Electron-Capture GLC Determination of Bromhexine in Human Plasma

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Received March 26, 1979, from the *Laboratoria voor Medische Biochemie en voor Klinische Analyse, Faculteit van de Farmaceutische Wetenschappen, Rijksuniversiteit, Academisch Ziekenhuis, 135 De Pintelaan, 9000-Gent, Belgium.* Accepted for publication July 12, 1979.

Abstract □ A specific and sensitive GLC analysis of bromhexine in human plasma is described. After addition of the internal standard and an aqueous triethanolamine solution, bromhexine is extracted at alkaline pH into *n*-hexane, transferred to an acidic aqueous solution, and back-extracted into *n*-hexane after alkalinization. Both compounds are derivatized with trifluoroacetic anhydride and quantified by GLC using a ^{63}Ni-electron-capture detector. The method has a sensitivity of ~1.0 ng/ml of plasma. Linearity of plasma working curves was good. The extraction recovery from spiked plasma was 90.1 ± 5.68% (*SD*). The within-run and within-day precisions (*CV*) were 6.0% (4.3 ng/ml, $n = 8$) and 8.6% (11.0 ng/ml, $n = 13$), respectively. The procedure was applied successfully to measurement of the plasma concentration–time profile in a human volunteer after oral drug administration.

Keyphrases □ Bromhexine—electron-capture GLC analysis, human plasma □ GLC—bromhexine, electron-capture detection, human plasma □ Mucolytic agents—bromhexine, electron-capture GLC analysis, human plasma

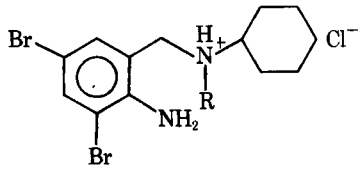
Bromhexine hydrochloride[1] [*N*-cyclohexyl-*N*-methyl-2-(2-amino-3,5-dibromo)benzylammonium chloride] (I) is used as a mucolytic agent (1, 2). The low drug dosage (3) sufficient for pharmacological effectiveness and its extensive metabolism (4, 5) result in low levels in circulating blood (nanograms per milliliter) (6, 7). Thus, to follow the rapidly declining levels of the unchanged drug, highly sensitive and specific methodology is required.

Studies on the absorption, metabolism, and excretion of ^{14}C-labeled I were performed by liquid scintillation spectrometry after extraction of the plasma and urine samples and TLC separation of the drug and metabolites (6–8). Eichler and Kreuger (9) described an electron-capture GLC assay for the quantitation of I residues in the milk and tissues of treated animals. A previous paper[2] described a new GLC assay for the determination of standard I solutions at the nanogram level. The chromatographic properties and electron-capture sensitivity of the trifluoroacetyl derivatives of I and a synthesized, structurally analogous internal standard, *N*-cyclohexyl-*N*-*n*- propyl-2-(2-amino-3,5-dibromo)benzylammonium chloride (II) were investigated.

The present report describes a GLC assay with electron-capture detection for the determination of plasma bromhexine in the low nanogram range.

EXPERIMENTAL

Reagents and Materials—The hydrochloride of I[3] was used as received, and II was synthesized as described (10). Pesticide grade *n*-hexane[4] and methanol[5], fluorometric grade ethyl acetate[6], and trifluoroacetic anhydride[7], obtained in 1-ml sealed glass ampuls, were used as supplied.



I: R = CH_3
II: R = $CH_2CH_2CH_3$

Aqueous 5 *N* NaOH[6], 2 *N* HCl[6], and 1 *N* triethanolamine[6] solutions were prepared with freshly double-distilled water.

Apparatus—GLC was conducted on a gas chromatograph[8] equipped with a 15-mCi ^{63}Ni-electron-capture detector[9] (pulsed, variable-frequency type). The stationary liquid phase, 4% SE-30[10], was coated on 100–120-mesh Gas Chrom Q[11] and packed into a 2.2-m × 2-mm i.d. spiral silanized glass column. The temperatures were: oven, 255°; injection port, 280°; and detector, 330°. Argon–methane (95:5) was the carrier and purge gas at a total flow rate of 28 ml/min.

Extraction—Plasma, 1.0 ml, was placed in a 20-ml polytef screw-capped test tube. The following were added by pipet: 80 μl of internal standard solution (1.2 ng of II/μl in methanol), methanol to a total volume of 200 μl/ml of plasma, 2 ml of 1.0 *N* aqueous triethanolamine, and 0.1 ml of 5.0 *N* NaOH. The solution was mixed well. *n*-Hexane, 15 ml, was added, and the tube contents were mixed on a rotary mixer[12] for 20 min. After centrifugation, the *n*-hexane was transferred to a tube containing 2 ml of 2.0 *N* HCl. The tube was shaken for 20 min, and the *n*-hexane was discarded after separation of the two layers. The extraction was repeated with 10 ml of *n*-hexane.

