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DETERMINATION OF BROMHEXINE AS ITS TRIFLUOROACETYL DERIVATIVE BY GAS–LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A specific gas–liquid chromatographic method with electron-capture detection is described for the determination of bromhexine at the nanogram level. Structurally analogous internal standards were synthesized and their suitability was investigited, based on their chromatographic properties on different stationary liquid phases. The electron-capture activity of the compounds is increased by trifluoroacetylation. Reaction conditions for this derivatization were studied. Calibration graphs in the range 0–57 ng (in a total of 100 μl of reaction mixture) showed good linearity.

INTRODUCTION

Bromhexine [N-cyclohexyl-N-methyl-(2-amino-3,5-dibromobenzyl)ammonium chloride; Bisolvon] is used as a mucolytic drug, and its pharmacological action has been reported in detail[1–3]. Pharmacokinetic studies with ^{14}C-labelled bromhexine, performed by liquid scintillation counting, showed that the compound and its metabolites are present in blood at low concentrations, typically in the nanogram per millilitre range[4,5].

A gas–liquid chromatographic (GLC) analysis with flame-ionization detection (FID) for bromhexine hydrochloride in pharmaceutical preparations has been reported[6]. This method is inadequate for the determination of the drug in biological fluids because of its lack of sensitivity. To our knowledge, only one paper has described a GLC procedure with electron-capture detection (ECD) for bromhexine residues in milk and tissues of treated animals[7].

This paper reports a new, sensitive and specific GLC system with electron-capture detection for the determination of bromhexine in standard solutions. This study is important with respect to applications to human plasma.

EXPERIMENTAL

Apparatus

GLC–FID analyses were carried out using a Hewlett-Packard (HP) Model

5750B gas chromatograph (Hewlett-Packard, Avondale, Pa., U.S.A.) with dual flame-ionization detectors. The carrier gas was nitrogen at a linear velocity of 5–6 cm/sec. The hydrogen and air flow-rates were adjusted to give optimal sensitivity and stability.

GLC–ECD analyses were carried out on an HP Model 5830A gas chromatograph equipped with a 15-mCi ^{63}Ni ECD (pulsed, variable-frequency type) at 330°. Argon–methane (95:5) was used as carrier and purge gas at a total flow-rate of 28 ml/min.

Both gas chromatographs were fitted with spiral silanized glass columns (180 or 220 cm × 2 mm I.D.), packed with different liquid stationary phases coated on 100–120 mesh Gas-Chrom Q (Supelco, Bellefonte, Pa., U.S.A.) by the filter fluidizing method[8]. All results were obtained under isothermal conditions. The injection and FID temperatures were kept a few degrees higher than the oven temperature.

UV spectra were recorded with a Pye Unicam SP1800 double-beam recording spectrophotometer (Pye Unicam, Cambridge, Great Britain).

IR spectra were recorded as potassium bromide pellets on a Pye Unicam SP1100 spectrophotometer.

A Varian HR-300 (300 MHz) instrument (Varian, Palo Alto, Calif., U.S.A.) was used to run NMR spectra with dimethyl sulphoxide as solvent and 1% tetramethylsilane as internal standard.

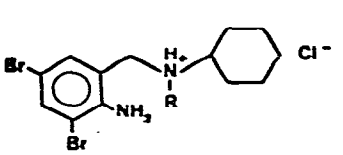
Low-resolution mass spectrometry (MS) and GLC–MS, with helium as carrier gas, were effected on an LKB 9000S gas chromatograph–mass spectrometer (LKB, Bromma, Sweden). The gas chromatograph was equipped with a 180 cm × 2 mm I.D. glass column packed with 1% OV-1 coated on 80–100 mesh Gas-Chrom Q. The temperatures of the oven, flash heater, separator and ion source were 205°, 265°, 270° and 270°, respectively. The ionization energy was 70 eV, the trap current 60 μA and the accelerating voltage 3.5 kV.

Solvents and reagents

Methanol of nanograde purity was purchased from Riedel-de Haën (Seelze-Hannover, G.F.R.). Fluorimetric-grade ethyl acetate and tetrahydrofuran were obtained from Merck (Darmstadt, G.F.R.). Trifluoroacetic anhydride (TFAA), heptafluorobutyric anhydride (HFBA), pentafluorobenzoyl chloride (PFB-Cl), all supplied in 1-ml sealed glass ampoules, were obtained from Pierce (Rockford, Ill., U.S.A.).

