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Short Communication

High-performance liquid chromatographic determination of ethacrynic acid in human plasma

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ABSTRACT

A high-performance liquid chromatographic method for the determination of ethacrynic acid (EA) in human plasma is described. Plasma was prepared for analysis by addition of 4-(2,4-dichlorophenoxy)-butyric acid as an internal standard followed by acidification with hydrochloric acid and extraction with ethyl acetate. Separation was by isocratic reversed-phase chromatography, the column effluent was monitored at 280 nm and quantitation was performed using peak-area ratios. The linear range for EA determination was from 0.5 to 25 μg/ml with a lower limit of detection of 0.1 μg/ml. The reported method is convenient, sensitive and reproducible, illustrating its usefulness for pharmacokinetic studies.

INTRODUCTION

Ethacrynic acid [2,3-dichloro-4-(2-methylenebutyryl)phenoxyacetic acid; EA] belongs to a class of drugs referred to as high-ceiling (loop) diuretics. High-ceiling diuretics are used clinically when a prompt and brisk diuretic effect is desired as in the case of acute pulmonary edema [1]. Currently, EA is not widely used as a diuretic agent because furosemide, another member of this drug class, has a superior toxicity profile [2]. However, recent findings have suggested a new use for EA.

EA is a substrate [3] and inhibitor [4] of a family of enzymes known as the glutathione S-transferases (GSTs). Recent evidence has correlated elevated GST

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levels to anticancer drug resistance [5] and other studies have shown that electrophilic anticancer drugs are substrates for the GSTs [6–8]. Tew *et al.* [9] showed that non-cytotoxic concentrations of EA enhanced the cytotoxicity of chlorambucil in resistant and sensitive Walker 256 rat breast carcinoma cells *in vitro*, and Clapper *et al.* [10] described the sensitization of human colon tumor xenografts to L-phenylalanine mustard *in vivo*, using the severe combined immunodeficient (SCID) mouse model. On the basis of these preclinical studies, a clinical trial of EA in combination with the alkylating agent N,N′,N″-triethylenethiophosphoramide (thio-TEPA) was initiated [11]. Since little is known about the pharmacokinetics of EA in humans, a convenient and reliable analytical method was desired. A recent gas chromatographic (GC) method, capable of measuring EA and its thiol adducts in guinea pig plasma, required electron-capture detection [12], and another method utilizing derivatization prior to GC [13] was considered too labor-intensive for the high sample throughput required for pharmacokinetics investigations. Thus, in this study we report a relatively simple high-performance liquid chromatographic (HPLC) procedure for the analysis of EA in human plasma.

EXPERIMENTAL

Chemicals

EA, 4-(2,4-dichlorophenoxy)butyric acid (DCPBA), triethylamine and hydrochloric acid were purchased from Sigma (St. Louis, MO, USA). HPLC-grade orthophosphoric acid (85%) was obtained from Fisher Scientific (Fair Lawn, NJ, USA). The extraction (ethyl acetate) and chromatography solvents (water, methanol and acetonitrile) were all HPLC grade and purchased from Burdick and Jackson (Muskegon, MI, USA). Control plasma was obtained from Interstate Blood Bank (Philadelphia, PA, USA).

Sample preparation

Plasma samples (1 ml) in 17 mm × 100 mm snap-top polypropylene tubes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ, USA) were prepared for extraction by addition of 40 μl of methanol for the plasma blank, or 20 μl each of the appropriate EA standard and the internal standard, DCPBA (750 μg/ml), dissolved in methanol. Patient samples were prepared by adding 20 μl of methanol and 20 μl of the DCPBA solution. After briefly vortex-mixing the samples, 3 ml of 1.0 *M* hydrochloric acid were added and the samples were again vortex-mixed. Organic extraction was performed by addition of 5 ml of ethyl acetate followed by rocking the samples for 5 min. The organic and aqueous layers were separated by centrifugation at 400 *g* for 5 min. Following centrifugation, three layers were apparent: (1) the bottom aqueous layer; (2) a middle, stiff gel-like layer; and (3) the upper ethyl acetate layer. The ethyl acetate layer (3.8 ml) could then be decanted into a clean 17 mm × 100 mm tube without disturbing the lower

gel and aqueous layers. The solvent was evaporated to dryness under a stream of nitrogen at 30°C. The residue was resuspended in 400 μl of a solution consisting of 30% acetonitrile and 70% buffer A (water containing 0.25% triethylamine and 0.25% orthophosphoric acid) and transferred to 12 mm × 32 mm crimp-top vial for analysis by HPLC.

