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High-performance liquid chromatographic determination of ambroxol in human plasma

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ABSTRACT

Ambroxol has been determined in biological fluids using a rapid and sensitive high-performance liquid chromatographic method. The samples prepared from plasma by liquid–liquid extraction were analysed on reversed-phase silica gel by competing-ion chromatography with ultraviolet detection. The method was applied to the determination of ambroxol levels in twelve healthy volunteers after oral administration of 90 mg of ambroxol in tablets of Mucosolvan and Ambrosan.

INTRODUCTION

Ambroxol, *trans*-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol hydrochloride (Fig. 1, compound **2**) is a pharmacologically active metabolite of bromhexine, N-cyclohexyl-N-methyl-(2-amino-3,5-dibromobenzyl)amine hydrochloride (Fig. 1, compound **1**) with potent mucolytic activity, for which it is used as an expectorant in therapeutics [1–5].

The high-performance liquid chromatographic (HPLC) determination of ambroxol in biological fluids has been described in a number of papers [6–8]. Vergin *et al.* [6] converted ambroxol in plasma samples into 6,8-dibromo-3-(*trans*-4-hydroxycyclohexyl)-1,2,3,4-tetrahydroquinazoline by treatments with formaldehyde (see Fig. 1, compound **3**), which was subsequently determined by HPLC using UV detection at 254 nm. Since the same tetraquinazoline derivative may be produced in the organism [4,5] it is possible

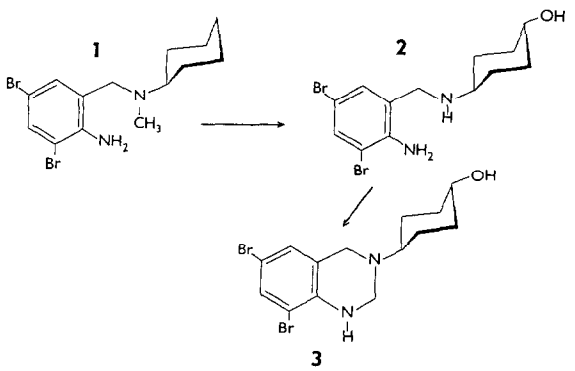

Fig. 1. Biotransformation of bromhexine (**1**) to ambroxol (**2**) and a 1,2,3,4-tetrahydroquinazoline derivative (**3**).

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that the determined plasma levels are the sum of the product formed in the body and in the course of the analytical procedure.

The method of Botterblom *et al.* [7] was tested in our laboratory; the extraction procedure (with minor modifications) was used in this study. Unfortunately, our attempts to reproduce the chromatography under the conditions used in original work failed, because interfering peaks appeared close to that of ambroxol.

Flores-Murrieta *et al.* [8] determined ambroxol in human plasma by reversed-phase HPLC with amperometric detection with an internal standard pelanserin (an arterial antihypertensive agent of 1,2,3,4-tetrahydroquinazoline-2,4-dione structure). Recoveries of ambroxol and of the internal standard from plasma samples were similar and ranged from 80 to 90%. The use of electrochemical detection improved the detection limit in comparison with UV detection, but the method required highly pure and degassed solvents and deionized water of high purity for the mobile phase. It was also necessary to maintain the column temperature at 40°C.

This paper describes a rapid competing-ion reversed-phase chromatographic determination of ambroxol at room temperature with a small consumption of acetonitrile for the mobile phase.

EXPERIMENTAL

Chemicals and solutions

Ambroxol hydrochloride (subst., lot 0809) and Ambrosan (ambroxol hydrochloride 30-mg tablets) were from PRO.MED.CS. (Prague, Czechoslovakia). Mucosolvan (ambroxol hydrochloride 30-mg tablets) was from Bender (Vienna, Austria). Acetonitrile (HPLC grade, Aldrich, Milwaukee, WI, USA), nonylamine (purum, Fluka, Buchs, Switzerland), methanol, phosphoric acid (85%), hydrochloric acid (35%), boric acid, potassium chloride, sodium hydroxide, diethyl ether (all analytical grade, Lachema, Brno, Czechoslovakia) were used.

Nonylamine buffer (0.02 *M*) was prepared by mixing 3.64 ml of nonylamine with 980 ml of redistilled water; the pH was adjusted to 2.4 with 2 *M* aqueous phosphoric acid, and the volume was finally adjusted to 1000 ml.

