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Determination of azosemide and its metabolite in plasma, blood, urine and tissue homogenates by high-performance liquid chromatography

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Abstract

High-performance liquid chromatographic methods were developed for the determination of azosemide and its metabolite, M1, in human plasma and urine and rabbit blood and tissue homogenates. The methods involved deproteinization of the biological samples: 2.5 volumes of acetonitrile were used for the determination of azosemide and 1 volume of saturated $Ba(OH)_2$ and $ZnSO_4$ for that of M1. A 50-μl aliquot of the supernatant was injected onto a C_{18} reversed-phase column in each instance. The mobile phases employed were 0.03 *M* phosphoric acid–acetonitrile (50:40, v/v) for azosemide and 0.03 *M* phosphoric acid/0.2 *M* acetic acid–acetonitrile (83:17, v/v) for M1. The flow-rate was 1.5 ml/min in both instances. The column effluent was monitored by ultraviolet detection at 240 and 236 nm for azosemide and M1, respectively. The retention times for azosemide and M1 were 6.0 and 8.3 min, respectively. The detection limits for both azosemide and M1 in both human plasma and urine were 50 ng/ml. The coefficients of variation of the assay were generally low (below 11.0%) for plasma, urine, blood and tissue homogenates. No interferences from endogenous substances or other diuretics tested were observed.

1. Introduction

Azosemide [5-(4-chloro-5-sulphamoyl-2-thenylaminophenyl) tetrazole] is a loop diuretic which resembles furosemide in its mode of action [1], and is also essentially equipotent to furosemide on a mass basis [2]. Fluorimetric [1] and high-performance liquid chromatographic (HPLC) [3–5] methods have been developed for the assay of azosemide and/or its metabolites. Total radioactivity was also measured after intravenous (i.v.) administration of [^{14}C]azosemide [6]. In the reported HPLC methods, azosemide in animal feed diet mixture [4], urine and serum [3] and azosemide and its metabolites, 5-(2-amino-4-chloro-5-sulphamoylphenyl) tetrazole (M1) and glucuronide conjugates of azosemide (M8) in plasma, urine and faeces [5] were determined. However, in the previous work [4,5], a tedious extraction procedure, a gradient system and 0.3 ml of serum [3] were employed.

This paper describes HPLC methods with simple preparation procedures for the determination of azosemide and M1 in human plasma

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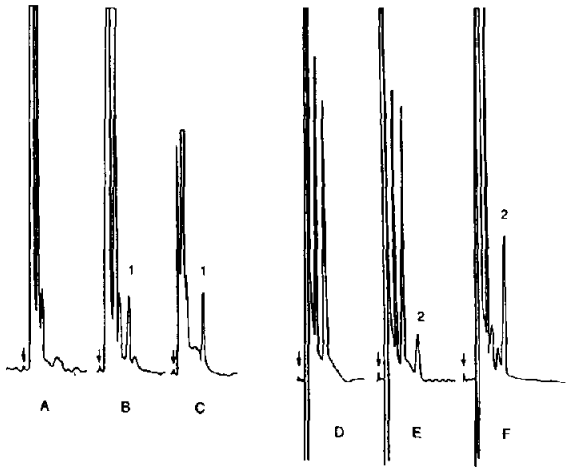

Fig. 1. Chromatograms of (A) drug-free human plasma, (B) human plasma spiked with 0.5 μg/ml of azosemide, (C) plasma obtained from a rat 240 min after 1-min intravenous administration of azosemide at 10 mg/kg, (D) drug-free human plasma, (E) human plasma spiked with 1.0 μg/ml of M1, and (F) plasma obtained from a rat 30 min after 1-min intravenous administration of M1 at 10 mg/kg. Peaks: 1 = azosemide (6.0 min); 2 = M1 (8.3 min). The arrows mark the points of injection. The detector sensitivity was set at 0.01 AUFS and recorder sensitivity was set at 10 mV (A, B, D and E), 20 mV (F) and 100 mV (C).

and urine and rabbit blood and tissue homogenates.

