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DETERMINATION OF BROMHEXINE IN PLASMA BY REVERSED-PHASE LIQUID CHROMATOGRAPHY

INTERFERENCE OF LIPOPROTEINS ON EXTRACTION

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SUMMARY

Extraction of the hydrophobic tertiary amine bromhexine from plasma using cyclohexane–heptafluorobutanol (99.5:0.5, v/v) was studied at different pH values. The extraction yield from buffer solutions was quantitative at pH > 4.1, but from plasma the extraction yield decreased with increasing pH. Furthermore, at pH 8.4 the extraction yield varied greatly (56–99%) in different human plasma. The addition of lipoproteins to phosphate buffer, at pH 8.1, decreased the extraction yields considerably. Quantitative extraction from plasma was obtained by using a very long extraction time at pH 8.4 or by decreasing the pH to 5.4. The chromatographic system consisted of a reversed-phase column (Nucleosil C_{18}, 5 μm) with an acidic mobile phase (methanol–phosphate buffer, pH 2) containing an aliphatic tertiary amine. UV detection at 308 or 254 nm was used. The limit of quantitation was 5 ng/ml using 3.00 ml of plasma and detection at 254 nm. The intra-assay precision for bromhexine was better than 3.6% at 5 ng/ml.

INTRODUCTION

Bromhexine hydrochloride (Fig. 1) is used as a mucolytic drug. The low drug dosage and the extensive metabolism [1] result in low concentrations of bromhexine in plasma. Bromhexine levels have previously been determined by gas chromatography with electron-capture detection [2], nitrogen-selective detec-

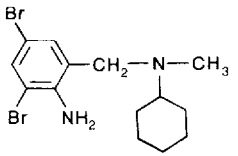

Fig. 1. Chemical structure of bromhexine.

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tion [3] or selected-ion monitoring [3,4]. Liquid chromatography (LC) with UV detection has also been used [5].

Existing data on the extraction yields of bromhexine from plasma showed large variations [2,5]. Extraction using cyclohexane–diethylamine from plasma at a pH of ca. 9 gave a recovery of only 60% compared with a buffer solution [5]. Extraction using hexane from plasma containing 20% methanol, sodium hydroxide and triethanolamine gave 90% recovery of bromhexine and its propyl homologue. However, without the addition of triethanolamine the recovery of the propyl homologue was only 32% [2]. The extractions into hexane, diethyl ether and toluene, respectively, were quantitative at pH values of 7.2 and 13, but not at pH 5.2 – unfortunately the data were only from pure buffer solutions [3].

The present study considers the extraction of bromhexine from plasma in the pH range 5–13 using cyclohexane with the addition of the strong hydrogen donor heptafluorobutanol. At pH 5.4 the extraction was quantitative from plasma. Addition of lipoproteins to phosphate buffer decreased the extraction yield to ca. 40% at pH 8.4 but not at pH 5.4.

EXPERIMENTAL

Chemicals

Bromhexine hydrochloride was purchased from Laboratori Mag (Milan, Italy). Dimethylhexylamine was obtained from Fluka (Buchs, Switzerland) and 1H,1H-heptafluorobutanol from Aldrich-Chemie (Steinheim, F.R.G.) The lipoproteins very-low-density (VLDL), low-density (LDL) and high-density (HDL) were dissolved in sodium chloride (0.15 *M* containing 0.01% EDTA, pH 7.4) and obtained from Sigma (St. Louis, MO, U.S.A.).

Apparatus

The LC system consisted of a Waters M6000 A pump (Waters Assoc., Milford, MA, U.S.A.), a Waters WISP 710B automatic injector and an LDC Spectro-Monitor III (Laboratory Data Control, Riviera Beach, FL, U.S.A.) with a variable-wavelength detector for measuring at 254 or 308 nm. The integrator was a Shimadzu C-R3A (Shimadzu, Kyoto, Japan). The column (150 mm×4.6 mm I.D.) was packed with Nucleosil C_{18}, 5 μm (Macherey-Nagel, Düren, F.R.G.).

