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Determination of 6-hydroxychlorzoxazone and chlorzoxazone in porcine microsome samples

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Abstract

A simple, accurate and sensitive HPLC method for the in vitro determination of 6-hydroxychlorzoxazone and chlorzoxazone in porcine microsome samples is described. Chromatography was performed on a YMC-Pack ODS-AQ column using a mobile phase of 0.05% phosphoric acid pH 3–methanol (60:40, v/v). UV detection was carried out at 287 nm. The detector response was linear over the concentration range 25–2000 ng/ml. This assay produced quick, accurate, and repeatable results.

Keywords: Hydroxychlorzoxazone; Chlorzoxazone

1. Introduction

The cytochrome P450 monooxygenase enzyme system is important in terms of its catalytic versatility and the sheer number of compounds it detoxifies or activates to reactive intermediates [1,2]. The largest concentration of P450 enzymes is located in the liver endoplasmic reticulum (microsomes), but they are located in virtually all other tissues in the body. These enzymes activate xenobiotics including drugs to toxic and/or tumorigenic metabolites, detoxify xenobiotics, and aid in determining intensity and duration of action of drugs. Therefore, it is essential to establish the activity and regulation of the P450 system for species selected for pharmacological and toxicological studies. In addition, impaired drug disposition in food-producing animals may lead to changes in residue levels of veterinary drugs and other xenobiotics in edible tissues, milk, or eggs. There is also concern about the increase in development of drug resistant bacterial strains in food animals. Much of the information known about P450 comes from studies conducted in rodents. However, the pig is becoming a popular alternative to traditional non-rodent species in pharmacological and toxicological testing [3]. The information on the P450 system for this species is limited.

P-450 enzymes are mainly responsible for phase I metabolism, which adds or exposes polar functional groups on a lipophilic substrate. In the investigation of P-450 mediated xenobiotic metabolism, individual

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Fig. 1. Structures of chlorzoxazone and 6-hydroxychlorzoxazone.

forms of P450 have been found to catalyze specific reactions with certain substrates, thus designating these activities as probes for these forms. To date, at least one marker activity exists for the majority of human P450 forms [4]. Cytochrome P450 2E1 is a major mammalian hepatic enzyme, which catalyzes the biotransformation of many low molecular mass xenobiotics. However, one of the challenges to studying 2E1 has been to identify a model substrate that is a sensitive and specific marker for the enzyme's activity. The conversion of chlorzoxazone (CZX) to 6-hydroxychlorzoxazone (OH-CZX) (Fig. 1) is one of the methods used to characterize human cytochrome P450 enzyme CYP2E1 activity both in vivo and in vitro. However, limited information exists in other mammalian species because of differences in biotransformation.

Several high-performance liquid chromatography (HPLC) methods have been developed to measure CZX and OH-CZX in biological fluids and microsomes [5–15]. Some of the methods involve the use of liquid–liquid extractions [5–7,10,12–15], while most use rat or human microsome samples.

We describe an easy and efficient method for the in vitro analysis of OH-CZX and CZX. This procedure provides a quick analysis that may facilitate the characterization of P450 metabolism in the pig. This characterization may allow the pig to be used in pharmacological and toxicological studies to determine detoxification of xenobiotics as well as aid in determining appropriate dosages and withdrawal times of drugs used in food animals.

2. Experimental

2.1. Reagents and standards

The methanol used was HPLC-grade; phosphoric acid was reagent grade while all other chemicals were enzyme grade. All of these chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA). Chlorzoxazone, glucose 6-phosphate (G-6-P), glucose 6-phosphate dehydrogenase (G-6-PDH), β-nicotinamide adenine dinucleotide phosphate (NADP) and phenacetin, the internal standard, were purchased from Sigma (St. Louis, MO, USA). Hydroxychlorzoxazone was purchased from RBI (St. Louis, MO, USA). Stock standard solutions of CZX (100, 5 and 1 μg/ml) and OH-CZX (100, 5, and 1 μg/ml) were prepared in methanol and stored at 4 °C. Solutions were stable for 6 months. Working standards were prepared fresh daily by dilution of the stock standards. A stock standard solution of phenacetin (100 μg/ml) was prepared in methanol and stored at 4 °C. This solution was also stable for 6 months. Phenacetin is light sensitive and all solution containers were wrapped in aluminum foil.

2.2. Apparatus

The analytical system consisted of a 626 solvent delivery system, a model 717 WISP autosampler, a 996 scanning UV detector and a computer equipped with Millennium software (Waters, Milford, MA, USA). The column was a YMC-Pack ODS-AQ (5 μm, 6×150 mm) equipped with a C_{18} Guard-Pak precolumn insert (Waters, Milford. MA, USA).