Phosphate buffer (0.01 *M*, pH 10) was prepared as follows: 6.183 g of phosphoric acid and 7.456 g of potassium chloride were dissolved in redistilled water to a volume of 1000 ml, and 50 ml of this solution were mixed with 43.9 ml of 0.1 *M* sodium hydroxide and diluted to 100 ml with water.

Volunteers

The pharmacokinetics after administration of two oral forms of ambroxol, Ambrosan (PRO.MED.CS.) and Mucosolvan (Bender) (both containing 30 mg in each tablet) was evaluated. The open, randomized, cross-over study was licensed by the regulatory governmental agency and the regional ethical committee.

Twelve healthy volunteers, who first signed the informed consent, were examined clinically and biochemically. The group consisted of nine males and three females, mean age 26.6 (range 18–37) years, mean weight 72.4 (52–96) kg, and mean height 176.6 (164–194) cm.

A single oral dose of 90 mg (three tablets of one of the preparations, Ambrosan or Mucosolvan) was administered after five days restriction of fat, alcohol and caffeine intake in the diet, and smoking prohibition (eleven were non-smokers). After two weeks the second dose of Mucosolvan or Ambrosan was administrated under the same conditions. The sequence of the preparations was random.

Blood samples were taken from the cubital vein at 0–24 h after administration into the heparinized syringe. Plasma was obtained by centrifuging at 3000 *g* for 10 min. Plasma specimens (2 ml) were stored at −20°C until analysis.

Sample preparation

To 1 ml of human plasma, 1 ml of 0.01 *M* buffer (pH 10) was added and the mixture was vigorously shaken for 10 s. After the addition of 4 ml of diethyl ether, the mixture was vortex-mixed for 10 min. After centrifugation (2600 *g*, 6 min) the diethyl ether layer was transferred to a clean glass tube, and 300 μl of 0.01 *M* hydro-

chloric acid were added. The mixture was vortex-mixed for 10 min and centrifuged at 2600 *g* for 6 min. The diethyl ether layer was discarded, and 50 μl of the acid layer were injected into the column.

Chromatography

The Spectra Physics chromatographic system consisted of an SP8800 ternary HPLC pump, a Spectra 100 UV–VIS detector, an SP4400 ChromJet integrator and an AT-computer (DRV, Dreieich, Germany) equipped with a Labnet interactive communications network controlled by WINner/286 software. A Rheodyne Model 7125 injection valve with a 50-μl injection loop was used.

A CGC (compact glass cartridge) analytical column (150 mm x 3.3 mm I.D.) and a CGC guard column (30 mm x 3.3 mm I.D.), both packed with Separon SGX C_{18}, 5 μm (Tessek, Prague, Czechoslovakia), were used.

The mobile phase, acetonitrile–nonylamine buffer (5:95, v/v), was mixed and degassed ultrasonically before use. The flow-rate was 0.9 ml/min (29–30 MPa), and chromatographic analyses were performed at room temperature. Detection was effected at 242 nm.

Calibration

The standard stock solution (prepared by dissolving 41.4 mg of ambroxol hydrochloride in 100 ml of water = 10^{-3} *M*) was diluted with 0.01 *M* hydrochloric acid to a calibration series of concentrations $2 \cdot 10^{-6}$, 10^{-6}, $5 \cdot 10^{-7}$, $2 \cdot 10^{-7}$ and 10^{-7} *M*. The same concentrations (except $2 \cdot 10^{-7}$ *M*) were used to make a calibration with drug-free plasma spiked with ambroxol. The extraction procedure was the same as described in *Sample preparation.*

Testing and statistical evaluation of the analytical procedure

Multilevel calibrations with six analyses in each concentration were performed. The on-line statistical processing of calibration analyses by the least-squares method was performed by the ChromJet integrator. The weighted linear regression was performed using the statistical program ADSTAT [12]

The linearity of the ambroxol calibration curve from solutions in 0.01 *M* hydrochloric acid (line A: $y = k_A x + q_A$) and from spiked human plasma extracts (line B: $y = k_B x + q_B$) was tested (x is the concentration of ambroxol in ng/ml, y is corresponding peak area, and q_A and q_B should approach zero). The accuracy was calculated as the percentage found on the linear standard curve (line B). The precision of the method, expressed as the relative standard deviation (R.S.D. = 100 S.D./mean) for line B was also calculated (see Results and Discussion).

