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ASSAY OF AMBROXOL IN BIOLOGICAL FLUIDS BY CAPILLARY GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and rapid method for the determination of ambroxol in biological fluids is described. It comprises a single extraction step, derivatization and selective determination with capillary gas–liquid chromatography (in split-mode) and electron-capture detection. The limit of quantification in plasma is ca. 3 ng/ml. The method is applied to the pharmacokinetics of ambroxol in humans.

INTRODUCTION

Ambroxol (Mucosolvan®, N-A 872 CL Thomae, Biberach, F.R.G.), *trans*-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol hydrochloride, is widely used as a secretolytic agent in human medicine [1].

Pharmacokinetic and metabolic investigations of ^{14}C-labelled ambroxol in humans and in animals have shown that ambroxol undergoes a rather simple metabolism, yielding mainly dibromanthranilic acid and conjugates of the parent compound [2,3]. On the other hand, ambroxol is one of the main metabolites of the mucolytic agent bromhexine [4].

Until now, only a few reports have been published concerning the semiquantitative and quantitative determination of Mucosolvan in biological material. Eichler and Kreuzer [5] have used gas chromatography (GC) with electron-capture detection (ECD) for analysing bromhexine residues and its metabolites (semiquantitatively) in tissues. Plasma levels have been reported by Seki et al. [6] using GC with nitrogen-selective flame ionization detection (N-FID). Jonckheere et al. [7] have published a qualitative assay of ambroxol in human urine after treatment with bromhexine [7]. Two liquid chromatographic (LC) methods are known, one by Schmid and Bozler [8] and one by Vergin et al. [9].

The published methods are not satisfactory for several reasons. Although mul-

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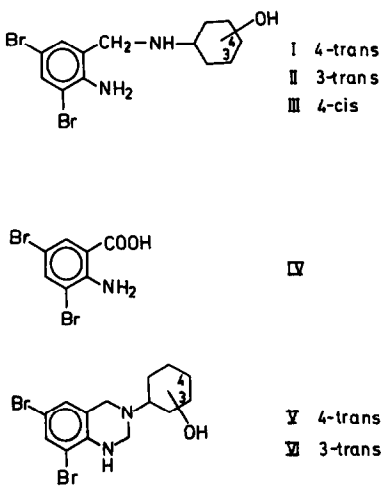

Fig. 1. Structural formulae of ambroxol (I), internal standard (II), metabolites III and IV and reference substances V and VI.

tiple extraction steps are performed no or no appropriate internal standard has been used [5–7], and neither accuracy nor precision has been evaluated. Of the two LC methods one is too insensitive [8] for plasma samples and the other [9] uses a lengthy clean-up procedure without an internal standard.

We have therefore developed a rapid method for the determination of ambroxol in human plasma and urine with appropriate precision, selectivity and limit of detection.

EXPERIMENTAL

Reagents and chemicals

Ambroxol [Mucosolvan, *trans*-4-(2-amino-3,5-dibromobenzylamino)-cyclohexanol hydrochloride (Fig. 1, I)], the internal standard [*trans*-3-(2-amino-3,5-dibromobenzylamino)cyclohexanol hydrochloride (Fig. 1, II)], the metabolites and the reference substances were of analytical grade.

The metabolites *cis*-4-(2-amino-3,5-dibromobenzylamino)cyclohexanol hydrochloride) (III) and dibromanthranilic acid (2-amino-3,5-dibromobenzoic acid, IV) and the further reference substances 6,8-dibromo-3-(*trans*-4-hydroxycyclohexyl)-1,2,3,4-tetrahydroquinazoline (V) and 6,8-dibromo-3-(*trans*-3-hydroxycyclohexyl)-1,2,3,4-tetrahydroquinazoline (VI) were all synthesized by Dr. J. Keck.

^{14}C-Labelled ambroxol (specific activity 42 mCi $\hat{=}$ 1.55 GBq/mmol) was synthesized in the isotope laboratory of Dr. Karl Thomae GmbH by H. Zipp. It was labelled at the benzylic position.