2. Experimental

2.1. Chemicals

Azosemide and M1 were kindly supplied by Sam Jin Pharmaceutical (Seoul, South Korea) and Boehringer (Mannheim, Germany), respectively. The other chemicals were of analytical-reagent grade or HPLC grade and were used as received.

2.2. Preparation of standard solutions

Stock standard solutions of azosemide and M1 (1 and 10 mg/ml) were prepared by dissolving the azosemide and M1 powder in minimum volume of 0.1 *M* NaOH, followed by appropriate dilutions with distilled water. Working standard solutions of azosemide and M1 in water, plasma or urine were prepared by spiking with the appropriate volume (less than 10 μl/ml) of the stock standard solution giving final concentrations of 100, 50, 20, 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0.05 μg/ml. HPLC assay results for only the 50, 5, 0.5, 0.05 μg/ml levels are given in Table 1. Recoveries from plasma, urine and tissue homogenates were calculated by dividing the peak heights of the drugs in biological samples by those in water. Response factors were calculated by dividing the peak heights (cm) of the drugs by their concentrations (μg/ml).

2.3. Sample preparation

To 50 μl of human plasma or urine samples were added either 125 μl of acetonitrile for the determination of azosemide or 50 μl of saturated $Ba(OH)_2$ and $ZnSO_4$ for the determination of M1. After vortex mixing and centrifugation at 9000 *g* for 10 min, 50 μl of the supernatant were injected directly onto the HPLC column. Rabbit tissues were homogenized with four volumes of 0.9% NaCl injectable solution using a tissue homogenizer (Tissuemizer, Model SDT-1800; Tekman, Cincinnati, OH, USA) and immediately centrifuged at 9000 *g* for 10 min. A 50-μl volume of supernatant was collected and treated as described for plasma and urine samples. Two volumes of distilled water were added to rabbit blood to facilitate the haemolysis of blood cells and to increase the reproducibility of the recovery of azosemide and M1 from whole blood [7,8]. After vortex mixing and centrifugation, a 50-μl aliquot of the supernatant was collected and processed as described for plasma and urine samples.

2.4. HPLC apparatus

The HPLC system consisted of a Model 7125 injector (Rheodyne, Cotati, CA, USA), a Model 1330 pump (Bio-Rad, Japan Servo, Tokyo, Japan), a reversed-phase C_{18} column, (30 cm × 3.9 mm I.D.; particle size 10 μm) (Waters, Milford, MA, USA), a Model 1306 UV detector (Bio-Rad) and a Model 1200 recorder (Linear,

Reno, NV, USA). The mobile phases, 0.03 *M* phosphoric acid–acetonitrile (50:40, v/v) for azosemide and 0.03 *M* phosphoric acid/0.2 *M* acetic acid–acetonitrile (83:17, v/v) for M1, were run at a flow-rate of 1.5 ml/min and the column effluent was monitored by UV detection at 240 nm for azosemide and 236 nm for M1. The retention times of azosemide and M1 were *ca.* 6.0 and 8.3 min, respectively.

3. Results and discussion

The UV absorption maxima of azosemide and M1 occurred at 240 and 236 nm, respectively, and were therefore used for the HPLC analysis. Fig. 1 shows typical chromatograms of drug-free human plasma, drug standards in human plasma and plasma from a rat receiving azosemide or M1; the corresponding chromatograms for human urine and rabbit tissue homogenates are shown in Figs. 2 and 3, respectively. No interferences from endogenous substances were observed in any of the biological samples. The peaks of azosemide and M1 were symmetrical and eluted at *ca.* 6.0 and 8.3 min, respectively. We also noted no interferences from some other commonly used diuretics that we tested, namely furosemide, bumetanide, hydrochlorothiazide, amiloride and spironolactone.

