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Note

Determination of the diuretic bumetanide in biological fluids by high-performance liquid chromatography

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Bumetanide (3-*n*-butylamino-4-phenoxy-5-sulphamoylbenzoic acid, Fig. 1) is a potent high-ceiling diuretic which is, on a weight for weight basis forty times more potent than frusemide [1]. Since the usual therapeutic dose of bumetanide is between 1 and 3 mg per day, resulting in peak serum levels of 10—120 ng ml^{-1}, a sensitive assay procedure is required to examine its pharmacokinetics in man.

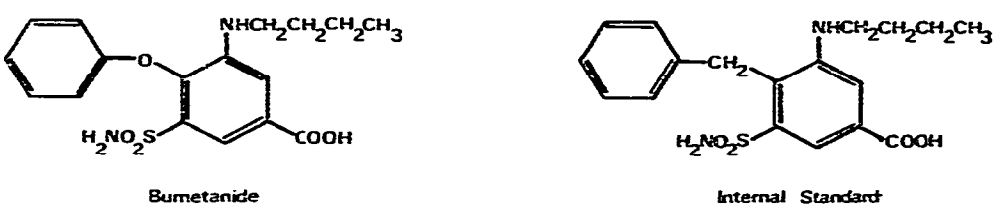

Fig. 1. Structural formulae of bumetanide and 4-benzyl-3-*n*-butylamino-5-sulphamoylbenzoic acid (internal standard).

Fluorimetric [2], gas—liquid chromatographic [3], radiometric [4] and radioimmuno [5] assays have been reported for the determination of bumetanide in biological fluids. However, all these methods have inherent disadvantages. Since bumetanide is partially metabolised in man [4] a specific and sensitive quanti-

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tative method is required for measuring its concentration in biological fluids.

The paper describes a quantitative high-performance liquid chromatographic (HPLC) method for the determination of bumetanide in biological fluids using a fluorescence detector and an internal standard.

MATERIALS AND METHODS

Chemicals and reagents

Bumetanide was supplied by Leo Labs. (Hayes, Great Britain). 4-Benzyl-3-*n*-butylamino-5-sulphamoylbenzoic acid (internal standard, Fig. 1) was supplied by Leo Pharmaceutical Products (Copenhagen, Denmark). All reagents and solvents were AnalaR grade (BDH, Poole, Great Britain) except the methanol which was HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain). Standard solutions of bumetanide (10—120 ng ml^{-1}) and internal standard (100 ng ml^{-1}) were prepared in water.

High-performance liquid chromatography

A Waters Assoc. high-performance liquid chromatograph equipped with a Model 6000A pump, UK6 injector and a Model 420 fluorescence detector was used. The column (30 cm × 0.39 cm I.D.) was prepacked with 10-μm ODS-silica (μBondapak C_{18}, Waters Assoc.). The mobile phase was methanol—water—acetic acid (70:30:1), pH 2.9 delivered isocratically at the rate of 1.5 ml min^{-1} at ambient temperature. The excitation filter wavelength was 340 nm and the emission filter wavelength, 425 nm. A pre-column packed with Bondapak C_{18}/Corasil (Waters Assoc.) was used to increase the life of the column.

Extraction procedure and sample preparation

Serum (1 ml) was placed in a glass stoppered test-tube along with internal standard (0.3 ml), phosphate—citrate buffer pH 5 (2 ml) and potassium chloride (0.5 g). For the calibration standards 1 ml of the buffer was replaced with a standard bumetanide solution (1 ml, 30 ng ml^{-1}). The mixture was extracted with diethyl ether (6 ml) by mechanically shaking for 2 min. The resultant mixture was centrifuged for 10 min at 1060 *g*, and the diethyl ether phase transferred to a tapered test-tube. A second volume of diethyl ether (5 ml) was added to the aqueous phase, and the extraction step repeated. The combined diethyl ether phases were evaporated to dryness on a water-bath at 50°C. The residue was reconstituted with mobile phase (120 μl), centrifuged and injected on to the column.

Similarly, urine (0.2 ml) was placed in a stoppered test-tube along with internal standard (0.6 ml), phosphate—citrate buffer pH 5 (2 ml), water (0.5 ml) and potassium chloride (1 g), and extracted as for serum. For the urine calibration standard 1 ml of buffer was replaced with standard bumetanide solution (1 ml, 60 ng ml^{-1}).

