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ASSAY OF BROMHEXINE IN HUMAN PLASMA BY CAPILLARY GAS—LIQUID CHROMATOGRAPHY WITH NITROGEN-SELECTIVE DETECTION AND SELECTED ION MONITORING

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SUMMARY

A specific, sensitive method for the determination of bromhexine in human plasma is described. It comprises a selective extraction procedure and a specific determination with capillary gas—liquid chromatography and nitrogen-selective flame ionization detection. The detection limit of the assay is about 0.5 ng/ml. The specificity of the assay was checked by gas chromatography—mass spectrometry. The method is applied to the pharmacokinetics of bromhexine in humans.

INTRODUCTION

Bromhexine (Bisolvon®, Thomae, Biberach, G.F.R.), N-cyclohexyl-N-methyl-(2-amino-3,5-dibromobenzyl)-amine, is widely used as a mucolytic agent in human and veterinary medicine [1—3].

Pharmacokinetic and metabolic investigations of ^{14}C-labelled bromhexine in man and in animals have shown that bromhexine undergoes extensive and rapid metabolism, yielding mainly dealkylated and hydroxylated metabolites and conjugates [4—7].

Successful therapy in man with bromhexine can be achieved using very low doses (8—15 mg). This low dosage, the rapid biotransformation, and the large distribution volume resulting from the lipophilic character of the drug [8] are the reasons for the very low plasma levels (nanogram range). Until now, only a few methods have been published concerning the quantitative determination of bromhexine in biological material. Eichler and Kreuzer [9] have used gas—liquid chromatography (GLC) with electron-capture (EC) detection for analysing bromhexine residues in animal tissues and in milk. Plasma levels have

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been determined by Matsumara et al. [10] using GLC with nitrogen-selective flame ionization detection (N-FID). De Leenheer and Vandecasteele-Thienpont [11] have reported on the GLC behaviour of bromhexine and derivatization methods, as well as on the determination of plasma levels in man [12] by EC-GLC.

The published methods are not satisfactory for several reasons. Although multiple extraction steps are performed, no [9] or no appropriate [10] internal standard has been used, and neither accuracy nor precision have been evaluated. On the other hand, in methods with good precision [12], no studies have been performed on the specificity. Based on our experience, the use of packed columns is not sufficient for the separation of the parent compound from its many similar basic metabolites; the stationary phases used were SE-30 [9, 12], OV-101 [10], mixed phase SE-30 and DEGS [9]. This was found also by Eichler and Kreuzer [9], and they therefore used a thin-layer chromatography clean-up step. In addition, the sensitivity of all the published methods is too low for detailed pharmacokinetic studies, with the exception of a recent gas chromatographic—mass spectrometric (GC—MS) assay using derivatisation and packed columns [13]. We have therefore developed a method for the determination of bromhexine in human plasma with appropriate precision, specificity and sensitivity.

EXPERIMENTAL

Reagents and chemicals

Bromhexine [Bisolvon®, N-cyclohexyl-N-methyl-(2-amino-3,5-dibromobenzyl)-amine (Fig. 1, I), the internal standard [N-cyclopentyl-N-methyl-(2-amino-3,5-dibromobenzyl)-amine] (Fig. 1, II), and the metabolites, named according to Schraven et al. [4], were of analytical grade. The most important metabolites are: M II = N-cyclohexyl-N-(2-amino-3,5-dibromobenzyl)-amine; M V = N-(*trans*-4-hydroxy-cyclohexyl)-N-methyl-(2-amino-3,5-dibromobenzyl)-amine; M VIII = N-(*trans*-4-hydroxy-cyclohexyl)-N-(2-amino-3,5-dibromobenzyl)-amine; and M XI = 6,8-dibromo-3-(*trans*-4-hydroxy-cyclohexyl)-1,2,3,4-tetrahydro-quinazoline.

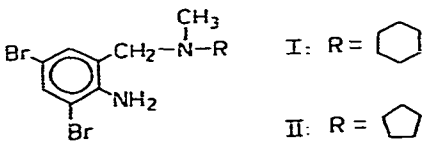

Fig. 1. Structural formulae of bromhexine (I) and the internal standard (II).