High-performance liquid chromatography

The chromatographic system consisted of a Hewlett-Packard (Palo-Alto, CA, USA) HP1090 series A liquid chromatograph equipped with an autosampler/autoinjector and an HP1040A diode-array UV detector. The injection volume was 200 μl and the column effluent was monitored at 280 nm (20 nm band width) where EA and DCPBA exhibit an absorption maximum (Fig. 1A). The chromatograph was operated with a Hewlett-Packard HP85B personal computer, and data were interpreted with a DPU multi-channel integrator. Chromatography was performed on a Hewlett-Packard reversed-phase C_{18} analytical column (Hypersil ODS, 5 μm, 100 mm × 4.6 mm I.D.) preceded by a 15 mm × 3.2 mm, 7 μm Aquapore C_{18} guard column (Brownlee Labs., Santa Clara, CA, USA).

EA ($k' = 15.0$) and DCPBA ($k' = 17.4$) were eluted, with retention times of 8.75 and 10.1 min, respectively, by an isocratic mobile phase at a flow-rate of 2 ml/min. The mobile phase consisted of 55% buffer A, 32% methanol and 13% acetonitrile. Standard curves consisting of five points (0.5, 1, 5, 10 and 25 μg/ml) were plotted as the peak-area ratio of EA to DCPBA *versus* concentration of EA. The linear regression lines were calculated by the method of least squares and were weighted by $1/x$.

RESULTS AND DISCUSSION

Following ethyl acetate extraction, HPLC of control plasma (Fig. 1A) yielded a chromatogram clear of interfering peaks at the retention times of EA and DCPBA, while the chromatogram of control plasma spiked with EA and DCPBA (Fig. 1B) demonstrated complete separation of EA from DCPBA.

As seen in Fig. 1C, the pre-treatment patient sample was void of interfering peaks and a post-treatment plasma sample (Fig. 1D) obtained 15 min after the end of drug infusion yielded a plasma concentration of 3.76 μg/ml. Quantifiable drug concentrations were detected in patient plasma up to 12 h following a single dose of EA.

There was a linear relationship between the peak-area ratio of EA to DCPBA and the concentration of EA from triplicate plasma standards following ethyl acetate extraction. The linear range was 0.5–25 μg/ml, with a lower limit of detection of 0.1 μg/ml. The minimal detectable concentration was determined from the EA peak resulting in a signal-to-noise ratio of 2 and was by calculated by direct measurement of blank and drug-containing plasma chromatograms obtained with the detector set at 1 ma.u.f.s. The average equation obtained from triplicate

