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Note

High-performance liquid chromatography with electrochemical detection of chlorzoxazone and its hydroxy metabolite in serum using solid-phase extraction

JAMES T. STEWART* and H. KEITH CARTER

Department of Medicinal Chemistry, College of Pharmacy, The University of Georgia, Athens, GA 30602 (U.S.A.)

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Chlorzoxazone (I, Fig. 1) is a very useful agent for the treatment of painful muscle spasms, especially in combination with acetaminophen. Clinical studies have indicated its therapeutic utility as a long-acting central muscle relaxant with no significant side-effects [1, 2]. Metabolism studies have shown that the major metabolite is 6-hydroxychlorzoxazone (II, Fig. 1) [3, 4]. There is rapid and complete absorption of I from the gastrointestinal tract with the drug essentially eliminated from the human body in 7 h.

CHLORZOXAZONE **6-HYDROXYCHLORZOXAZONE**

Fig. 1. Chemical structures of chlorzoxazone (I) and 6-hydroxychlorzoxazone (II).

Analytical methods to determine I in biological fluids have utilized spectrophotometry [3] and high-performance liquid chromatography (HPLC) [5]. Other titrimetric [6], gas chromatographic (GC) [7, 8] and thin-layer chromatographic (TLC) [9] procedures have also been reported for the analysis of I in other types of samples. The HPLC method was developed in this laboratory and is the only procedure that separates and quantitates both I and its hydroxy metabolite II from a single serum sample. Sample preparation involved protein denaturation, centrifugation, extraction, quick-freeze, filtering, and

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evaporation steps prior to the chromatographic separation. It was of interest to delete much, if not all, of the time-consuming sample preparation steps by investigating the use of solid-phase extraction techniques. Preliminary experiments conducted in our laboratory had also indicated that both I and II would oxidize at the glassy carbon electrode in a flowing stream. Thus it seemed possible to improve our previous HPLC methodology for I and II by utilizing both the increased efficiency and speed of solid-phase extraction columns and increased sensitivity of electrochemical oxidation.

In this paper, an HPLC method with electrochemical detection (ED) for I and II in serum is reported. The compounds are separated from serum components using a C_{18} bonded-phase extraction column. The eluent is injected onto an octadecylsilane HPLC column and the solutes are detected at the glassy carbon electrode using a cell potential of +1300 mV. The overall analysis time was halved and the detectability of I and II was increased almost 30-fold compared to our previous UV method.

EXPERIMENTAL

Materials

Powdered samples of chlorzoxazone and 6-hydroxychlorzoxazone (McNeil Pharmaceuticals, Spring House, PA, U.S.A.) were used in the preparation of standard solutions. Drug-free human serum was obtained as lyophilized material (Fisher Scientific, Pittsburgh, PA, U.S.A.) and was reconstituted with distilled water prior to use. Disposable 1-ml C_{18}, C_8, cyanopropyl, cyclohexyl and phenyl solid-phase extraction columns (Bond-Elut, Analytichem International, Harbor City, CA, U.S.A.) were used to separate I and II from serum components. All other chemicals and solvents were the highest grade of commercially available materials.

HPLC conditions

The HPLC analyses were performed using a Spectroflow 400 solvent delivery system (Kratos, Ramsey, NJ, U.S.A.), a Model 7125 injector equipped with a 50-μl fixed loop (Rheodyne, Cotati, CA, U.S.A.), a Kel-F electrochemical cell containing a glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.) controlled by a Tacussel Model ED-110 electrochemical detector (ASI, Santa Clara, CA, U.S.A.), and a Microscribe recorder (Bausch and Lomb, Houston, TX, U.S.A.). The column was a 5-μm Nova-pak C_{18} (150 mm × 3.9 mm I.D., Waters Assoc., Milford, MA, U.S.A.) equipped with a direct-connect guard column containing an Adsorbosphere C_{18} cartridge (Alltech Assoc., Deerfield, IL, U.S.A.). Column temperature was 22 ± 1°C, the mobile phase was 25:75 acetonitrile—aqueous 0.05 *M* sodium dihydrogen phosphate (pH 4.5, adjusted if necessary with 1 *M* sodium hydroxide) and was deaerated by sonication before use. The flow-rate was set at 1.0 ml/min (80 bar), the glassy carbon cell potential was set at +1300 mV, and the chart recorder was set at 1 V. Peak heights of each solute were measured manually in mm.