^{14}C-Labelled bromhexine (specific activity = 23.4 μCi/mg = 8.65 · 10^5 dps per mg) was synthesized in the isotope laboratory of Dr. Karl Thomae GmbH. It was labelled in the benzylic position.

n-Hexane (Nanograde quality, No. 4159) was from Mallinckrodt (Wesel, G.F.R.). All other reagents were purchased from E. Merck (Darmstadt, G.F.R.): p.a. quality, sulfuric acid (No. 731), sodium hydroxide (No. 6498) and 1 *M* KH_2PO_4 solution (No. 4881) were used. The buffer solutions for the partition studies were made by mixing 1 *M* KH_2PO_4 with 1 *M* H_3PO_4 (No. 552)

(pH range 1—4) and 1 *M* KH_2PO_4 with 1 *M* Na_2HPO_4 (No. 6589) (pH range 4.5—7). The perchlorate solutions were made by mixing 1 *M* $NaClO_4$ (No. 6564) and 1 *M* $HClO_4$ (No. 519).

The internal standard (Fig. 1, II) was used in a concentration of 10 ng/ml in hexane.

Apparatus

The gas chromatograph used was a Hewlett-Packard 5840 equipped with a nitrogen—phosphorus-sensitive-detector, Model No. 18848A, and an autosampler No. 7672.

The column was a 25 m × 0.3 mm I.D. WCOT capillary column with OV-17 as the stationary phase (Perkin-Elmer, Überlingen, G.F.R.).

Operating conditions were injector temperature 240°C, detector temperature 260°C. After injection in the splitless mode, the split was opened 1 min later and the temperature program was started. The column temperature was programmed from 60°C to 140°C at 10°C/min and then to 230°C at 5°C/min. Gas flow-rates were helium (carrier) 2 ml/min, hydrogen 3 ml/min, air 50 ml/min, helium (auxiliary gas) 35 ml/min.

The gas chromatograph—mass spectrometer was a Finnigan 3300 with 6015 data system. The gas chromatograph was equipped with the same column as the GC—N-FID system. The operating conditions in this case were injector temperature 260°C. After injection in the splitless mode, the split was opened 1 min later and the temperature program was started. The column was programmed from 60°C to 230°C at 20°C/min. The gas flow-rate was about 2 ml/min. The column was directly connected to the transfer line.

The mass spectra of bromhexine and the internal standard are depicted in Fig. 2. The following ions were monitored: m/z = 293 [M^+ — cyclohexyl (C_6H_{11}) = 83 mass units] and m/z = 264 [M^+ — N-methyl-cyclohexyl ($C_7H_{14}N$) = 112 mass units).

The helium was always purified by an Oxisorb-Kit purchased from Messer-Griesheim (Frankfurt, G.F.R.) to improve the service life of the column. The evaporation of the organic extracts was carried out with a Vortex-Evaporator (Searle-Buchler, Fort Lee, NJ, U.S.A.).

Analytical procedures

Extraction. The blood was sampled using heparinized syringes (Monovette®, Sarstedt, Nümbrecht, G.F.R.), centrifuged and the plasma kept at −30°C in glass tubes. It was thawed at room temperature and 1-ml portions were pipetted into 25-ml centrifuge tubes. For plasma levels lower than 5 ng/ml, 2 ml of plasma were used, and for levels above 40 ng/ml 0.5-ml samples were used. Then 2 ml of buffer and 10 ml of internal standard solution were added to the plasma. The pH attained was about 5.2. After extraction and phase separation the aqueous layer was discarded and the organic layer was extracted with 1.0 ml of 2 *N* sulfuric acid. After phase separation the aqueous phase was washed once with 5 ml of hexane. Then the aqueous phase was adjusted to pH 12.5 with 0.5 ml of 5 *N* sodium hydroxide. The aqueous phase was extracted twice with 5 ml of hexane.