Standards

Bromhexine hydrochloride (I) was kindly provided by Boehringer (Ingelheim, G.F.R.). Two structurally analogous compounds, N-cyclohexyl-N-ethyl-(2-amino-3,5-dibromobenzyl)ammonium chloride (II) and N-cyclohexyl-N-*n*-propyl-(2-amino-3,5-dibromobenzyl)ammonium chloride (III), potential internal standards, were synthesized according to methods described in the literature[9,10].



R = CH_3	I
CH_2CH_3	II
$CH_2CH_2CH_3$	III

The principal spectral characteristics of II and III are as follows. UV: II, λ_{max}^{MeOH} = 250 and 319 nm (ε = 11003 and 3423 cm²/mmole) and λ_{min}^{MeOH} = 282 nm; III, λ_{max}^{MeOH} = 249 and 318 nm (ε = 11444 and 3328 cm²/mmole) and λ_{min}^{MeOH} = 282 nm;

IR: II and III, 3030, 3000 (aromatic CH), 1640, 1450 (C=C), 850 (aromatic H), 2940, 2860 (aliphatic CH), 3460, 3320 (primary NH), 2700–2250 (*tert.*-amine salt) cm^{-1};

NMR, δ (ppm): II, 7.74 (1,d,J = 2.25 cps, aromatic H), 7.67 (1,d,J = 2.25 cps, aromatic H), 6.37 (2,br,NH_2), 4.38 (1,d,J = 13.50 cps, aryl-CH_2-N^+-), 4.29 (1,d,J = 13.50 cps, aryl-CH_2-N^+-), 1.22 (3,t,J = 7.00 cps, CH_3), 3.18 (2,m,-N^+-CH_2-), 2.95 (1,m,H_{ax_1}), 2.24 (1,d,J = 10.00 cps, H_{eq}), 2.12 (1,d,J = 10.00 cps, H_{eq}), 1.82 (2,d,J = 12.50 cps, H_{eq}), 1.58 (3,m,H_{eq4},H_{ax}), 1.25 (3,m,H_{ax}); III, 7.74 (1,d,J = 2.25 cps, aromatic H), 7.67 (1,d,J = 2.25 cps, aromatic H), 5.57 (2,br,NH_2), 4.38 (1,d,J = 15.00 cps, aryl-CH_2-N^+-), 4.31 (1,d,J = 15.00 cps, aryl-CH_2-N^+-), 0.78 (3,t,J = 7.00 cps, CH_3), 2.82 (2,m,-N^+-CH_2-CH_2-), 3.05 (2,m,-N^+-CH_2-), 3.22 (1,t,J = 12.00 cps, H_{ax_1}), 2.22 (1,m,H_{eq}), 2.04 (1,d,J = 12.50 cps, H_{eq}), 1.82 (2,m,H_{eq}), 1.58 (3,m,H_{eq4},H_{ax}), 1.22 (3,m,H_{ax}).

MS, m/z (relative intensity): II, 390 (10.9) $C_{15}Br_2N_2H_{22}^+$, 361 (3.2) $(M-C_2H_5)^+$, 347 (5.0) $(M-C_3H_7)^+$, 319 (33.7) $(M-C_5H_{11})^+$, 307 (30.4) $(M-C_6H_{11})^+$; 264 (38.5) $(M-C_8H_{16}N)^+$, 140 (15.2) $(M-C_6H_4Br_2N)^+$, 126 (55.0) $(M-C_7H_6Br_2N)^+$, 112 (9.3) $(M-C_8H_8Br_2N)^+$, 98 (8.2) $(M-C_9H_{10}Br_2N)^+$, 84 (100.0) $(M-C_{10}H_{12}Br_2N)^+$, 56 (22.7) $(M-C_{12}H_{16}Br_2N)^+$; III, 404 (4.9) $C_{16}Br_2N_2H_{24}^+$, 375 (7.3), 361 (2.4), 333 (10.0), 321 (9.7), 154 (5.2), 140 (17.8), 112 (38.8), 98 (55.4) $(M-C_{10}H_{12}Br_2N)^+$, 72 (7.0) $(M-C_{12}H_{14}Br_2N)^+$, 56 (17.9) $(M-C_{13}H_{18}Br_2N)^+$, 30 (100.0) $(M-C_{15}H_{20}Br_2N)^+$.

