thiazolidine with thiol groups in the skin cannot be determined at this time.

Thus, a series of thiazolidine derivatives of progesterone and testosterone were prepared and characterized. Except for VII the thiazolidines were found to be inactive or less active than their parent steroids in animal models. This lack of activity can be explained by the rapid distribution of the thiazolidines out of blood followed by a slow release of the parent, such that the biological concentration of steroid is always too low to be biologically effective.

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High-Performance Liquid Chromatographic Assay for Bumetanide in Plasma and Urine

DAVID E. SMITH

Received May 26, 1981, from the *University of Michigan, College of Pharmacy, Ann Arbor, MI 48109.* Accepted for publication August 20, 1981.

Abstract □ A new high-performance liquid chromatographic (HPLC) method was developed for the analysis of bumetanide in plasma and urine. A reversed-phase column was fitted to the instrument and fluorescent (excitation λ = 338 nm, emission λ = 433 nm) and UV (254 nm) detectors were utilized to monitor simultaneously bumetanide and the internal standard, acetophenone, respectively. The assay is rapid, sensitive, and specific. Plasma bumetanide concentrations can be detected as low as 5 ng/ml using a 0.20-ml sample. Time-consuming extraction and/or derivatization steps are not required. The only clean-up procedure involved is the precipitation of plasma proteins with acetonitrile.

Keyphrases □ Bumetanide—high-performance liquid chromatographic determination in plasma and urine □ High-performance liquid chromatography—determination of bumetanide in plasma and urine □ Diuretics—bumetanide, determination in plasma and urine by high-performance liquid chromatography

Bumetanide (3-*n*-butylamino-4-phenoxy-5-sulfamoylbenzoic acid) is a new, high-ceiling diuretic with a pharmacological action similar to furosemide (1–4). However, on a molecular weight basis, bumetanide is 40–60 times more potent (1–4). Studies describing the pharmacokinetics and disposition of bumetanide have been sparse due to the lack of a sensitive and specific assay method. Fluorimetric (1), GLC (5), radioactive (6, 7), radioimmune (8), and high-performance liquid chromatographic (HPLC) (9) assays currently are available for the determination of bumetanide in biological fluids. However, all of these methods have inherent disadvantages, including time-consuming extraction, derivatization, or incubation steps (1, 5–9), large sample requirements (1, 5–7, 9), poor sensitivity (1, 5), or lack of specificity (1, 8).

Therefore, a rapid, sensitive, and specific HPLC assay was developed, without requiring prior extraction and/or derivatization, for the measurement of bumetanide in plasma and urine. The analytical method is suitable for bioavailability and pharmacokinetic studies in dogs and human subjects.

EXPERIMENTAL

Chemicals—Bumetanide[1], acetophenone[2], acetic acid[3], and phosphoric acid[4] were used as received. The methanol[3], acetonitrile[5] (glass-distilled), and deionized water[6] were filtered and degassed prior to use.

Standard Solutions—Bumetanide (4.12 mg) was dissolved in 50% acetonitrile–distilled water to yield a stock solution of 41.2 μg/ml. This stock solution was diluted 20- (2.06 μg/ml) and 100-fold (0.412 μg/ml) to give the working standard solutions for urine and plasma, respectively. Acetophenone was diluted in 50% acetonitrile–distilled water to yield a 0.50 mg/ml stock solution for urine and a 0.25 mg/ml stock solution for plasma.

Instrumentation—Samples were analyzed using a high-performance liquid chromatograph[7] equipped with a U6-K universal injector[8], a flu-

[1] Hoffmann-La Roche, Nutley, N.J.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Baker Analyzed Reagent, J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Certified ACS, Fisher Scientific Co., Fair Lawn, N.J.
[5] MCB Manufacturing Chemists, Cincinnati, Ohio.
[6] Milli-Q Reagent-Grade Water System, Millipore Corp., Bedford, Mass.
[7] Model 6000A, Waters Associates, Milford, Mass.
[8] Model U6-K, Waters Associates, Milford, Mass.