Acetonitrile, silylation quality (No. 20062) and hexamethyldisilazane (No. 84770) were purchased from Pierce (Rockford, IL, U.S.A.); toluene, nanograde quality (No. 8092) from Mallinckrodt (Wesel, F.R.G.); 13 m*M* borate buffer pH

8 (No. 38747) and 13 m*M* citrate buffer pH 4 (No. 38743) from Riedel (Seelze, F.R.G.); formaldehyde stabilized with 10% methanol (No. 47629) from Fluka (Buchs, Switzerland).

The internal standard solution was prepared as follows: 1.00 mg of II (hydrochloride) was dissolved in 100 ml of water. This stock solution was diluted with water to a final concentration of 1 μg/ml.

Apparatus

The following equipment was used: gas chromatograph, Varian 6000 with a capillary split injector equipped with an electron-capture detector; autosampler, Hewlett-Packard No. 7672; integrator, Spectra-Physics Type 4100; vortex evaporator, Searle Buchler (Fort Lee, NJ, U.S.A.); DB-1701 quartz capillary column, J & W Scientific (local supplier ICT, Frankfurt, F.R.G.), 30 m×0.32 mm I.D., film thickness 0.25 μm (No. 122-0732).

The GC operating conditions were: injector temperature, 300°C; detector temperature, 300°C; column temperature, 200 to 280°C at 20°C/min, final temperature hold for 19 min; split ratio, 1:30; injection volume, 2 μl; auxillary gas flow-rate (argon–methane, 95:5), 30 ml/min; carrier gas flow-rate (helium) 40 cm/s (200°C). The carrier gas was always purified by an Oxisorb-Kit purchased from Messer-Griesheim (Frankfurt, F.R.G.) to improve the service life of the column.

Extraction procedures

Plasma. The plasma was thawed at room temperature, and a 1-ml volume was pipetted into a 25-ml centrifuge tube. Then 2 ml of buffer pH 8 were added (the pH should be 7.0–8.0, cf. for urine) followed by 100 μl of the internal standard solution, containing 1 μg/ml internal standard, and 10 μl of formaldehyde (cf. for urine). The tube was shaken briefly by hand, then kept at 20°C for 10 min before 8 ml of toluene were added. The tubes were cupped, then shaken for 15 min at 120 strokes per min and centrifuged for 20 min at 1800 *g* at 10°C. The phases were separated by freezing out the aqueous phase at −35°C and decanting the organic layer into an other tube.

Urine. The urine was thawed at room temperature, and a 50-μl volume was pipetted into a 25-ml centrifuge tube. Then 0.5 ml of pH 4 buffer was added, followed by 100 μl of the internal standard solution, containing 1 μg/ml internal standard, and 10 μl of a diluted formaldehyde solution (1 part of formaldehyde to 49 parts of water). The tube was shaken briefly by hand, then kept at 20°C for 10 min. Then 2.0 ml of buffer pH 8 (the pH should be 7.0–8.0) were added, followed by 8 ml of toluene. The tubes were cupped, and the plasma procedure then followed.

Silylation. The organic phase was evaporated at 40°C, in the vortex evaporator, and the residue redissolved in a mixture of 50 μl of acetonitrile and 50 μl of hexamethyldisilazane. The tubes were carefully cupped, and heated for 1 h at 80°C. The mixture was cooled in an ice-bath and centrifuged (5 min, 1800 *g*) to concentrate the solution in the tip of the tube. The contents of the vial were evaporated to dryness at 40°C in the evaporator, and 30 μl of dry toluene containing

5% hexamethyldisilazane (important!) were added. A 2-μl aliquot was injected into the chromatograph.

Preparation of the calibration curve

The calibration curve was constructed by preparing an aqueous stock solution containing 1.00 mg of ambroxol hydrochloride in 100 ml (10 μg/ml). From this aqueous solution 250 μl were diluted with blank plasma up to 25 ml (100 ng/ml). After incubation at 37°C for 2 h, this plasma was diluted with blank plasma to obtain a concentration range of 10–100 ng/ml. The standard samples were analysed by the same procedure as described above. For urine samples the same procedure was applied, except that a concentration range of 250–2000 ng/ml was chosen and 50 μl of urine were used.

