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Sensitive high-performance liquid chromatographic determination of chlorzoxazone and 6-hydroxychlorzoxazone in plasma

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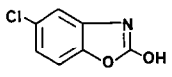
ABSTRACT

A rapid and sensitive high-performance liquid chromatographic assay was developed for the quantitation of chlorzoxazone and its major metabolite 6-hydroxychlorzoxazone in plasma. These compounds, as well as the internal standard 5-fluorobenzoxazolone, were extracted from plasma (0.5 ml) using C_{18} solid-phase extraction columns. The extracts were analyzed on a 10-μm Waters C_{18} μBondapak column with a mobile phase of acetonitrile–tetrahydrofuran–0.1 *M* ammonium acetate (22.5:5.5:72). The assay utilized ultraviolet detection (283 nm) which provided sensitivity and specificity sufficient to simultaneously quantify $\geq$ 100 ng/ml chlorzoxazone and 6-hydroxychlorzoxazone in plasma. The mean correlation coefficient of the multi-level standard curves for each compound was 0.996 or greater over a concentration range of 100–3000 ng/ml. The inter-day and intra-day coefficients of variation were $<6\%$. This method has been used by our laboratory to provide the unattended overnight analysis of chlorzoxazone and 6-hydroxychlorzoxazone in plasma samples obtained from human study subjects.

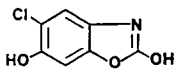
INTRODUCTION

Chlorzoxazone (CX, Fig. 1) is a skeletal muscle relaxant effective in the treatment of muscle spasms [1,2]. Recently, however, this compound has been suggested for use as a chemical probe to assess the activity of cytochrome P-450 2E1 *in vitro* [3]. Studies have shown that the major metabolite produced from human hepatic microsomal metabolism of chlorzoxazone as well as from *in vivo* metabolism is 6-hydroxychlorzoxazone (HCX, Fig. 1) [4,5]. Moreover, *in vitro* studies performed with selective antibodies to this enzyme have shown that this particular reaction is highly correlated with cytochrome P-450 2E1 activity [3]. This enzyme catalyzes the metabolism of numerous low-molecular-mass solvents such as ethanol, propylene glycol, diethyl ether, and acetone to name but a few [6]. Cytochrome P-450 2E1 is also known to be responsible for the activation of many pro-toxins and -carcinogens such as carbon tetrachloride, benzene, N-nitrosodimethylamine, and aniline [3,7].

In an effort to characterize the pharmacokinetics of chlorozoxazone ($t_{1/2}$ = 66 min) in human subjects, and in effect the *in vivo* activity of cytochrome P-450 2E1, a sensitive and specific assay



CHLORZOXAZONE



6-HYDROXYCHLORZOXAZONE

Fig. 1. Molecular structures of chlorzoxazone and 6-hydroxychlorzoxazone.

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for CX and HCX in plasma was required. Since some of our studies involve the co-administration of caffeine, dapsone, mephenytoin, and debrisoquine, in addition to chlorozoxazone, the assay had to be selective for CX and HCX and devoid of interference from the other drugs or their metabolites. Several column liquid chromatographic methods for the analysis of CX and HCX have been described [8–11]. The modes of detection used for these assays are ultraviolet and electrochemical. The major drawback to these methods is limited sensitivity based on their reported limits of quantitation and calibration curve ranges.

This paper describes a simple, specific, and sensitive method using UV detection for the simultaneous quantitation of CX and HCX in plasma. Samples are processed via C_{18} solid-phase extraction and have an HPLC run time of 18 min. This method is suitable for pharmacokinetic studies (7-h sampling period) in subjects dosed with as little as 250 mg of CX.

EXPERIMENTAL

Reagents and chemicals

CX was purchased from Aldrich (Milwaukee, WI, USA) while HCX and 5-fluorobenzoxazolone (internal standard) were generously provided by Dr. G. R. Wilkinson, Vanderbilt University. Acetonitrile, tetrahydrofuran, and ammonium acetate were of HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA, USA). All water used in the analysis was obtained from a four-bowl Milli-Q reagent water system (Millipore, Bedford, MA, USA). β-Glucuronidase (Type G0751) and analytical-grade sodium acetate were obtained from Sigma (St. Louis, MO, USA). Solid-phase extraction columns (C_{18} Bond elut, 3 ml/200 mg) were purchased from Analytichem International (Harbor City, CA, USA). Polypropylene snap cap microcentrifuge tubes (2 ml) were obtained from Sarstedt (Newton, NC, USA). Blank plasma used for the preparation of standards and quality control (QC) samples was obtained from in house donors as well as through the local blood bank.

