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DETERMINATION OF ESTRAMUSTINE AND ITS 17-KETO METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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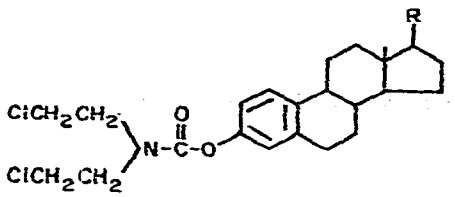
SUMMARY

A rapid, sensitive and specific high-performance liquid chromatographic (HPLC) assay was developed for the determination of estramustine and its 17-keto metabolite in plasma. The assay involves extraction of the compounds into hexane from plasma buffered to pH 9.0, the residue obtained by evaporation of the hexane extract is dissolved in the mobile phase hexane—ethanol (92.5:7.5) with HPLC analysis performed on a 5-μm silica gel column using a fluorescence detector with excitation at 195 nm and emission at wavelengths greater than 250 nm. The overall recoveries and limits of sensitivity for estramustine and the 17-keto metabolite are 74.7% and 40 ng/ml of plasma and 85.1% and 50 ng/ml of plasma, respectively. The method was used to obtain plasma concentration—time profiles in three subjects with prostatic cancer following oral administration of a single 7 mg/kg dose of estramustine phosphate.

INTRODUCTION

Estramustine phosphate (I), estradiol-3N-bis(2-chloroethyl) carbamate-17β-dihydrogen phosphate (Fig. 1), a nitrogen mustard derivative of estradiol-17β-phosphate has been shown to be effective in the treatment of prostatic carcinoma [1—5]. Studies on the distribution, absorption and metabolism of the drug in man, rat and dog have been reported [6—10]. In man, orally administered estramustine phosphate is rapidly dephosphorylated in the gastrointestinal tract to yield estramustine (II) [8]. Estramustine (II) and its 17-keto analogue (III), estrone-3N-bis(2-chloroethyl) carbamate, (Fig. 1) are the major metabolites found in plasma [9].

A radioimmunoassay was recently reported and used for the determination of intact estramustine phosphate following intravenous administration to dogs [10]. In addition, a gas—liquid chromatographic (GLC) method using flame ionization detection (FID) to quantitate II and III has been developed [9]. The



R	Compound
$-OP(OH)_2$ (=O)	Estramustine Phosphate, I
$-OH$	Estramustine, II
$=O$	Estrone-3N-bis(2-Chloroethyl) carbamate, III

Fig. 1. Chemical structures of estramustine phosphate (I), estramustine (II) and estrone-3N-bis(2-chloroethyl) carbamate (III).

GLC assay utilizes selective extraction of the compounds into diethyl ether, purification of the extract by hexane washing, alumina chromatography, and GLC—FID of II and III as the silyl derivative and intact compound, respectively.

The present communication describes a high-performance liquid chromatographic (HPLC) assay with fluorescence detection capable of simultaneous measurement of the two unconjugated metabolites II and III in plasma. The assay is rapid and is performed directly on an extract of plasma and eliminates the time-consuming clean-up procedures and derivatization steps required for the GLC assay [9]. The HPLC assay has comparable sensitivity to the GLC assay [9] with the advantage that only 1 ml of plasma is required. The method was used to obtain the plasma concentration—time profiles of II and III in three subjects following single 7 mg/kg oral doses of estramustine phosphate (I).

EXPERIMENTAL

Column

The column used was a 25 cm × 4.6 mm I.D. stainless-steel prepacked column containing 5 μm Partisil PXS 5/25 silica gel (Whatman, Clifton, NJ, U.S.A.).

Instrumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector (Waters Assoc., Milford, MA, U.S.A.) and a Model FS-970 fluorescence detector (Schoeffel Instrument, Westwood, NJ, U.S.A.). A deuterium lamp was used to provide monochromatic excitation at 195 nm while fluorescence emission greater than 250 nm was measured through a UV 28 ultraviolet transmitting filter with a quartz window photo multiplier tube. The isocratic mobile phase was a mixture of hexane—ethanol (92.5:7.5) at a column head pressure of 1000 p.s.i. and a flow-rate of 1.5 ml/min. Under these conditions, the retention time of compound II was 7.2 ($k' = 2.2$) and that of com-

pound III was 5.4 min (k' = 1.4). The fluorescence detector attenuation was 0.1 μA full scale with a time constant of 3.0 sec. The chart speed was 1 cm/min on the 1.0-mV Omniscribe recorder (Houston Instrument, Austin, TX, U.S.A.). Under these conditions 140 ng of compound II and 200 ng of compound III per 20 μl injected give approximately full scale response. The minimum detectable amounts of both compounds is approximately 10 ng injected on column. A typical chromatogram for 90 ng of II and 80 ng of III per 20 μl injected is shown in Fig. 2.

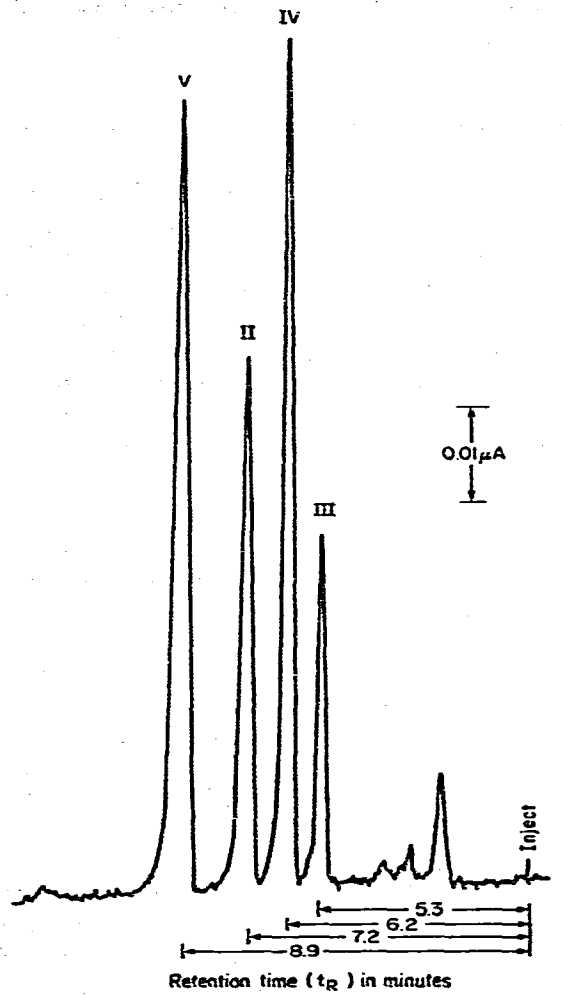


Fig. 2. Chromatographic separation of 90 ng estramustine (II), 80 ng estrone-3N-bis(2-chloroethyl) carbamate (III), 110 ng estrone (IV), and 20 ng estradiol (V).

Analytical standards

Compound II, [estramustine; estradiol-17β-3N-bis(2-chloroethyl) carbamate, $C_{23}H_{31}O_3NCl_2$, MW 440.41, m.p. 104—105°C] and compound III, [estrone-3N-bis(2-chloroethyl) carbamate, MW 439.40] of pharmaceutical grade purity (> 99%) are used as analytical standards.

Prepare stock solutions of compounds II and III in separate 50-ml volumetric flasks by dissolving 5.0 mg of each compound in 50 ml of ethanol. These stock solutions (containing 100 μg/ml) are used to prepare two sets of four mixed standard solutions (Nos. 1—4) containing 400, 600, 800 and 1000 ng of II and 500, 1000, 3000 and 5000 ng of III per ml of ethanol or hexane—ethanol (92.5:7.5). Aliquots (100 μl) of the ethanolic solutions are added to plasma to construct the calibration curve for the determination of the concentration in the unknowns and for the determination of percent recovery. Aliquots (20 μl) of the hexane—ethanol (92.5:7.5) solutions are injected as the external standards for establishing the HPLC parameters using fluorescence detection.