Partition studies

For the partition studies, ca. 50 μg of the ^{14}C-labelled compound were dissolved in buffer solutions and 2 ml of these solutions were extracted by 8 ml of organic solvent by shaking for 15 min with a shaking machine. After centrifugation, aliquots of both phases were measured by liquid scintillation counting.

Recovery studies

For the recovery studies we used plasma spiked with 10 and 100 ng/ml ^{14}C-labelled ambroxol and extracted as described under *Extraction procedures*. The aqueous phase, as well as the organic derivative, were measured with the scintillation counter.

Stability studies

For stability studies ex vivo standards and spiked standards were prepared. To prepare the ex vivo standards, 100 ml of plasma from one volunteer, who had received 30 mg ambroxol orally, were taken 3 h after dosing. This plasma was portioned in aliquots of 2.5 ml, frozen at −25°C and analysed on different days in duplicate. To prepare the spiked standards, 100 ml of blank plasma were spiked (procedure according calibration curve) with ca. 110 ng/ml (resp. 22 ng/ml) ambroxol hydrochloride, portioned and frozen at −25°C.

Statistics

The statistics were performed by Dr. Trieb (Dept. Res. Administration). For the estimation of the precision (reproducibility) of the assay under routine conditions, duplicates of a bioavailability study (ca. 155 pairs) were statistically analysed according to the method of Haeflinger and Wall [10].

RESULTS AND DISCUSSION

Principle of the method

The derivatization of ambroxol is performed in two steps (Fig. 2): the first is the quinazoline formation with formaldehyde in plasma prior to the extraction and the second is the silylation of the alicyclic alcohol function.

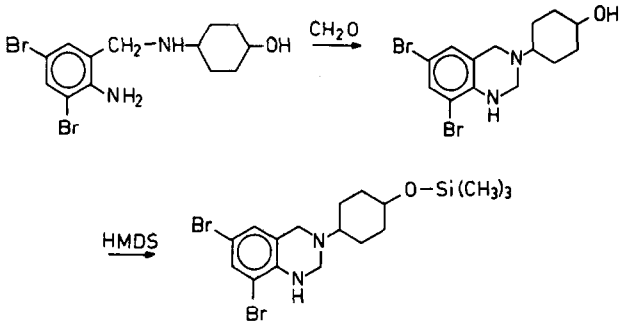

Fig. 2. Principles of the derivatization procedure.

Extraction procedure

The experiments presented in Table I show that diethyl ether and toluene are favourable extraction media. Toluene was chosen, as phase separation by freezing out the aqueous phase is better with this solvent and water is removed azeotropically during evaporation, a step that is important for the subsequent derivatization.

Fig. 3 indicates the pH dependence of the partition behaviour between toluene and buffer solutions. Ambroxol is a base, $pK_{a_1} = 8.0$ (benzylamino group), $pK_{a_2} = -0.3$ (anilino group). The values for the quinazoline derivative (V, Fig. 1) are $pK_{a_1} = 5.8$ and $pK_{a_1} < 1$. In addition, this compound is more lipophilic than ambroxol.

The distribution coefficients log P for octanol–water, calculated according to Hansch and Leo [16], are 5.8 for compound V and only 3.5 for ambroxol.

In order to avoid coextraction, and especially to prevent reaction of the labile conjugates of ambroxol [8], it is reasonable to extract at the lowest possible pH. Fig. 3 shows that ring formation before the extraction is very favourable owing to the lower pK_a and the higher lipophilicity of the quinazoline (V).

Derivatization

The ambroxol molecule has three derivatizable sites. Attempts to chromatograph ambroxol without derivatization are unsatisfactory owing to tailing. The

TABLE I

PARTITION RATIOS OF THE QUINAZOLINE DERIVATIVE (V) BETWEEN DIFFERENT SOLVENTS AT DIFFERENT pH VALUES OF THE AQUEOUS PHASE

Aqueous phase	Organic solvent	Patrition ratio (C_{org}/C_{aq})
Phosphate buffer (pH 5.2)	Hexane	0.16
	Diethyl ether	23.0
	Toluene	10.7
Ammonia (pH 11.2)	Hexane	2.50
	Diethyl ether	48.2
	Toluene	78.5

