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Short communication

Sensitive method for the determination of ambroxol in body fluids by capillary electrophoresis and fluorescence detection

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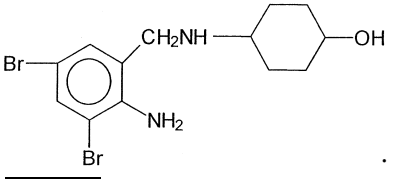
Abstract

A sensitive and rapid capillary electrophoretic method combined with laser-induced fluorescence detection has been developed for the determination of ambroxol. Samples were derivatized with $5 \cdot 10^{-4}$ *M* fluorescein isothiocyanate. A linear relationship between concentration and peak area was obtained in the concentration range 0.008–42 $\mu g \quad ml^{-1}$ with a correlation coefficient of 0.9999. The applicability of the method to serum and urine samples was demonstrated. The method is also useful for the determination of ambroxol in pharmaceutical preparations.

Keywords: Ambroxol; Capillary electrophoresis; Laser-induced fluorescence; Body fluids; Pharmaceuticals

1. Introduction

Ambroxol is a metabolite of bromhexine and has similar action and uses. It is administered as ambroxol hydrochloride in a daily dose of 30–120 mg by mouth divided into 2 or 3 doses. Similar doses can be given by inhalation, injection, or rectally. In order to carry out pharmacokinetic studies of ambroxol a selective and sensitive analytical method was needed, which could allow its determination in plasma and urine samples



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Several different methods have been used to the determination of ambroxol in pharmaceutical preparations, including ultraviolet spectrometry [1], high-performance liquid chromatography (HPLC) [1,2], gas–liquid chromatography (GLC) [1] and flow-injection analysis using a continuous liquid–liquid extractor [3].

Prior to developing a system for the quantitative analysis of ambroxol in biological fluids it was noticed that, to date, two GLC methods [4,5] and four HPLC methods [6–9] have been reported. The HPLC methods make use of ternary solvent mixture, 5-μm stationary phases, relatively high-flow-rates and, in one case, an ion-pairing reagent. Derivatization with trifluoroacetic anhydride at 90°C for 30 min and hexamethyldisilaze at 80°C for 60 min were used in the GLC methods.

Capillary electrophoresis (CE) is a relatively new mode of analytical separation with great potential and is already applied to a wide variety of molecules,

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ranging from simple ions to larger particles, and for ionized as well as neutral compounds. This technique has already attracted a considerable amount of attention in areas such as analytical biochemistry, molecular biology, analytical chemistry and medical biology [10,11]. Trace analysis at sub-ppb levels can become feasible only if sample preconcentration or improved detection systems are used. Laser-induced fluorescence detection (LIFD) is the most sensitive detection system in CE; however, most of the compounds of interest do not exhibit native fluorescence and have to be derivatized with fluorescence tags. Fortunately, there is a large variety of fluorochromes with high affinity for primary amine or carboxylic groups, which are abundant in biologically active compounds. To our knowledge, no attempt to apply CE–LIFD to the analysis of ambroxol has been made.

Ambroxol is a highly substituted aniline derivative with a primary amine group which can be derivatized with different fluorochromes. In this work, fluorescein isothiocyanate (FITC) was used because it has an excellent quantum efficiency and its absorption peak matches very well the blue line of argon ion laser. The aim of this contribution was developed a rapid and sensitive CE–LIFD method for the determination of ambroxol in biological fluids.

2. Experimental

2.1. Reagents and solutions

Ambroxol hydrochloride, fluorescein isothiocyanate isomer I and all inorganic chemical were obtained from Sigma (St. Louis, MO, USA). Deionized water from a Milli-Q water-purification system (Millipore Ibérica, Madrid, Spain) was used in the preparation of the solutions.

