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Short communication

Determination of chlorzoxazone and 6-hydroxychlorzoxazone in human plasma and urine by high-performance liquid chromatography

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Abstract

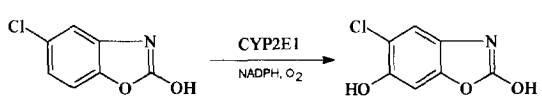
A sensitive and reproducible method is described for the determination of the cytochrome P450 enzyme 2E1 substrate chlorzoxazone and its primary metabolite 6-hydroxychlorzoxazone in human plasma and urine. Plasma or diluted urine were acidified, incubated with β-glucuronidase and then were extracted with diethyl ether. Separation of the analytes was achieved on a C_{18} column with UV detection set at 283 nm. Excellent linearity was observed over the concentration ranges of 100–3000 ng/ml and 4–400 μg/ml in plasma and urine, respectively. The intra-assay variability was ≤5.1% and the inter-assay variability was ≤8.2% for each compound in each matrix. The method presented is applicable to pharmacokinetic and pharmacogenetic studies utilizing chlorzoxazone.

Keywords: Chlorzoxazone; 6-Hydroxychlorzoxazone

1. Introduction

Chlorzoxazone (CZX) is a centrally acting skeletal muscle relaxant that is used in the treatment of muscle spasms [1]. CZX primarily undergoes hydroxylation to form 6-hydroxychlorzoxazone (HCZX; Fig. 1), a reaction that has been shown in vitro to be catalyzed by the cytochrome P450 enzyme 2E1 (CYP2E1) [2]. CZX is extensively metabolized (>99%) and rapidly eliminated, making it an attractive drug to use as a probe of in vivo CYP2E1 activity in humans.

Several HPLC methods have been reported for the analysis of CZX and HCZX in biological fluids [3–10]. In humans, CZX exists in the unchanged form in plasma but is not generally found (<1% of a dose) in urine, whereas HCZX is predominantly present in both plasma and urine as a glucuronide conjugate [3–5,9]. Some of the assay methods reported previously are only for the measurement of CZX in plasma [3,9] or HCZX in urine [8]. A common limitation of the other methods reported is



I II

Fig. 1. Metabolism of chlorzoxazone (I) to 6-hydroxychlorzoxazone (II) by CYP enzyme.

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insufficient sensitivity for low dose (250 mg) CZX pharmacokinetic studies [5–7]. We previously reported a sensitive method for the determination of CZX and HCZX in plasma that utilized solid phase extraction [4]. While this method was sensitive and specific for the quantitation of CZX and HCZX in plasma, we experienced unacceptable variability in the recovery of HCZX in subsequent lots of extraction columns. This led to the modification reported here, whereby samples are processed by a simple one-step extraction. The method retains excellent sensitivity and is also applicable to urine samples without modification.

2. Experimental

2.1. Reagents and chemicals

CZX, phenacetin (urine internal standard), β-glucuronidase (Type G0751), reagent-grade ammonium acetate, sodium chloride and sodium acetate were obtained from Sigma (St. Louis, MO, USA). 3-Aminophenyl sulfone (plasma internal standard) was obtained from Aldrich (Milwaukee, WI, USA). HCZX was a generous gift from McNeil Consumer Products (Ft. Washington, PA, USA). HPLC-grade acetonitrile, tetrahydrofuran and diethyl ether were obtained from Baxter Scientific (McGaw Park, IL, USA). All water used in the analyses was purified with a four-bowl Milli-Q reagent water system (Millipore, Bedford, MA, USA).

2.2. Equipment

The HPLC system consisted of a Waters Model 501 solvent delivery pump, a Model 712 Wisp automatic sample processor and a Model 486 UV detector which was set at 283 nm. Signal output was captured with the Waters Maxima 820 chromatography workstation (Waters, Milford, MA, USA).

2.3. Chromatographic conditions

The mobile phase used for the analysis consisted of 0.1 *M* ammonium acetate, acetonitrile and tetrahydrofuran (72:22.5:5.5, v/v) delivered at a flow-rate of 1.0 ml/min (80 bar). The mobile phase was degassed and filtered through a 0.22-μm Nylon 66 membrane before use. Separation of CZX and HCZX was achieved with an Alltech Associates (Deerfield, IL, USA) direct-connect guard column (20×2 mm, I.D.) filled with pellicular C_{18} packing connected to either an Alltech 300×3.9 mm I.D. Alphabond 10 μm C_{18} analytical column (plasma) or to a Waters 300×3.9 mm I.D. μBondapak 10 μm C_{18} analytical column (urine). Sample preparation and analysis were performed at ambient temperature.

