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DETERMINATION OF BUMETANIDE IN THE PLASMA OF NON-HUMAN PRIMATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple, sensitive and specific method for the determination of bumetanide in the plasma of non-human primates has been developed using high-performance liquid chromatography in a reversed-phase mode. The limit of accurate measurement for bumetanide in plasma was 1 ng/ml although lower concentrations could be detected. The method has been applied to plasma samples obtained from cynomolgus monkeys after intravenous doses of bumetanide of 0.03 mg/kg. In this species, mean plasma concentrations declined from 300 ng/ml at 2 min after dosing to 1 ng/ml at 180 min; the half-life of the terminal linear phase was 43 min.

INTRODUCTION

Bumetanide (3-*n*-butylamino-4-phenoxy-5-sulphamoyl benzoic acid) (Fig. 1) is a potent "high ceiling" (loop) diuretic which produces the rapid onset and short duration of action characteristic of this class [1—4]. Since doses of bumetanide in clinical usage are low, a sensitive and highly specific method of analysis for the measurement of this drug in plasma is necessary.

A wide variety of methods have been reported for the quantitative estimation of bumetanide, many of which employed the radiolabelled drug and thin-layer chromatography but did not distinguish between bumetanide and its

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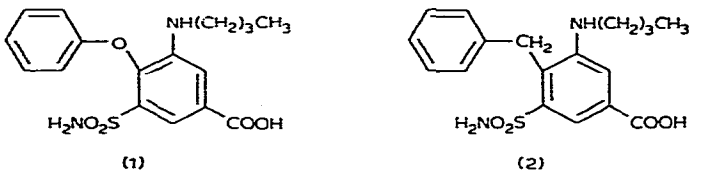

Fig. 1. Chemical structure of bumetanide (1) and internal standard (2).

metabolites; other methods are not sufficiently sensitive for the accurate determination of circulating levels of the drug. Published methods include those based on gas–liquid chromatography [5], thin-layer chromatography with fluorimetry [4] and radiometric assay [6].

In order to investigate the pharmacokinetics of bumetanide in non-human primates, an analytical method was developed using high-performance liquid chromatography (HPLC) coupled with fluorimetric detection. Fluorimetry is an ideal technique for bumetanide analysis, in that it is particularly sensitive and can be highly specific when coupled with chromatographic separation. A similar method has recently been published [7]. The major advantage of the method described in this paper is the greater sensitivity, giving a limit of accurate measurement of 1 ng/ml of plasma. The improved sensitivity is achieved by the use of a scanning fluorimeter which facilitates the measurement of the drug by using both the excitation and emission maxima of the compound. Sensitivity is further enhanced by buffering the mobile phase to pH 4.0 at which value the fluorescence of bumetanide is at a maximum.

EXPERIMENTAL

Materials

All reagents were of analytical grade and all inorganic reagents were prepared in fresh glass-distilled water. Diethyl ether was freshly redistilled prior to use. Standard solutions of bumetanide were prepared at concentrations of 0.1 μg/ml, 1.0 μg/ml and 10.0 μg/ml in methanol. A stock solution of 4-benzyl-3-*n*-butylamino-5-sulphamoyl benzoic acid (Fig. 1) for use as internal standard was prepared at a concentration of 1 μg/ml in methanol. All standard solutions were stored at 4°C.

Extraction procedure

Plasma samples (1 ml) were transferred to 10-ml pointed centrifuge tubes and spiked with internal standard (30 μl, containing 30 ng). Sulphuric acid (200 μl, 1 *N*) was added and the mixture was extracted by vortex mixing with diethyl ether (5 ml). After centrifugation of the extract at 2000 *g* for 10 min, the organic layer was transferred to another pointed centrifuge tube and evaporated to dryness under a stream of dry nitrogen at 37°C. The residue was washed to the bottom of the tube with a small volume of diethyl ether, which was again evaporated to dryness. The dry residue was redissolved in methanol (30 μl) and the total sample was injected into the liquid chromatograph.

High-performance liquid chromatography

The chromatograph consisted of an M6000A pump (Waters Assoc., Northwich, Great Britain) fitted to a Perkin-Elmer 3000 fluorimeter (Perkin-Elmer, Beaconsfield, Great Britain) operated at an excitation wavelength of 338 nm and an emission wavelength of 440 nm (the fluorescence maximum for bumetanide dissolved in mobile phase). Injection was made by syringe via a U6K universal injector (Waters Assoc.). The column was constructed of stainless steel (30 cm × 4 mm I.D.) prepacked with μBondapak C_{18}, particle size 10 μm (Waters Assoc.). A pre-column (7 cm × 2 mm I.D.) constructed of stainless steel and dry-packed with pellicular Co:Pell® ODS (particle diameter 25—37 μm, Whatman, Maidstone, Great Britain) was installed in front of the analytical column.

