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Note

Determination of bromhexine in human plasma and urine by high-performance liquid chromatography

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Bromhexine hydrochloride [N-cyclohexyl-N-methyl-2-(2-amino-3,5-dibromo)benzylammonium chloride] is an expectorant drug, promoting bronchial secretion and having mucolytic properties.

Until recently, no specific analytical method has been available for the assessment of the unchanged drug in plasma and urine, although bromhexine has been on the market for many years. Low clinical dosages and extensive metabolism resulting in low levels in circulating blood (low nmol/l range) may be an explanation, as this requires development of a highly sensitive method. This is now made available both by a gas—liquid chromatographic (GLC) assay with electron-capture detection of trifluoroacetylated bromhexine [1] and by high-performance liquid chromatographic (HPLC) assay with UV detection, described in the present paper. Both methods cover the determination of plasma bromhexine in the low nmol/l range and the present HPLC method also comprises an assay of bromhexine in urine.

MATERIALS AND METHODS

Apparatus

The chromatographic system consisted of a solvent delivery system Model 6000A, a universal injector Model U6K with a 2-ml injection loop, and a UV-absorbance detector Model 440 equipped with a 254-nm filter, all supplied by Waters Assoc. (Milford, MA, U.S.A.). Chromatograms were recorded on a Perkin-Elmer Model 561 recorder.

Chromatography

The column (30 cm × 3.9 mm I.D.) was pre-packed with μBondapak C_{18}

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(10 μm), manufactured by Waters Assoc. As protection column was used a stainless-steel column (5 cm × 4.6 mm I.D.) packed with Nucleosil C_{18} (10 μm) from Macherey, Nagel & Co.

The mobile phase consisted of acetonitrile—methanol—0.01 *M* phosphate buffer, pH 7 (40:40:20 for plasma and 41:41:18 for urine). The organic solvents were filtered through a 0.5-μm FH-Millipore filter and the phosphate buffer through a 0.5-μm HA-Millipore filter. The mobile phase was degassed ultrasonically.

The flow-rate was 2.5 ml/min and the chromatography was performed at room temperature.

Reagents and glassware

Distilled water was used throughout. Acetonitrile "zur Rückstandanalyse" was from Merck (Darmstadt, G.F.R.). Methanol, cyclohexane, diethylamine and phosphates were all of analytical reagent grade from Merck. Phosphate buffer (pH 7) was prepared by dissolving 2.89 g of KH_2PO_4 and 5.12 g of $Na_2HPO_4 \cdot 2H_2O$ in 1000 ml of water. Phosphate buffer (pH 9.5) was a saturated solution of $Na_2HPO_4 \cdot 2H_2O$ in water.

Test tubes of glass for blood sampling (10 ml) were pre-treated by heparinization. Urine was collected in glass containers. Plasma and urine were stored deep-frozen in glass-stoppered test-tubes until analysed.

Extraction tubes were of 10 ml capacity with glass stoppers.

Standard solutions

Standard stock solution: 1.5 mg of bromhexine hydrochloride dissolved in 100 ml of methanol. Standard working solution: 2 ml of the standard stock solution diluted with 98 ml of water. This solution was used to determine the calibration curves. Internal standard stock solution: 5 mg of amitriptyline hydrochloride dissolved in 100 ml of methanol. Internal standard working solution: 5 ml of the internal standard stock solution diluted with 95 ml of water.

Procedure

To 3 ml of plasma or urine were added 0.5 ml of phosphate buffer (pH 9.5), 100 μl of internal standard working solution and 3 ml of cyclohexane—diethylamine (149:1).

The mixture was rotated at 30 r.p.m. for 60 min on a Rotamix test-tube rotator, and then centrifuged at 850 *g* for 15 min. A 2.5-ml volume of the organic layer was evaporated to dryness at room temperature by a stream of nitrogen.

The tube wall was washed with 300 μl of cyclohexane, which was then evaporated. The residue was dissolved in 50 μl of methanol. This solution remained stable for one week in a refrigerator. A 25-μl volume of the solution was finally injected. The retention times for bromhexine were ca. 9 and 7 min for plasma and urine, respectively. The relative retention time bromhexine/internal standard was ca. 0.7.

Standard solutions of bromhexine in plasma or urine were treated and analysed simultaneously with samples. The sample concentration was calculated on the basis of the peak-height ratio of bromhexine and internal standard, by

reference to the standard curve obtained by linear regression (conversion factor: 1 nmol/l equals 0.41 ng/ml).

Testing of the analytical procedure

The accuracy, precision and linearity of the method were determined using spiked samples of human plasma and urine analysed at random. Total recovery of bromhexine and the internal standard was determined as the response from analysed standards, relative to the response of methanolic solutions, directly injected into the chromatograph.

The stability of samples was tested from spiked human plasma and urine and from samples obtained after bromhexine administration. The samples were stored deep-frozen for two months.