Chromatographic system

Bromhexine was eluted on the Nucleosil C_{18} column with a mobile phase consisting of 5 m*M* of dimethylhexylamine in methanol–phosphate buffer of pH 2 (μ=0.1) (59:41, v/v). The flow-rate was 1.0 ml/min and the column was used at room temperature.

Determination of distribution data

The distribution data were determined by extraction of equal volumes of cyclohexane–heptafluorobutanol (99.5:0.5, v/v) and phosphate buffer (pH 2.0–2.6, μ=0.1). The initial concentration of bromhexine in the buffer solution was 28 μg/ml. After extraction for 15 min and centrifugation, 2.00 ml of the organic

phase were evaporated and redissolved in 25.00 ml of mobile phase. Before and after extraction, 2.00 ml of the aqueous phase were diluted to 25.00 ml with mobile phase. Measurement was then done with the LC system described above.

Determination of extraction yield from plasma

A 2-ml volume of the appropriate phosphate buffer (μ=1.0) was added to the plasma sample (3.00 ml). The mixture was extracted at different times using 3.00 ml of cyclohexane–heptafluorobutanol (99.5:0.5, v/v). After centrifugation, 2.00 ml of the organic phase were evaporated to dryness under nitrogen at 50°C. The residue was redissolved in 200 μl of mobile phase, and 100 μl were injected onto the column. Immediately after elution of bromhexine, 500 μl of a mixture of 5 m*M* dimethylhexylamine in methanol–phosphate buffer pH 2 (90:10, v/v) was injected onto the column to decrease the retention of late-eluting endogenous plasma compounds. The extraction yields were compared with those of buffer solutions extracted either in the same way (relative recovery) or by direct injection of bromhexine dissolved in the mobile phase (absolute recovery).

Ultrafiltration

In order to study the protein binding, preliminary experiments were performed with different ultrafiltration systems. Bromhexine (50 ng/ml) was diluted in a phosphate buffer of pH 7.4 (μ=0.1) or pH 9.0 (μ=0.1). The solution was centrifuged for 8 min at 150 *g* in Amicon YMT ultrafiltration membranes for MPS-1 or in Amicon Centriflo ultrafiltration membrane cones (CF 25) (Amicon, Danvers, MA, U.S.A.). Furthermore, a cellulose dialysis tube was used according to ref. 6. The concentration of bromhexine was determined by direct injection onto the column of an acidified mixture of the initial solution, the supernatant and the filtrate, respectively.

Extraction of plasma samples

A 3-ml volume of plasma was mixed with 2.00 ml of citrate buffer of pH 5.0 (μ=1.0). After extraction for 15 min using 3.00 ml of cyclohexane–heptafluorobutanol (99.5:0.5, v/v) the mixture was centrifuged, and 2.00 ml of the organic phase were evaporated. The residue was redissolved in 200 μl of mobile phase, and 100 μl were injected onto the column.

A calibration curve was prepared in the range 5–100 ng/ml by spiking 3.00 ml of plasma.

RESULTS AND DISCUSSION

Extraction

Bromhexine is a weak lipophilic base with pK_{a1} 8.5 and pK_{a2} −0.3 [3]. The octanol–water distribution constant is $10^{6.2}$ [3]. The distribution of bromhexine in three different organic phases [cyclohexane, 5% (v/v) butanol and 0.5% (v/v) heptafluorobutanol in cyclohexane] with phosphate buffers as the aqueous

phase, gave the same value of 2.1 for the product of the distribution constant and the acid dissociation constant ($-\log K_d \times K'_{HA}$). A quantitative extraction should then be obtained at pH > 4.1 using equal phase volumes. Owing to the high distribution constant, back-extraction of bromhexine was impractical. At pH 1, an aqueous phase volume eight times the organic phase volume is necessary to obtain a theoretical recovery of 99.0%. Evaporation of bromhexine in cyclohexane and redissolution in the mobile phase resulted in losses. These were avoided by the addition of 5% (v/v) butanol or 0.5% (v/v) heptafluorobutanol in cyclohexane. These proton-donating agents are known to increase the extraction of amines [7,8] and to decrease the adsorption losses [9,10] of lipophilic amines. Extraction at pH 5.4 using 0.5% heptafluorobutanol in cyclohexane was used in this assay, since it gave cleaner chromatograms than 5% butanol in cyclohexane.