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Table I—Interday Variability of Slopes and Intercepts Derived from the Standard Curves of Bumetanide

Curve No.	Slope	Y-Intercept	r^2
	In Plasma[a]		
1	0.0235	0.0075	0.9998
2	0.0251	0.0237	0.9997
3	0.0249	0.0130	0.9997
4	0.0232	0.0082	0.9999
5	0.0234	0.0142	0.9997
Mean	0.0240	0.0133	0.9998
SD	0.0009	0.0065	0.0001
	In Urine[b]		
1	1.674	0.014	0.9997
2	1.804	0.022	0.9997
3	1.829	0.018	0.9993
4	1.626	0.011	0.9998
5	1.700	0.011	0.9999
Mean	1.727	0.015	0.9997
SD	0.087	0.005	0.0002

[a] Standard curves were constructed on five different days over a 12-day period. [b] 11-day period.

orescence spectrophotometer[9], and a variable wavelength UV absorbance detector[10]. A reversed-phase column[11] (25 cm × 4.6-mm i.d.) was fitted to the instrument and a dual-pen recorder[12] was used at a chart speed of 20 cm/hr. Fluorescent (excitation λ = 338 nm, emission λ = 433 nm) and UV (254 nm) detection were used to monitor simultaneously bumetanide and the internal standard, respectively.

Plasma Assay of Bumetanide—Bumetanide stock solution (0.412 μg/ml) was added in volumes of 0, 2.5, 5, 15, 30, 50, 75, and 100 μl to 0.20 ml of blank plasma to provide calibration standards of 0 (no bumetanide added), 5.2, 10.3, 30.9, 61.8, 103, 154, and 206 ng/ml. A 50-μl aliquot of the internal standard (0.25 mg/ml) was added to the mixture which was then shaken on a vortex mixer[13]. Precipitation of plasma proteins was accomplished by addition of 0.40 ml of acetonitrile. The mixture was shaken again on a vortex mixer and then sonicated[14] for ~2 min. After 10 min of centrifugation[15] (0.75 speed), the supernate was transferred to a clean test tube. An appropriate aliquot was then injected directly into the loop injector. Plasma samples were prepared in an identical manner except for the addition of bumetanide stock solution.

The mobile phase consisted of methanol–water–acetic acid (70:30:1), pumped isocratically at a flow rate of 1.5 ml/min, at ambient temperature.

Urine Assay of Bumetanide—Bumetanide stock solution (2.06 μg/ml) was added in volumes of 0, 2, 5, 15, 30, 50, 75, and 100 μl to 0.10 ml of blank urine and 0.10 ml distilled water to provide calibration standards of 0 (no bumetanide added), 0.0412, 0.103, 0.309, 0.618, 1.03, 1.54, and 2.06 μg/ml. A 50-μl aliquot of the internal standard (0.50 mg/ml)

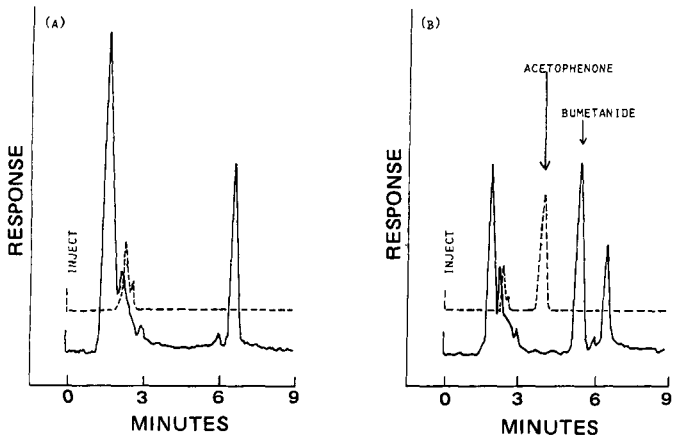

Figure 1—*Chromatograms for blank plasma (A) and for plasma spiked with bumetanide and the internal standard, acetophenone (B). Detection: fluorescence (——); UV (- - -).*

[9] Model 650-10S, Perkin-Elmer, Mountainview, Calif.
[10] Model 450, Waters Associates, Milford, Mass.
[11] Partisil-10 ODS-3, Anspec Co., Ann Arbor, Mich.
[12] Model 585, Linear, Irvine, Calif.
[13] Thermolyne Maxi-mix, VWR Scientific, Detroit, Mich.
[14] Bransonic Model 12, VWR Scientific, Detroit, Mich.
[15] Model HN-SII, VWR Scientific, Detroit, Mich.