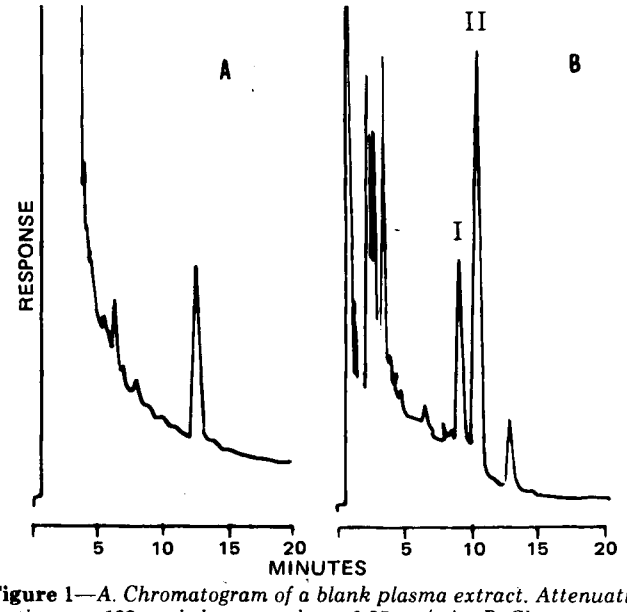


Figure 1—*A. Chromatogram of a blank plasma extract. Attenuation setting was 128, and chart speed was 0.25 cm/min. B. Chromatogram of a plasma extract 90 min after oral administration of 4 mg of bromhexine hydrochloride, representing a level of 3.9 ng of I/ml. Attenuation setting was 256.*

[1] Bisolvon.
[2] To be published.
[3] Supplied by Boehringer, Ingelheim, West Germany.
[4] Carlo Erba, Milan, Italy.
[5] Riedel-de Haën A.G., Seelze-Hannover, West Germany.
[6] E. Merck A.G., Darmstadt, West Germany.
[7] Pierce Eurochemie B.V., Rockford, Ill.
[8] Model 5830 A, Hewlett-Packard, Avondale, Pa.
[9] Model 18803 B, Hewlett-Packard, Avondale, Pa.
[10] R. S. L., St. Martens-Latem, Belgium.
[11] Supelco Inc., Bellefonte, Pa.
[12] Cenco Instrumenten B.V., Breda, The Netherlands.

0022-3549/80/0100-0099$01.00/0

Table I—Recovery of I in Human Plasma by Electron-Capture GLC Analysis (n = 3)

I Added to 1.0 ml of Plasma, mg	Mean I Amount Recovered after Extraction, ng	Mean Recovery ± *SD*, %
6.6	6.5	98.5 ± 3.02
13.3	11.6	87.5 ± 8.90
26.6	23.4	88.0 ± 1.84
53.1	45.9	86.3 ± 4.75
Mean		90.1 ± 5.68

The acid solution was made basic with 1.5 ml of 5 *N* NaOH and was extracted twice with 5 ml of *n*-hexane during 20 min. After centrifugation, the organic layer was evaporated in a 3-ml glass-stoppered conical vial in a heating block[13] at 45° under a nitrogen stream. The residue was redissolved in ~100 μl of ethyl acetate. A slight excess of trifluoroacetic anhydride was added and mixed. After 5 min, excess trifluoroacetic anhydride was removed at 45° under nitrogen. The residue was dissolved in 100 μl of methanol, and ~2 μl was injected with a 10-μl syringe[14] onto the GLC column.

Linearity—Linearity was tested from 1.6 to 12.7 ng/ml and from 7.0 to 115.8 ng/ml. Solutions of I in methanol were added to plasma to give 1.6, 3.2, 6.4, and 12.7 ng/ml and 7.0, 11.6, 23.2, 46.3, 69.5, 92.7, and 115.8 ng/ml. An adjusted constant amount of II was added. The samples were extracted as described and analyzed by GLC. Peak height ratios of I to II were plotted *versus* the concentration ratio of I to II.

Recovery—Known I amounts were added to 1.0 ml of drug-free plasma to give drug concentrations as detailed in Table I. The internal standard was added after the extraction just before derivatization. Percentage recoveries were calculated from a standard curve of the same amounts of I and II derivatized without extraction.