The *n*-alkane (n-C_{25}) standard was purchased from Poly-Science Corp. (Evanston, Ill., U.S.A.).

Derivatization and linearization

An amount of 17.8 mg of bromhexine hydrochloride was dissolved in 25.0 ml of methanol and serial dilutions were made to give final concentrations of 0.0356, 0.0712, 0.1424, 0.2848, 0.4272 and 0.5696 μg/ml. Of each solution a 100-μl aliquot and 70 μl of III (1.016 μg/ml) were carefully evaporated to dryness under nitrogen in a glass-stoppered test-tube. The residue was dissolved in about 100 μl of ethyl acetate and a slight excess of TFAA was added with mixing. After reaction, excess of TFAA was removed at 45° with the aid of a gentle stream of nitrogen. The residue was finally dissolved in 100 μl of methanol. An 1-μl aliquot was injected with a 10-μl syringe on top of the GLC column.

RESULTS AND DISCUSSION

Although the ECD responses for bromhexine and the proposed internal standards II and III were good, we investigated the electron affinity after derivatiza-

tion of the aromatic amine with TFAA, HFBA and PFB-Cl. The last reagent did not yield derivatives suitable for quantitative GLC determinations. The incorporation of a higher number of fluorine atoms by heptafluorobutyrylation in comparison with trifluoroacetylation did not result in a considerable increase in electron-capture activity. On the other hand, the response ratio for TFA-derivatized to underivatized bromhexine was about 2, with, for the former compound, a minimum detectable amount of about 4 pg. This bromhexine TFA derivative was identified by combined GLC–MS: *m/z* (relative intensity) 472 (25.0) M^+, 403 (4.1) $(M-CF_3)^+$.

Reaction conditions, *i.e.*, the influence of the reaction time, reaction temperature and amount of derivatizing reagent, were investigated by GLC–FID. For this purpose, an *n*-alkane (*n*-C_{25}) in tetrahydrofuran was used as internal standard. Measurement of peak-height ratios (bromhexine-TFA to *n*-C_{25}) indicated that derivatization was complete in 1 min at room temperature when a slight excess of TFAA was used. Moreover, the TFA derivatives were found to be stable in the dry state but relatively unstable in methanol. To obtain reproducible calibration graphs we therefore chromatographed the TFA derivatives within 0.5 h after dissolution in the solvent methanol.

Bromhexine and the two synthesized internal standards were chromatographed on following liquid stationary phases: OV-1, SE-30 (methyl silicone polymer), Dexsil-300 (carborane–methyl silicone polymer), OV-17 (methyl phenyl silicone polymer), OV-25 (methyl phenyl silicone polymer), QF-1 (fluoroalkyl silicone polymer), XE-60 (nitrile silicone gum), NPGS (neopentyl glycol succinate), STAP (reaction product of Carbowax 20M and succinic acid), FFAP (reaction product of Carbowax 20M and *m*-nitroterephthalic acid) and DEGA (diethylene glycol adipate). Retention data are summarized in Table I.

TABLE I

RETENTION TIMES OF BROMHEXINE AND II AND THEIR TFA DERIVATIVES ON DIFFERENT COLUMN SYSTEMS

Column system	*Column length (cm)*	*Oven temperature (°C)*	*Retention time (min)*			
			Bromhexine	*II*	*Bromhexine-TFA*	*II-TFA*
5% OV-1	180	230	14.6	15.9		
3.8% SE-30	180	220	8.9	9.7	7.6	8.0
3% Dexsil-300	180	255	11.2	12.0	9.2	9.2
3% OV-17	180	210	18.1	19.0		
		250	7.0	7.3		
1% OV-25	180	220	10.1	10.4	7.4	7.4
3% QF-1	180	200	10.6	11.3	10.3	10.3
5% XE-60	180	210	6.9	7.4	11.0	11.4
1% NPGS	180	215	9.9	9.9		
1% STAP	180	220	9.4	9.4		
1% FFAP	180	210	3.7	3.7		
2% DEGA	180	200	16.2	16.2	19.9	18.7