RESULTS

The analytical procedure for bromhexine in plasma and urine has been found to be accurate, precise and linear (Table I). The accuracy was 102% (range 99—107%) for plasma and 102% (range 99—104%) for urine, calculated as percentage found on the basis of the linear standard curve. The precision, expressed as relative standard deviation (S.D.%), was 10% (range 6—13%) and 6% (range 3—8%) for plasma and urine, respectively. At the detection limit of ca. 10 nmol/l in plasma the precision was 23%. The analytical procedure for both plasma and urine was linear, as the deviation of accuracy from 100% was smaller than the precision at all concentration levels tested. The regression line, however, for plasma had an intercept of ca. 5 nmol/l.

Total recovery of bromhexine was ca. 100% for urine and ca. 60% for plasma. The total recovery of the internal standard for both urine and plasma was ca. 100%.

Frozen plasma and urine samples remained stable for at least two months.

TABLE I

ACCURACY AND PRECISION OF THE DETERMINATION OF BROMHEXINE

Spiked human plasma and urine were used for six determinations at each level.

Plasma				Urine			
Added (nmol/l)	Found $\bar{x}$ (nmol/l)	Accuracy (found, %)	Precision (S.D., %)	Added (nmol/l)	Found $\bar{x}$ (nmol/l)	Accuracy (found, %)	Precision (S.D., %)
0	0.0	—	—	0	0.0	—	—
12.2	12.4	102	(23.3)*	24.4	24.2	99	8.3
24.4	26.1	107	12.0	122	122	100	7.2
61.0	61.5	101	12.8	610	622	102	4.3
122.0	120.7	99	5.8	2439	2529	104	3.2
Mean		102	10.2	Mean		102	5.8

*Not included in mean.

DISCUSSION

Extraction of bromhexine from plasma has been a main problem in the development of the analytical procedure. According to Vessman [2], extraction of amine compounds from plasma may be difficult, a phenomenon which also was observed by De Leenheer and Vandecasteele-Thienpont [1] for a bromhexine analogue used as the internal standard, but not for bromhexine itself. Initially we found low recovery even with relatively polar organic solvents such as toluene, ethyl acetate and methanol—cyclohexane, similar to the principle of homogeneous extraction [3]. The extraction period was found to be very important, and cyclohexane gave a relatively clean extract. The recovery, however, from plasma was still relatively low (ca. 60%), contrary to the 100% obtained by extraction from water. Pre-treatment with the proteolytic enzyme Subtilisin A® (A/S Novo, Copenhagen, Denmark) did not increase the recovery, and salting-out with sodium chloride decreased the recovery. Bromhexine was found to adsorb to plastic but not to glass, not even during the evaporation as described, for example, for femoxetine [4]. Because of the recovery of ca. 60% from plasma the use of an individual plasma standard curve was preferred. The procedure is simple, comprising only one extraction, compared to the GLC assay [1], which uses an additional acidic and alkaline extraction for cleaning-up (recovery ca. 90%, *n*-hexane as organic phase and an initial addition of methanol and triethanolamine to plasma).

The detection limits of ca. 10 nmol/l and ca. 5 nmol/l (equals ca. 5 and 2.5 ng/ml) for plasma and urine respectively, can only be obtained at 0.001 a.u.f.s. The determining factor for the detection limit was the tailing from the preceding peak in the chromatogram (Fig. 1). The separation was very sensitive to small changes in the water content of the mobile phase. Only for urine could a better separation be achieved by increasing the water content in the mobile phase.

Bromhexine is metabolized by hydroxylation of the cyclohexyl ring, N-demethylation and cyclization to tetrahydroquinazolines [5, 6]. None of these metabolites were available to us for specificity studies, but the method is expected to be specific, due to the rather unpolar extraction solvent used and the ability of the chromatographic system to separate secondary amines from tertiary amines (e.g. relative retention nortriptyline/amitriptyline = 1.65).

Further, the plasma concentration—time curve (Fig. 2), obtained by this method after oral administration of bromhexine to man [7] is comparable to that described in connection with the GLC assay [1].

The plasma assay has been used in a bioequivalence study (32 mg of bromhexine hydrochloride orally administered to man); and the unknown bromhexine first-pass effect and half-life were estimated on the basis of the combined plasma and urine data [7]. The sensitivity of the plasma assay (ca. 10 nmol/l) was not sufficient for more sophisticated pharmacokinetic calculations, due to the rapidly declining plasma concentration. For this purpose a sensitivity which is more than ten times higher would be preferable. The sensitivity of the GLC assay [1] was reported to be 2 (up to 5) nmol/l, when using 1 ml of plasma.

