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Note

Gas chromatographic determination of the high-ceiling diuretic ethacrynic acid and its thiol adducts in plasma of guinea pigs

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Ethacrynic acid, 2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxyacetic acid (I, Fig. 1), is a high-ceiling diuretic [1]. Although it has been available for over twenty years [2], assay methodology for its estimation in plasma has only recently been published [3]. A UV spectrophotometric method employing 1-ml samples has been used [4] to study binding of I to dog plasma proteins and to bovine serum albumin, and two procedures have been published for its assay in pharmaceutical formulations. Gupta et al. [5] published a high-performance liquid chromatographic (HPLC) method which was suitable for quantities in the

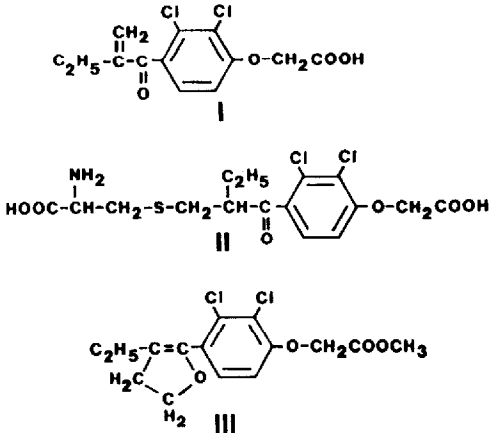

Fig. 1. Structures of ethacrynic acid (I), cysteine adduct of ethacrynic acid (II) and esterification by-product of ethacrynic acid (III).

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0.5–3 μg range while Goerlitzer and Hoebbel [6] titrated the iodine released from KI after I had been reacted with excess potassium bromide–potassium bromate and hydrochloric acid. Stueber et al. [3] described a procedure in which I is extracted from 1.0 ml of plasma and derivatized with pentafluorobenzyl bromide followed by gas chromatographic–mass spectrometric (GC–MS) measurement. Levels in the ng/ml and μg/ml range could be measured and the limit of detection was between 10 and 20 ng/ml of plasma.

Studies in our laboratory on the ototoxic interaction of I with aminoglycoside antibiotics [7] indicated a need for methodology suitable to measure I and its cysteine adduct (II, Fig. 1), a metabolite [8] which has potent diuretic activity, in small plasma samples as well as in cochlear perilymph samples from guinea pigs treated with I. Since the procedures cited above are not suitable for the small samples (less than 20 μl, containing less than 1 ng/μl I) of guinea pig perilymph available for assay, we have developed a GC method and are now reporting on its application to plasma.

Midha et al. [9] studied the derivatization of I by diazomethane and by hydrogen chloride in methanol. With diazomethane I gave as the major product a methyl ester (III, Fig. 1) containing an additional $-CH_2$ moiety as part of a new ring. With methanolic hydrogen chloride I gave predominantly its expected methyl ester plus a minor product having a longer retention time, corresponding to III.

The condensation of I with L-cysteine to form II is readily reversible [10] and we have found that an alkaline solution of II releases I which, after acidification, can be extracted into butyl chloride (in which II is insoluble) and measured. This provides a means for assaying of II in the presence of I.

EXPERIMENTAL

Reagents and supplies

Solvents. Glass-distilled butyl chloride, methanol and 2,2,4-trimethylpentane (TMP) were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). HPLC-grade water was purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.).

Reagents. DuPont reagent-grade sulfuric and hydrochloric acids were purchased from VWR Scientific (San Francisco, CA, U.S.A.). Reagent-grade sodium bicarbonate was from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and sodium hydroxide from American Scientific & Chemical (Portland, OR, U.S.A.). Reagent-grade sodium phosphates, monobasic and dibasic, were from Mallinckrodt (St. Louis, MO, U.S.A.).