Calibration curves and extraction yields

For the quantitative determination of bumetanide in serum and urine, standard curves were prepared by adding either bumetanide (10—60 ng) and internal standard (30 ng) to blank serum or bumetanide (20—120 ng) and internal

standard (60 ng) to blank urine. The samples were extracted and chromatographed as described above and the peak height ratios plotted against the bumetanide concentration.

The recovery of bumetanide and internal standard after either a single extraction or a double extraction with diethyl ether was obtained by adding a constant amount of each to either blank serum or blank urine and treating them in the same way as the calibration standards.

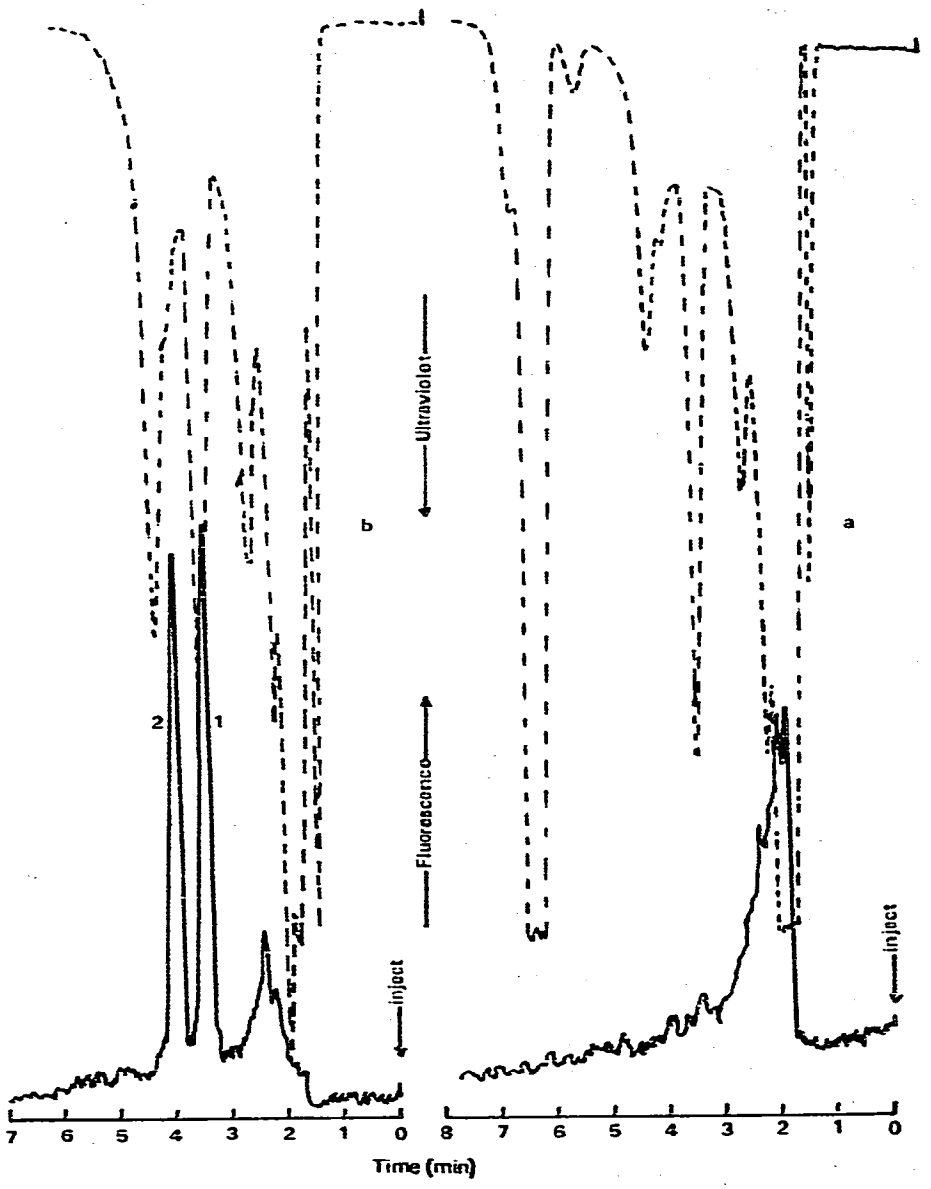

Fig. 2. HPLC chromatograms of (a) serum blank and (b) bumetanide (1) and internal standard (2) recovered from serum. Conditions as described in Materials and Methods. Detection: UV — — —; fluorescence ——.