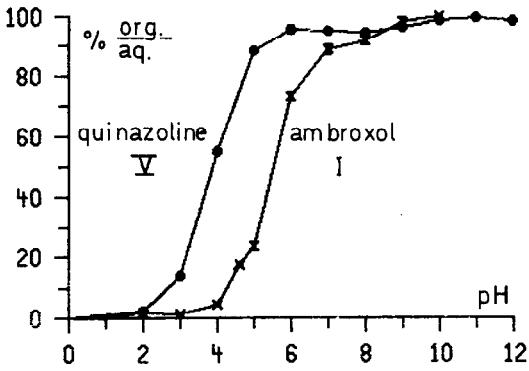

Fig. 3. Partition values of ambroxol and the quinazoline derivative (V) between organic phase (toluene) (8 ml) and aqueous phase (2 ml).

hydroxyl function is responsible for this behaviour rather than the amino groups. Two approaches to derivatization are possible: trifluoroacetylation and silylation. Trifluoroacetylation (a procedure described by Jonckheere et al. [7]) causes and additional ECD response. This, however, is not required for sensitivity reasons because the ambroxol molecule has two bromine atoms itself and is counterproductive from the selectivity point of view, as coextracts become sensitive to ECD.

Silylation of the alcohol function is sufficient for good chromatographic performance. But it was realized that analyte I in plasma extracts readily underwent ring-closure (Fig. 2) with traces of aldehyde from the solvents used during extraction or from the coextracts during injection into the GC system. Even stabilization with hydroxylamine or other aldehyde-capturing compounds could not prevent this ring-formation. We therefore forced this ring-closure by addition of formaldehyde to plasma or urine samples. The amounts of aldehyde needed for the two biological fluids are different, however (cf. *Extraction procedures*). Of all the silylation reagents tried, we found that hexamethyldisilazane was superior owing to its selectivity towards alcohol functions and because it forms only volatile decomposition products that are not sensitive to ECD. However, a small amount (ca. 10%) of O,N-bis-silylation by-products with retention times of 14.3 and 18.3 min (Fig. 4) are formed. It is noteworthy that the quinazolines V and VI are stable under the silylation procedure.

Chromatographic system

For ultra-trace analysis in the lower parts per 10^9 range, every means to gain sensitivity must be used. Capillary GC reduces peak width to a few seconds. This means that for nearly all types of GC detector the limit of detection with capillary columns is lower than with packed columns. The theoretical background has been described by Yang and Cram [11] and Franzen [12]. The gain in sensitivity is about four-fold using N-FID and mass spectrometry (MS), but about 80-fold with ECD [11].

A second consideration leads to the use of capillary columns. In plasma samples

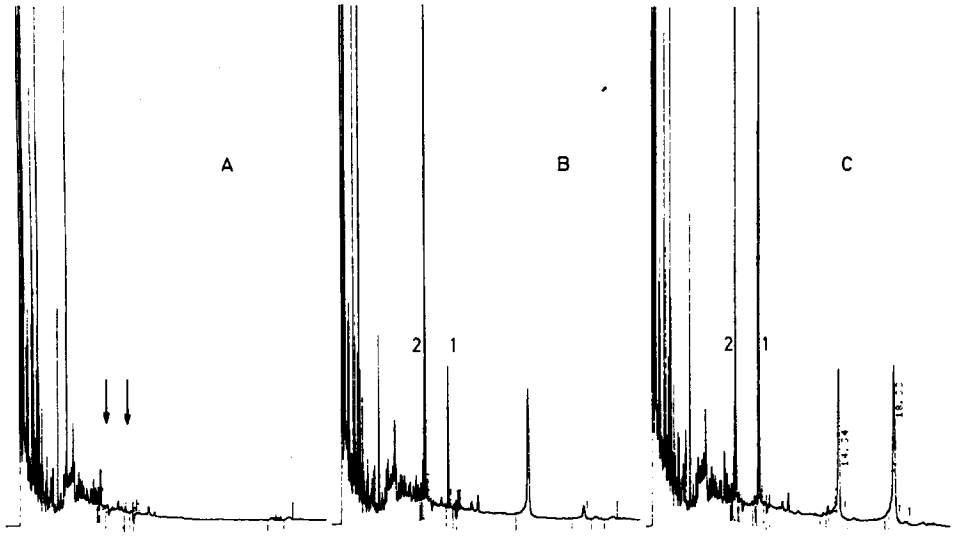

Fig. 4. Chromatograms of extracts from human plasma (ex vivo) showing blanks (A) and ambroxol levels of 10.6 ng/ml (B) and 104 ng/ml (C). Peaks: 1 = ambroxol (I) derivative; 2 = internal standard (II) derivative.