Standard solutions for calibration curve

Stock solutions of I and II (125 μg/ml) were prepared by dissolving weighed

amounts of each powder in acetonitrile—aqueous 0.05 *M* sodium dihydrogen phosphate, pH 4.5 (50:50). In addition, an internal standard stock solution (250 μg/ml) of phenacetin was also prepared in the same solvent.

Calibration curves for I and II in serum

Into individual 15-ml centrifuge tubes were placed 1-ml aliquots of drug-free human serum. Accurately measured volumes (Hamilton microliter syringes, Reno, NV, U.S.A.) of 10, 20 and 40 μl of I and II stock solutions were added such that the final concentrations of both I and II in each sample were 1.25, 2.5 and 5.0 μg/ml, respectively. Internal standard stock solution (20 μl) and 0.25 *M* sulfuric acid (500 μl) were also added to each tube followed by mixing on a vortex mixer (30 s).

Each entire spiked serum sample was quantitatively transferred with the aid of one 1.5-ml portion of distilled water to a disposable solid-phase extraction column attached to a vacuum manifold (Vac-Elut, Analytichem International). The extraction columns had been prerinsed with two column volumes of methanol followed by two column volumes of distilled water. The serum sample and washing were drawn through the column and manifold under reduced pressure. Then, a 1.5-ml quantity of distilled water was drawn through the column, still maintained at reduced pressure. Normal pressure was restored, and 1-ml volumetric flasks were inserted into the manifold for collection of samples. The compounds I and II and the internal standard were then eluted from the column using 2—400-μl portions of 50:50 acetonitrile–0.05 *M* sodium dihydrogen phosphate (pH 4.5) under reduced pressure. Normal pressure was again restored, the 1-ml flasks were removed from the manifold, and the volume of each was adjusted to 1 ml using eluting solvent. The flasks were manually shaken to ensure homogeneity and a 50-μl sample was injected into the liquid chromatograph.

Determination of I and II in human serum

Samples (1 ml) of human serum containing I and II were placed in individual 15-ml centrifuge tubes to which 500 μl of 200 Sigma units/ml β-glucuronidase in phosphate buffer (Sigma, St. Louis, MO, U.S.A.) were added. The samples were vortexed for 15 s and allowed to stand overnight at ambient temperature (23 ± 1°C). Then 20 μl of internal standard stock solution and 500 μl of 0.25 *M* sulfuric acid were added to each tube and the samples vortexed for 30 s. Each serum sample was then treated in the same manner as described above. A blank consisting of drug-free human serum sample was also performed. Triplicate injections of 50 μl were injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Modification in the HPLC analysis of I and II in human serum necessitated the examination of two separate problems: (a) the separation of I, II and internal standard from serum components using a solid-phase extraction column; (b) the development of operating parameters for the oxidation of I and II at the glassy carbon electrode. Initially, the extraction of I and II from serum was studied using commercially available C_{18} solid-phase extraction

TABLE I

RECOVERIES OF CHLORZOXAZONE (I) AND HYDROXYCHLORZOXAZONE (II) FROM SERUM ON C_{18} AND C_8 SOLID-PHASE EXTRACTION COLUMNS

Solid phase	Sample preparation and extraction conditions	Recovery* (%)	
		I	II
C_{18}	Denature with trichloroacetic acid (TCA); elute with 50:50 acetonitrile—phosphate buffer, pH 4.5	22.59 ± 0.07 (n = 3)	23.51 ± 0.37 (n = 3)
C_{18}	Denature with TCA; elute with 75:25 acetonitrile—phosphate buffer, pH 4.5	19.19 ± 0.31 (n = 4)	22.09 ± 0.42 (n = 4)
C_{18}	Denature with ammonium sulfate; elute with 50:50 acetonitrile—phosphate buffer, pH 4.5	26.13 ± 2.86 (n = 3)	29.87 ± 0.59 (n = 3)
C_{18}	Denature with TCA; elute with 50:50 methanol—phosphate buffer, pH 7.0	13.50**	46.47**
C_{18}	Denature with TCA; elute with 60:40 methanol—phosphate buffer, pH 4.5	23.30**	40.85**
C_{18}	Denature with TCA; elute with absolute methanol	56.02**	49.16**
C_{18}	Denature with ammonium sulfate; elute with absolute methanol	22.45**	24.17**
C_{18}	No denaturation step; elute with 50:50 acetonitrile—phosphate buffer, pH 4.5	90.24 ± 4.28 (n = 9)	86.06 ± 3.58 (n = 9)
C_8	No denaturation step; elute with 50:50 acetonitrile—phosphate buffer, pH 4.5	54.41 ± 2.92 (n = 3)	10.98 ± 0.45 (n = 3)