Unexpectedly, the extraction yield of bromhexine decreased as the pH of the plasma phase was raised (Fig. 2), although, as mentioned before, the theoretical extraction yield from the buffer is greater than 99% at a pH above 4.1. The addition of bromhexine to blank plasma from thirteen different humans gave highly varied extraction yields (56–99%; mean, 84%), when the extraction was performed at pH 8.4. Lowering the pH to 5.4 increased the extraction yield to 98–108% (mean 102%) (Table I). A prolongation of the extraction time to 22 h increased the extraction yield to greater than 95% (Fig. 3) at pH 8.4, while the extraction yield was greater than 95% after 15 min at pH 5.4. One possible reason

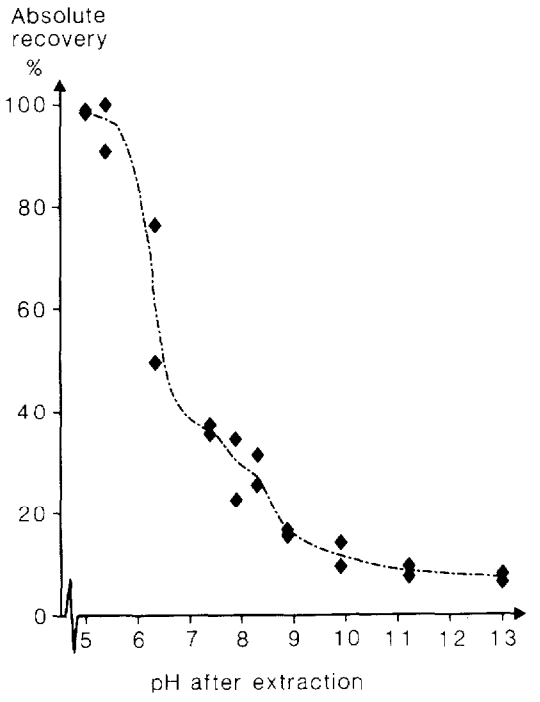

Fig. 2. Extraction yields of bromhexine from pooled plasma adjusted to different pH values with a phosphate buffer (see Experimental). The concentration of bromhexine in plasma was 88 ng/ml. The pH values were determined after the extraction.

TABLE I

EXTRACTION YIELDS OF BROMHEXINE FROM DIFFERENT HUMAN PLASMA, EXTRACTED AT pH 8.4 OR 5.4

Concentration of bromhexine in plasma, 88 ng/ml.

Human	Relative recovery*	
	pH 8.4	pH 5.4
1	99	100
2	91	108
3	90	98
4	76	–
5	86	106
6	87	100
7	85	100
8	57	102
9	95	102
10	56	103
11	89	100
12	81	–
13	95	–
Mean	84	102

*Compared with extraction from a phosphate buffer at the respective pH.

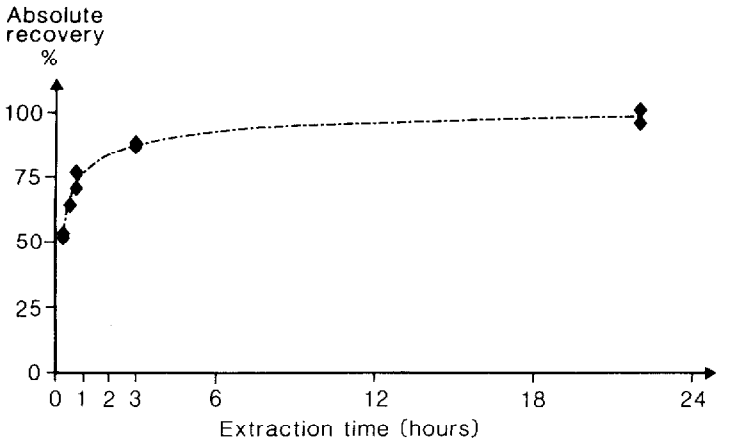

Fig. 3. Extraction yield of bromhexine from plasma, at pH 8.4, after different extraction times. The concentration of bromhexine in plasma was 84 ng/ml.