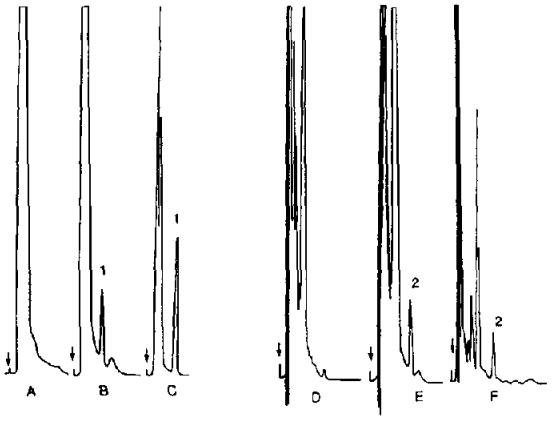

Fig. 2. Chromatograms of (A) drug-free human urine, (B) human urine spiked with 0.5 μg/ml of azosemide, (C) urine obtained from a rat collected between 0 and 8 h after 1-min intravenous administration of azosemide at 10 mg/kg, (D) drug-free human urine, (E) human urine spiked with 0.5 μg/ml of M1, and (F) urine obtained from a rat collected between 0 and 8 h after 1-min intravenous administration of azosemide at 20 mg/kg. Peaks: 1 = azosemide (6.0 min); 2 = M1 (8.3 min). The arrows mark the points of injection. The detector sensitivity was set at 0.01 AUFS and recorder sensitivity was set at 10 mV (A, B, D and E) and 100 mV (C and F).

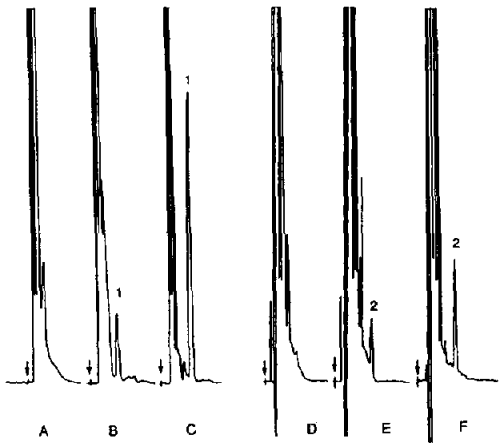

Fig. 3. Chromatograms of (A) drug-free rabbit liver homogenate, (B) rabbit liver homogenate spiked with 0.5 μg/ml of azosemide, (C) liver homogenate obtained from a rat 30 min after 1-min intravenous administration of azosemide at 20 mg/kg, (D) drug-free rabbit small intestine homogenate, (E) rabbit small intestine homogenate spiked with 0.5 μg/ml of M1, and (F) small intestine homogenate obtained from a rat 30 min after 1-min intravenous administration of azosemide at 20 mg/kg. Peaks: 1 = azosemide (6.0 min); 2 = M1 (8.3 min). The arrows mark the points of injection. The detector sensitivity was set at 0.01 AUFS and recorder sensitivity was set at 10 mV (A, B, D and E), 20 mV (F) and 50 mV (C).

The detection limits for both azosemide and M1 in both human plasma and urine were 50 ng/ml (Table 1), based on a signal-to-noise ratio of 3.0. The mean within-day coefficients of variation (C.V.s) of azosemide in human plasma and urine were 3.15% (range 1.64–5.55%) and 2.82% (range 1.67–4.24%), respectively, and the corresponding values for M1 were 5.75% (range 3.46–8.32%) and 3.76% (range 1.72–7.88%) (Table 1). The between-day C.V.s for the analysis of the same samples on five days for azosemide were 1.68 and 1.51% in human plasma and urine, respectively, and the corresponding values for M1 were 1.03 and 1.89%. Mean analytical recoveries of spiked azosemide

Table 1
Response factors and recoveries of azosemide and M1 at various concentrations in human plasma and urine

