditions as for Fig. 2.

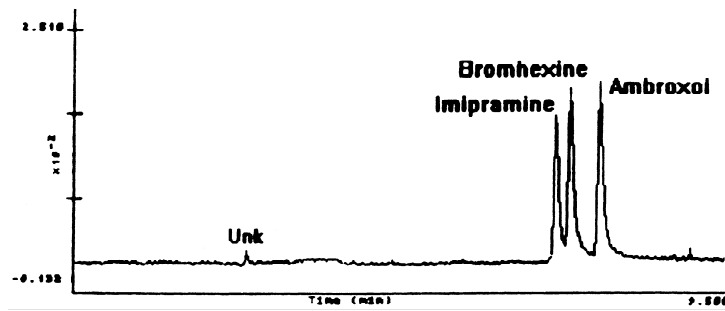


Fig. 4. Electropherogram of serum-1 sample. Hydrodynamic injection (2 s). Other conditions as for Fig. 2.

treated following the procedure described in Section 2.

The relative recoveries from the determination of BXH and ABX in urine and blood serum are summarized in Table 2. As can be seen, the results obtained were within the range 94.6–101.2% of the amounts added in the preparation of synthetic samples.

Peak confirmation and peak purity evaluation were achieved by comparison of the absorption spectra obtained from the electropherograms of the two

analytes in the real samples with those of the standards in water and also by comparison of the migration time with that of the standard solution.

4. Conclusions

This study has demonstrated that CE is suitable for separating BHX and ABX. The CZE procedure yields good repeatability of both migration time and peak area. The relative responses of BHX and ABX

Table 2
Recoveries of bromhexine and ambroxol in real samples

Sample	Bromhexine			Ambroxol		
	Added (ppm)	Mean recovery (%)	R.S.D. (%)	Added (ppm)	Mean recovery (%)	R.S.D. (%)
Urine 1	5.0 (3) ^a	97.1	2.1	5.0 (3)	97.5	1.6
Urine 2	10.0 (3)	97.8	2.6	10.0 (3)	100.9	2.2
Urine 3	20.0 (4)	100.2	1.4	20.0 (4)	101.2	2.4
Serum 1	10.0 (4)	97.61	1.8	10.0 (4)	95.5	2.2
Serum 2	20.0 (4)	95.61	1.5	20.0 (4)	94.6	1.1

^aNumber of samples is in parentheses.

to the internal standard are linear in a wide range and the peaks are symmetrical.

There are some advantages in using CZE for the separation of BHX and ABX. Compared with HPLC, the overall running time of the method is short and solvent and sample consumption are low. In addition, the thermal degradation of analytes is much reduced compared with the degradation which takes place with GC.

The proposed method is a promising one for the simultaneous analysis of both BHX and ABX in pharmaceutical preparations and urine and serum specimens.

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References

- [1] G. Indrayanto and R. Handayani, *J. Pharm. Biomed. Anal.*, 11 (1993) 781.
- [2] M.I.R.M. Santoro, M.D. Dos Santos and J.F. Magalhaes, *J. Assoc. Off. Anal. Chem.*, 67 (1984) 532.
- [3] A.P. de Leenheer and L.M.R. Vandecasteele-Thienpont, *J. Chromatogr.*, 175 (1979) 301.
- [4] O.W. Lan and Y.M. Cheung, *Analyst*, 115 (1990) 1349.
- [5] L. Colombo, F. Marcucci, M.G. Marini, P. Pierfederici and E. Mussini, *J. Chromatogr.*, 95 (1990) 141.
- [6] J.L. Kumar, W.C. Mann and A. Rozanski, *J. Chromatogr.*, 249 (1982) 373.
- [7] E.V. Rao, G.R. Rao, S. Raghuvveer and P. Khadgpathi, *Analyst*, 112 (1987) 311.
- [8] F.J. Flores-Murrieta, C. Hoyo-Vadillo, E. Hong and E. Castaneda-Hernández, *J. Chromatogr.*, 83 (1989) 464.
- [9] M. Eisman, M. Gallego and M. Valcarcel., *J. Anal. At. Spectrom.*, 7 (1992) 1295.
- [10] M. Eisman, M. Gallego and M. Valcarcel., *J. Pharm. Biomed. Anal.*, 11 (1993) 301.
- [11] T. Pérez-Ruiz, C. Martínez-Lozano, A. Sanz and S. Mondejar, *J. Pharm. Biomed. Anal.*, 13 (1995) 1101.
- [12] T. Pérez-Ruiz, C. Martínez-Lozano, A. Sanz and M.T. San Miguel, *Talanta*, 43 (1996) 1029.
- [13] A.P. de Leenheer and L.M.R. Vandecasteele-Thienpont, *J. Pharm. Sci.*, 69 (1980) 99.
- [14] E. Bechgaard and A. Nielsen, *J. Chromatogr.*, 228 (1982) 392.
- [15] S.F.Y. Li, *Capillary Electrophoresis*, Elsevier, Amsterdam, 1992, pp. 438–355.
- [16] D. Grossman and J.C. Colburn, *Capillary Electrophoresis*, Academic Press, San Diego, CA, 1992, pp. 330–332.
- [17] N.W. Smith and M.B. Evans, *J. Pharm. Biomed. Anal.*, 12 (1994) 579.
- [18] C.E. Uboh, J.A. Rudy, L.R.Soma, M. Fennell, L.May, R. Sams, F.A. Railing, *J. Pharm. Biomed. Anal.*, 9 (1991) 33.
- [19] H.D. Renovanz and H.J. Merker, *Pneumologie*, 148 (1973) 125.
- [20] R.L. Chien and D. Burgi, *J. Chromatogr.*, 18 (1991) 153.