for low extraction yields of bromhexine from plasma is pH-dependent protein binding. Attempts to determine the extent of protein binding by ultrafiltration experiments proved, however, to be difficult owing to excessive adsorption to the ultrafiltration systems (Amicon and cellulose tubes) at both pH 7.4 and pH 9. However, separation of the proteins by ultrafiltration of blank plasma and addition of bromhexine to the filtrate before extraction gave a yield of over 95% at

pH 9. The addition of the VLDLs, LDLs and HDLs to the phosphate buffer at pH 7.4 (μ=0.2) decreased the extraction yield considerably at pH 8.1±0.2 but gave quantitative extraction at pH 5.0±0.1 (Table II). Neither orosomucoid nor albumin, the main transport proteins in plasma, affected the extraction yield at pH 5.0 or 8.1.

These results indicated that the low extraction yield of bromhexine from plasma may be due to binding to lipoproteins. At a pH below 7.5, bromhexine is charged. This may decrease the binding to lipoproteins and, furthermore, increase the extraction yield by ion-pair extraction of bromhexine with different plasma anions, e.g. chloride. The different lipoprotein classes (VLDLs, LDLs and HDLs) contain various amounts of protein, triglycerides, cholesterol esters, cholesterol and phospholipids [11]. The neutral lipids are sequestered in a central core whose surface is stabilized by phospholipid polar groups and proteins, which are in contact with the aqueous environment [12]. However, structural changes occurred in rabbit HDL apoproteins in the pH range 6.3–10.1 [13] and in human LDL fractions, but not in the VLDL fraction, in the temperature range 20–40°C [14,15]. These observations offer a further explanation for the results obtained. Several other lipophilic drugs are also known to be bound to lipoproteins, e.g. quinidine [16–19], chloropromazine [20], imipramine [20,21], tetracycline [22] diclofenac [23], propranolol [18,19], 5-methoxypsoralen [24], amitriptyline [25] and nortriptyline [25].

The reasons behind the increasing extraction yield to increasing extraction times are not known, but it may due to slow conformational changes of the proteins under the influence of the extraction conditions. Another possibility is that the amine is occluded in the hydrophobic parts of the protein and that the diffusion from this environment is slow.

TABLE II

EXTRACTION YIELDS OF BROMHEXINE AFTER ADDITION OF DIFFERENT PROTEINS TO PHOSPHATE BUFFER pH 7.4 (μ=0.2)

Concentration of bromhexine, 188 ng/ml. VLDL=pre β- lipoprotein; LDL=β-lipoprotein; HDL=α-lipoprotein.

Protein	Concentration (g/l)		Absolute recovery (%) extracted at	
	In human plasma	Added to phosphate buffer, pH 7.4	pH 8.1±0.2	pH 5.0±0.1
Albumin	45*	45	96	95
Orosomucoid	0.8**	0.8	99	100
VLDL	1.2***	0.28	77	100
LDL	4.6***	1.4	44	96
HDL	2.9***	1.9	71	103
VLDL+LDL+HDL+orosomucoid		0.28+1.4+1.9+0.8	36	96
VLDL+LDLD+HDL		0.28+1.4+1.9	38	95

*Geigy Scientific Tables [11], p.141, men 20–39 years.
**Geigy Scientific Tables [11], p.142, men 20–39 years.
***Geigy Scientific Tables [11], pp.116 and 119; danish population 31–40 years.

This has been reported for hydrophobic tertiary amines, such as imipramine, amitriptyline, clomipramine and trimipramine, extracted with methylene chloride. This solvent is, however, known to give heavy protein precipitation. Quantitative extractions of these hydrophobic amines were obtained, in fact, within 15 min using hexane–pentanol (95:5, v/v) [27].