The hexane was removed in silanized tubes by the evaporator. The residue

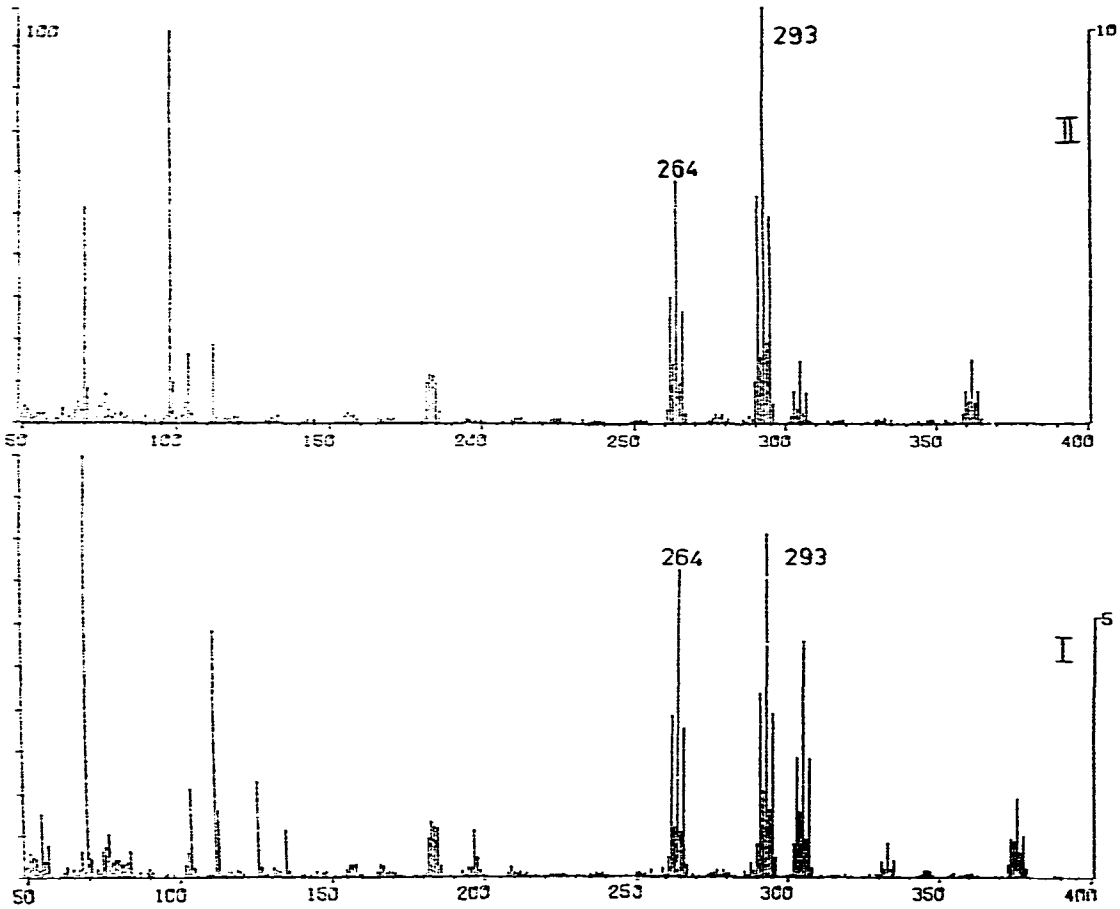

Fig. 2. Electron-impact (70 eV) mass spectra of bromhexine (I) and internal standard (II).

was then dissolved in 25 μl of hexane and transferred into autosampler vials; 2 μl were injected into the chromatograph. For GC—MS, the volume was reduced by concentration to about 5 μl and 2 μl were injected.

All extraction steps were performed with a shaking machine (120 strokes per min) for 15 min. Phase separation was always performed by freezing out the aqueous phase at −20°C and decanting the organic layer.

Preparation of the calibration curve. The calibration curve was constructed by preparing an aqueous stock solution containing 400 ng/ml bromhexine hydrochloride. From this aqueous solution 2.5 ml were added to 47.5 ml of human plasma. After incubation (2 h, 37°C), this plasma (20 ng/ml) was diluted with non-spiked plasma to obtain a concentration range of 1—20 ng/ml. An equilibrium of bromhexine binding with plasma protein was thus established, as the equilibration time for protein binding is in the range of seconds [14]. The standard samples were analysed by the same procedure as described above.