Stock standard solution of ambroxol (4160 $\mu g\ ml^{-1}$) was prepared by dissolving the required amount of ambroxol in 250 ml water. Working standard solutions of lower concentrations were freshly prepared by appropriate dilution of the stock solution. The FITC stock solution was prepared by dissolution of 19.47 mg FITC in 50 ml acetone. The separation buffer consisted of 100 m*M* boric acid adjusted to pH 9 by the addition of sodium hydroxide.

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

2.2. Apparatus and running conditions

A P/ACE Model 5000 instrument (Beckman Instruments, Palo Alto, CA, USA) with a laser-induced fluorescence detector was used for all experiments. The excitation was performed by an air-cooled argon ion laser (3 mW) at a wavelength of 488 nm. The emission intensities were measured at a wavelength of 520 filtered by a band pass filter. The instrument was controlled and data were collected with the SYSTEM GOLD data station. Unless otherwise specified, separation took place in a uncoated fused-silica capillary [57 cm (50 cm to the detector)×75 μm I.D.] thermostated at 25°C. Sample injection was accomplished by pressure (0.5 p.s.i.; 1 p.s.i.= 6894.76 Pa) for a time of 5–10 s. The capillary was conditioned every morning before starting a sequence of runs by rinsing in the high-pressure mode for 5 min with 0.1 *M* sodium hydroxide, 5 min with water and 10 min with the running buffer. After every run it was rinsed for 5 min with the electrophoretic buffer.

The voltage applied was 25 kV, and the current was ~150 μA with the running buffer described above.

2.3. Derivatization procedure

The derivatization procedure consisted of mixing 10 μl of blank, standard or sample with 990 μl of 0.2 *M* carbonate buffer, pH 9, and 1000 μl of $5 \cdot 10^{-4}$ *M* FITC solution. The mixture was incubated for 12 h in the dark. This solution was diluted 100-fold in water prior to analysis.

2.4. Determination of ambroxol in serum

Serum samples were spiked with appropriate amounts of ambroxol to obtain a final concentration within its therapeutic range. A volume of 500 μl of each sample was placed in a test tube and 100 μl of 1.2 *M* tricloroacetic were added. The mixture was

vortexed for 1 min and allowed to stand for 5 min and then centrifuged. A volume of 10 μl of the liquid supernantant was derivatized following the above described procedure for the standard solutions.

2.5. Determination of ambroxol in urine

Urine samples spiked with different amounts of ambroxol were centrifuged for 10 min at 3800 rpm. A 10-ml volume of clear supernantant urine was transferred into a 25-ml calibrated flask and diluted to the mark with deionized water. This solution was stored and maintained below 5°C. A volume of 10 μl was analysed following the procedure for the standard solutions.

2.6. Determination of ambroxol in pharmaceutical preparations

Syrups were appropriately diluted with deionized water so that the ambroxol concentration was in the working range. A volume of 10 μl of the diluted sample was analysed by the above described procedure.

3. Results and discussion

Ambroxol has a primary amine group which can be derivatized with different fluorescent labelling reagents. In this work, FITC was selected because it offered the follows advantages: (i) the excitation wavelength exactly matches the 488 nm light provided by the argon laser; (ii) the use of long wavelength excitation light reduces problems with light scattering and thus enhances sensitivity and (iii) sensitivity is very high because fluorescein derivative molecules are among those which show the highest fluorescence quantum efficiency.

3.1. Optimization of derivation conditions

In initial experiments, we used borate, carbonate and phosphate buffers at several concentrations in order to ascertain the influence of the type and concentration of the buffer solution on the efficiency of the derivation reaction. Peak height and shape depended on the type and concentration of the buffer. The best results were obtained with 0.2 *M* carbonate buffer, pH 9. Working without any buffer components gave very low derivatization efficiencies and totally disturbed peak shapes.