The recovery was determined as the ratio of the slopes of line B and line A (see *Calibration*), multiplied by 100: recovery (%) = 100 (k_B/k_A).

The detection limit (at an ambroxol signal-to-noise ratio of 2) was calculated from six chromatograms.

RESULTS AND DISCUSSION

Chromatography

Competing-ion chromatography has been often used for the separation of basic compounds (drugs and their metabolites) by reversed-phase HPLC [9,10].

It is known that the addition of a primary amine with a long alkyl chain (nonylamine) to the mobile phase has a strong influence on the separation efficiency, peak width and symmetry. In the acid mobile phase the protonated nonylamine masks residual silanols at the surface of the solid phase. The silanol–alkylammonium interaction is at the second part of the nonylamine molecule stabilized by hydrophobic interaction of the nonyl and octadecyl moieties. This dynamic blockade of residual silanol groups of the ODS silica gel reduces polar silanol–drug interactions, shortens the retention time of drug and improves the shape and symmetry of the peak.

Fig. 2 demonstrates the influence of nonylamine in the mobile phase on the retention time of ambroxol. The mobile phase was acetonitrile–buffer pH 2.4 (5:95, v/v) containing various concentrations of nonylamine: 0, 0.001, 0.0025,

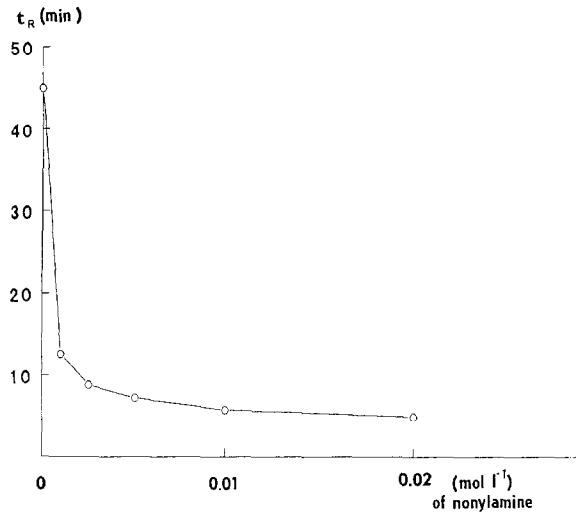

Fig. 2. Influence of the nonylamine concentration in the buffer of the mobile phase on the retention time of ambroxol.

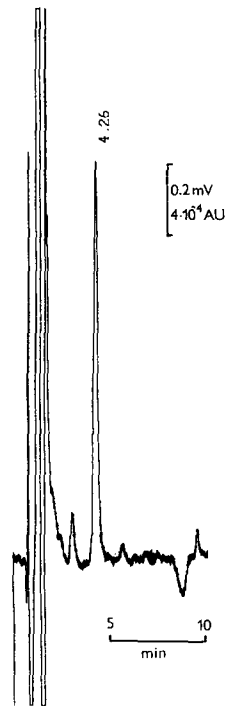

Fig. 3. Chromatogram of a human plasma extract. The peak at t_R = 4.26 min corresponds to 17 ng of ambroxol. The negative peak at *ca.* 9 min is caused by a trace of diethyl ether in the acid reextract. Flow-rate, 0.9 ml/min.

0.005, 0.01 and 0.02 *M*. The retention time of ambroxol in the mobile phase without the nonylamine was 45–49 min. In the mobile phase with 0.001 *M* nonylamine the retention time of ambroxol was reduced to 12.6 min; further addition of nonylamine (0.02 *M*) led to a retention time of 4.3 min (Fig. 3).

The analytical procedure is accurate and precise. Two calibration curves (A, samples from aqueous acidic solutions; B, samples from spiked human plasma) were found to be linear; weighted linear regression allowed (on the basis of standard deviations of intercepts q_A and q_B) both curves to be expressed as linear relationships, omitting the intercepts ($y = k_A x$, with k_A = 335.16, S.D. (k_A) = 2.34, r = 0.9996; $y = k_B x$ with k_B = 276.36, S.D. (k_B) = 2.62, r = 0.9992).

The accuracy and precision of ambroxol determination in spiked human samples were found to be 95.63% (range 92.3–98.7%) and 4.6% (range 4.0–5.2%), respectively.

The total recovery of ambroxol (calculated from calibration curves) was 82.5%, and the detection limit was 4 ng/ml (mean of six determinations).