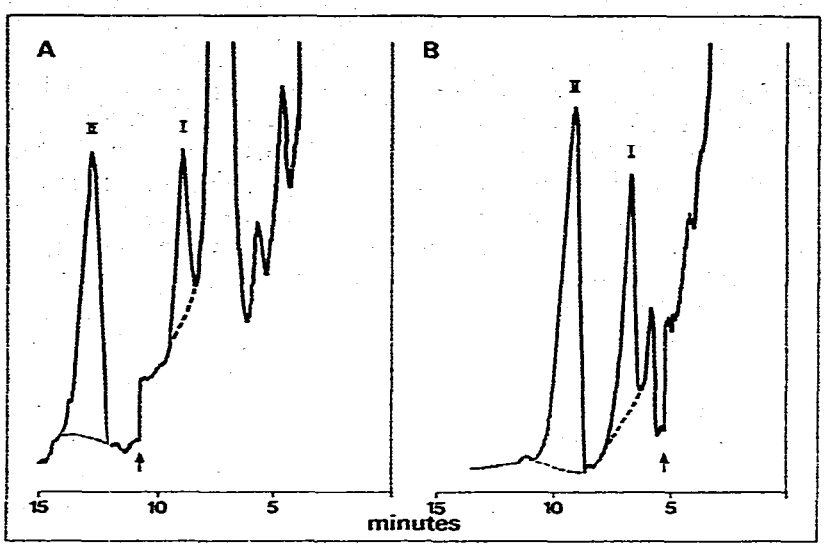

Fig. 1. Typical chromatograms of extracts from blank plasma (A) and blank urine (B) spiked with 61 and 122 nmol/l bromhexine, respectively. (– – –) Blank, I = Bromhexine, II = internal standard (amitriptyline). The arrow indicates a change in sensitivity from 0.001 to 0.002 a.u.f.s.

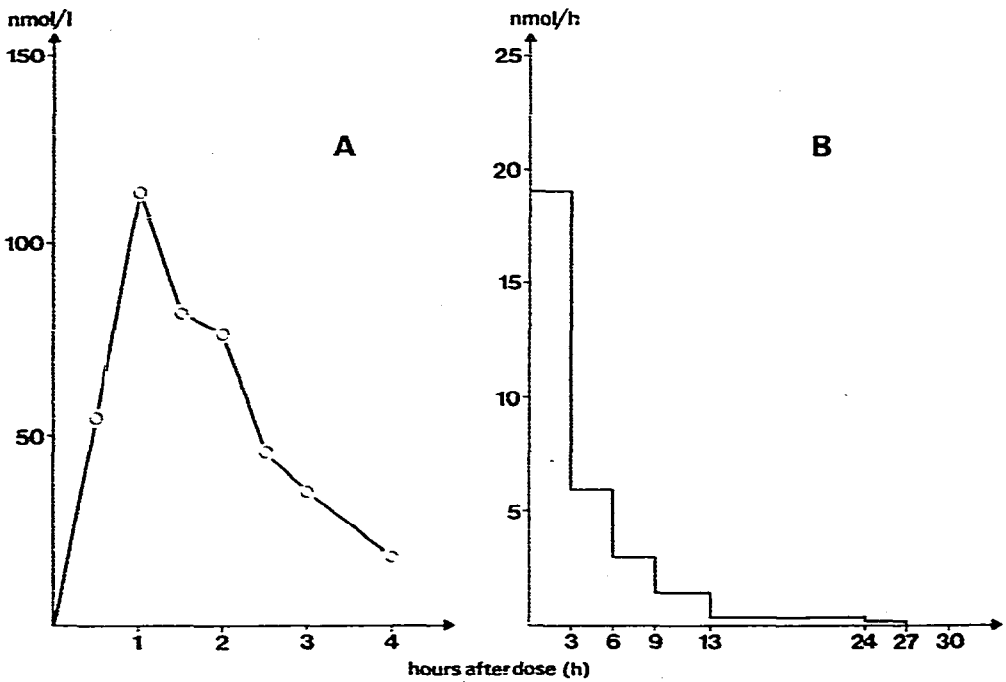

Fig. 2. Plasma levels (A) and urinary elimination rates (B) of bromhexine in a healthy volunteer after a single oral administration of three Bromhexin tablets, DAK 8 mg (77.6 μmol).

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REFERENCES

1 A.P. De Leenheer and L.M.R. Vandecasteele-Thienpont, J. Pharm. Sci., 69 (1980) 99.
2 J. Vessman, Symposium on Drug Analysis in Biological Material — Principal Aspects, Stockholm, November 1—3, 1978, p. 474.
3 C.-G. Hammar, G. Freij, S. Strömberg and J. Vessman, Acta Pharmacol. Toxicol., 36 (Suppl. III) (1975) 33.
4 E. Bechgaard and J. Lund, J. Chromatogr., 133 (1977) 147.
5 R. Jauch and R. Hankwitz, Arzneim.-Forsch., 25 (1975) 1954.
6 E. Schraven, F.W. Koss, J. Keck and G. Beisenherz, Eur. J. Pharmacol., 1 (1967) 445.
7 E. Bechgaard and A. Nielsen, submitted for publication.