The derivatization of standard solutions with $5\cdot10^{-4}$ *M* FITC gave higher peaks of ambroxol than derivatization with $5\cdot10^{-5}$ *M* FITC. However, more 'ghost peaks' were also observed with $5\cdot10^{-4}$ *M* FITC; these peaks corresponded to different chemical species of fluorescein. As the ambroxol–FITC peak was firstly eluted and well separated from the FITC peaks, a $5\cdot10^{-4}$ *M* FITC solution was used in order to achieve better sensitive (Fig. 1).

The dependence of derivatization on time is show in Fig. 2. A reaction time over the range 10–16 h was long enough to obtain the maximum peak height. Parallel experiments in the presence of pyridine have shown that this activator [12] does not influence the quantity ambroxol–FITC derivative formed. In addition, pyridine impurities produced additional FITC derivates; therefore, derivatization in absence of pyridine was chosen.

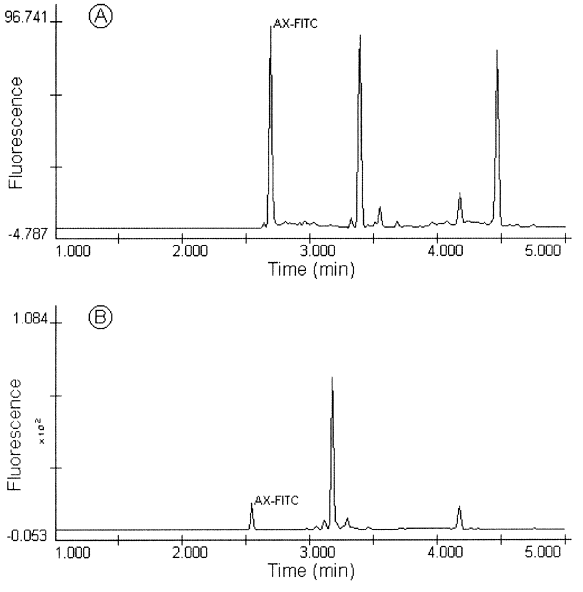

Fig. 1. Influence of FITC concentration. (A) Derivatization of 41.6 μg ml^{-1} ambroxol standard solution for 12 h with $5\cdot10^{-4}$ *M* FITC. (B) Derivatization with $5\cdot10^{-5}$ *M* FITC.

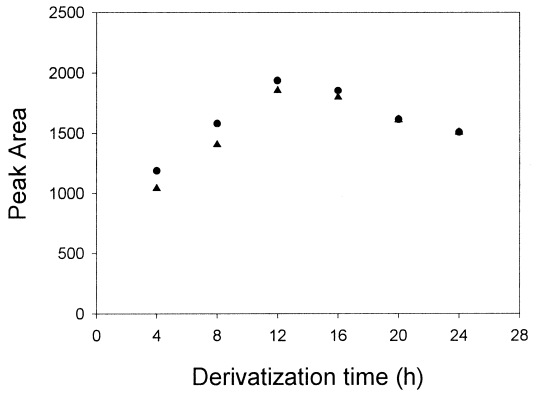

Fig. 2. Plot of peak area of ambroxol-FITC as a function of derivatization time with (●) and without (▲) pyridine.

3.2. Optimization of analytical conditions

To optimize the electrophoretic separation three main parameters were considered: the pH and the type and concentration of the running buffer.

Based on the width of the ambroxol–FITC peak and its separation from the other peaks corresponding to different species of fluorescein, the best results were obtained with a 0.1 *M* borate buffer, pH 9. Under these alkaline conditions, the electroosmotic flow is high enough to permit the elution of the ambroxol–FITC derivate in <3 min, although the adduct has a negative charge due to the presence of the carboxylic group on the label.

A study was performed to determine the degree of trace enrichment for different injection times and any resulting sacrifice in resolution. It was possible to perform pressure injections up to 10 s with no loss in resolution and with satisfactory peak area linearity against injection time (analyte quantity). It was observed that injection times >12 s resulted in poor peak areas.

3.3. Calibration graph

A calibration graph was obtained by injecting standard solutions of ambroxol in the concentration range 0.008–42 μg ml^{-1} which had previously been derivatized with $5 \cdot 10^{-4}$ *M* FITC following the procedure described in Experimental.