Reagents

All reagents are of analytical grade purity and are prepared in deionized, distilled water.

Borate—KCl—Na_2CO_3 buffer (1.0 M, pH 9.0). Dissolve 61.8 g of boric acid (H_3BO_3) and 74.6 g KCl per liter of distilled water. Dissolve 106 g of Na_2CO_3 per liter of distilled water. To 630 ml of the boric acid—KCl solution add 370 ml of the Na_2CO_3 solution to make 1 l of buffer solution. Shake well and adjust to pH 9.0 if necessary with the Na_2CO_3 solution. This solution is 1 *M* with respect to H_3BO_3—Na_2CO_3—KCl. The solution should be stored at 35—37°C to prevent crystallization of the salts from the solution. Other reagents included hexane (UV grade, Burdick and Jackson Labs., Muskegon, MI, U.S.A.) and ethanol (200 Proof, Pharmaco, Publicker Industries, Philadelphia, PA, U.S.A.).

Assay

Into a 50-ml stoppered-centrifuge tube (PTFE No. 16, stoppered) add 100 μl of ethanol, 1.0 ml of unknown plasma, and 2.0 ml of 1 *M* borate—KCl—Na_2CO_3 buffer (pH 9.0) and mix well. Extract the samples with 12 ml of hexane by slowly shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.), centrifuge the samples in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon-IEC, Needham, MA, U.S.A.) at 5°C for 10 min at approximately 1200 *g*. Transfer a 10-ml aliquot of the hexane extract into a tapered 15-ml stoppered centrifuge tube (PTFE No. 13 stoppered), and evaporate to dryness at 50°C in a N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean, dry nitrogen. Dissolve the residue in 100 μl of hexane—ethanol (92.5:7.5) and inject a 20-μl aliquot for HPLC analysis. Along with the samples, a 1.0-ml specimen of control plasma and four 1.0-ml specimens of control plasma containing 100 μl of ethanolic solutions Nos. 1—4, equivalent to 40, 60, 80 and 100 ng of compound II and 50, 100, 300 and 500 ng of compound III per ml of plasma, are processed. These standards are used to establish the calibration curve for the direct quantitation of the unknowns using the peak height vs. concentration technique. Appropriate corrections for any changes in sample aliquots or serial dilutions used must be performed. Typical chromatograms of plasma extracts are shown in Fig. 3.

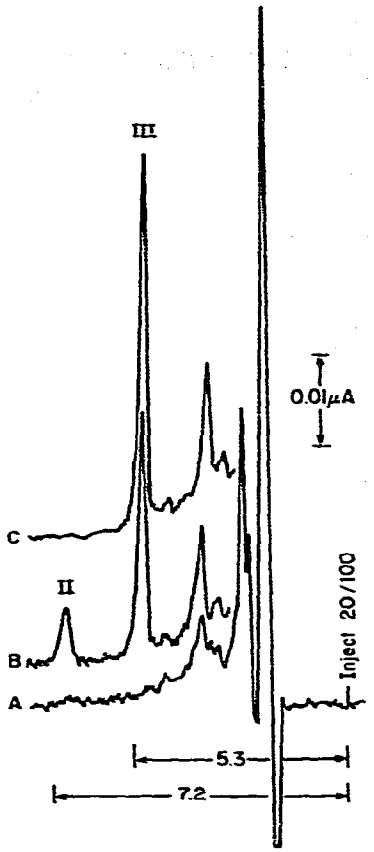


Fig. 3. Chromatograms for the HPLC analysis of hexane extracts of (A) control plasma, (B) control plasma containing 100 ng II per ml and 500 ng III per ml added authentic standard, and (C) human plasma 2 h post 7 mg/kg dose of estramustine phosphate.

