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Short Communication

Measurement of bumetanide in plasma and urine by high-performance liquid chromatography and application to bumetanide disposition

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ABSTRACT

A high-performance liquid chromatographic method for the measurement of bumetanide in plasma and urine is described. Following precipitation of proteins with acetonitrile, bumetanide was extracted from plasma or urine on a 1-ml bonded-phase C_{18} column and eluted with acetonitrile. Piretanide dissolved in methanol was used as the internal standard. A C_{18} Radial Pak column and fluorescence detection (excitation wavelength 228 nm; emission wavelength 418 nm) were used. The mobile phase consisted of methanol–water–glacial acetic acid (66:34:1, v/v) delivered isocratically at a flow-rate of 1.2 ml/min. The lower limit of detection for this method was 5 ng/ml using 0.2 ml of plasma or urine. Nafcillin, but not other semi-synthetic penicillins, was the only commonly used drug that interfered with this assay. No interference from endogenous compounds was detected. For plasma, the inter-assay coefficients of variation of the method were 7.6 and 4.4% for samples containing 10 and 250 ng/ml bumetanide, respectively. The inter-assay coefficients of variation for urine samples containing 10 and 2000 ng/ml were 8.1 and 5.7%, respectively. The calibration curve was linear over the range 5–2000 ng/ml.

INTRODUCTION

Bumetanide (3-*n*-butylamino-4-phenoxy-5-sulfamoylbenzoic acid) is a potent inhibitor of Na^+–K^+ co-transport in the thick ascending limb of the loop of Henle [1]. Published high-performance liquid chromatographic (HPLC) methods for determination of bumetanide concentrations in plasma and urine have required relatively large sample sizes, dual detection, and/or methodology for different biological fluids [2–7]. We have developed a sensitive, simple microanalyt-

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ical method for bumetanide using solid-phase extraction and fluorescence detection that is applicable to both plasma and urine samples.

EXPERIMENTAL

Standards

Bumetanide and piretanide, the internal standard, were donated by Hoffman-LaRoche (Nutley, NJ, USA). Stock solutions were prepared by dissolving of 50 mg of bumetanide in 100 ml of methanol and 1 mg of piretanide in 10 ml of methanol. The stock solutions were stable at −10°C for up to three months. Working solutions were prepared by serial dilution of stock solutions. All solvents used to develop the assay were HPLC grade. ACS grade KCl and K_2HPO_4 (Sigma, St. Louis, MO, USA) were used to prepare a 0.1 *M* phosphate buffer (pH 5.0) as previously described [4].

Sample preparation

To each 0.2-ml plasma or urine sample, 20 or 100 ng, respectively, of piretanide was added. Next, acetonitrile (0.4 ml) was added to precipitate proteins. Each sample was vortex-mixed for 2 min, sonicated for 2 min in a water bath and centrifuged for 10 min at 1000 *g*. The supernatant was transferred into clean tubes and 4 ml of phosphate buffer were added. Prior to introduction of samples, a 1-ml bonded-phase C_{18} column (Bond Elut, Analytichem International, Harbor City, CA, USA) was washed sequentially with 0.6 ml of acetonitrile, 2 ml of methanol and 3 ml of non-organic deionized water. The samples were passed over the column while a constant negative pressure was applied by a vacuum pump. The column was then washed with 3 ml of reagent-grade water. Bumetanide and piretanide were eluted from the column with 0.3 ml of acetonitrile, then evaporated to dryness under a constant stream of nitrogen at 37°C. Samples were reconstituted in 0.2 ml of the HPLC eluent and 100 μl were injected onto the analytical column.