Chromatography was performed in a reversed-phase mode using a mobile phase of 60% (v/v) methanol in aqueous potassium dihydrogen orthophosphate (0.1%, w/v); the pH of the final mixture was adjusted to 4.0 with phosphoric acid. The mobile phase was passed through the column at a flow-rate of 2 ml/min.

Chromatograms were recorded using a 3380A computing integrator (Hewlett-Packard, Slough, Great Britain). Peak height measurements were used in preference to peak area measurements due to the inability of the integrator to assign correct areas to the smaller peaks.

Under the conditions described, bumetanide had a retention time of 5.7 min and the internal standard a retention time of 8.0 min (Fig. 2).

Calibration procedure

The calibration line was constructed from peak height ratio measurements of bumetanide to internal standard against concentration, over the concentration range 1—100 ng/ml. Samples of blank plasma (1 ml) were spiked with bumetanide at concentrations of 1, 5, 15, 30, 60, and 100 ng/ml, and with internal standard at a fixed concentration of 30 ng/ml. Samples were taken through the extraction procedures described previously.

Studies in monkeys

Five cynomolgus monkeys (*Macaca fascicularis*) were dosed with the commercial preparation of bumetanide, Burinex® (Leo Labs., Hayes, Great Britain). Each animal was dosed intravenously with 0.03 mg bumetanide per kg of bodyweight (corresponding on a mg/kg basis to the human therapeutic dose).

The animals were fasted for at least 12 h preceding drug administration and for 6 h following drug administration. Blood samples were taken from the femoral veins of the animals at 0 (pre-dose), 2, 5, 10, 20, 30, 45, 60, 80, 100, 120, 150, 180, 240, 300, 450, and 1440 min after dosing. The samples were taken into heparinised tubes and the blood cells separated by centrifugation and discarded. The separated plasma was stored at −20°C until analysis by the method described. Where less than 1 ml of plasma from the dosed animals was required for analysis, the volume was made up to 1 ml with control plasma.

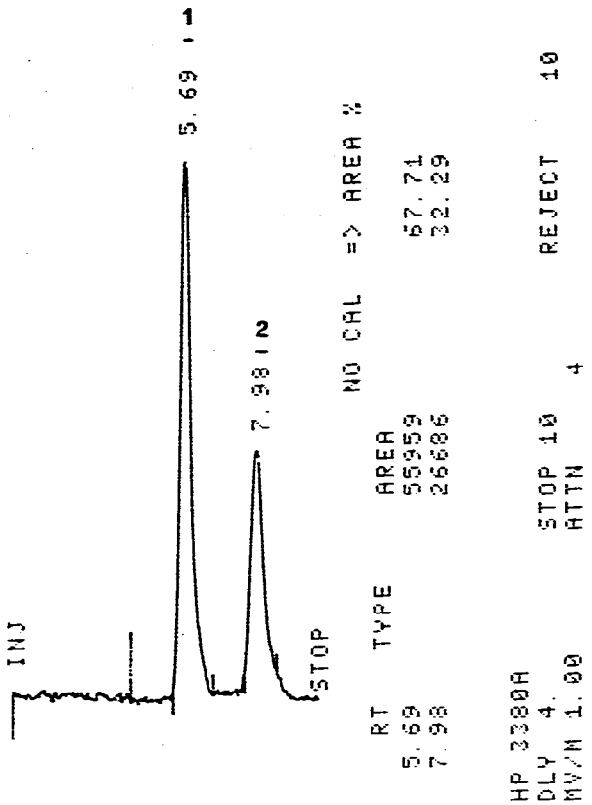

Fig. 2. Chromatogram of a standard mixture containing bumetanide (1) and internal standard (2). Column, μBondapak C_{18} (Waters Assoc.); mobile phase, 60% (v/v) methanol in aqueous potassium dihydrogen orthophosphate (0.1%, w/v), pH 4.0; flow-rate, 2 ml/min; detector, fluorescence, excitation 338 nm, emission 440 nm.