Instrumentation

The HPLC system consisted of a Waters Model 510 solvent delivery pump, Model 712 Wisp automatic sample processor, and Model 484 UV–VIS detector operated at a wavelength of 283 nm. Signal output was computed with a Hewlett-Packard (Santa Clara, CA, USA) Model 3354A integrator. Separation of the compounds of interest was achieved with an Alltech Assoc. (Deerfield, IL, USA) direct connect guard column (20 mm × 2 mm I.D.) filled with pellicular C_{18} packing connected to a Waters 300 mm × 3.9 mm I.D. μBondapak 10-μm C_{18} analytical column. The mobile phase used for the analysis consisted of acetonitrile–tetrahydrofuran–0.1 *M* ammonium acetate (pH 7.0) (22.5:5.5:72) at a flow-rate of 1 ml/min (76 bar). The mixture was degassed and filtered through a 0.22-μm Nylon 66 membrane before use.

Preparation of stock solutions and spiked plasmas

A 1.0 mg/ml CX stock solution was prepared by dissolving 10 mg of CX in 10 ml of methanol. A 1.0 mg/ml stock solution of HCX was prepared in the same manner. The internal standard 5-fluorobenzoxazolone was prepared as a 0.04 mg/ml solution in methanol. These solutions were stable for more than six months when stored at 4°C. A 10 μg/ml solution of CX and HCX was prepared on a daily basis by adding 20-μl aliquots of each stock solution to 1.96 ml of water. This solution was used to spike plasma samples in the daily preparation of standards. The amount of this solution added to blank plasma samples ranged from 0 to 150 μl per 0.5 ml of plasma. QC samples were prepared by adding aliquots of the stock solutions to 50-ml volumes of blank plasma which were subsequently divided into 1.5-ml aliquots and stored at −80°C. A 400-ng amount of 5-fluorobenzoxazolone (10 μl) was used as internal standard in patient plasma samples as well as in plasma standards and QC samples which contained up to 3000 ng/ml CX and HCX.

Extraction of plasma samples

To 0.5 ml of plasma (spiked standards, QC

samples, patient samples) were added 1 ml of 0.2 *M* sodium acetate buffer (pH 4.75) and 1000 U of β-glucuronidase dissolved in 0.5 ml of 0.2% sodium chloride. Samples were vortex-mixed and incubated at 37°C for 3 h. After incubation, samples were loaded onto solid-phase extraction columns which had been preconditioned with one column volume of methanol followed by one column volume of acidified water (50 μl of glacial acetic acid per liter of water). Columns were subsequently washed with 1 ml of acidified water and the retained components eluted with two 500-μl aliquots of acetonitrile into 2-ml polypropylene microcentrifuge tubes. Eluents were evaporated to dryness at 40°C under a stream of nitrogen. The residues were reconstituted with 250 μl of 40% acetonitrile in 0.1 *M* ammonium acetate, vortex-mixed, and centrifuged for 1 min at 13 600 *g* in a Model 235C microcentrifuge (Fisher Scientific). The supernatants were transferred to Wisp microinserts and 50-μl aliquots injected into the HPLC column.

Calibration and linearity

Calibration curves using six different concentrations of CX and HCX in plasma, ranging from 100 to 3000 ng/ml, were obtained daily for three days by calculating the peak-height ratios of these compounds to that of the internal standard *versus* the respective concentrations of CX and HCX. The concentrations of the standards evaluated were 100, 200, 800, 2000, and 3000 ng/ml. All standards were run as duplicates.

Precision and accuracy

The precision and accuracy of the assay was determined through the analysis of CX- and HCX-spiked QC plasma samples. The concentrations of the controls analyzed were 500 and 2500 ng/ml. Five QC samples at each concentration were analyzed daily for days 1–4 after which the inter- and intra-day means, standard deviations, and coefficients of variation (C.V.) were calculated.