Concentration (μg/ml)	Human plasma		Human urine	
	Response factor[a]	Recovery[b] (%)	Response factor[a]	Recovery[b] (%)
Azosemide				
50	6.05 (2.56)	96	5.20 (2.04)	82
5	5.66 (2.08)	100	5.23 (2.37)	93
0.5	6.03 (4.52)	105	5.28 (2.23)	92
0.05	6.10 (2.99)	103	5.22 (4.24)	88
M1				
50	1.34 (3.46)	27	3.94 (3.10)	79
5	1.39 (6.47)	29	3.99 (2.91)	83
0.5	1.34 (6.38)	32	3.94 (4.09)	93
0.05	1.36 (8.10)	27	4.02 (5.96)	79

Values in parentheses are C.V. (%); $n = 6$.
[a] Peak height (cm) divided by its concentration (μg/ml); mean.
[b] Relative recovery compared with water.

Table 2
Response factors and recoveries of azosemide and M1 at various concentrations in rabbit blood and tissue homogenates

Sample[a]	Concentration (μg/ml)	Azosemide		M1	
		Response factor[b]	Recovery[c] (%)	Response factor[b]	Recovery[c] (%)
Blood	1	4.51 (4.26)	78	0.88 (5.33)	21
	0.1	4.45 (4.49)	74		
Liver	1	4.06 (1.92)	70	2.51 (1.06)	60
	0.1	4.58 (8.65)	77	2.83 (4.08)	54
Lung	1	3.63 (4.49)	62	2.94 (0.59)	70
	0.1	4.25 (9.63)	71	3.15 (8.66)	61
Heart	1	2.89 (5.60)	50	3.37 (7.57)	80
	0.1	2.73 (6.93)	46	3.43 (6.06)	66
Brain	1	2.80 (1.29)	48	4.03 (2.65)	95
	0.1	3.02 (5.82)	50	4.23 (4.92)	81
Kidney	1	4.12 (1.91)	71	2.82 (7.24)	67
	0.1	3.80 (4.74)	64	2.63 (7.75)	51
Muscle	1	3.80 (3.23)	66	3.04 (5.24)	72
	0.1	4.15 (7.52)	69	3.37 (7.48)	65
Stomach	1	4.75 (2.97)	82	4.00 (1.28)	95
	0.1	4.42 (2.61)	74	4.20 (2.38)	81
Intestine	1	4.28 (2.74)	74	3.31 (3.70)	79
	0.1	4.68 (8.29)	78	3.73 (4.09)	72
Spleen	1	5.05 (6.75)	87	2.25 (1.33)	53
	0.1	4.60 (9.96)	77	2.77 (11.0)	53

Values in parentheses are C.V. (%); $n = 3$.
[a] Tissue samples were homogenized with four volumes of normal saline.
[b] Azosemide or M1 peak height (cm) divided by its concentration (μg/ml); mean.
[c] Relative recovery compared with water.

from human plasma and urine were 100% (range 96–105%) and 92% (range 82–100%), respectively, and the corresponding values for M1 were 28% (range 25–33%) and 84% (range 77–96%) (Table 1). The exact reason for the low recovery of M1 from human plasma is unknown. However, the recovery of M1 from rat plasma was *ca.* 85%, hence M1 might coprecipitate during the deproteinization procedures and this could be the result of binding and/or adsorption of M1 to the endogenous compounds in human plasma.

The HPLC method was also successful for the determination of azosemide and M1 in rabbit tissues (Table 2). The detection limits for azosemide were 100 ng/ml for blood, heart, kidney and stomach and 50 ng/ml for liver, lung, brain, muscle, intestine and spleen. The detection limits for M1 were 500 ng/ml for blood and 100 ng/ml for all other tissues. The mean within-day C.V.s for azosemide ranged from 3.59% (brain) to 7.84% (lung), and the values for M1 were from 1.48% (stomach) to 6.57% (kidney). The mean analytical recoveries of spiked azosemide ranged from 48.2% (heart) to 82.9% (spleen) and the values for M1 were from 21.1% (blood) to 90.1% (brain).