Partition studies. For the partition studies, about 10 μg of the compounds were dissolved in the organic solvents (5 ml) and the extraction with aqueous phase (5 ml) was performed by shaking for 15 min with a shaking machine.

After centrifugation, aliquots of both phases were measured. The concentrations were determined photometrically or by liquid scintillation counting if ^{14}C-labelled compounds were used.

Recovery studies. For the recovery studies, 100 ng and 10 ng of ^{14}C-labelled bromhexine were used and extracted as described under Extraction, but without the addition of internal standard.

Human studies

Five healthy volunteers participated in the study: R.R., 23 years, female, 58 kg; H.D.S., 30 years, male, 72 kg; J.B., 25 years, male, 68 kg; J.E., 26 years, male, 75 kg; R.K., 24 years, female, 60 kg.

During the test no other drugs were taken. The formulation used was Lot No. 6203. An amount of 15 mg (7.5 ml) was administered intravenously by a continuous 45-sec infusion. Two hours later, the volunteers received a standard breakfast (tea and toast with ham). Four hours later a normal dinner was served.

RESULTS AND DISCUSSION

Extraction procedure

The experiments presented in Table I show that hexane is a very favourable extraction medium. The result at pH 5.2 is particularly interesting. We attribute this behaviour to the highly lipophilic character of the compound. A similar effect was observed for methaqualone [15].

TABLE I

PARTITION RATIOS (C_{org}/C_{aq}) OF BROMHEXINE BETWEEN DIFFERENT SOLVENTS AND DIFFERENT pH VALUES OF THE AQUEOUS PHASE

Aqueous phase	Organic solvent		
	Hexane	Diethyl ether	Toluene
NaOH, 0.1 *N*	99	>100	100
Phosphate buffer, pH 7.2	>100	>100	100
Phosphate buffer, pH 5.2	23	95	83
H_2SO_4, 0.2 *N*	<0.01	<0.01	0.04

In Fig. 3 the pH dependence of the partition behaviour between hexane and phosphate buffer, hexane and perchlorate solution, and chloroform and perchlorate is plotted. Bromhexine is a weak base, $pK_{a1} = 8.5$ (benzylamino group) $pK_{a2} = -0.3$ (anilino group), and a very lipophilic compound. The calculated log P for octanol—water, according to Hansch and Leo [16] is 6.2. From the results of Fig. 3, the true partition coefficient, P_t, was calculated from measurements of the apparent partition coefficient, P_a, the pK_a and the pH according to $\log P_t = \log P_a + pK_{a1} - pH$. The $\log P_t$ calculated in this way is about 6.4. The relatively low values of P_a in Fig. 3 at a pH above 6 is due to the great influence of small amounts of ^{14}C-labelled degradation products having a lower partition coefficient than bromhexine. These results mean that only the system

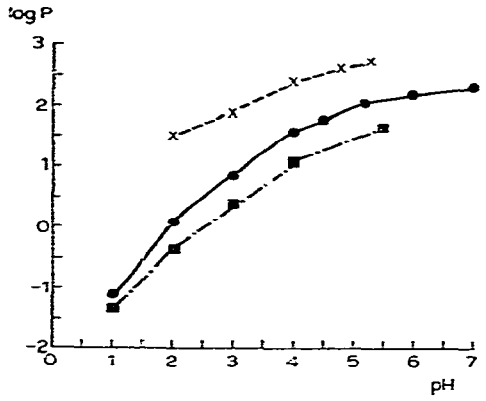

Fig. 3. Log P values of bromhexine between chloroform and perchlorate (×), hexane and phosphate (•), and hexane and perchlorate (▪).

TABLE II

PARTITION RATIOS (C_{org}/C_{aq}) OF BROMHEXINE METABOLITES BETWEEN THE ORGANIC PHASES AND PHOSPHATE BUFFER AT pH 5.2

Substance	Hexane	Toluene
Bromhexine	23	83
M II	23	100
M V	0.43	16.6
M VIII	0.26	0.61

chloroform—perchlorate reveals ion-pair mechanisms.

The use of silanized glass is strongly recommended (see under Assay parameters — recovery) to minimize losses caused by adsorption. This is of special importance since we used hexane without adding alcohol. Silanization was done according to the vapor phase procedure of Fenimore et al. [17].