Other supplies. Ethacrynic acid, I, and the internal standard, 2,3-dichloro-4-(2-ethylidene-1-oxobutylphenoxyacetic) acid, the next higher homologue of I [11], were gifts from Dr. E.J. Cragoe, Jr. of Merck Sharp & Dohme (West Point, PA, U.S.A.). They were dissolved in HPLC-grade water containing a slight molar excess of sodium bicarbonate. The cysteine adduct, II, was prepared as reported by Sprague and Schultz [12]. Screw-capped thick-walled glass vials (Reacti-Vial™) and a siliconizing fluid (Surfasil™) were obtained from Pierce (Rockford, IL, U.S.A.).

Gas chromatography

A Hewlett-Packard Model 5830A gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The glass column was 1.8 m × 2 mm I.D., packed with 3% OV-225 on 80–100 mesh Chromosorb W HP. Carrier gas was methane–argon (5:95) at a flow-rate of about 45 ml/min. Column oven temperature was set at 225 °C, injection chamber at 275 °C and detector at 325 °C. Recently we have been using a fused-silica 530-μm capillary column, HP-225 (25% cyanopropyl silicone) with good results.

Measurement of I in guinea pig plasma

Plasma intended for assay was promptly mixed with an equal volume of 0.1 *M* hydrochloric acid and refrigerated until assayed, normally within three days. Free I was determined first. Acidified plasma (20 μl) was added to 10 μl of solution containing internal standard (1 ng/μl) in a 300-μl vial followed by 100 μl butyl chloride. After brief agitation with a vortex mixer, the upper layer was removed with a Hamilton syringe and transferred to a clean siliconized 1000-μl vial. The extraction was repeated with two additional 100-μl portions of butyl chloride. The combined organic solvent layers were evaporated in a stream of nitrogen at 40 °C, then dried for 2 h or longer (preferably overnight) in a vacuum desiccator containing anhydrous calcium chloride. To the dry residue was added 100 μl of 10% hydrogen chloride in methanol which had been prepared by dissolving gaseous hydrogen chloride (from mixing concentrated hydrochloric acid with sulfuric acid) in glass-distilled methanol. The vial was capped and heated in an oven at 65–70 °C for 15 min, then chilled to below room temperature. To the cold reaction mixture were added 100 μl of trimethylpentane and 400 μl of 1 *M* phosphate buffer, pH 7.0, and the (reclosed) vial contents were mixed. After brief centrifugation at 850 *g* to facilitate separation of the layers, aliquots (usually 1–2 μl) of the upper layer were removed with a clean 10-μl Hamilton syringe and injected into the gas chromatograph. Areas under the I peaks at retention times of approximately 3.5 and 6.2 min were integrated and their sum divided by the area under the internal standard peak at retention time 5.5 min. From this ratio the I content of the sample was calculated using the slope and intercept derived from the least-squares regression line obtained with spiked plasma samples of known I content. Standard curves were linear over the range 0–50 ng/μl. Alkali-releasable I was then determined. A 20-μl volume of 0.1 *M* sodium hydroxide was added to the aqueous layer remaining after removal of free I, and the mixture was agitated for I min. Brief mixing with 5 μl of 1 *M* hydrochloric acid was then followed by addition of 10 μl internal standard and extraction with three 100-μl portions of butyl chloride. The I assay was completed as described above.

In vivo studies

Guinea pigs under allobarbital–urethane anesthesia received intravenous doses of 40 mg/kg I or equimolar doses of II. Blood samples were removed at the times indicated in Table III and plasmas promptly prepared from them for assay.

RESULTS AND DISCUSSION

Fig. 2 shows chromatograms of methyl esters from (a) control guinea pig plasma to which 10 μl of the internal standard (1 ng/μl) had been added, (b) plasma to which both I (10 ng/μl) and internal standard (1 ng/μl) had been added and (c) plasma from a guinea pig 1 h after injection of I, from which free I had been removed and the (apparently) S-bound I then released by 0.1 *M* sodium hydroxide treatment.