there is always interfering material of various origins: endogenous compounds, nutritional components and smoking (flavours, caffeine, nicotine and metabolites thereof, etc.) and drug metabolites. The lower the blood levels of the drug, the greater the probability that there are interfering endogenous compounds. A chromatographic system with extreme separation power, however, reduces the possibility of interference simply on probability considerations. Furthermore, capillary columns favour the application of internal standards that are structurally very similar to the drug. These factors have been summarized recently by VandenHeuvel et al. [13].

The sample injection process is a critical step in quantitative high-resolution GC analysis. As "only" a sensitivity of ca. 5 ng/ml is required the split-mode can be used. This type of injection gives excellent peak shapes and can be easily used with an autosampler. As an aliquot of only ca. 2 μl of plasma reaches the column (2 μl of a total of 30 μl and a split ratio of 1:30), no clean-up of the extracts is necessary. Contamination of the column and the detector with lipids is avoided. This consideration is valid for the excess of derivatization reagent, too. However, the insert of the injector has to be cleaned after 100 injections. The column was cleaned after ca. 1000 injections by breaking off 50 cm of the beginning of the column and flushing the remainder with lipophilic solvents by means of an HPLC pump.

Detection

The detector used for drug monitoring must be sensitive, selective, robust and compatible with capillary columns. These requirements are fulfilled perfectly by the electron-capture detector. It is sensitive, with a limit of detection for pure

TABLE II

REPEATABILITY OF AMBROXOL DETERMINATION ON ONE DAY

For between-day results, see Fig. 5.

Ambroxol added (ng/ml)	Ambroxol found (ng/ml)	n	C.V. (%)
20	19.7	4	4.7
50	49.6	4	3.7
100	101.5	5	1.0

substances of less than 2 pg, and very selective. To maintain this selectivity, derivatization with reagents sensitive to ECD such as trifluoroacetic anhydride must be avoided. In the split-mode the detector is robust. Recent developments in detector design have dramatically improved the compatibility with capillary columns [14].

Assay parameters

The analytical method described corresponds to the IFCC recommendations [15] concerning sensitivity, selectivity, accuracy and precision. Fig. 4 shows the chromatograms of extracts from plasma compared with the plasma blanks, which are very low ($\ll$1 ng/ml). The limit of quantification of the assay in plasma is 3 ng/ml and the limit of detection is determined by the volume injected. A procedure with back-extraction and splitless injection would increase the limit of detection to ca. 100 pg/ml.

The calibration curve of ambroxol is linear in the range 10–100 ng/ml. It can be described by the equation

$$y = 9.90 \cdot 10^{-3} x + 1.32 \cdot 10^{-2}$$

where y = [ambroxol]/[I.S.] and x = [ambroxol]. The coefficient of correlation r is 0.9993.

The calibration curve for urine was linear in the range from 250 ng/ml to 2 μg/ml when 100 μl of urine were used. It can be described by the equation:

$$y = 1.023 \cdot 10^{-3} x$$

where y = [ambroxol]/[I.S.] and x = [ambroxol]. The coefficient of correlation r is 0.9990.

The repeatability was studied by analysing plasma samples on one day. These results are shown in Table II.

The recovery of ambroxol was carefully established by using liquid scintillation counting. The recovery in the organic extract of ^{14}C-labelled ambroxol from human plasma was 89.3 $\pm$ 3.50% (n=6) at a concentration of 10 ng/ml and 90.3 $\pm$ 1.67% at the 100 ng/ml level. A total recovery of 93.1 and 93.9% was found for 10 and 100 ng of ambroxol, respectively, when we also measured the aqueous phase. This satisfactory result we attribute to the use of silanized glass tubes.