Azosemide was administered at 10 mg/kg (total injection volume *ca.* 1 ml) in 1 min via the jugular vein to Sprague–Dawley rats (260–305 g, $n = 8$). Approximately 0.12 ml of blood was collected at appropriate time intervals and 50 μl of plasma sample were stored in a freezer prior to the HPLC assay of azosemide. The mean arterial plasma concentration–time profile of azosemide is shown in Fig. 4. The mean terminal half-life, total body clearance, apparent volume of distribution at the steady state and mean residence time of azosemide were 44.3 min, 3.96 ml/min/kg, 140 ml/kg and 37.6 min, respectively.

Blood partition studies were performed [7,8] using blood from three New Zealand White rabbits. The mean blood partition (plasma-to-blood concentration ratio) value of azosemide was 1.36 at azosemide whole blood concentrations of 1–20 μg/ml, and azosemide was found to reach equilibrium between plasma and blood cells immediately after spiking of azosemide to whole blood. The tissue concentrations of azosemide and M1 were also measured at 30 min after a 1-min intravenous administration of azosemide at 20 mg/kg to one rat; the values for azosemide were 64.9, 55.0, 12.6, 9.20, 60.4, 1.05, 1.41, 37.3, 4.39 and 2.76 μg/g tissue for plasma, liver, lung, heart, kidney, muscle, stomach, small intestine, large intestine and spleen, respectively, and the values for M1 were 0.165, 0.679, 0.917, 8.28 and 0.259 μg/g tissue for plasma, liver, kidney, small intestine and large intestine, respectively (Table 3).

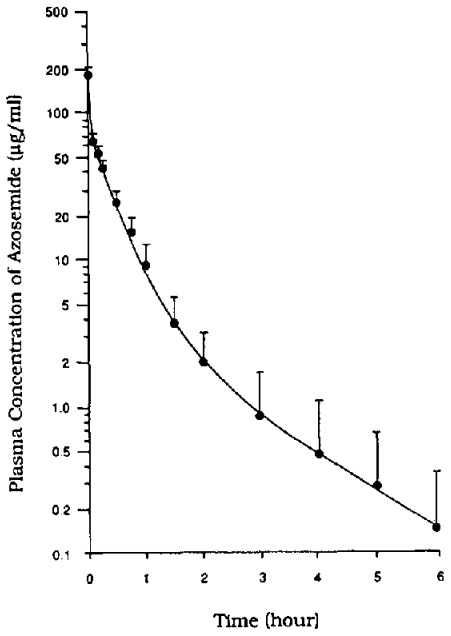

Fig. 4. Mean arterial plasma concentration–time profile of azosemide after 1-min intravenous administration of azosemide at 10 mg/kg to eight Sprague–Dawley rats. Bars represent standard deviations.

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Table 3
Amounts of azosemide and M1 remaining in each organ (μg/g tissue) at 30 min after intravenous administration of azosemide at 20 mg/kg to a rat

Organ	Azosemide		M1	
	Amount (μg/g tissue)	T/P ratio[a]	Amount (μg/g tissue)	T/P ratio[a]
Plasma	64.9	1.00	0.165	1.00
Liver	55.0	0.847	0.679	4.12
Lung	12.6	0.194	N.D.	N.C.
Heart	9.20	0.142	N.D.	N.C.
Brain	N.D.[b]	N.C.[c]	N.D.	N.C.
Kidney	60.4	0.931	0.917	5.56
Muscle	1.05	0.0162	N.D.	N.C.
Stomach	1.41	0.0217	N.D.	N.C.
Small intestine	37.3	0.575	8.28	50.2
Large intestine	4.39	0.0676	0.259	1.57
Spleen	2.76	0.0425	N.D.	N.C.

[a] Tissue to plasma ratio.
[b] N.D. = not dectectable.
[c] N.C. = not calculable.

and Dr. E. Besenfelder (Boehringer) for the kind donation of azosemide and M1, respectively.

5. References

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