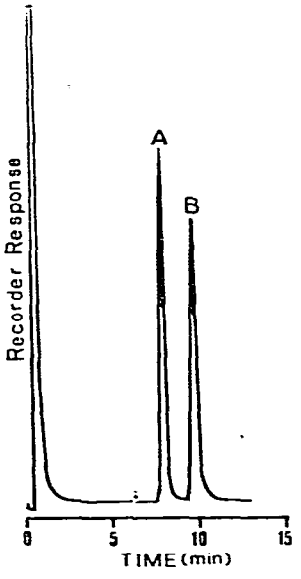
No acceptable resolution between bromhexine and II, both underivatized or even less as the TFA derivative, was obtained on either column system. Thus, for quantitative GLC we decided to use III as the internal standard. Separations between

TABLE II

RELATIVE RETENTION TIMES OF III AND ITS TFA DERIVATIVE ON DIFFERENT COLUMN SYSTEMS

Column system	*Column length (cm)*	*Oven temperature (°C)*	*Relative retention time*	
			III (bromhexine = 1.00)	*III-TFA (bromhexine-TFA = 1.00)*
4% SE-30	220	250	1.23	1.16
3% OV-17	180	225	1.19	1.07
1% FFAP	180	200	1.18	1.00

bromhexine and III, underivatized or as the TFA derivatives, were determined on apolar, mediumpolar and polar stationary liquid phase, as shown in Table II. The apolar 4% SE-30 was selected as the most suitable stationary phase. A typical example of a gas chromatogram of bromhexine and III before and after derivatization on this column system is shown in Figs. 1 and 2.



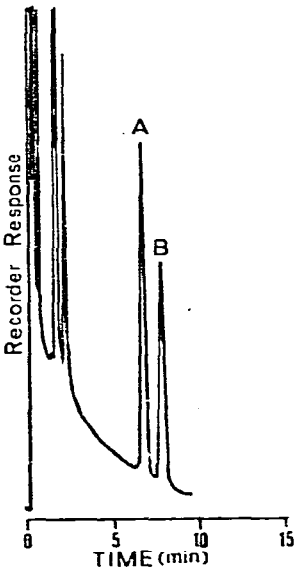


Fig. 1. Gas chromatogram of about 531 pg of underivatized bromhexine (A) and 621 pg of III (B) on a 4% SE-30 column. Attenuation setting 512.

Fig. 2. Gas chromatogram of about 531 pg of bromhexine-TFA (A) and 621 pg of III-TFA (B) on a 4% SE-30 column. Attenuation setting 1024.

Stock solutions of bromhexine hydrochloride and III were prepared and derivatized as described earlier. The calibration graph was obtained by plotting peak-height ratios against the bromhexine concentration. For the range 0–57 ng per 100 μl,

a linear relationship with a zero intercept was obtained as shown in Fig. 3. This offers a new possibility for the development of a sufficiently sensitive assay for pharmacokinetic studies.

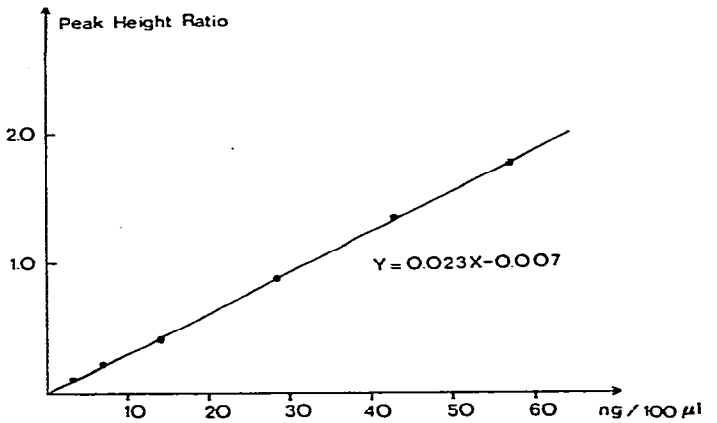


Fig. 3. Typical calibration graph of the TFA derivatives of bromhexine and III. Peak-height ratio of bromhexine-TFA to III-TFA plotted against bromhexine concentration (ng per 100 µl).

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