Chromatographic properties of bromhexine and metabolites

The chromatographic properties of bromhexine and its derivatives have been described by Eichler and Kreuzer [9] and De Leenheer and Vandecasteele-Thienpont [11]. They demonstrated that bromhexine can be satisfactorily separated without derivatisation. These observations are confirmed by our chromatograms. We conclude that the chromatographic behaviour of free bromhexine is due to the fact that both amino groups are shielded sterically and that the anilino group has a weak basic character (pK_a of anilino-hydrochloride is −0.3). As the N-FID sensitivity is not decisively increased by derivatisation steps, we used underivatized bromhexine.

The retention times of bromhexine, the internal standard and the metabolites are summarized in Table III. The figures demonstrate that a sufficient separation between bromhexine (I) and M II is achieved only on the OV-17 phase.

TABLE III

RETENTION TIMES OF BROMHEXINE (I), INTERNAL STANDARD (II) AND METABOLITES ON OV-101 AND OV-17

The length of the column was 25 m in each case.

Compound	Retention time (min)		
	OV-101 (70—220°C, 20°C/min)	OV-17 (isothermal, 230°C)	OV-17 (actual temperature programme)
I	9.95	2.55	20.63
II	9.28	2.21	19.12
M II	9.93	2.89	21.15
M V	12.83	6.79*	—
M VIII	13.08	6.96*	—
M XI	15.35	7.71*	—

*M V, M VIII, M XI had no appropriate behaviour on this column and only showed peaks at very high concentrations.

Chromatographic system

For ultra trace analysis in the lower parts per 10^9 range, every means to gain sensitivity must be used. Capillary GLC reduces peak width to a few seconds. This means that for nearly all types of GLC detectors the detection limit with capillary columns is lower than with packed columns. The theoretical background has been published by Yang and Cram [18] and Franzen [19]. The gain in sensitivity is about four times using an N-FID and MS, but about 80 times in the case of an EC detector [18].

A second consideration leads to the use of capillary columns. In plasma samples 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 probability of interference on just probability considerations. Furthermore, capillary columns favour the application of internal standards which are structurally very similar to the drug. These facts have been recently summarised [20].

The sample injection process is a critical step in quantitative high-resolution GC analysis. For trace analysis [21] the split mode cannot be used. The direct sampling and the falling-needle device are not yet usable for automatic sampling. Only splitless injection can be performed with an autosampler and this therefore appears to be the method of choice for day and night operation.

Detection

The detector used for drug monitoring must be sensitive, selective, robust and compatible with capillary columns. The nitrogen detector used meets all these requirements. Its high sensitivity (25 pg of pure substance) reduces the plasma volumes needed for analysis. The detection limit of the GC—MS systems was about 100 pg; the detection limit of the EC detector was not established.

The robust performance of a detector reduces the servicing time. The FID and N-FID are particularly insensitive to depositions since all substances eluting from the column are combusted by an oxygen-rich flame. This is the major advantage of this kind of detector as compared to a mass spectrometer or EC detector. Additionally, the small dead volume of the N-FID does not spoil the resolving power of capillary columns [18].

Assay parameters

The analytical method described corresponds to the IFCC recommendations [22] concerning sensitivity, specificity, accuracy and precision.

Fig. 4 shows the chromatograms of extracts from plasma compared with the plasma blanks, which are very low (< 0.2 ng/ml at the detection limit of the system) and nearly constant for all volunteers. The detection limit of the assay is about 0.5 ng/ml and for the pure substance about 25 pg. The detection limit is determined by the volume of blood available (2 ml) and the fact that only one-tenth (2 μl from 20 μl) of the extract can be injected. The back-extraction with sulfuric acid in necessary for two reasons. Firstly, we gain selectivity because interfering non-basic compounds are not extracted. Without back-extraction we have a detection limit of about 2 ng/ml. Secondly, this procedure is necessary to improve the life-time of the column, because otherwise it would