Conditions of analysis

A Waters Assoc. (Milford, MA, USA) HPLC apparatus with the following instrumentation was used: Waters intelligent sample processor (WISP), Waters Model 600E system controller, Model 712 injector, Model A 740 data module and a Model 470 fluorescence spectrophotometer. A 10 cm × 8 mm, 5 μm Radial Pak C_{18} column (reversed phase) with a C_{18} guard column was used. The mobile phase consisted of methanol–water–glacial acetic acid (66:34:1, v/v) delivered isocratically at a flow-rate of 1.2 ml/min. All solutions were prefiltered through a 0.22-μm filter (Millipore, Milford, MA, USA) and the eluent was degassed with helium sparging.

All samples were analyzed in duplicate at room temperature (20–22°C). The WISP was programmed to inject 100 μl of the extracted samples. Fluorescence

detection (excitation wavelength 228 nm; emission wavelength 418 nm) was used to monitor bumetanide and piretanide over a total run time of 10 min.

Variability and stability

Standard curves were prepared in plasma containing 10, 50 or 250 ng/ml and urine containing 10, 200 or 2000 ng/ml bumetanide. Spiked plasma and urine samples used to prepare standard curves were extracted and prepared for analysis as previously described. Inter-assay variability was assessed by analysis of eight extracted plasma or urine samples at each concentration.

Intra-assay variability was assessed by determination of the peak-area ratios for plasma samples ($n = 5$) containing 50 or 250 ng/ml bumetanide and urine samples ($n = 5$) containing 10, 100 or 2000 ng/ml bumetanide.

Stability studies were performed on refrigerated (4°C) and frozen (−70°C) aliquots of human plasma containing 5, 50 or 250 ng/ml bumetanide. Similar studies were performed on refrigerated (4°C) and frozen (−70°C) aliquots of human urine containing 10, 100 or 1000 ng/ml bumetanide.

Selectivity

Drugs that are commonly co-administered with bumetanide were tested for potential interference (Table I). Analytical standards for each drug were prepared in phosphate buffer. Duplicate samples were processed as described for bumetanide.

Recovery

Recovery experiments were conducted using known concentrations of bumetanide in methanol, plasma and urine. Plasma samples ($n = 3$) were prepared using 10 and 100 ng/ml bumetanide and urine samples were prepared using 10, 100 and 1000 ng/ml bumetanide. Plasma and urine samples were extracted as previously described. Piretanide was added and aliquots of extracted plasma and urine were injected onto the analytical column. Bumetanide in methanol (10, 100 or 1000 ng/ml) was evaporated to dryness and reconstituted with 200 μl of eluent. Piretanide was added and aliquots were injected. Recoveries of bumetanide from plasma and urine after solid-phase extraction were determined by comparison of peak-area ratios obtained from biologic fluids with those of non-extracted solutions of bumetanide in methanol.

RESULTS

Elution characteristics

A typical chromatographic tracing of a sample urine is reproduced in Fig. 1. With a constant flow-rate of 1.2 ml/min and chart recorder speed of 1 cm/min, bumetanide and piretanide eluted with retention times of 6.5 and 5.1 min, respectively. Both compounds consistently produced symmetrical peaks with minimal

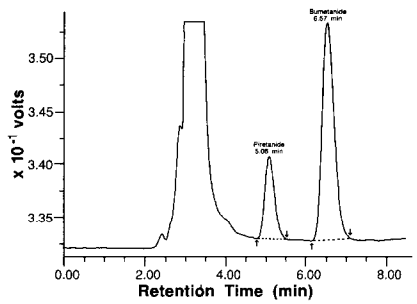

Fig. 1. Representative chromatographic tracing after extraction of 200 μl of urine. The urine sample was collected between 5 and 6 h administration of bumetanide (0.10 mg/kg) to a 3.78-kg neonate. The concentration of bumetanide, as determined by comparison of the bumetanide/piretanide peak-area ratio with the standard curve, was 162 ng/ml.

tailing. No interfering peaks were observed after extraction and injection of drug-free plasma or urine.