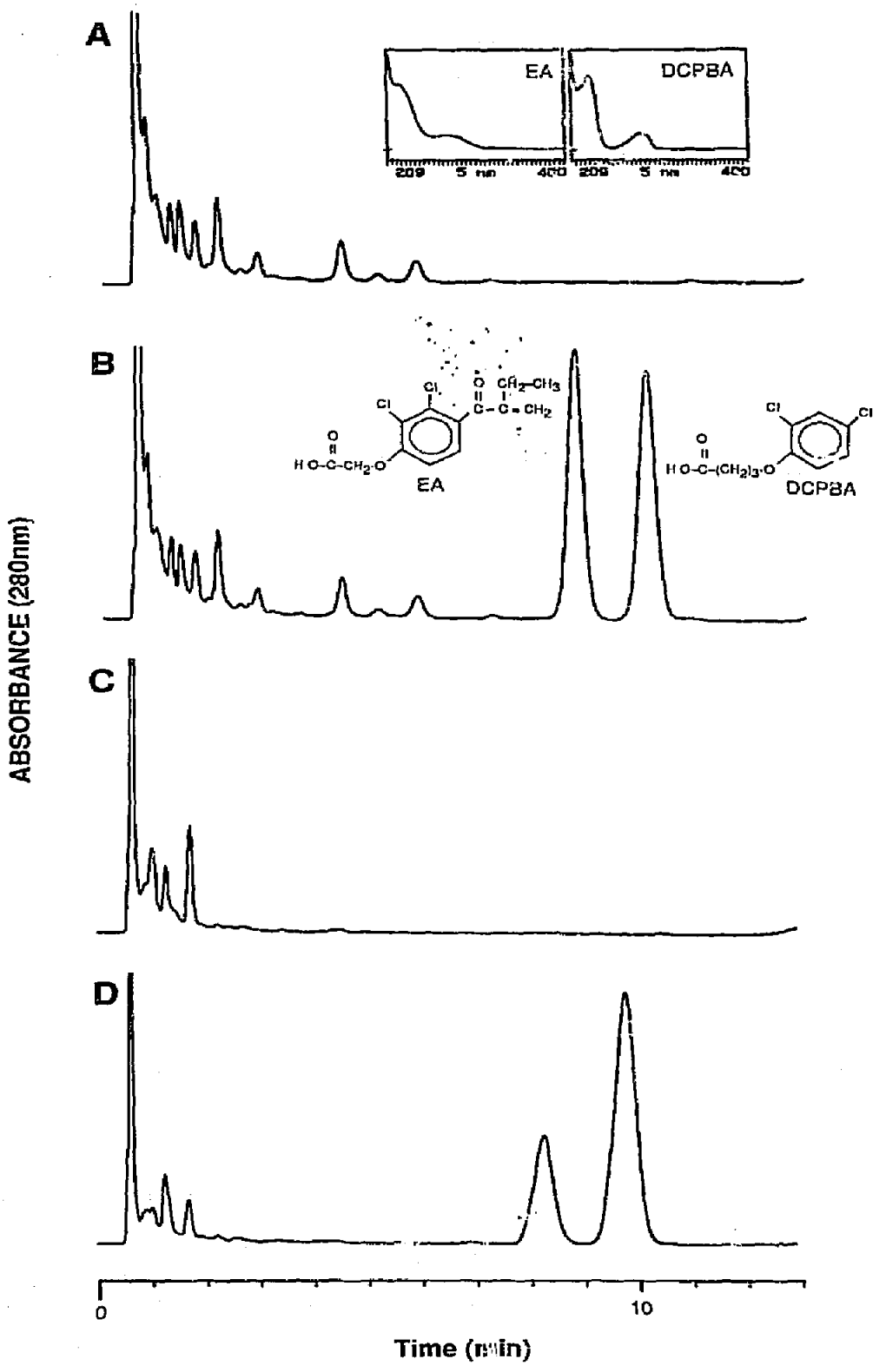

Fig. 1. Chromatograms of blank and EA-containing plasma samples. (A) Blank samples; insets of EA and DCPBA UV spectra. (B) Plasma spiked with EA (10 μg/ml) and DCPBA (15 μg/ml); injection volume 200 μl, detector at 35 ma.u.f.s. (C) Pre-treatment patient plasma. (D) Post-treatment patient plasma obtained 30 min after the beginning of a 15-min zero-order intravenous infusion (EA dose = 100 mg); injection volume 200 μl, detector at 35 ma.u.f.s.

TABLE I

INTER-DAY AND INTRA-DAY ASSAY VARIABILITY AND ACCURACY

Error = [(assay concentration − spiked concentration)/spiked concentration] × 100. Differences between days of means and S.D.s were evaluated using the T and F statistics, respectively. No significant differences were found.

Spiked concentration (μg/ml)	Found (mean ± S.D.) (μg/ml)	C.V. (%)	Error (%)
Day 1 (n=3)			
20.26	19.89 ± 1.560	7.8	−1.8
2.03	2.07 ± 0.054	2.6	2.0
Day 2 (n=3)			
20.26	19.97 ± 0.918	4.6	−1.4
2.03	2.12 ± 0.024	1.1	4.4
Day 3 (n=3)			
20.26	19.03 ± 1.140	6.0	−6.1
2.03	1.98 ± 0.019	1.0	−2.5

standard curves run on three consecutive days was $y=0.108x+0.005$, $r=0.9998$. The coefficients of variation (C.V.) of the slopes obtained from these curves ($n=9$) was 5.9%.

The accuracy and precision of the analytical method were evaluated using two different EA-containing plasma standards, prepared separately and stored frozen at −20°C prior to analysis. Each standard was run in triplicate on three consecutive days along with the three standard curves described above. The results, given in Table I, indicate that the method is accurate and reproducible. The percentage difference between the actual *versus* the found concentration (accuracy) was no greater than 6% (absolute value) and the C.V. (precision) was less than 7.8.%. Statistical analysis of these data revealed no differences between any of the values obtained on three consecutive days.

The extraction efficiency was determined by comparing the peak areas of standards from triplicate extractions to unextracted standards injected directly into the HPLC system. The average peak area of DCPBA following direct injection was 907.4 mAU ($n=5$). After ethyl acetate extraction the DCPBA peak area was 719.7 mAU ($n=15$) for an extraction efficiency of 79%. The recovery of EA was calculated in an identical manner. Across the range of the standard curve the recovery was 78%. These results indicate that DCPBA fulfills the requirements of a good internal standard, on the basis of its similarity to EA in recovery and chromatographic behavior.

We have described an accurate, reproducible and convenient assay for the analysis of EA in human plasma by HPLC. Application of this method for the analysis of EA from patients in a Phase I anticancer drug trial will be performed

to determine if sufficient concentrations of EA are obtained systemically to inhibit cellular GSTs and enhance the activity of chemotherapeutic agents.

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