2.4. Preparation of stock solutions and spiked standards

Stock solutions containing 0.2, 1.0 and 2.0 mg/ml of CZX and HCZX were made in methanol and stored at 4°C. These solutions were stable for at least six months when stored at 4°C. Standards and quality control samples were prepared daily by appropriate dilution of the stock solutions with plasma or urine. The plasma internal standard, 3-aminophenyl sulfone, was prepared as a 400-μg/ml stock solution in methanol and the urine internal standard, phenacetin, was prepared as a 0.5-μg/ml solution in methanol.

2.5. Preparation of plasma and urine samples

To 0.5 ml of plasma 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, diethyl ether (5 ml) was added. The samples were shaken for 10 min and then centrifuged at 2000 *g* for 10 min. The upper organic layer was transferred to a clean tube and evaporated at 40°C under a stream of nitrogen. The residues were reconstituted in 200 μl of mobile phase, transferred to Wisp microinserts, and 50-μl aliquots were injected into the HPLC system. Urine samples were first diluted 1:1 (v/v) with water and then processed as described for plasma samples, except that residues were reconstituted in 500 μl of mobile phase.

2.6. Calibration and linearity

Plasma standards containing both HCZX and CZX at concentrations of 100, 200, 400, 800, 2000 and

3000 ng/ml were prepared by dilution of stock solutions with human plasma. Urine standards containing only HCZX were prepared at concentrations of 4, 20, 50, 100, 200 and 400 μg/ml through dilution of the stock solution with interference-free urine. Urine standards and controls did not include CZX, since unchanged CZX is not excreted in urine [11,12]. For each matrix, a standard curve was prepared and analyzed daily for three days. For each curve, the peak-height ratio of drug to internal standard was calculated and plotted against the respective concentrations of CZX or HCZX. All standards were run as duplicates.

2.7. Precision and accuracy

The precision and accuracy of the assay was determined through the analysis of CZX and HCZX plasma QC samples spiked at concentrations of 500 and 2500 ng/ml or HCZX urine QC samples spiked at concentrations of 40 and 400 μg/ml. Multiple QC samples at each concentration were analyzed daily for three days and the intra- and inter-day means, standard deviations and coefficients of variation were calculated.

2.8. Extraction recovery

The extraction recoveries of HCZX and CZX from plasma and HCZX from urine were determined by comparing the peak height(s) obtained from extracted QC samples with the peak height(s) observed after direct injection of an aqueous solution containing the same analyte concentration(s). Percent extraction recovery was estimated as the difference between the individual peak heights of extracted QC samples and the unextracted standards. The means and standard deviations of percent extraction recovery were determined in each matrix from five replicates of the low and high QC concentrations.

3. Results and discussion

Representative chromatograms of a spiked standard and a patient plasma sample are shown in Fig. 2 and those of a urine sample are shown in Fig. 3. Each was obtained after a 250-mg oral CZX dose. In plasma, the retention times of HCZX, internal standard and of CZX were 7.2, 12.1 and 17.5 min, respectively, while in urine, the retention times of HCZX and internal standard were 7.6 and 10.9 min, respectively. Calibration curves for HCZX and CZX were generated by weighted ($1/y^2$) linear regression analysis. Linear calibration curves were obtained for HZCX and CZX in plasma over the concentration range 100–3000 ng/ml and for HCZX in urine over the concentration range of 4–400 μg/ml. The correlation coefficients (r) were greater than 0.999. The intra- and inter-day precision and %CV were determined for HCZX and CZX in plasma (Table 1) and for HCZX in urine (Table 2) and were 8.2% or less.

The modification of our previously reported method retains excellent sensitivity and yields more consistent recovery of HCZX. The plasma internal standard was changed from our original method, where 5-fluorobenzoxazolone was used. This compound is not commercially available and so was replaced with the readily available 3-aminophenyl sulfone. The retention time of this new internal standard was similar to 5-fluorobenzoxazolone and was devoid of interferences.

The average extraction recoveries of HCZX and CZX from plasma at the low (500 ng/ml) concentration were 95±6.0% and 103±2%, respectively, and were 79±6% and 92±4% at the high (2500 ng/ml) concentration, respectively. The average recovery of HCZX from urine at the low (40 μg/ml) and high (400 μg/ml) concentration was 65±2% and 78±2%, respectively. The lower limit of quantitation in plasma for both CZX and HCZX was 100 ng/ml (at a signal-to-noise ratio of more than 5:1), which is sufficient to measure CZX and HCZX concentrations for 6 to 10 h following oral administration of a 250-mg tablet. In addition to increased sensitivity in plasma, our method also differs from previously published methods for plasma or urine in the time of incubation with β-glucuronidase. The time used in our method was 3 h, which is considerably shorter than the overnight incubations used by Lucas et al. [5] (at 37°C) or Zhang and Stewart [6] (ambient temperature). Incubation time trials in our laboratory using 1000 units of β-glucuronidase indicate that hydrolysis is complete by 2 h.