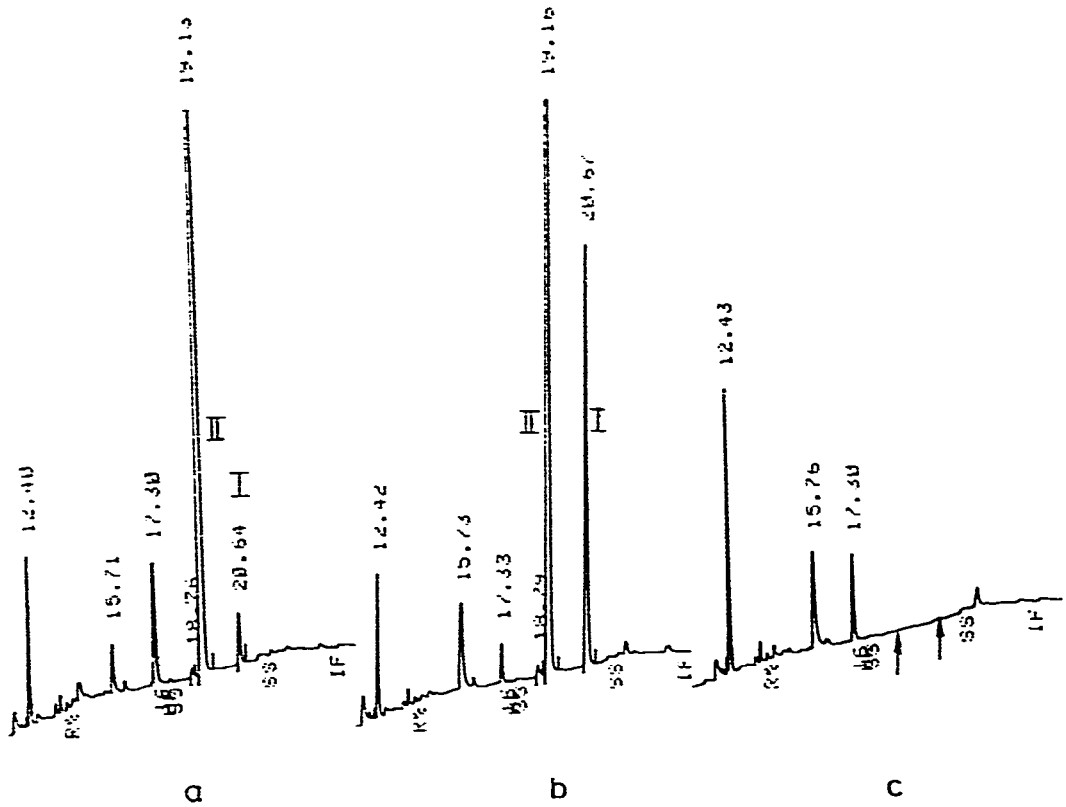

Fig. 4. Chromatograms of human plasma showing bromhexine at a level of (a) 1.5 ng/ml, (b) 18.7 ng/ml, (c) blank. I = bromhexine, II = internal standard.

become polluted with about 200 μg of triglycerides and 100 μg of cholesterol after each injection. Up to now the life-time of the column is about 900 injections with the back-extraction procedure.

The calibration curve is linear in the range 1—20 ng/ml bromhexine. It can be described by the equation $y = 1.84 \cdot 10^{-2} x - 5.5 \cdot 10^{-3}$. The coefficient of correlation is $r = 0.9978$.

The repeatability was studied by preparing plasma samples containing different amounts of bromhexine and analysing them on different days. The results are shown in Table IV.

TABLE IV

REPRODUCIBILITY OF BROMHEXINE DETERMINATION ON ONE DAY (A) AND BETWEEN DAYS (B)

	Bromhexine added (ng/ml)	Bromhexine found (ng/ml)	No. of determinations	S.D. (%)
A	2.0	2.03	4	4.2
	7.5	7.68	6	4.6
	15.0	15.16	5	3.5
B	10.0	9.66	7	5.7

Using liquid scintillation counting, the recovery of the ^{14}C-labelled bromhexine was 83.4 ± 5.9% (n = 4) at a concentration of 10 ng/ml and 85.1 ± 0.9% (n = 4) at 100 ng/ml. This experiment was performed without internal standard to check losses due to adsorption. A recovery of 96.5 ± 1.0% was found for 100 ng and 101.7 ± 5.0% for 10 ng of bromhexine when we measured all partition phases. This satisfactory result is due to the use of silanized glass tubes.