Recovery

Recovery of CX and HCX was determined by comparing the peak-height ratios of the eluates passed through the Bond Elut to the peak-height ratio of the aqueous solution not passed through the Bond Elut. Spiked plasma samples were treated as described previously except that the internal standard was not added until the evaporation step.

Stability

Plasma samples spiked with CX and HCX at 500 and 2500 ng/ml were subjected to three

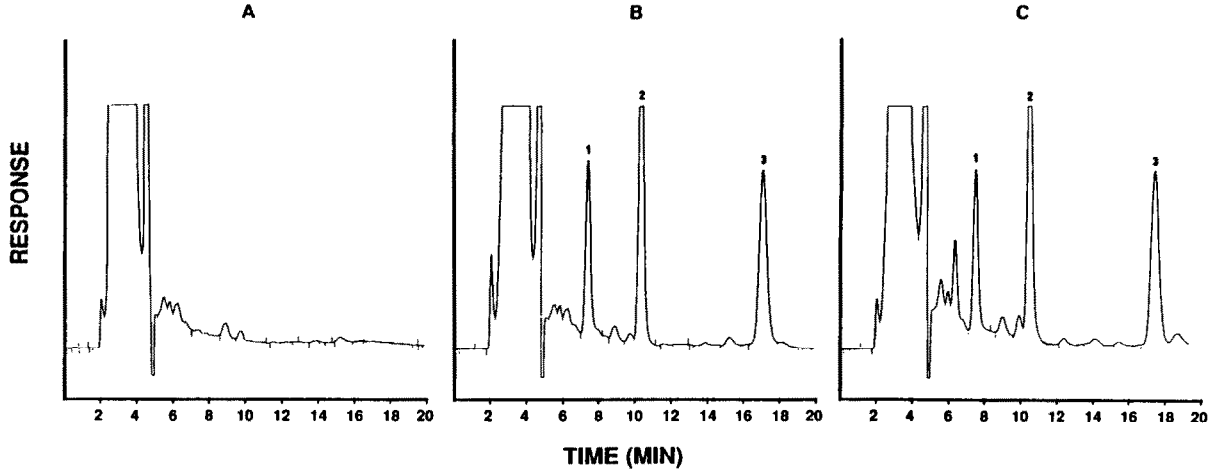

Fig. 2. Chromatograms of (A) blank plasma, (B) plasma spiked with 500 ng/ml chlorzoxazone and 6-hydroxychlorzoxazone, and (C) a patient plasma sample obtained 4 h after a 250-mg oral dose of chlorzoxazone. Peaks: 1 = 6-hydroxychlorzoxazone; 2 = internal standard; 3 = chlorzoxazone.

TABLE I

INTRA- AND INTER-DAY PRECISION AND ACCURACY FOR CX AND HCX QUALITY CONTROLS

Compound	Concentration (ng/ml)		C.V. (%)	% Deviation (found *versus* added)
	Added	Found (mean ± S.D.)		
Intra-assay reproducibility[a]				
CX	500	498.0 ± 8.4	1.7	−0.40
	2500	2491.1 ± 8.6	0.34	−0.36
HCX	500	502 ± 13	2.6	0.40
	2500	2568 ± 44	1.7	2.7
Inter-assay reproducibility[b]				
CX	500	506 ± 14	2.8	1.2
	2500	2490 ± 73	2.9	−0.40
HCX	500	517 ± 19	3.6	3.4
	2500	2514 ± 140	5.4	0.56

[a] Five samples per concentration.

[b] Five samples per day per concentration for four days.

freeze–thaw cycles (−80°C–room temperature). The samples were subsequently analyzed for the concentrations of these two compounds.