We are currently using CZX as an in vivo probe of

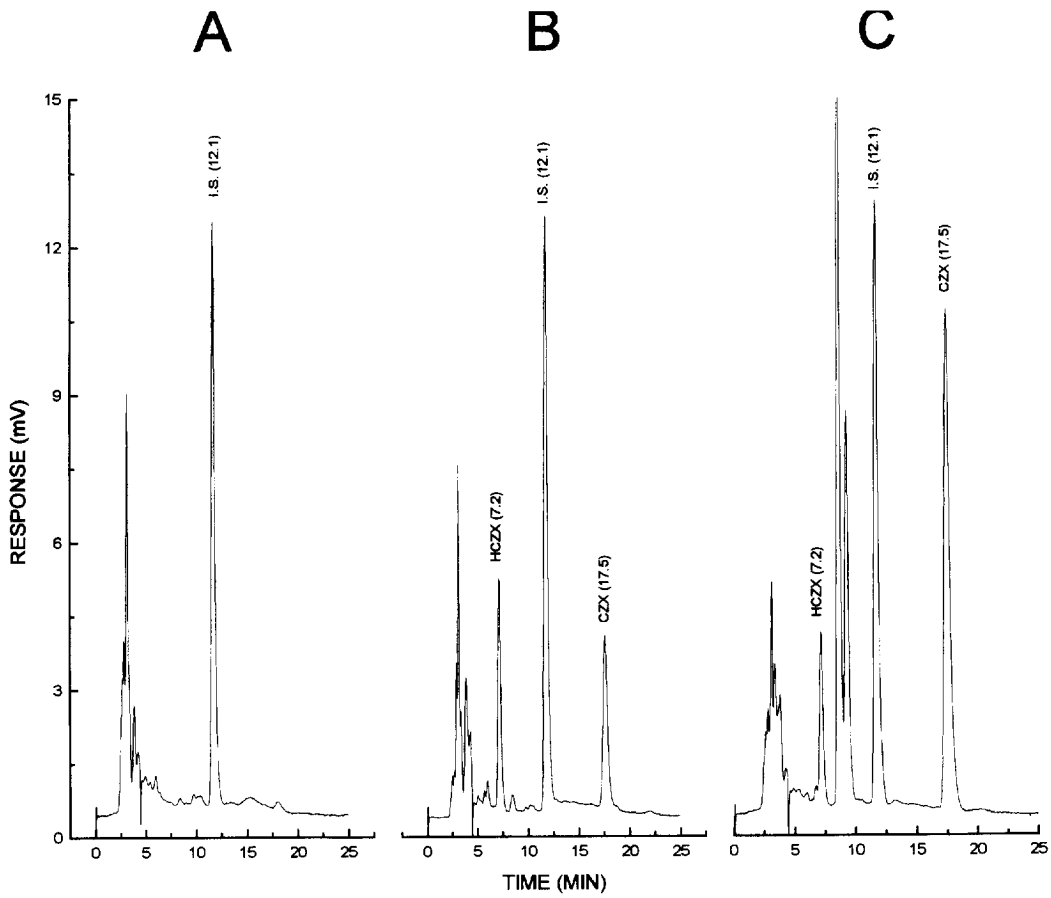

Fig. 2. Typical HPLC chromatograms of (A) blank human plasma; (B) spiked plasma containing 800 ng/ml of CZX and HCZX; (C) patient plasma sample obtained 4 h after a 250-mg dose containing 2228.6 ng/ml of CZX and 570.1 ng/ml of HCZX. The large peaks between HCZX and CZX are from the co-administered probe drug, dapsone, and its monoacetylated metabolite.

Table 1
Intra- and inter-day precision and accuracy for chlorzoxazone and 6-hydroxychlorzoxazone in plasma

Compound	Concentration (ng/ml)		C.V. (%)	Deviation (%)
	Added	Found (mean±S.D.)		
Intra-assay[a]				
CZX	500	507.5±8.8	1.7	1.5
	2500	2441.4±17.4	0.7	−2.3
HCZX	500	563.2±18.0	3.2	12.6
	2500	2395.8±41.2	1.7	−4.2
Inter-assay[b]				
CZX	500	503.4±25.9	5.1	0.7
	2500	2458.0±43.9	1.8	−1.7
HCZX	500	520.1±42.7	8.2	4.0
	2500	2372.3±69.2	2.9	−5.1

[a] Seven samples per concentration.
[b] Five samples per day per concentration for three days.