Gas chromatography—mass spectrometry

Gas chromatography—mass spectrometry (GC—MS) was carried out using a Pye 104 gas chromatograph (Pye-Unicam, Cambridge, Great Britain) linked via a single-stage, glass jet separator to a Micromass 16F mass spectrometer (VG Analytical, Altrincham, Great Britain). The mass spectrometer was operated in the electron impact mode of ionisation, with an electron beam energy of 70 eV, a trap current of 100 μA and a source temperature of 200°C. Mass spectra were obtained at 10-sec intervals and the data stored using a Display Digispec data system (VG Analytical) using floppy diskettes.

For the analysis of bumetanide samples, the GC oven was fitted with a glass column (1 m × 3 mm I.D.) packed with 1% OV-1 on Diatomite CLQ (100—120 mesh) and was operated at 230°C. Helium was used as the carrier gas at a flow-rate of 20 ml/min. The temperature of the GC—MS interface was 250°C.

Bumetanide samples for GC—MS were evaporated to dryness and the residues (ca. 2 μg) were methylated by dissolving them in Methelute (0.2 *M* trimethylanilinium hydroxide in methanol; 10 μl) (Pierce and Warriner (U.K.), Chester, Great Britain); 2.5-μl aliquots were injected for on-column reaction.

Specificity of the analytical method

Initial studies on bumetanide reported in the literature suggested that there was no metabolism of the drug in man [2, 4, 8]; more recent work shows that there is N-debutylation and oxidation in the butyl side-chain of the molecule [6,9—11]. In the absence of analytical standards of the proposed metabolites, it was not possible to test for the co-elution of these compounds with the parent drug in the chromatographic system described. However, the literature available suggests that no metabolites of bumetanide have been reported after acid—ether extraction of the drug from biological fluids, while Pentikainen et al. [6] reported than an ether extraction from an acid medium quantitatively separated bumetanide from its more polar metabolites.

Profiles of the methylated compound produced by the mass spectrometer (Fig. 3) were similar for the pure drug and for extracts of post-dose plasma samples measured as bumetanide by the HPLC method described. Three major ions corresponding to *m/z* 420 (molecular ion), *m/z* 377 ($M - C_3H_7$) and *m/z* 58 [$(CH_3)_2\overset{\oplus}{N} = CH_2$, formed by rearrangement] were observed in all samples, with no other major ions occurring in the plasma extracts (as would be expected if metabolites were present). Similarly, the gas chromatograms for the samples each yielded only one major peak, with the same retention time as authentic bumetanide.

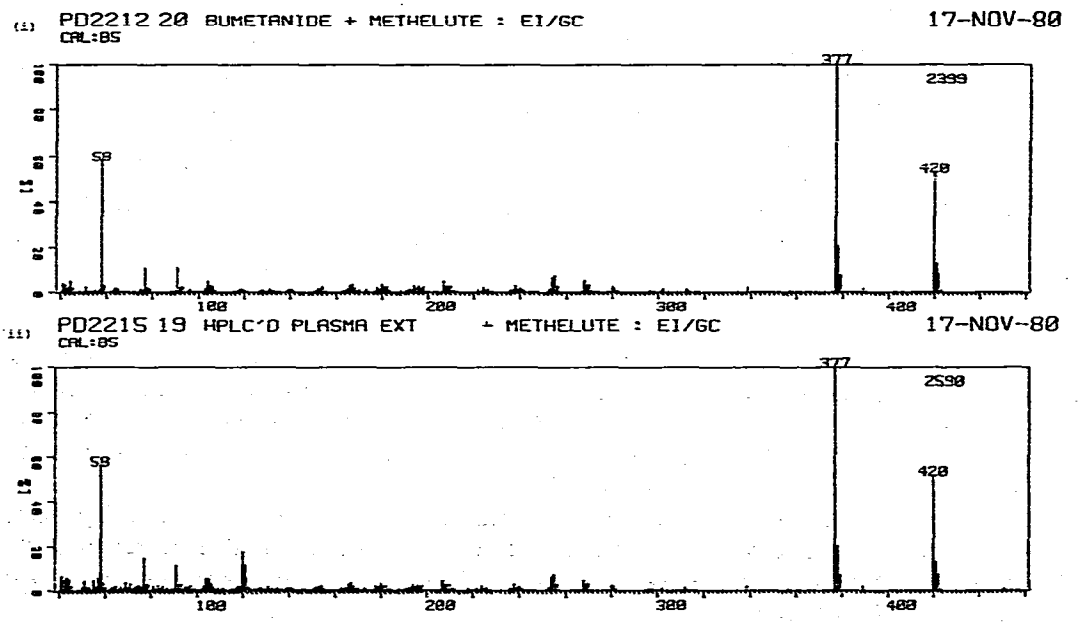

Fig. 3. Mass spectra of (i) authentic bumetanide (methylated) and (ii) post-dose plasma extract of bumetanide, separated by HPLC then methylated.