The repeatability of the method was studied by measuring the migration times, peak area and peak heights of eleven injections of each solution containing 4.16 and 10.4 μg ml^{-1} of ambroxol. The relative standard deviation (RSD) of the migration time were 0.3 and 0.4%, respectively. Both peak area and peak height were reproducible with an RSD$\leq$ 1%.

3.4. Analytical applications

To demonstrate the applicability of the proposed method, it was applied to the determination of ambroxol in blood serum, urine and pharmaceutical preparations.

The linearity and precision of the assay in serum were tested using spiked samples. The linearity was confirmed in the range 0.04–30 μg ml^{-1} using a 10-μl sample. The precision of the method was assessed by determining three concentrations within the range 1.04–20 μg ml^{-1} in six independent series of samples; RSD values $<3.7\%$ were always obtained. The lower limit of quantitation was 3.7 ng ml^{-1}. The correlation coefficient of the regression lines was 0.999 or higher. Fig. 3A shows a representative electropherogram of a processed serum blank and indicates that no endogenous FITC derivates exist. Day-to-day precision data were obtained over a period of five working days by daily derivatizing with FITC aliquot of serum samples containing 1.04 and 4.16 μg ml^{-1} of ambroxol. The values of RSD obtained were 4.4% (lowest concentration) and 2.8% (highest concentration). Table 1 shows the recoveries obtained for ambroxol in three different serum samples.

Parallel experiments using urine samples demonstrated that there was linearity between peak area and ambroxol concentration in the range 0.04–30 μg ml^{-1} (correlation coefficient of the regression lines 0.999 or higher). The quantification limit was 3.2 ng ml^{-1}. Endogenous peaks were absent in urine samples treated with FITC. The repeatability was very good, and RSD values $<1.2\%$ were obtained using ten replicate injections of two urine samples containing 1.04 and 4.16 μg ml^{-1} of ambroxol. Day-to-day precision was evaluated analysing two samples containing 1.04 and 4.16 μg ml^{-1} of ambroxol. The RSD values were 3.7 and 2.4%, respectively. The results obtained in the