Table 2
Intra- and inter-day precision and accuracy for 6-hydroxychlorzoxazone in urine

Compound	Concentration (μg/ml)		C.V. (%)	Deviation (%)
	Added	Found (mean±S.D.)		
Intra-assay[a]				
HCZX	40	45.4±2.3	5.1	13.4
	400	417.3±12.2	2.9	4.3
Inter-assay[b]				
HCZX	40	44.1±3.0	6.8	10.3
	400	418.1±26.5	6.3	4.5

[a] Three samples per concentration.
[b] Three samples per day per concentration for three days.

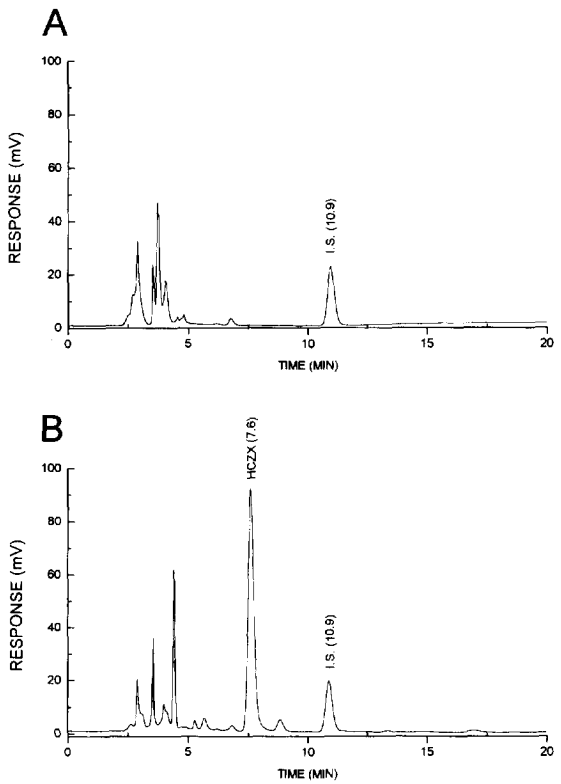

Fig. 3. Typical HPLC chromatograms of (A) blank human urine and (B) a human urine sample containing 276.4 μg/ml of HCZX obtained from a 0–8 h collection following a 250-mg dose.

CYP2E1 activity in humans. The activity of CYP2E1 can be estimated using either the ratio of HCZX to CZX in a 4-h plasma sample or the HCZX formation clearance, which is calculated using the total urinary recovery of HCZX and the plasma clearance of CZX [7,12,13]. Thus, the ability to accurately measure CZX and HCZX concentrations in plasma and HCZX concentrations in urine are critical. Of note, using this method we are able to measure HCZX in the plasma of all subjects following a 250-mg CZX dose. This is in contrast to the report of Dreisbach et al. [10], who were able to measure HCZX in only four out of ten patients following a 500-mg oral dose of CZX. CZX and HCZX concentration–time profiles, along with the cumulative urinary recovery of HCZX in a representative subject, are shown in Fig. 4.

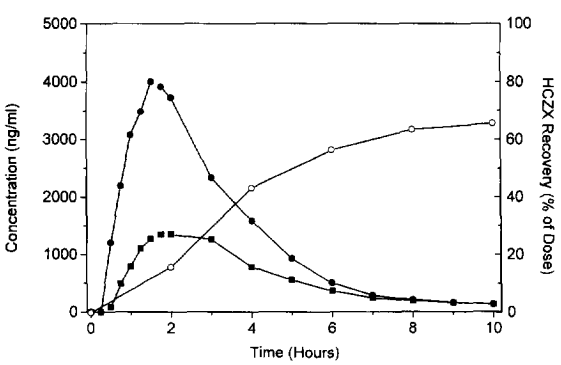

Fig. 4. Plasma concentration–time profiles of CZX (●) and HCZX (■) and the cumulative urinary recovery of HCZX (○) following a single oral 250-mg dose of CZX.

In order to estimate the activity of multiple CYP enzymes in a single experimental session, we are simultaneously administering CZX with the probe drugs caffeine, dapsone, debrisoquine and mephenytoin to estimate the in vivo activity of the CYP enzymes 2E1, 1A2, 3A4, 2D6 and 2C19, respectively. No interfering peaks are present from these co-administered drugs or their metabolites in either plasma or urine. In addition, no interferences have been observed in samples from multiple patient populations, including patients with hepatic or renal disease, patients with cancer and from liver transplant patients.

In conclusion, we have described a sensitive and reproducible method for the determination of CZX and HCZX in plasma or urine. The method described is currently being used to support pharmacokinetic and pharmacogenetic studies utilizing CZX.

Acknowledgments

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