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each concentration was repeated on six

TABLE I

PRECISION OF MEASUREMENT AND RECOVERY OF BUMETANIDE FROM PLASMA

Concentration added to plasma (ng/ml)	Mean peak height ratio (bumetanide/ internal standard)	Coefficient of variation (%)	Recovery (%)
1	0.06	17	77
5	0.26	4	74
15	0.80	3	68
30	1.64	1	69
60	3.08	2	69
100	5.26	2	69

occasions over the calibration range. The precision of the method for the measurement of bumetanide in plasma was indicated by the coefficients of variation of peak height ratios (Table I) which were ± 17% at 1 ng/ml, ± 1% at 30 ng/ml and ± 2% at 100 ng/ml.

Accuracy

The calibration line for the measurement of bumetanide in plasma was constructed over the range 1—100 ng/ml; six replicate extractions were made at each concentration over the range. The plot of peak height ratios against concentration was linear ($Y = a + bX$, where $a = 0.0087$ and $b = 0.0523$, correlation coefficient $r = 0.9995$) where Y is the peak height ratio and X is the concentration (ng/ml) of bumetanide. The accuracy of the method as defined by the 95% confidence limits of the least-squares regression line, i.e. taking the calibration line as an estimate of the concentration of bumetanide in plasma, was ± 44%, ± 8% and ± 2% at 5, 30 and 100 ng/ml, respectively.

Recovery

The recovery of internal standard (30 ng/ml) from plasma (1 ml) was determined by comparison of peak height ratio measurements of internal standard to bumetanide of standards taken through the extraction procedure, to those injected into the chromatograph without extraction. The mean recovery of internal standard was 66 ± 5% S.D. ($n = 5$).

The mean recovery of bumetanide from plasma was determined by comparison of peak height ratio measurements of non-extracted standards to those of extracted standards corrected for 100% recovery of internal standard, and was 71 ± 4% S.D. (Table I).

Stability of bumetanide in plasma

The stability of bumetanide in plasma under the storage conditions used (−20°C) was tested by storing 50 ng/ml plasma standards for 16 weeks. No decomposition of bumetanide was detected over this period.

Limits of detection

No interfering peaks with retention times similar to bumetanide were present

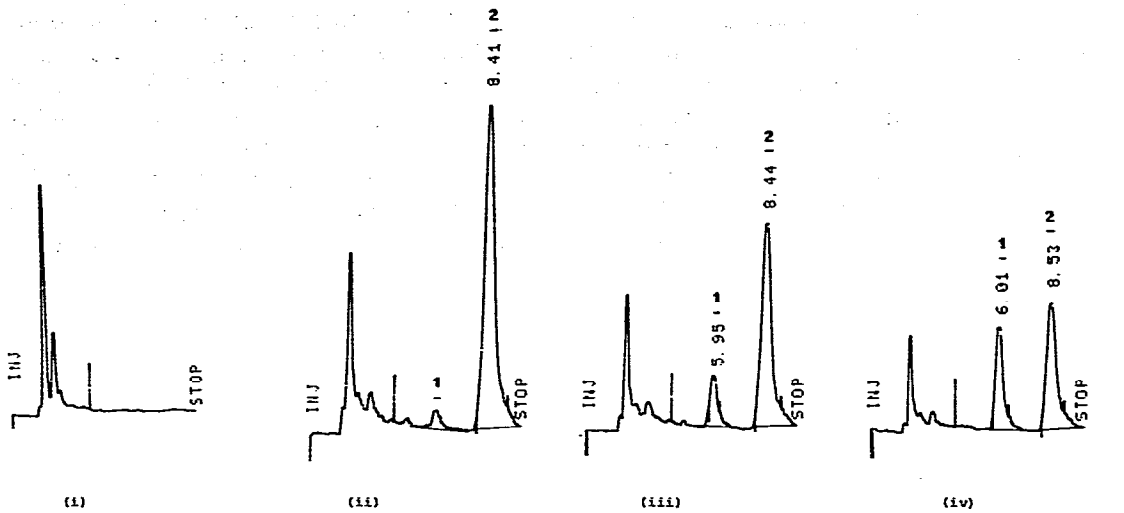

Fig. 4. Chromatograms of (i) pre-dose control plasma, and (ii—iv) plasma samples containing bumetanide at 1, 5, and 15 ng/ml, respectively, and 30 ng of internal standard (2).