Standard curve and limit of detection

Standard curves ($n = 5$) were prepared from 0.2-ml plasma and urine samples as previously described. Linear regression analysis was performed on each set of data obtained from the standard curves and the following tests were conducted: (1) a correlation coefficient (r), H_0 being that x (bumetanide plasma concentration in ng/ml) and y (the peak-area ratio, calculated by dividing the area under the bumetanide peak by the area under the piretanide peak) are independent; (2) analysis of variance, H_0 being that the regression of y on x is not linear. The equation for the unweighted least-squares fit of the line representing the standard curve in plasma was $y = 0.017x + 0.009$ ($r = 0.993$; $p < 0.001$). The equation for the unweighted least-squares fit of the line representing the standard curve in urine was $y = 0.0874x + 1.254$ ($r = 0.995$; $p < 0.001$). The calibration curves were linear over the range 5–500 and 10–2000 ng/ml for plasma and urine samples, respectively.

Variability and stability

The inter-assay coefficients of variation were 7.6, 6.9 and 4.4% for plasma samples containing 10, 50 and 250 ng/ml bumetanide, respectively. The inter-assay coefficients of variation for urine samples containing 10, 200 and 2000 ng/ml bumetanide were 8.1, 8.1 and 5.7%, respectively. The intra-assay coefficients of variation for both plasma and urine samples were consistently less than 2%. No instability was detected (*i.e.* stored samples $\geq 90\%$ of the original concentration detected) for up to 48 h in refrigerated samples and up to six weeks in frozen samples.

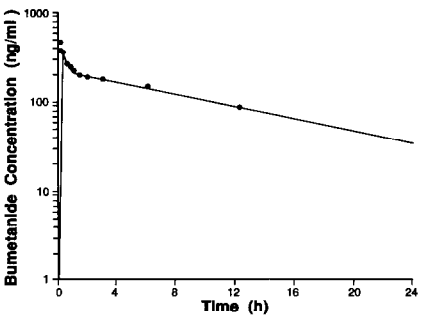

Fig. 2. Plasma concentration *versus* time curve after intravenous administration of bumetanide (0.10 mg/kg) in a neonate receiving ECMO therapy. The biexponential curve fit was generated using SIPHAR®/Base.

Selectivity

Table I contains the retention times for compounds which are often administered concurrently with bumetanide. Among the compounds studied, only nafcillin eluted near the retention times for bumetanide or piretanide.

TABLE I

POTENTIAL INTERFERING SUBSTANCES

Peak areas less than 5000 (below limit of assay sensitivity) were recorded as N.D. (not detected).

Drug	Concentration	Retention time (min)
Dopamine · HCl	40 mg/ml	4.5.
Carbamazepine	500 μg/ml	N.D.
Phenytoin sodium	50 mg/ml	N.D.
Midazolam base	100 μg/ml	N.D.
Desmethyldiazepam	10 μg/ml	N.D.
Diazepam	1 mg/ml	N.D.
Lorazepam	250 ng/ml	N.D.
Ranitidine	25 mg/ml	N.D.
Gentamicin	10 μg/ml	N.D.
Amikacin	30 μg/ml	4.5
Erythromycin	1 mg/ml	N.D.
Ampicillin	1 mg/ml	N.D.
Nafcillin	100 μg/ml	6.2
Ticarcillin sodium	1 mg/ml	N.D.
Cefotaxime	330 mg/ml	N.D.
Ceftazidime	180 mg/ml	N.D.
Ceftriaxone	100 mg/ml	N.D.
Vancomycin	50 mg/ml	N.D.
Clindamycin	150 mg/ml	N.D.
Theophylline	200 μg/ml	N.D.
Digoxin	100 μg/ml	N.D.
Heparin sodium	100 USP units/ml	2.8 and 3.6
Furosemide	2 mg/ml	4.1