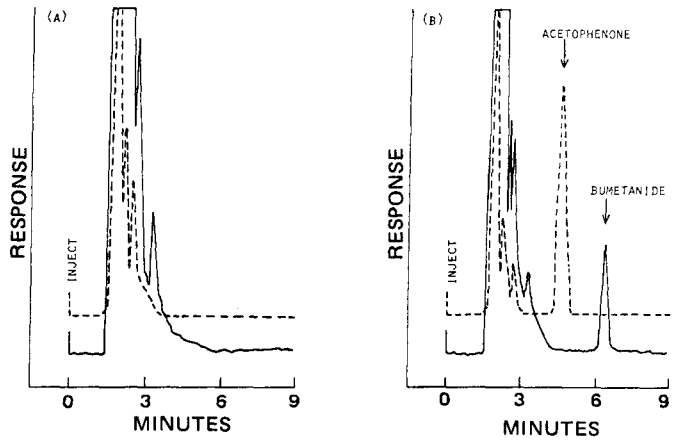

Figure 2—*Chromatograms for blank urine (A) and for urine spiked with bumetanide and the internal standard, acetophenone (B). Detection: fluorescence (——); UV (- - -).*

was added to the mixture which was then shaken on a vortex mixer. An appropriate volume was injected directly into the loop injector. Urine samples were prepared in an identical manner except for the addition of bumetanide stock solution.

The mobile phase consisted of 50% acetonitrile in 0.015 *M* phosphoric acid aqueous solution, pumped isocratically at a flow rate of 2.0 ml/min, at ambient temperature.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram for the analysis of bumetanide in plasma. Using the appropriate mobile phase, as described previously, the retention times for bumetanide and acetophenone were 5.5 and 4.0 min, respectively. A representative standard curve of the bumetanide–acetophenone peak height ratio over the bumetanide plasma concentration range 5.2–206 ng/ml resulted in the following linear least-squares regression equation: $Y = 0.0235X + 0.0075$; $r^2 = 0.9998$. With fluorescence detection, concentrations as low as 5 ng/ml were measured for bumetanide (peak to noise ratio ≥5) using 0.20-ml plasma samples.

Figure 2 shows a typical chromatogram for the analysis of bumetanide in urine. A different mobile phase than that used for the plasma assay was necessary for the analysis of bumetanide in urine, due to the presence

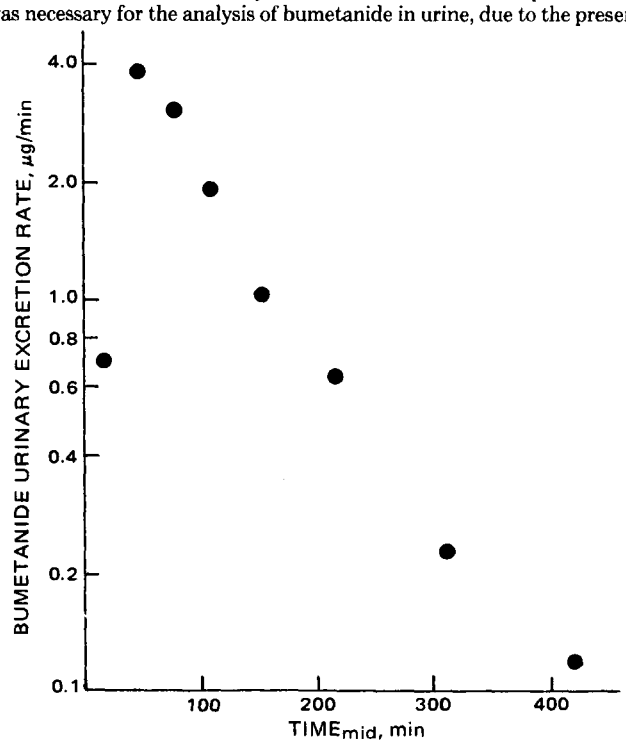

Figure 3—*Urinary excretion rate* versus *midpoint time plot of unchanged bumetanide after oral administration of 1.0 mg of bumetanide to a healthy volunteer.*

Table II—Intraday and Interday Variability of Bumetanide Concentration in Plasma Samples

Intraday[a]				Interday[b]			
Added, ng/ml		Measured, ng/ml	% Bias[c]	Added, ng/ml		Measured, ng/ml	% Bias[c]
5.2	Mean:	4.8	−7.7	5.2	Mean:	4.8	−7.7
	SD:	0.1			*SD*:	0.2	
	CV, %:	2.1			*CV*, %:	4.2	
61.8	Mean:	62.9	1.8	61.8	Mean:	62.2	0.6
	SD:	1.4			*SD*:	1.6	
	CV, %:	2.2			*CV*, %:	2.6	
154.0	Mean:	152.0	−1.3	154.0	Mean:	153.0	−0.6
	SD:	2.6			*SD*:	3.0	
	CV, %:	1.7			*CV*, %:	1.9	

[a] Mean values represent five different plasma samples. [b] Mean values represent plasma samples analyzed on 5 different days over a 12-day period. [c] % Bias = 100 × (measured concentration − added concentration)/added concentration.