Pharmacokinetics

Fig. 4 shows the mean plasma concentration–time curve calculated from the plasma levels of twelve volunteers. The relative standard errors of the mean (S.E.M.) (indicated as vertical bars) exhibit relatively great absolute deviations, mostly in peak values. This fact has been observed earlier [6], and can probably be ascribed to variations in body weight of the volunteers (52–96 kg), who were given the same dose (90 mg per volunteer).

The area under curve (AUC, determined via the linear trapezoidal rule from t = 0 to t = 24 h), the area under the first moment curve (AUMC) and the mean time (as the sum of the mean residence time and the mean absorption time) were calculated for each volunteer. The maximum of the concentration–time curve, c_{max}, and the corresponding time, t_{max}, were obtained as the maximum of the Reinsch spline function [11]. The differences in these parameters for Ambrosan and Mucosolvan for the same individuals

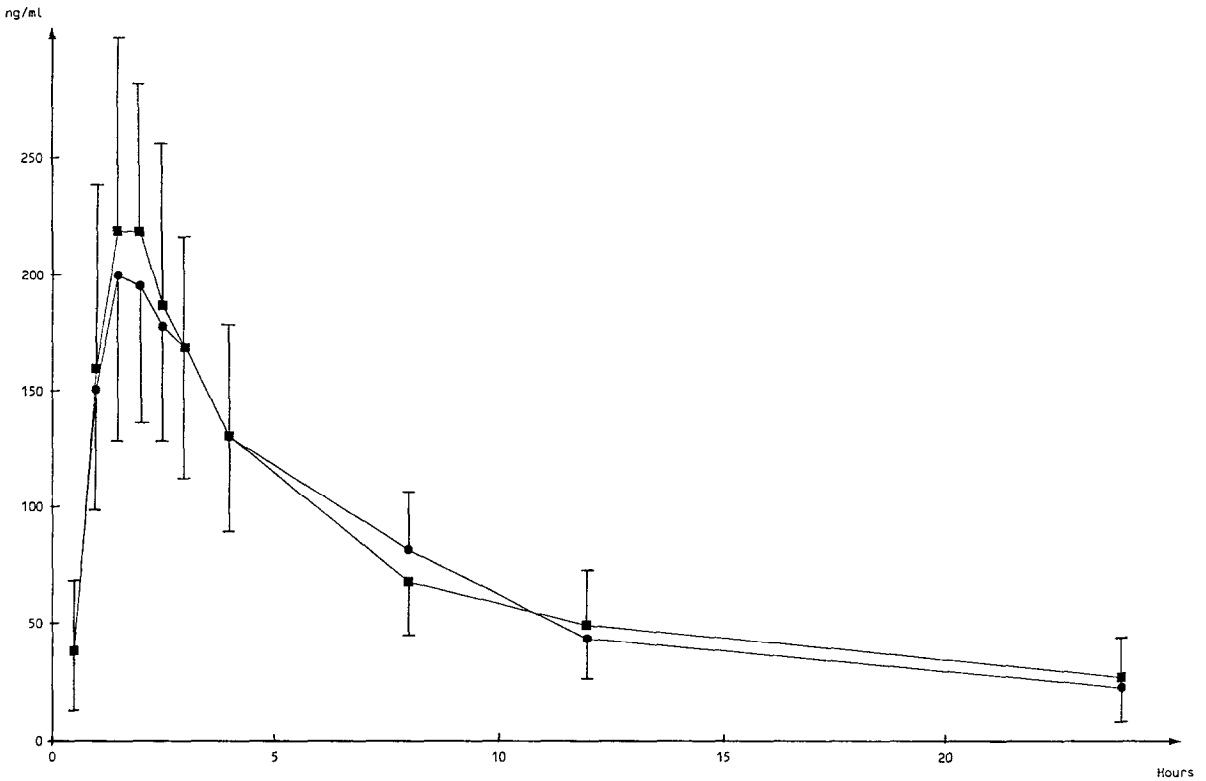

Fig. 4. Pharmacokinetics of ambroxol after oral administration of 90 mg in tablets of Mucosolvan (●) and Ambrosan (■). Mean values from twelve volunteers; vertical bars represent standard error of the mean (S.E.M.)

at comparable time intervals were determined by paired Student's t-test. Differences in the parameters of oral pharmacokinetics of Ambrosan and Mucosolvan, respectively, were not significant at the $\alpha_1 = 0.01$ and $\alpha_2 = 0.05$ probability level.

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