RESULTS AND DISCUSSION

Calibration curve, accuracy, and precision

Linear calibration curves were obtained for CX and HCX over the concentration range 100–3000 ng/ml. CX by least-squares regression analysis gave a mean linear correlation coefficient of $r^2 = 0.999$ with an y-intercept of 0.00483 and a slope of 0.00102. For HCX, regression analysis gave a mean linear correlation coefficient of $r^2 = 0.996$ with an y-intercept of 0.00559 and a slope of 0.00114. A weighting factor of $1/y$ was used for both regression analyses. The limit of quantitation for both CX and HCX was 100 ng/ml. The actual quantity of each compound per HPLC injection volume (50 μl) at this concentration was 10 ng. Fig. 2 portrays typical chromatograms for blank plasma, plasma spiked with CX and HCX (500 ng/ml), and a clinical plasma sample (subject DS1) collected 4 h after administration of a 250-mg CX tablet. The calculated concentrations of CX and HCX in the patient plasma sample were 512.6 and 457.7 ng/ml, respectively. Retention times for HCX, internal standard, and CX were 7.45, 10.53 and 17.65 min, respectively.

The precision of the method over the entire working concentration range was determined with the analysis of spiked QC samples. Table I shows the intra- and inter-day precision and C.V. for two concentrations of CX and HCX. As indicated, the C.V.s for the intra-day and inter-day studies were below 6%.

Recovery and stability

Results from the recovery study of CX and

TABLE II

RECOVERY OF CX AND HCX FROM PLASMA ($n = 3$)

Compound	Concentration (ng/ml)	Recovery (%)
CX	200	94.3
	800	99.0
	2000	95.2
HCX	200	72.0
	800	80.5
	2000	83.2

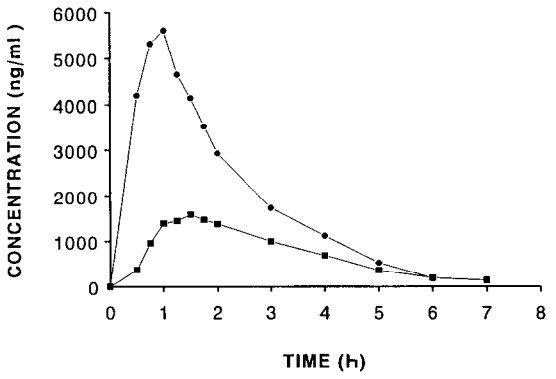

Fig. 3. Plasma concentrations of chlorzoxazone (●) and 6-hydroxychlorzoxazone (■) (subject CH3) following a single 250-mg dose of chlorzoxazone.

HCX from plasma appear in Table II. The mean recovery (n = 3) for CX at concentrations of 200, 800, and 2000 ng/ml was ≥ 94% while the recovery for HCX at the same concentrations was 72%. Incubation of patient plasma samples with β-glucuronidase was critical to the accurate determination of HCX concentrations since this compound exists mainly as the glucuronide in plasma. In a study conducted with a single patient plasma sample, hydrolysis of the HCX glucuronide conjugate was complete after 2 h of incubation at 37°C since no change in the concentration of HCX resulted in samples incubated for longer time periods. In an unincubated sample, the calculated concentration of HCX was below the limit of quantitation for the assay but was estimated at *ca.* 35 ng/ml. However, in a sample incubated with enzyme for 2 h the calculated concentration of HCX increased to 1858.3 ng/ml. Enzyme incubation has essentially no effect on the calculated concentrations of CX. Plasma samples spiked with CX and HCX and subjected to three freeze–thaw cycles showed no appreciable degradation with the concentration of each compound being ≥ 95% of the theoretical concentration.

Pharmacokinetic studies

The method we are reporting is currently being used in the study of CX pharmacokinetics. Fig. 3 is a representative example of plasma concentration *versus* time data for CX and HCX after the administration of a 250-mg CX tablet. Pharmacokinetic analyses derived from regression fitting of the data revealed a CX half-life of 1.1 h with a clearance of 21.2 l/h and an area under the curve (AUC) of 11.8 mg · h/l. The half-life of HCX was 1.3 h and the AUC 5.24 mg · h/l. As one can note, the plasma concentration of CX in this patient, as in all other patients, was always greater than that of the metabolite and above the assay limit of quantitation (100 ng/ml) for a sampling period of 7 h. In addition, this assay has the desired specificity for the measurement of CX and HCX in plasma collected from subjects administered a cytochrome P-450 drug cocktail probe consisting of caffeine, mephenytoin, debrisoquine, dapsone, and CX. Currently, we are conducting additional studies with CX in a series of subjects to evaluate its utility as an *in vivo* probe for assessing cytochrome P-450 2E1 activity.

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