Although only a single peak, showing a retention time of 3.6 min, is obtained when samples containing methyl ester from 200 pg or less of I are chromatographed, larger amounts (e.g. 1 ng) give rise to two peaks, the second of which has retention time of 6.3 min. It is probable that the second peak is due to the by-product III since GC–MS showed the masses to differ by 14 units. The area under the second peak usually accounts for about 25–30% of the sum of all peaks due to I.

Tables I and II present the results of assays of plasmas to which I or II had been added. The columns A–E contain data from experiments performed several days apart. Recoveries are excellent in the range 1–50 ng/μl and standard curves are reproducible.

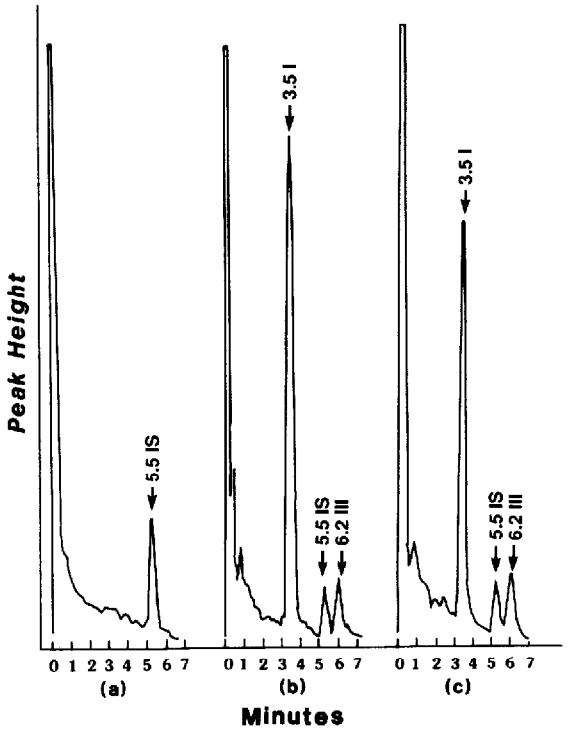

Fig. 2. Gas chromatograms of (a) control guinea pig plasma to which 1 ng/μl internal standard (IS) was added, (b) plasma to which 10 ng/μl ethacrynic acid (I) and 1 ng/μl IS were added and (c) plasma from a I-treated guinea pig showing peaks due to I released by alkali after removal of free I and to added IS (1 ng/μl).

TABLE I

RECOVERY OF ETHACRYNIC ACID (I) FROM SPIKED GUINEA PIG PLASMA

Added (ng/μl)	Recovered* (ng/μl)			Recovery (mean ± S.D.) (%)
	A	B	C	
0	0.3 ± 0.0	0.0	0.2 ± 0.2	–
1	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	113.8 ± 10.2
10	9.6 ± 1.2	9.2 ± 0.7	10.0 ± 0.7	100.2 ± 11.6
20	20.2 ± 2.8	20.8 ± 1.4	19.2 ± 0.2	100.3 ± 8.7
35	34.6 ± 2.9	33.7 ± 0.5	35.4 ± 3.4	98.8 ± 6.8
50	50.3 ± 5.9	50.7 ± 3.3	49.9 ± 2.2	100.7 ± 7.1
1–50				102.8 ± 6.2

*Mean ± S.D. ($n = 3$) of experiments on separate days (A, B, C).

TABLE II

RECOVERY OF CYSTEINE ETHACRYNIC ACID (II) FROM SPIKED GUINEA PIG PLASMA

Added (ng/μl)	Recovered* (ng/μl)		Recovery (mean ± S.D.) (%)
	D	E	
10	10.3 ± 0.8	10.8 ± 0.6	105.5 ± 7.1
20	20.0 ± 0.8	19.8 ± 0.8	99.5 ± 3.7
35	34.2 ± 5.6	32.1 ± 1.7	94.7 ± 11.1
50	50.5 ± 4.8	52.0 ± 0.8	102.5 ± 6.4
10–50			100.6 ± 8.1

*After conversion to I (see Experimental). Mean ± S.D. ($n = 3$) of experiments on separate days (D, E).