RESULTS AND DISCUSSION

The advantage of fluorescent detection over UV detection in liquid chromatography is increased specificity, resulting in increased sensitivity since UV absorbing endogenous materials which are co-extracted with the drug and internal standard are not detected with the fluorescence detector (Fig. 2). Although the emission maxima for bumetanide and 4-benzyl-3-*n*-butylamino-5-sulphamoylbenzoic acid (internal standard) obtained with an excitation wavelength of 340 nm are 446 nm and 442 nm respectively, the wavelengths used in the method were dictated by the choice of filters available with the instrument.

Using a reversed-phase C_{18} system, bumetanide and the internal standard are well resolved from the void volume peak in extracts from either serum (Fig. 2) or urine (Fig. 3d). The retention times for bumetanide and internal standard are 3.6 and 4.3 min respectively, and it has been demonstrated that the desbutyl and aliphatic acid metabolites [4] are eluted in the void volume (unpublished results) with the HPLC system described in this paper.

An improved extraction yield was obtained for both bumetanide and internal standard from serum with a second extraction step (Table I) thus increasing the sensitivity of the method. It was not necessary to carry out a double extraction from urine since the extract yield for bumetanide was 95%. The reproducibility of the method based on ten replicate analyses of bumetanide (9 ng

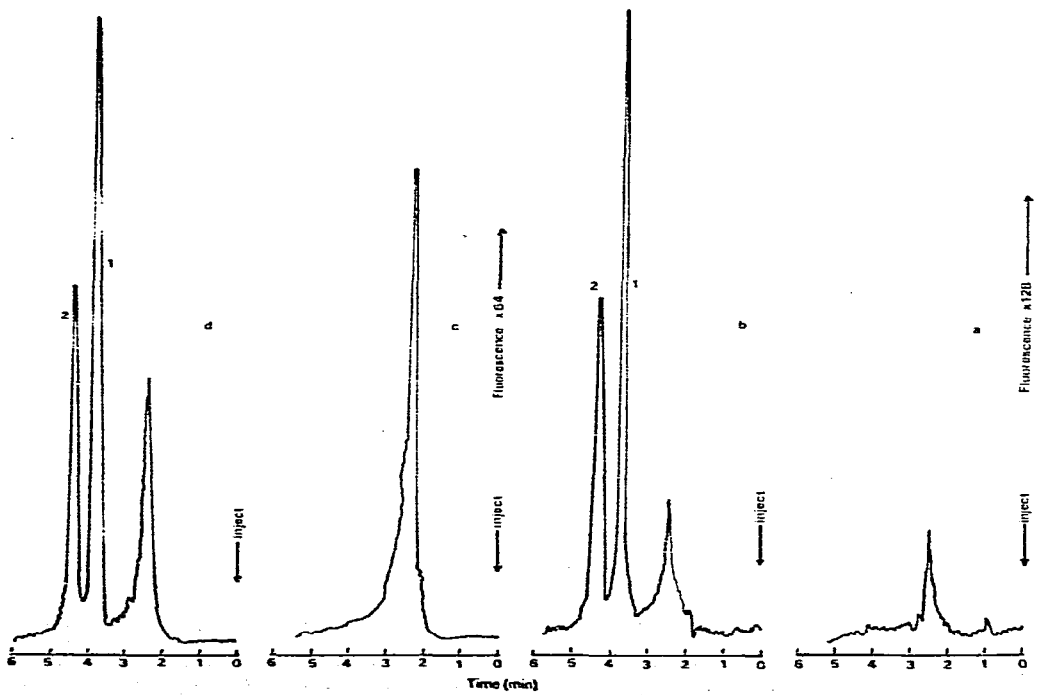

Fig. 3. Chromatograms of serum and urine samples from a subject who received bumetanide (1 mg) orally. (a) Control serum extract; (b) test serum extract; (c) control urine extract; (d) test urine extract. Peaks: 1 = bumetanide; 2 = internal standard. Conditions as described in Materials and Methods.