Compounds containing primary amino groups may give Schiff bases with endogenous aldehydes or ketones [27–30]. The extraction yields of tocainide could be increased by the addition of hydroxylamine, which probably acts as a competitor for carbonyl groups in the biological sample [31]. A correspondingly increased extraction yield could not, however, be obtained with bromhexine by the addition of 100 m*M* hydroxylamine to plasma 30 min before extraction.

Chromatography

Hydrophobic amines often give tailing peaks in reversed-phase liquid chromatography. The addition of an aliphatic tertiary amine to an acidic mobile phase has been shown to improve the peak symmetry [32]. Bromhexine gives a peak asymmetry of 2.3 in a mobile phase consisting of methanol–phosphate buffer pH 2 (μ=0.1) (55:45 v/v). The addition of 0.05 m*M* dimethyloctylamine improves the peak symmetry to 1.5. A further increase in dimethyloctylamine to 5 m*M* did not improve the peak symmetry, but decreased the retention slightly. Influence on the retention may be due to competition between bromhexine and the aliphatic tertiary amine for the same sites on the solid phase. Dimethyloctylamine decreased the retention of bromhexine more than dimethylhexylamine, owing to stronger adsorption of the former.

A mobile phase consisting of 5 m*M* dimethylhexylamine in methanol–phosphate buffer pH 2 (μ=0.1) (59:41, v/v) gave a suitable retention time for bromhexine.

Blank plasma samples gave late-eluting endogenous compounds (Fig. 4). The

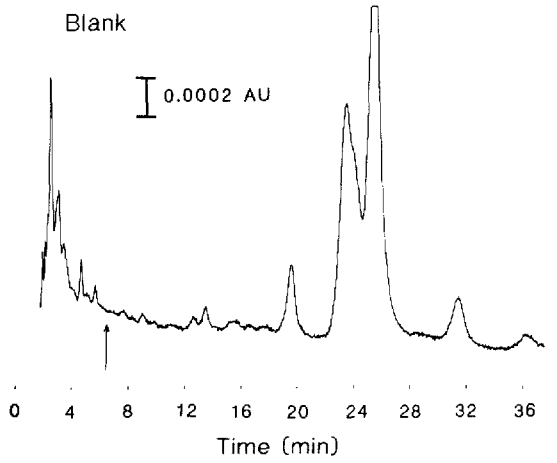

Fig. 4. Chromatogram from blank plasma, with UV detection at 308 nm. The arrow gives the retention time of bromhexine. Chromatographic conditions: column, Nucleosil C_{18} 5 μm; mobile phase, 5 m*M* dimethylhexylamine in methanol–phosphate buffer pH 2 (59:41, v/v).

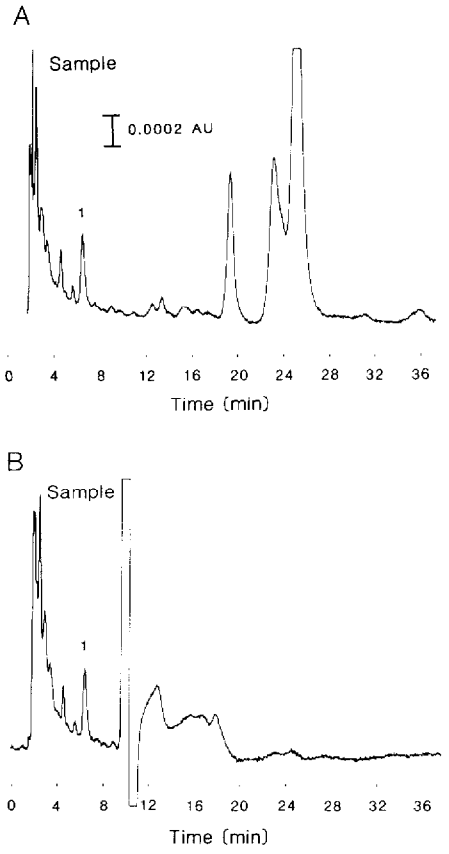

Fig. 5. Comparisons of plasma chromatograms monitored at 308 nm (A) without and (B) with injection of a step gradient of 500 μl of 5 m*M* dimethylhexylamine in 90% (v/v) methanol in phosphate buffer pH 2 (μ=0.1). The injection was performed after 8 min. The plasma contained 34 ng/ml bromhexine. Chromatographic conditions as in Fig. 4.