RESULTS AND DISCUSSION

Figures 1A and 1B show representative chromatograms of drug-free plasma and an extract after a single 4-mg oral dose, respectively. Back-extraction from an organic solvent into an acidic aqueous solution was essential for sample cleanup prior to electron-capture GLC. Based on its retention on the 4% SE-30 stationary phase, the extraneous peak at a retention time of 12.6 min was supposed to represent dioctyl phthalate, used as a plasticizer. The component also was present after extraction

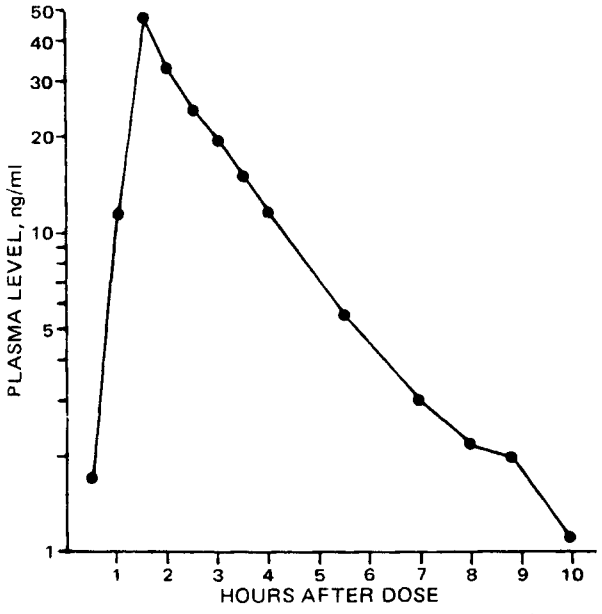


Figure 2—*Mean plasma bromhexine levels in a human volunteer following oral administration.*

[13] Dri-Bath, Thermolyne, Dubuque, Iowa.
[14] Hamilton 701, Bonaduz, Switzerland.

Table II—Assay Precision

Within-Day Precision, ng/ml		Day-to-Day Precision	
		Day	ng/ml
	4.3	1	10.2
	4.3	2	11.6
	4.4	3	10.3
	4.4	4	11.5
	4.1	5	11.9
	4.1	6	10.1
	4.2	7	11.2
	4.9	8	10.7
		9	11.0
Mean	4.3	10	10.1
SD	0.26	11	13.0
CV, %	6.0	12	12.0
		13	9.8
		Mean	11.0
		SD	0.95
		CV, %	8.6

of an aqueous solution. Therefore, it was not an endogenous compound. This peak can be eliminated by solvent distillation and storage in glass equipment.

Compound II was synthesized and chosen as the internal standard for GLC experiments[2] on the basis of its structural similarity to I. However, the extraction characteristics from plasma of I and II were different. In contrast, the extraction recovery from water was the same for I and II. Plasma extracts alkalinized only with sodium hydroxide without the triethanolamine solution afforded a relative recovery for II of only 32.1 ± 2.70% (*SD*); after the addition of the triethanolamine solution, the recovery increased to 81.3 ± 6.51% (*SD*). A plot of the response ratios of I to II *versus* the plasma concentration ratio of I to II in the 1.6–12.7- and 7.0–115.8-ng/ml ranges demonstrated a linear relationship (regression lines: $y = 2.5x + 0.04$, $r = 0.9997$, and $y = 2.3x + 0.01$, $r = 0.9996$, respectively).

A detection limit of ~1.0 ng of I/ml was obtained using 1.0 ml of plasma for the analysis.

The within-run precision of the overall procedure (*CV*) of 6.0% was obtained on analyzing eight samples of pooled plasma having a mean value of 4.3 ± 0.26 (*SD*) ng/ml. The day-to-day precision (*CV*) over 8 weeks was 8.6% at a mean concentration of 11.0 ± 0.95 (*SD*) ng/ml. For this study, plasma samples were stored at −20°. These data are presented in Table II. The I recovery studies in the 6.6–53.1-ng/ml range showed a mean of 90.1 ± 5.68% (*SD*) (Table I).

The utility of the technique developed for the determination of I was demonstrated in the establishment of plasma levels in a volunteer after oral administration of 24 mg of I as tablets. Two standard curves were constructed by adding quantities of I to plasma samples in the range of the expected levels, as mentioned under the linearity experiments. A typical time course for the I concentration is shown in Fig. 2. For an exact estimate of the pharmacokinetics of the drug after oral administration in humans, the levels below 3 ng/ml are of limited value since they are too close to the detection limit.

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ACKNOWLEDGMENTS

Supported by the I.W.O.N.L. through a bursary to one author.