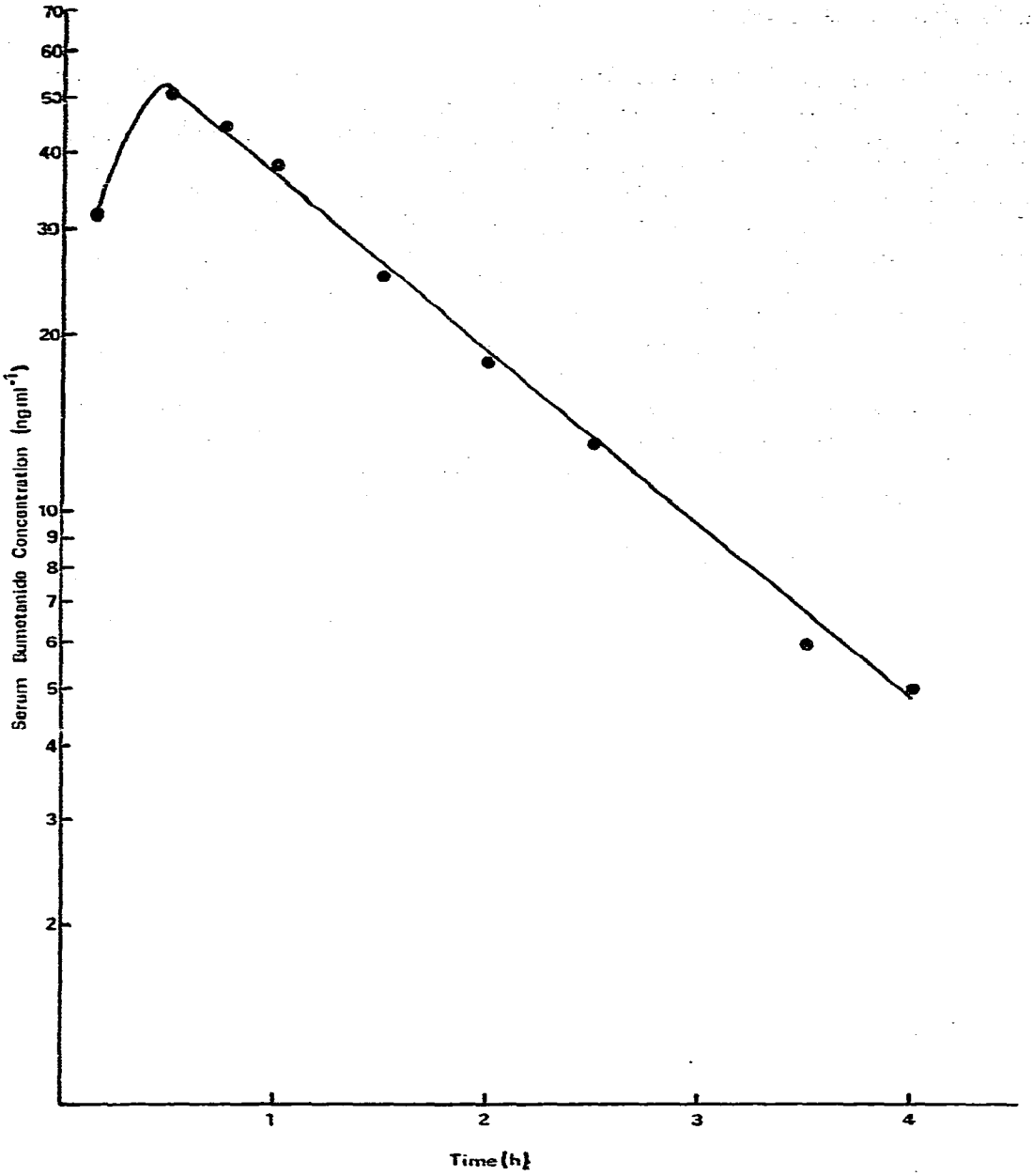

Fig. 4. Serum concentration—time curve for bumetanide assayed in duplicate.

ml^{-1}) in serum and bumetanide (66 ng ml^{-1}) in urine was 5.2% and 3.2% respectively. There was a rectilinear relationship between the peak height ratio and bumetanide concentration up to 60 ng ml^{-1} in serum and 120 ng ml^{-1} in urine.

Fig. 3 shows typical chromatograms obtained from serum and urine extracts from a subject who had received 1 mg of bumetanide orally. The biological half-life for bumetanide for this subject calculated from the serum concentration—time curve was 1 h (Fig. 4). Of the dose administered 52% was excreted in 24 h.

TABLE I

RECOVERIES OF BUMETANIDE AND 4-BENZYL-3-*n*-BUTYLAMINO-5-SULPHAMOYL-BENZOIC ACID (INTERNAL STANDARD) FROM SERUM AND URINE

$n = 6$.

	Extraction yield (%) (mean ± S.D.)	
	Bumetanide	Internal standard
Serum		
single extraction	71 ± 7	73 ± 7
double extraction	91 ± 7	86 ± 6
Urine		
single extraction	95 ± 6	89 ± 5

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