retention times of these were not affected by changing the concentration of dimethylhexylamine or by using dimethyloctylamine, indicating that they were non-protolytic compounds. However, an increase of the methanol content by injecting 5 m*M* dimethylhexylamine in methanol–phosphate buffer pH 2 (90:10, v/v) drastically decreased the retentions of the endogenous compounds. The injection of the methanol solution after the elution of bromhexine reduced the run time for each plasma sample from 32 to 15 min (Fig. 5).

Selectivity, sensitivity and precision

The eluate was monitored at 254 or 308 nm. The signal-to-noise ratio was ca. 3.6 times higher at the more unselective wavelength of 254 nm than at 308 nm. The detection limit at 254 nm, defined as a signal-to-noise ratio of 3, was 1.4 ng of bromhexine.

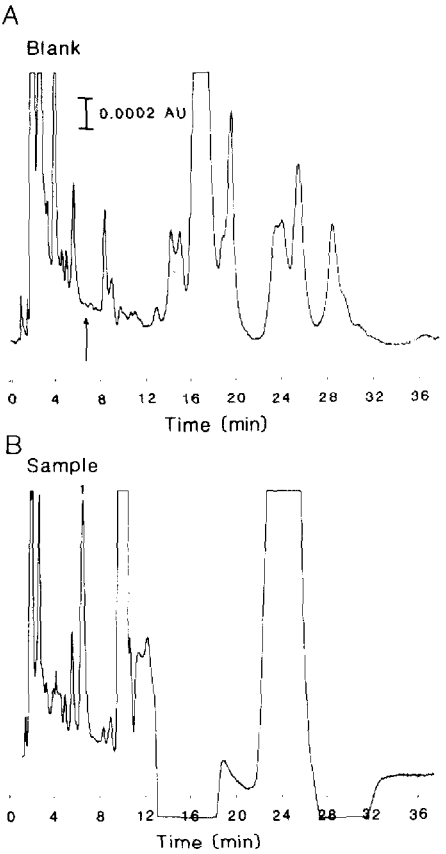

Fig. 6. Comparison of plasma chromatograms monitored at 254 nm (A) without and (B) with injection of a step gradient of 500 μl of 5 m*M* dimethylhexylamine in 90% (v/v) methanol in phosphate buffer pH 2 (μ=0.1). The injection was performed after 8 min. The plasma contained 34 ng/ml bromhexine. Chromatographic conditions as in Fig. 4.

TABLE III

RELATIVE STANDARD DEVIATIONS (R.S.D.) AND ABSOLUTE RECOVERIES OF BROMHEXINE AT TWO PLASMA CONCENTRATIONS

Concentration added (ng/ml)	R.S.D. (%)	*n*	Absolute recovery (%)	Used wavelength (nm)
5.0	3.6	8	99.4	254
55.5	1.9	8	97.5	308

At 254 nm, several late-eluting endogenous compounds were detected although the selectivity for bromhexine was good at both wavelengths. Injection of a step gradient of 5 m*M* dimethylhexylamine in 90% methanol in a phosphate buffer of pH 2 (μ=0.1) was tried, but interference still remained up to about the same retention time as without the step gradient (Fig. 6, cf. Fig. 5). This means that,

when monitoring at the more sensitive wavelength of 254 nm, a run-time of 34 min had to be used, compared with 15 min for 308 nm. The limit of quantification was, however, significantly lower at 254 nm: plasma levels down to 5 ng/ml could be measured. The absolute recoveries and the precisions are shown in Table III. The absolute recoveries are obtained by comparison with direct injection of bromhexine dissolved in the mobile phase.

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