Table III—Intraday and Interday Variability of Bumetanide Concentration in Urine Samples

Intraday[a]				Interday[b]			
Added, ng/ml		Measured, ng/ml	% Bias[c]	Added, ng/ml		Measured, ng/ml	% Bias[c]
41.2	Mean:	39.6	−3.9	41.2	Mean:	40.5	−1.7
	SD:	1.5			*SD*:	2.1	
	CV, %:	3.8			*CV*, %:	5.2	
618.0	Mean:	626.0	1.3	618.0	Mean:	632.0	2.3
	SD:	15.8			*SD*:	10.0	
	CV, %:	2.5			*CV*, %:	1.6	
1540.0	Mean:	1510.0	−1.9	1540.0	Mean:	1530.0	−0.6
	SD:	7.1			*SD*:	33.6	
	CV, %:	0.5			*CV*, %:	2.2	

[a] Mean values represent five different urine samples. [b] Mean values represent urine samples analyzed on 5 different days over an 11-day period. [c] % Bias = 100 × (measured concentration − added concentration)/added concentration.

Table IV—Recovery of Bumetanide from Plasma

Concentration, ng/ml	Peak Height Ratio, Plasma *versus* Water, %	
5.2		97.1
		96.6
		95.1
		93.0
		101.0
	Mean	96.6
	SD	3.0
61.8		
		96.4
		102.0
		95.8
		100.0
		97.3
	Mean	98.3
	SD	2.6
154		
		100.0
		99.3
		102.0
		101.0
		99.4
	Mean	100.0
	SD	1.0

of endogenous interference peaks. With the appropriate solvent system, as described previously, bumetanide and acetophenone had retention times in urine of 6.5 and 4.5 min, respectively. A representative standard curve of bumetanide–acetophenone peak height ratio over the bumetanide urine concentration range 0.0412–2.06 μg/ml resulted in the following linear least-squares regression equation: $Y = 1.674X + 0.014$; $r^2 = 0.9997$.

Both the plasma and urine assays were specific with respect to possible metabolite interference. Several metabolites of bumetanide (2′-alcohol, 3′-alcohol, 4′-alcohol, and 3′-acid), which have been reported recently in healthy volunteers (6), were obtained[1] and found to elute in the void volume prior to the elution of the compounds of interest (bumetanide and acetophenone). The desbutyl derivative was not available but should also elute in the void volume.

Standard curves of bumetanide in plasma (5.2–206 ng/ml) were constructed on 5 different days to determine the variability of the slopes and intercepts (Table I). The results show little day-to-day variability of slope and intercept as well as good linearity ($r^2 > 0.999$) over the plasma concentration range studied. The coefficient of variation for the slope was 3.8%. Standard curves of bumetanide in urine (0.0412–2.06 μg/ml) were also constructed (Table I). The coefficient of variation for the slope was 5.0% and all five curves showed good linearity ($r^2 > 0.999$) over the urine concentration range studied.

Table II shows the intra- and interday precision and accuracy for the plasma assay of bumetanide, assessed at three concentrations. The precision of the assay, as determined by the coefficient of variation, was ≤4.2% intraday as well as interday. In addition, the assay was quite accurate even at plasma concentrations as low as 5.2 ng/ml (bias = 7.7%). Other plasma concentrations had a bias ≤1.8%. A similar comparison was made for the urine assay of bumetanide as shown in Table III. The method was found to be precise (CV ≤5.2%) and accurate (bias ≤3.9%) intraday as well as interday.

The recovery of bumetanide from plasma proteins was assessed by comparing the bumetanide–acetophenone peak height ratio in plasma samples *versus* samples prepared in water. Five comparisons at three different concentrations were made. As shown in Table IV, the recovery of bumetanide from plasma was essentially complete at all concentrations.

Stability studies of plasma spiked with bumetanide (12.0, 50.0, and

Table V—Stability of Bumetanide in Plasma

Added Concentration, ng/ml	Measured Concentration/Added Concentration, % Days					
	0	1	3	8	21	31
12.0	96.7	100.0	—	104.0	108.0	97.5
50.0	104.0	98.4	97.6	99.6	96.2	95.0
140.0	102.0	99.3	97.1	99.3	100.0	101.0

140 ng/ml) were performed over a 31-day period (Table V). Plasma samples were stored in the freezer at −20° until the time of analysis. The results demonstrate that bumetanide can be stored frozen in plasma for at least 1 month without degradation.