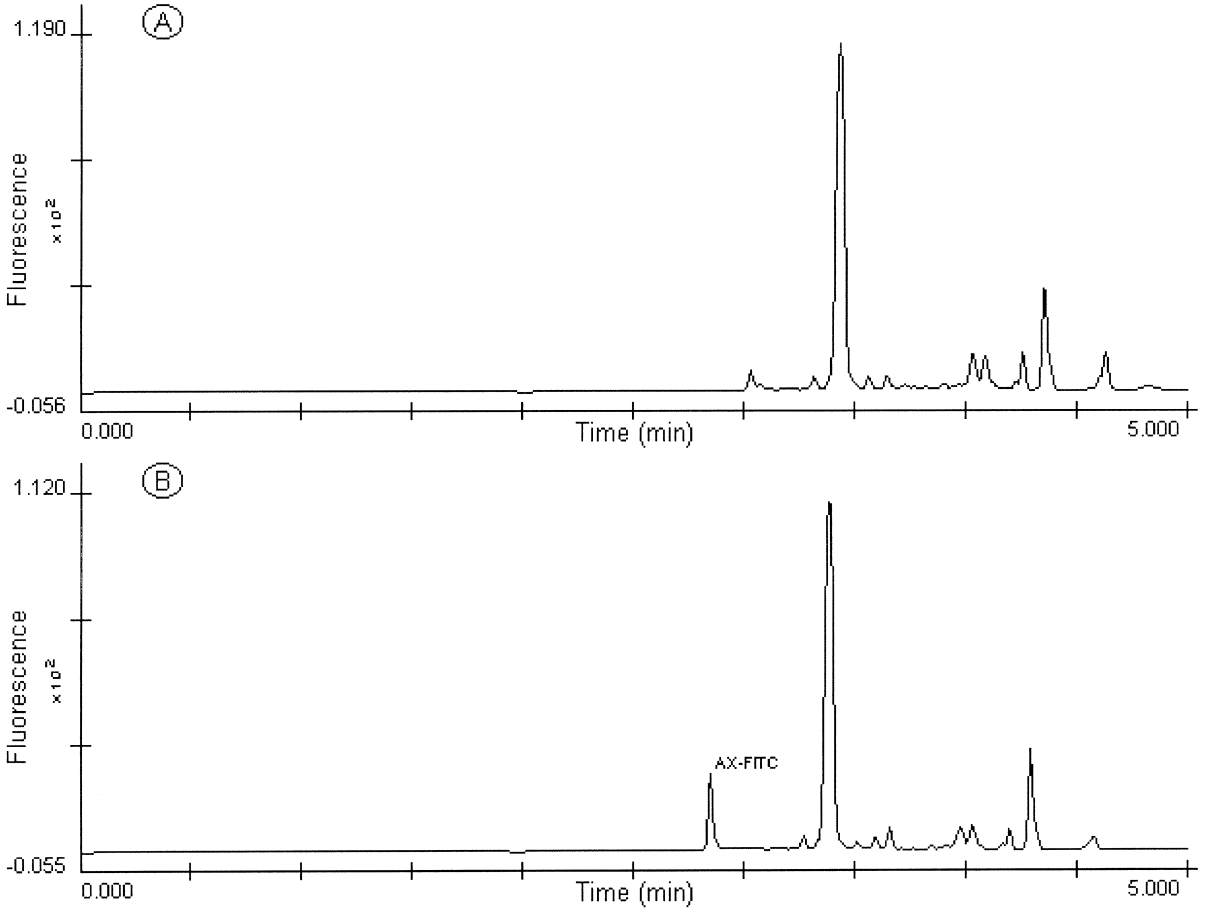

Fig. 3. Electropherograms of (A) blank serum; (B) blank serum spiked with ambroxol (4.16 μg ml^{-1}). Derivatization conditions: 0.2 *M* carbonate buffer, pH 9, FITC ($5 \cdot 10^{-4}$ *M*). Electrophoretic conditions: a 100 m*M* borate running buffer, pH 9; applied voltage 25 kV; injection pressure 5 s (0.5 p.s.i.).

analysis of three different samples are indicated in Table 1.

The proposed method was also used to determine ambroxol in pharmaceutical preparations. Additives and excipients did not interfere. The quantification of ambroxol in pharmaceuticals was carried out from the calibration graph obtained using the standard solutions. The data in Table 2 were in good agreement with values for the nominal contents. The

Table 1
Recoveries of ambroxol in real samples

Sample	Amount added	Amount found[a]	Recovery (%)
Serum 1	1.04	0.94±0.06	90.8
Serum 2	4.16	3.79±0.05	91.1
Serum 3	20.8	21.11±1.04	101.5
Urine 1	1.04	1.01±0.06	97.1
Urine 2	4.16	414±0 10	99.5
Urine 3	20.8	20.55±1.73	98.8

[a] Mean of four injections±standard deviation.

Table 2
Determination of ambroxol in real pharmaceuticals formulations

Preparation	Supplier	Amount (mg ml^{-1})	
		Theoretical	Experimental[a]
Naxpa	Norag	3	2.94±0.22
Mucosan	Fher	3	2.90±0.06
Mucibron	Hosbon	3	3.09±0.22

[a] Mean of four samples±standard deviation.

recoveries obtained for ambroxol added to each pharmaceutical formulation were in the range 96.6–103.0%.

4. Conclusions

The combination of CE with LIFD is a good method for determining ambroxol in serum and urine samples after derivatization with fluorescein isothiocyanate. The high sensitivity of the method permitted this drug to be determined up to a 10^{-9} *M* concentration level.

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