TABLE III

GUINEA PIG PLASMA LEVELS OF FREE AND ALKALI-RELEASABLE I FOLLOWING INJECTION OF I OR II

I = Ethacrynic acid; II = cysteine adduct of I.

Time after injection (min)	Concentration (mean ± S.D.) (μg/ml)			
	After 40 mg/kg I		After 56 mg/kg II*	
	Free I	I released by alkali	Free I	I released by alkali
12	–	–	27.3	80.8
17	14.8 ± 4.1	14.3 ± 2.0	–	–
23	–	–	21.2	67.3
30	4.4 ± 0.4	18.3 ± 0.0	–	–
60	2.2 ± 0.1	7.5 ± 0.3	–	–
80	–	–	2.9	4.8
120	0.6 ± 0.1	3.3 ± 2.4	–	–
154	–	–	0.5	1.2

*Mole equivalent of 40 mg/kg I.

In Table III are reported data from guinea pigs treated with equimolar doses of I and II. They show that both free and alkali-releasable I are present after intravenous injection of either drug. We have observed that the glutathione adduct, a I metabolite which is excreted into bile [13], also releases I on brief exposure to alkali. In the absence of specific identifications, we cannot assume that II is the sole source of all I released by alkali; other alkali-labile adducts are possible.

First-order analysis of the plasma decay of I in Table III gave a half-life of 15 min. Similar analysis of II gave 22 min. The more rapid elimination of I is probably a result of its more rapid redistribution and metabolism over that of II. However, the interconvertibility of I and II would complicate analysis of these pharmacokinetic processes.

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REFERENCES

1 I.M. Weiner and G.H. Mudge, in A.G. Gilman, L.G. Goodman, T.W. Rall and F. Murad (Editors), Goodman and Gilman's Pharmacological Basis of Therapeutics, MacMillan, New York, 7th ed., 1985, pp. 896–900.
2 M. Windholz (Editor), The Merck Index, Merck, Rahway, NJ, 10th ed., 1983, p. 539.
3 W. Stueber, E. Mutschler and D. Steinbach, J. Chromatogr., 227 (1982) 193–198.
4 E. Ronwin and A.G. Zacchei, Can. J. Biochem., 45 (1967) 1433–1443.
5 V.D. Gupta, C.W. Gibbs and A.G. Ghanekar, Jr., Am. J. Hosp. Pharm., 35 (1978) 1382–1385.
6 K. Goerlitzer and G. Hoebbel, Arch. Pharm., 312 (1979) 631–632.
7 R.E. Brummett and K.E. Fox, in E. Whelton and H.C. Neu (Editors), The Aminoglycosides, Marcel Dekker, New York, 1982, pp. 437–446.
8 K.H. Beyer, J.E. Baer, J.K. Michaelson and H.F. Russo, J. Pharmacol. Exp. Ther., 147 (1965) 1–22.
9 K.K. Midha, J.W. Hubbard, C. Charette and H.W. Jun, J. Pharm. Sci., 67 (1978) 975–978.
10 E.J. Cragoe, Jr., in E.J. Cragoe, Jr. (Editor), Diuretics: Chemistry, Pharmacology and Medicine, Wiley, New York, 1983, p. 214.
11 E.M. Schultz, E.J. Cragoe, Jr., J.B. Bicking, W.A. Bolhofer and J.M. Sprague, J. Med. Pharm. Chem., 5 (1962) 660–662.
12 J.M. Sprague and E.M. Schultz, U.S. Pat., 3 453 312 (1969), example 19.
13 C.D. Klaasen and T.J. Fitzgerald, J. Pharmacol. Exp. Ther., 191 (1974) 548–556.