The specificity of the method with respect to known metabolites is summarized in Table III, with respect to the blanks shown in Fig. 4. We further analysed bromhexine by GC—MS. In Fig. 5 the multiple ion detection traces of a plasma extract containing 2.9 ng/ml bromhexine show the sensitivity of the GC—MS assay.

Fig. 6 shows the regression line of the bromhexine levels from a pharmacokinetic study as determined by GC—N-FID and simultaneously by GC—MS. The regression line was calculated by the sum of least squares. The regression coefficient is 0.996 with a slope of 0.99 (both calculated from non-logarithmic data) and is therefore compatible with the assumption that there are no systematic deviations due to unknown metabolites.

The GC—N-FID method is useful for serial analyses. As the extraction steps are performed in centrifuge tubes and the phase separation is done by freezing out, 30 samples can be analysed per day by one technician. The advantages of phase separation by centrifuging and freezing out [23] have been emphasized recently [24]. Using an autosampler, the time needed for one run (35 min) is of minor importance since day and night operation is possible.

Fig. 7 shows the mean plasma level of five human volunteers after administration of 15 mg of bromhexine by a continuous 45-sec infusion. The values measured are fitted by a three-compartment model with two side-compart-

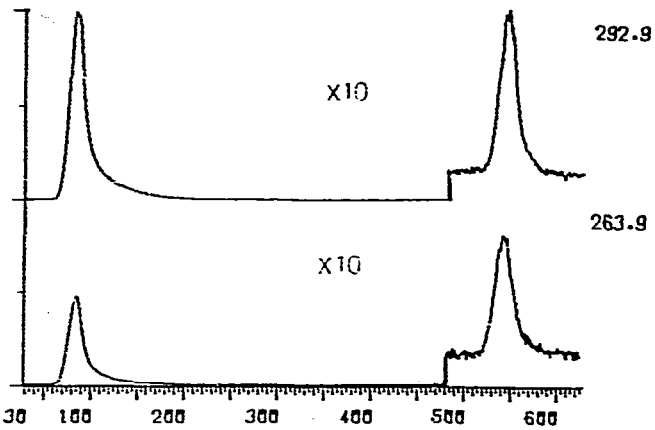

Fig. 5. Multiple ion detection traces of a plasma extract containing 2.9 ng/ml bromhexine, the actual retention times being 14.05 min and 16.32 min.

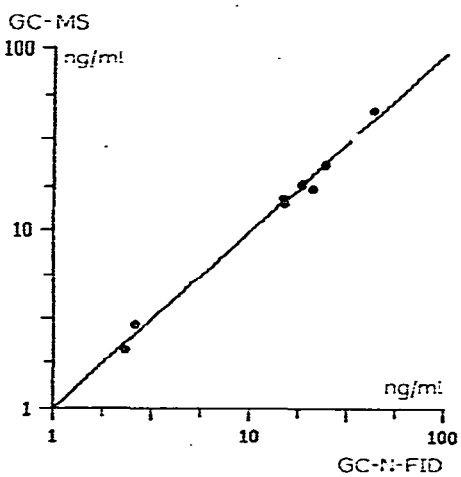

Fig. 6. Regression line of the bromhexine levels from a pharmacokinetic study determined by GC—N-FID and GC—MS.

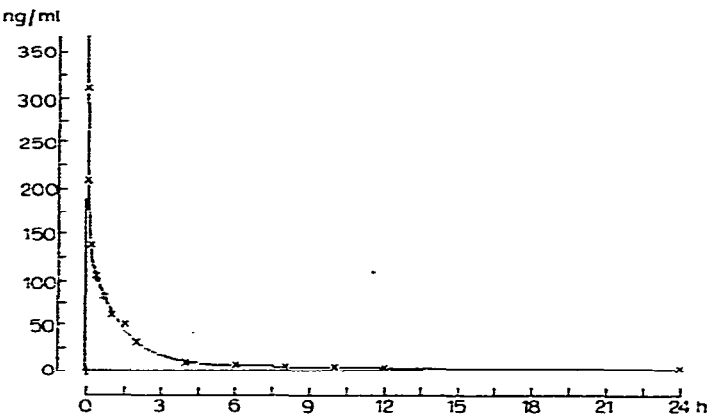

Fig. 7. Plasma levels of bromhexine in human subjects (n = 5) following intravenous administration of 15 mg by an infusion lasting 45 sec. (X), Experimental values; (—), computer-fitted curve.