The long-term stability of the assay was tested in two ways: (1) spiked plasma samples were analysed for a period of 60 days (Fig. 5); (2) samples from a pharmacokinetic study were analysed twice and the determinations were statistically

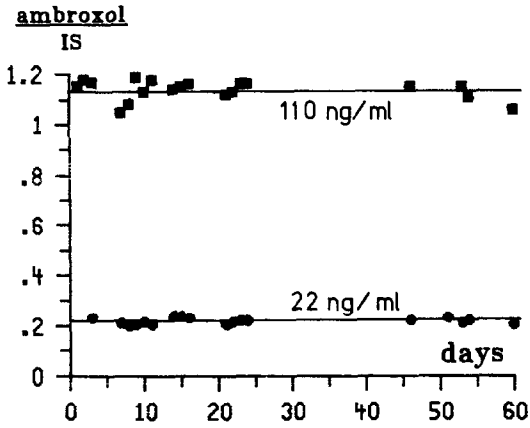

Fig. 5. Stability and reproducibility of samples of 110 ng/ml (top) and 22 ng/ml (bottom) over 60 days.

analysed according to the method of Haeflinger and Wahl [10]. The results are shown in Table III. These two procedures revealed that the assay is stable for a long period, that the coefficient of variation (C.V.) between duplicate samples is ca. 3% and is nearly independent in the range between 10 and 100 ng/ml of the plasma level and that day-to-day variations do not contribute to the variation of the procedure.

TABLE III

PERFORMANCE DATA OF DUPLICATES UNDER FIELD CONDITIONS

Calculated according to Haeflinger and Wahl [10].

Concentration range (ng/ml)	Mean C.V. (%)	Frequency of duplicate (C.V. $\leqslant$ 5%)
0- 4.99	10.5	3/5
5- 9.99	8.64	2/5
10- 19.99	3.63	12/17
20- 49.99	2.34	73/84
50-100.0	2.64	36/42
(>100)	3.75	1/2

TABLE IV

RETENTION TIMES OF DERIVATIZED AMBROXOL, INTERNAL STANDARD AND THE TWO KNOWN METABOLITES

Compound	Retention time (s)
Ambroxol derivative (V)	517
Internal standard (II)	419
Dibromanthranilic acid (IV)	No stable derivative
cis-Ambroxol (III)	432

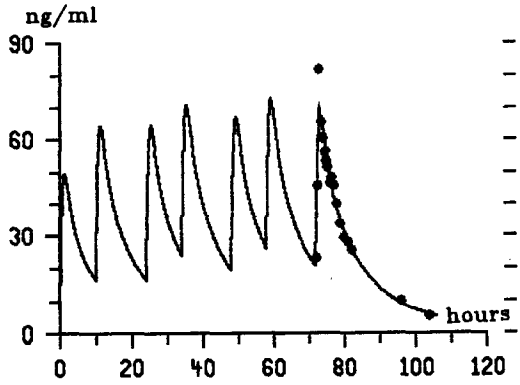

Fig. 6. Plasma levels of ambroxol in human subjects ($n=3$) after a seven-fold oral administration of 30 mg of ambroxol. The points are experimental values and the dashed line is a computer-fitted curve.

The selectivity of the method with respect to known metabolites is summarized in Table IV and with respect to blanks in Fig. 4.

Stability of biological samples

At −25°C, spiked plasma samples and ex vivo samples are stable for at least two months. At 4°C, ex vivo plasma samples are stable for at least one week. At 20°C, ex vivo plasma samples are stable for only one day, after which ambroxol values increase (presumable owing to labile conjugates). Derivatized extracts are stable in toluene at 4°C for one week, and at 20°C for one to two days.

Kinetics

The GC–ECD method is useful for serial analyses. As the extraction steps are performed in centrifuge tubes and the phase separation is done by freezing out, 40 samples can be analysed per day by one technician. Fig. 6 shows the mean plasma level for three human volunteers after administration of 30 mg of ambroxol orally. The total plasma clearance of ambroxol is ca. 450 ml/min and is mainly represented by phase II metabolic clearance. The terminal elimination half-life is ca. 10 h and represents back-diffusion from tissue compartments.

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