Statistical evaluation of the method

The method was evaluated over a concentration range of 45.3–113.2 ng of II and 48.6—486 ng of III per ml of plasma. These concentration ranges were based upon the plasma levels of the two compounds measured in human subjects in a pilot study during the initial developmental stages of the assay. Four sets of four individual samples (total, 16 samples) of II and III in the above concentration ranges were added to 1 ml plasma. These spiked samples were then taken through the analytical procedure and the recovery data obtained are shown in Table I. The data for II and III were best described by linear least-square equations of the form $y = mx + b$ ($y = 0.0195\ x - 0.194$ and $y = 0.0150\ x - 0.0917$) with correlation coefficients r of 0.960 and 0.994, respectively indicating the high degree of linearity of the procedure for both compounds. The coefficients of variation for II and III over their respective concentration ranges were 5.7 and 5.6% respectively. The recovery of compounds II and III (after correction for 10/12 aliquot taken for analysis) calculated against pure standards of the compounds prepared in hexane—ethanol (92.5: 7.5) was 74.7 ± 6.0% and 84.9 ± 4.1%, respectively.

TABLE I

LINEARITY AND PRECISION OF THE HPLC ASSAY OF COMPOUNDS II AND III RECOVERED FROM PLASMA

Each determination was performed with four individual samples.

Concentration added (ng/ml)	Mean concentration found ± S.D.	Coefficient of variation (%)	Recovery (%)
*Compound II**			
45.3	46.3 ± 2.7	6.0	68.2
68.0	69.3 ± 1.1	1.6	77.3
90.6	84.7 ± 4.6	5.4	70.1
113.2	116.7 ± 11.7	10.1	83.2
Mean ± S.D.		5.7	74.7 ± 6.0
*Compound III***			
48.6	52.6 ± 2.4	4.5	80.0
97.2	101.6 ± 6.4	6.3	85.8
291.6	273.6 ± 21.6	7.9	32.7
486.0	495.5 ± 18.4	3.7	91.1
Mean ± S.D.		5.6	84.9 ± 4.1

*$y = 0.0195\,x - 0.194$; $r = 0.960$.
**$y = 0.0150\,x - 0.0917$; $r = 0.994$.

Specificity of the method

The specificity of the plasma assay for II and III was established by the absence of any interfering peaks occurring in the retention volume of these compounds following extraction of control (drug-free) human plasma from different sources and from treated subjects under the chromatographic separation conditions described above. Estramustine phosphate (I) is not extracted from plasma and does not give a chromatographic peak under the described conditions. Estrone (t_R = 6.2 min, k' = 1.8) and estradiol (t_R = 8.9 min, k' = 3.0), which are known metabolites in the dog [11], are chromatographically resolved from II and III (Fig. 1).

No suitable internal standard was found for this assay procedure. Of the many steroids evaluated as reference compounds, only equilenin, equilin and estradiol-3-methyl ether demonstrated similar fluorescence properties, however, these compounds were not chromatographically resolved from III under the chromatographic conditions described.

RESULTS AND DISCUSSION

Spectrofluorometric analysis of estrogens in urine typically measure the resultant fluorescence of these compounds generated in solutions containing a high percentage of concentrated phosphoric or sulfuric acid (excitation/emission, 410—450/500 nm) [12,13]. The native fluorescence of the estrogens (excitation/emission, 280/310 nm) has been used for the assay of estrogens in pregnancy urine [14]. HPLC assay of estrogens utilizing reversed-phase chromatography with UV detection for placental estriol in urine [15], normal-

phase chromatography with UV detection for estrogenic steroids in pregnancy urine [16], and ion-exchange chromatography with radiometric detection for estrogen conjugates in plasma [17] have all been recently reported.

The present study utilizes normal-phase HPLC and the measurement of the intrinsic fluorescence of the estrogen derivatives II and III for a highly sensitive and specific assay of the two components. A significant increase in fluorescence sensitivity in hexane—ethanol (92.5:7.5) was obtained by using a deuterium lamp at 195 nm as the excitation source compared to a xenon lamp. This wavelength (195 nm) coincides with the high-energy output range of the deuterium lamp and the UV absorbance maxima of the compounds, hence the fluorescence yield measured shows a dramatic increase in sensitivity. Reversed-phase chromatography of II (t_R = 7.4 min, k' = 3.8) and III (t_R = 8.4 min, k' = 4.4) is also possible in a solvent system of methanol—water (80:20) at this excitation wavelength. However, the overall sensitivity in this medium for estramustine (II) is only 25% of that measured in hexane—ethanol (92.5:7.5).