Figure 3 shows a urinary excretion rate *versus* midpoint time plot of unchanged bumetanide after oral administration of 1.0 mg of bumetanide to a healthy volunteer. The biological half-life, as determined by linear regression using the last four data points from the log-linear terminal portion of the curve, was 84.5 min. The fraction of the oral dose excreted unchanged in the urine was 0.445. Both of these parameters are in agreement with values from other studies (5, 6–8, 10).

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Application of the Ammonia Gas-Sensing Electrode: Determination of Drugs Having a Carboxyamide Group by Decomposition with Acid

SHOICHIRO TAGAMI ˣ and MASAKO FUJITA

Received June 8, 1981, from the *Toyama Medical and Pharmaceutical University, Sugitani, Toyama, Japan.* Accepted for publication August 19, 1981.

Abstract □ A simple potentiometric method for the determination of drugs having a carboxyamide group is described. Ethenzamide, niacinamide, pyrazinamide, or salicylamide was refluxed with 20% HCl and the carboxyamide was hydrolyzed. The ammonia evolved at a pH >11 and was determined without separation from the decomposition solution using an ammonia gas-sensing electrode. A linear calibration plot was obtained with drugs in the range of 2×10^{-5}–1×10^{-2} *M*. This method was applied to the analysis of injection and powder-containing auxiliary compounds.

Keyphrases □ Ammonia gas-sensing electrode—analysis of drugs with a carboxyamide group by decomposition with acid □ Potentiometry—determination of ammonia using an ammonia gas-sensing electrode □ Carboxyamide groups—determination of carboxyamide moiety using ammonia gas-sensing electrode analysis

Methods for the determination of drugs having a carboxyamide group are based on the determination of ammonia liberated by refluxing the carboxyamide compounds in alkaline solution. The ammonia is distilled and determined by titration. The methods described in the United States (1), British (2), and Japanese (3) Pharmacopoeias are based on these principles. Spectrophotometric methods of analysis for the determination of carboxyamide having a pyridine ring are based on the König reaction (4) of pyridine derivatives with cyanogen bromide. These methods are sensitive and relatively free from interference; however, they have the disadvantage of using the extremely toxic cyanogen bromide. Polarographic and microbiological methods (5) have also been employed, but they are tedious and time consuming. The gas-permeable membrane electrode is advantageous because of its simplicity, accuracy of assay, and lower cost compared with the conventional methods. However, its applications to drug analysis have not been widely reported in the literature, although the analysis of *N*-unsubstituted carbamates and meprobamate has been studied (6).

This paper describes a potentiometric method for the determination of drugs having a carboxyamide group. Assay methods for ethenzamide (o-ethoxybenzamide), salicylamide, niacin (nicotinamide), and pyrazinamide were developed. The samples were refluxed with 20% HCl and the carboxyamide was hydrolyzed, liberating an equivalent amount of ammonium ion. After alkalinization, the ammonia was determined with an ammonia gas-sensing electrode.

EXPERIMENTAL

Apparatus—The ammonia gas-sensing electrode[1] consisted of an ammonia gas-permeable membrane, a pH glass-electrode, and a silver–silver chloride reference electrode. The potential measurement system consisted of a pH/mV meter[2] and recorder[3].

All measurements were carried out at 20° in an 80-ml cell equipped with a magnetic stirrer.

Reagents—Ethenzamide[4], salicylamide[4], and pyrazinamide[4] were purified twice by recrystallization from water and then dried *in vacuo* at room temperature for 5 hr. Niacinamide[5] was dried *in vacuo* at room temperature for 4 hr. Other chemicals used were reagent grade.

A stock solution of 0.1 *M* NH_4Cl was prepared for testing the ammonia response of the electrode, and 5 *M* NaOH was employed to adjust the pH of the solution to within the operating range of the electrode.

[1] Model 5002-05 T, Horiba, Co., Kyoto, Japan.
[2] Model F-7ss, Hitachi-Horiba Instruments, Horiba Co., Kyoto, Japan.
[3] Model EPR-22A, Toa-Denpa Co., Tokyo, Japan.
[4] Tokyo Kasei Co., Tokyo, Japan.
[5] Japanese Pharmacopoeia Reference Standard.

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