TABLE II

PHARMACOKINETIC PARAMETERS CALCULATED AFTER A 0.10 mg/kg DOSE OF BUMETANIDE IN A 3.78-kg NEONATE RECEIVING ECMO THERAPY

Pharmacokinetic parameter[a]	Value
$AUC_{0-24\ h}$ (μg/ml h)	1.84
Cl_{total} (ml/min/kg)	0.59
Cl_{renal} (ml/min/kg)	0.12
$Cl_{non\text{-}renal}$ (ml/min/kg)	0.47
Vd_{ss} (l/kg)	0.42
Vd_{β} (l/kg)	0.43
Vd_c (l/kg)	0.17
$t_{1/2\alpha}$ (h)	0.17
$t_{1/2\beta}$ (h)	8.45

[a] AUC = area under the plasma concentration *versus* time curve; Cl_{total} = plasma clearance; Cl_{renal} = renal clearance; $Cl_{non\text{-}renal}$ = non-renal clearance; Vd_{ss} = steady-state volume of distribution; Vd_{β} = volume of distribution, elimination phase; Vd_c = volume of the central compartment; $t_{1/2\alpha}$ = half-life, distribution phase; $t_{1/2\beta}$ = half-life, elimination phase.

Recovery

Mean recoveries from 200-μl plasma samples were 90 and 100% for bumetanide concentrations of 10 and 100 ng/ml, respectively. For urine bumetanide concentrations of 10, 100 and 1000 ng/ml, the corresponding mean recoveries were 80, 86 and 90%.

Application: bumetanide disposition in a neonate

To demonstrate the clinical applicability of this method, bumetanide disposition was studied in neonates treated with extracorporeal membrane oxygenation (ECMO). This clinical investigation was approved by the Human Research Advisory Committee at the University of Arkansas for Medical Sciences. A 3.78-kg neonate received a single dose of bumetanide (0.10 mg/kg) intravenously over 2 min. A log-linear concentration *versus* time plot is shown in Fig. 2. The apparent peak serum concentration (477.4 ng/ml) was observed 2 min after the termination of the infusion. Pharmacokinetic parameters were estimated using SIPHAR®/Base (SIMED, Creteil, France). The disposition of bumetanide was best described by an open, two-compartment model. Calculated pharmacokinetic parameters are shown in Table II. Urinary recovery (24 h) of bumetanide in this patient was 12.6%.

DISCUSSION

Previous methods for measurement of bumetanide in body fluids have re-

quired large sample volumes, serial detection of bumetanide and internal standards, or cumbersome and time-consuming extractions [2–7]. Methods which require larger sample volumes [2,6,7] are of limited usefulness for adaptation to pharmacokinetic studies in neonates, infants and small children. Unless microanalytical procedures are developed, drug disposition cannot be studied in the segments of the population in which drug elimination is likely to be altered by age-dependent differences in renal clearance and/or hepatic biotransformation. Our method allows for precise measurement of bumetanide in small volumes of blood, a prerequisite for serial plasma sampling necessary for accurate characterization of drug disposition.

Older methods for measurement of bumetanide employed dual detection schemes [3]. Although bumetanide was detected using a fluorescence detector, acetophenone, the internal standard, required ultraviolet detection. By using piretanide, a compound that is structurally similar to bumetanide and has similar fluorescent properties, use of a dual detection system is not necessary. As furosemide was easily detected with our method and has a retention time markedly different from bumetanide, it might also be useful as an internal standard. Alternatively, it is possible that this assay might be used to detect furosemide using bumetanide as an internal standard, although this method has not been evaluated using the technique and system described.

Compared with methods which use liquid-phase extractions, solid-phase extraction provided a rapid, simple approach to achieve separation of bumetanide. Solid-phase extraction has been reported to be useful in urine samples [5], but we also achieved adequate separation using plasma samples. A major advantage of the present method is applicability to both plasma and urine without significant modification of extraction technique, eluent delivery or detection technique.

CONCLUSION

We have developed a simple, rapid microanalytical method for measurement of bumetanide in plasma and urine. The utility of the method was demonstrated in a clinical investigation of bumetanide disposition in an infant. Further studies using the analytical method described are in progress to assess bumetanide disposition in specialized pediatric populations.

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