Application of the method to biological specimens

The HPLC assay described was applied for the analysis of plasma samples from three subjects with prostatic cancer following single oral administration of 7 mg/kg of estramustine phosphate (I). Peak levels of III ranging from 408 to 904 ng/ml were measured at 2-h post dosing and levels of III were measurable for 10—12 h (Table II). The levels of II were non-measurable (≤ 40 ng/ml)

TABLE II

PLASMA LEVELS (ng/ml) OF III IN THREE HUMAN SUBJECTS FOLLOWING A SINGLE 7 mg/kg ORAL DOSE OF ESTRAMUSTINE PHOSPHATE (I)

Time (h)	Concentration of III (ng/ml)		
	Subject A	Subject B	Subject C
1	95	401	294
2	408	474	904
3	223	271	689
4	136	132	723
6	119	76	565
8	69	n.a.*	316
10	53	37**	232
12	n.a.*	29**	102

*n.a. = not analyzed.
**Analysis of 40/100 μl of reconstituted extract.

during the same experimental period which suggests that extensive oxidation of II at the 17-position occurs during its "first pass" through the liver and/or gastrointestinal tract. This procedure with minor modifications has recently been applied to the analysis of II, III, estrone, and estradiol in man following multiple dosing (8.6—15.4 mg/kg/day) of estramustine phosphate (I) [18].

REFERENCES

1 L. Andersson, F. Edsmyr, G. Jonsson and I. Konyves, Cancer Res., 60 (1977) 73.
2 G. Jonsson and B. Hogberg, Scand. J. Urol. Nephrol., 5 (1971) 103.
3 G. Jonsson, B. Hogberg and T. Nilsson, Scand. J. Urol. Nephrol., 11 (1977) 231.
4 A. Mittleman, S.H. Shukla and G.P. Murphy, J. Urol., 115 (1976) 469.
5 R. Nagel and C.P. Kolln, Brit. J. Urol., 49 (1977) 73.
6 R.Y. Kirdani, A. Mittleman, G.P. Murphy and A.A. Sandberg, J. Clin. Endocrinol. Metab., 41 (1975) 305.
7 R.Y. Kirdani, G.P. Murphy and A.A. Sandberg, Proc. Soc. Exp. Biol. Med., 155 (1977) 94.
8 G. Plym Forshell, J. Muntzing, A. Ek, E. Lindstedt and H. Dencker, Invest. Urol., 14 (1976) 128.
9 P.O. Gunnarsson, T. Nilsson and G. Plym Forshell, Urol. Res., (1979) in press.
10 B. Forsgren, P.O. Gunnarsson, S. Johansson and T. Kant, Acta Pharm. Suecica, 15 (1978) 200.
11 J. Carbone and R. Dixon, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 38 (1979) 442.
12 S. Udenfriend, Fluorescence Assay in Biology and Medicine, Academic Press, New York, 1962, pp. 353—359.
13 C. Goutle-Coussieu, G. Habrioux, D. Eichenberger and M. Jayle, Clin. Chem., 25 (1979) 230.
14 J. Bramhall and A.Z. Britten, Clin. Chim. Acta, 68 (1976) 203.
15 G.R. Gottelli, J.H. Wall, P.M. Kabra and L.J. Marton, Clin. Chem., 24 (1978) 2132.
16 R.J. Dolphin and P.J. Pergande, J. Chromatogr., 143 (1977) 267.
17 P.I. Musey, D.C. Collins and J.R.K. Preedy, Steroids, 31 (1978) 583.
18 R. Dixon, M.A. Brooks and G. Gill, Res. Commun. Chem. Pathol. Pharmacol., 27 (1980) 17.