ments using our Thomae-Topfit program [25]. In accordance with older studies [6] in which only total radioactivity was measured, we find that bromhexine is rapidly metabolised. The total clearance [26] is about 1.1 l/min. The terminal elimination half-life is about 15 h and is determined by the back-diffusion of bromhexine from the tissues.

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REFERENCES

1 R. Engelhorn and S. Püschmann, Arzneim.-Forsch., 13 (1963) 474.
2 E.M. Boyd and P. Sheppard, Arch. Int. Pharmacodyn. Ther., 163 (1966) 284.
3 H. Geide, Tieraerztl. Umsch., 9 (1967) 478.
4 E. Schraven, F.W. Koss, J. Keck and G. Beisenherz, Eur. J. Pharmacol., 1 (1967) 445.
5 Z. Kopitar, R. Jauch, R. Hankwitz and H. Pelzer, Eur. J. Pharmacol., 21 (1973) 6.
6 R. Jauch and R. Hankwitz, Arzneim.-Forsch., 25 (1975) 1954.
7 H.T. Nilson, C.G.A. Persson and T. Tegner, Acta Pharm. Suecica, 13 (1976) 241.
8 J. Schmid, to be published.
9 D. Eichler and H. Kreuzer, Arzneim.-Forsch., 25 (1975) 615.
10 R. Matsumara, T. Yoshida, S. Kyui, S. Kobajashi, M. Toyoda, A. Nagakura and H. Kohei, Iyakukin Kenkyu, 10 (1979) 279.
11 A.P. De Leenheer and L.M.R. Vandecasteele-Thienpont, J. Chromatogr., 175 (1979) 301.
12 A.P. De Leenheer and L.M.R. Vandecasteele-Thienpont, J. Pharm. Sci., 69 (1980) 99.
13 J.A.A. Yonckheere, L.M.R. Thienpont, A.P. De Leenheer, P. De Backer, M. Debackere and F.M. Belpaire, Biomed. Mass Spectrom. 7 (1980) 582.
14 W. Scholtan, Arzneim.-Forsch., 28 (1978) 1037.
15 D. Chin and E. Fastlich, Clin. Chem., 20 (1974) 1382.
16 C. Hansch and A. Leo, Substituent Constants for Correlation Analysis in Chemistry and Biology, Wiley, New York, 1979, p. 18.
17 D.C. Fenimore, C.M. Davies, J.H. Whitford and C.A. Harrington, J. Anal. Chem., 48 (1976) 2289.
18 F.J. Yang and S.P. Cram, in R.E. Kaiser (Editor), 3rd Int. Symp. on Capillary Chromatography, Hindelang, April 29—May 3, 1979, Institute of Chromatography, Bad Dürkheim, 1979, pp. 509—523.
19 J. Franzen, in R.E. Kaiser (Editor), 3rd Int. Symp. on Capillary Chromatography, Hindelang, April 29—May 3, 1979, Institute of Chromatography, Bad Dürkheim, 1979, p. 131.
20 W.J.A. VandenHeuvel and J.S. Zweig, J. High Resolut. Chromatogr. Chromatogr. Commun., 3 (1980) 381.
21 F.J. Yang and S.P. Cram, presented at the 13th Int. Symp. on Chromatography, Cannes, June 30—July 4, 1980.
22 J. Büttner, J. Clin. Chem. Clin. Biochem., 4 (1976) 265.
23 H.W. Diekmann, Arzneim.-Forsch., 26 (1976) 727.
24 J. Schmid, J. Chromatogr., 222 (1981) 129.
25 G. Bozler, G. Heinzel, F.W. Koss, R. Hammer and M. Wolf, Arzneim.-Forsch., 27 (1977) 895.
26 U. Gibaldi, R.N. Boyes and S. Feldmann, J. Pharm. Sci., 60 (1971) 1338.