*Mean ± standard deviation based on replicate determinations.
**Based on a single determination of spiked serum sample.

columns. Percentage recovery data are shown in Table I. In some of the investigations, serum samples containing spiked quantities of I and II were pretreated prior to addition to the column with various protein denaturation reagents such as trichloroacetic acid and saturated ammonium sulfate solution. While the data indicated that recovery of both I and II was higher at acid pH, the use of a protein denaturation reagent always resulted in low extraction efficiencies presumably owing to loss of the compounds in the precipitates that normally result from the denaturation and centrifugation steps. Extraction of either compound I or compound II was not significantly improved using varied proportions of acetonitrile—water or methanol—water eluents at pH 4.5 or 7.0. Based on preliminary data, it was decided to investigate the extraction of I and II on the C_{18} column without the use of a protein denaturation step. The percentages recoveries were comparable to those reported previously by this laboratory using a protein denaturation—ether extraction procedure of serum [5]. Optimization of the extraction parameters also involved studying the

recoveries of I and II on other solid-phase extraction columns such as C_8, cyanopropyl, cyclohexyl and phenyl. Significantly lower extractions of I and II were obtained with C_8 and no measurable recoveries of compounds from serum were obtained using either cyanopropyl, cyclohexyl or phenyl phases. Thus, the recovery data indicated that both I and II could be efficiently extracted from an acidified serum sample using a C_{18} solid-phase extraction column and a 50:50 acetonitrile—water eluent (pH 4.5).

Hydrodynamic voltammograms were run on I and II in acetonitrile—aqueous phosphate buffer (pH 4.5). It was found that both compounds oxidized at the glassy carbon electrode and that +1300 mV was the best cell potential to obtain maximum sensitivity for I and II (see Fig. 2). Further investigations revealed that the compounds and internal standard were most propitiously separated on an octadecylsilane column using acetonitrile—aqueous 0.05 *M* sodium dihydrogen phosphate (25:75) at pH 4.5. Fig. 3 shows a chromatogram of the separation of I, II and phenacetin (internal standard) in a serum sample from a patient to whom I was administered. Under the extraction and chromatographic conditions chosen, endogenous serum constituents do not interfere with the assay.

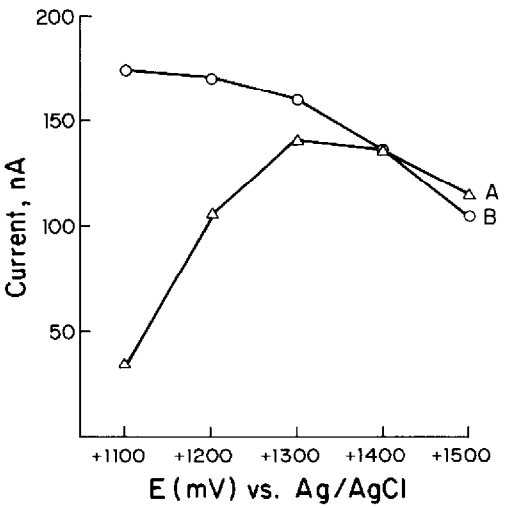

Fig. 2. Hydrodynamic voltammograms of hydroxychlorzoxazone (A) and chlorzoxazone (B) at the glassy carbon electrode in 25:75 acetonitrile—aqueous 0.05 *M* sodium dihydrogen phosphate (pH 4.5) at a flow-rate of 1 ml/min. The concentrations of A and B were 2.5 μg/ml.

A flow-rate of 1.0 ml/min (80 bar) allowed the HPLC separation to be obtained in approximately 10 min. The void volume of the column was 1.0 ml. Total analysis time per sample is about 20 min, which includes solid-phase extraction of I, II and internal standard from serum followed by HPLC separation and quantitation.