TABLE II

CONCENTRATION OF BUMETANIDE IN THE PLASMA OF FIVE CYNOMOLGUS MONKEYS AFTER A SINGLE INTRAVENOUS DOSE OF 0.03 mg/kg

Concentrations are given in ng/ml.

Time (min)	Animal number					Mean ± S.D.
	1	2	3	4	5	
2	230	360	280	360	270	300.0 ± 57.9
5	120	180	140	130	100	134.0 ± 29.7
10	90	80	60	80	50	72.0 ± 16.4
20	50	30	20	50	20	34.0 ± 15.1
30	40	12	14	20	13	19.8 ± 11.7
45	24	6	8	12	6	11.2 ± 7.6
60	14	4	6	8	4	7.2 ± 4.1
80	8	3	5	4	4	4.8 ± 1.9
100	6	3	4	2	2	3.4 ± 1.7
120	4	2	3	2	2	2.6 ± 0.9
150	3	<1	3	1	<1	1.4 ± 1.5
180	3	<1	2	<1	<1	1.0 ± 1.4
240	2	<1	2	<1	<1	<1
300	1	<1	1	<1	<1	<1
450	<1	<1	<1	<1	<1	<1

in pre-dose (blank) plasma (Fig. 4). The limit of detection of bumetanide in plasma was less than 1 ng/ml and was set by instrumental noise.

Concentrations of bumetanide in the plasma of cynomolgus monkeys

The mean concentrations of bumetanide in the plasmas of five cynomolgus monkeys after an intravenous dose of 0.03 mg/kg are shown in Table II and

Fig. 5. A peak mean plasma concentration of 300 ng/ml bumetanide was measured at the first time of sampling (2 min); thereafter, mean plasma concentrations declined to the limit of accurate measurement (1 ng/ml) at 180 min after dosing. The half-life of the terminal linear section of the mean plasma bumetanide concentration—time relationship was 43 min compared with reported half-lives of 10 min in the rat [12], 9—15 min in the dog [4,12], and 60—90 min in humans [6—8,10,12]. The bumetanide half-life of 43 min is greater than that of 29 min reported for frusemide (another high-ceiling diuretic in clinical usage) in the same species [13].

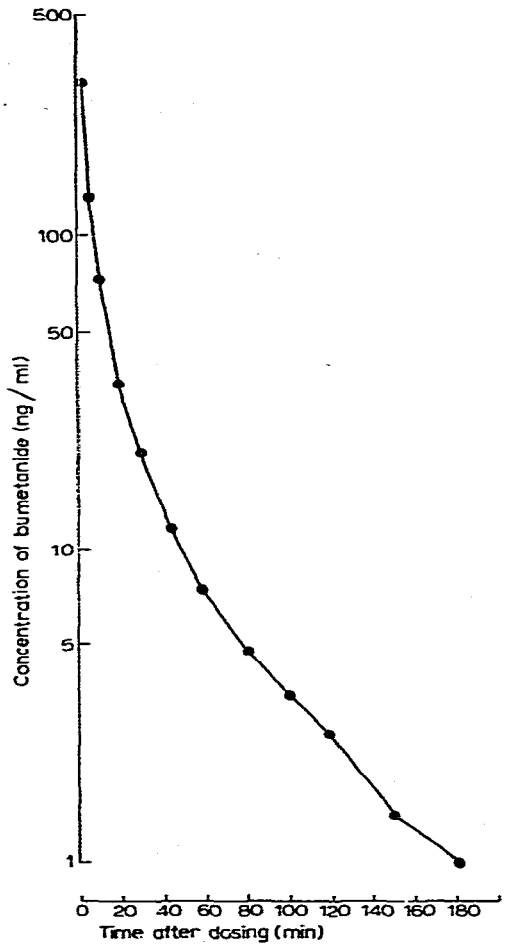

Fig. 5. Mean plasma concentrations of bumetanide after single intravenous doses of bumetanide (0.03 mg/kg) to five cynomolgus monkeys.

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