Calibration curves for I and II in the anticipated serum concentration range (1—5 μg/ml) were performed. Ratios of peak heights of each compound to that of the internal standard (D/IS) were calculated for each chromatogram. Regression analysis of these data at the various concentrations of I and II gave

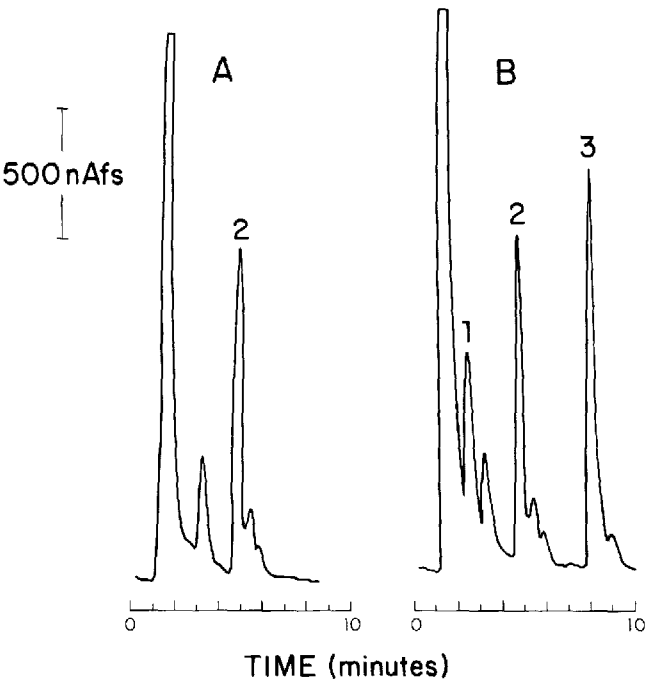

Fig. 3. (A) Chromatogram of a serum sample taken from a 82-kg male patient prior to drug administration. (B) Chromatograms of hydroxychlorzoxazone (1), phenacetin (2) (internal standard) and chlorzoxazone (3) in a human serum sample obtained from the patient 2 h after a single 250-mg dose of chlorzoxazone had been administered. Conditions: column, octadecylsilane (150 mm × 3.9 mm I.D.); eluent, acetonitrile– aqueous 0.05 *M* sodium dihydrogen phosphate, pH 4.5 (25:75); flow-rate, 1 ml/min (80 bar); glassy carbon electrode set at +1300 mV; column temperature, 22 ± 1° C.

TABLE II

ANALYSIS OF CHLORZOXAZONE AND HYDROXYCHLORZOXAZONE IN SPIKED SERUM SAMPLES

Mixture	Components	Initial concentration (μg/ml)	Concentration found* (μg/ml)	Relative standard deviation (%)	Relative error (%)
A	Chlorzoxazone	1.875	1.895 ± 0.127	6.70	1.07
	Hydroxychlorzoxazone	1.875	1.903 ± 0.094	4.94	1.49
B	Chlorzoxazone	3.75	3.860 ± 0.200	5.18	2.93
	Hydroxychlorzoxazone	3.75	3.845 ± 0.075	1.95	2.53

*Mean ± S.D. based on ten determinations of each sample.

slopes, intercepts and correlation coefficients of 0.3477, —0.0756 and 0.9930 (n = 21) and 0.2395, 0.0457 and 0.9929 (n = 15), respectively. The limits of detection for I and II using this HPLC—ED procedure are 2.5 ng (equivalent to 50 ng/ml using a 50-μl injection at 100 nA f.s.) based upon extraction of each compound from serum.

Human serum samples containing spiked quantities of I and II within the serum concentration range of each were chromatographed concurrently with the calibration solutions and the ratios of peak heights of each component

to internal standard were calculated. The slope and intercept data calculated from linear regression analysis for I and II calibration samples were used to calculate the concentrations of I and II in the spiked samples: D/IS = (slope × concentration) + intercept. The data in Table II demonstrate the quantitative results obtained from these spiked serum samples. The utility of this improved HPLC assay for I and II in serum using electrochemical detection is clearly demonstrated, with a relative error of $< 3\%$.

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