2.3. Chromatography

The mobile phase consisted of a mixture of (A) 0.05% phosphoric acid, pH 3.0 and (B) methanol. The mixture was pumped as a gradient starting at 60% A and 40% B and was maintained for 12 min. Over a 2-min period the mixture changed to 58% A and 42% B which was maintained for 5 min and then returned to initial conditions over the final 3-min period. The system was ready for the next injection without further equilibration. The mobile phase was prepared fresh daily using double-distilled deionized water, filtered (0.22 μ*M*) and degassed before use. The flow-rate was 1.7 ml/min. Column temperature was ambient and UV detection was measured at 287 nm.

2.4. Sample treatment

Spiked samples were prepared by addition of appropriate volumes of both CZX and OH-CZX. The

internal standard, phenacetin (30 μl of 100 μg/ml) was added. Appropriate amounts of the solutions used in microsomal preparations were added to produce a 0.5-ml final volume. Samples were vortex-mixed and a 190-μl sample injected onto the liquid chromatograph.

Microsomal samples were prepared using Lake's ultracentrifugation method [16]. Incubation mixtures contained 0.5 mg of microsomal protein, 100 m*M* phosphate buffer at pH 7.4 containing 6 m*M* magnesium chloride, 1 m*M* EDTA, and a NADPH-generating system (1 m*M* NADP, 10 m*M* G-6-P and 0.7 U of G-6-PDH) in a total volume of 0.5 ml. Incubation mixtures contained CZX and inhibitors and the reactions were initiated by addition of the NADPH-generating system after a 5-min pre-incubation step at 37 °C. Reactions were quenched with 0.1 ml of ice cold acetonitrile after 20 min in a 37 °C shaking water bath and then placed on ice for 1 h. Samples were centrifuged at 16 000 *g* for 15 min. The supernatant was removed and stored at −80 °C until analysis could be performed. Reaction rates were linear with incubation time under these conditions. Frozen samples were thawed on ice and vortex-mixed before use. Phenacetin (30 μl of 100 μg/ml) was added to a 0.5-ml microsome sample and vortex-mixed. Samples, which contained particulates, were centrifuged for 5 min at 16 000 *g* in an Eppendorf centrifuge (Brinkman Instruments, New York, NY, USA). A 190-μl aliquot of the supernatant was injected onto the liquid chromatograph.

3. Results

A blank chromatogram, which is everything but drug added, for a microsomal sample is shown in Fig. 2A with a large peak at 2.32 min and a small peak at 2.95 min. These peaks are the result of NADPH generating solution used in the preparation of the microsome sample; however, they do not interfere with the peaks of interest. The *x*-axis on chromatograms 2B and 2C start roughly at 5 min in order to eliminate the large NADPH peak and provide a better image. The chromatogram in Fig. 2B represents a 500 ng/ml standard. The retention times were 6.79, 11.78 and 20.28 min for OH-CZX, phenacetin and CZX, respectively. The chromatogram in Fig. 2C represents a porcine liver microsome sample after incubation with 10 μ*M* of CZX. Retention times for OH-CZX, phenacetin and CZX were 6.84, 11.92 and 20.67 min.

The detection response was linear for the concentration range of 25–2000 ng/ml for CZX and its metabolite, with correlation coefficients ranging from 0.998 to 0.999 for both compounds. Replicate analyses performed on the same day for microsomal samples spiked with specific concentrations of CZX produced coefficients of variation (C.V.) of 4.6% for 60 ng/ml, 2.9% for 800 ng/ml and 1.8% for 1700 ng/ml. The metabolite's C.V. was 3.2, 0.3, and 0.9% for the same concentrations (Table 1). Day-to-day variability for microsomal replicates appears in Table 2. Mean recoveries of CZX were 96, 101, 102, 97, 98, 99, and 95% for 25, 50, 100, 250, 500, 1000, and 2000 ng/ml. Mean recoveries of OH-CZX were 104, 96, 94, 98, 95, 98, and 99% for 25, 50, 100, 250, 500, 1000, and 2000 ng/ml. The detection limit for CZX was 20 ng/ml and that for its metabolite was 10 ng/ml. This represents a peak approximately three times that of baseline noise.

Numerous drugs and chemicals used in inhibition studies were tested for interference with the chromatographic procedure (Table 3). Initially, quinidine was found to be co-elute with OH-CZX; however, using the current chromatography and a change of pH to 3.2 eliminated this problem. Furafylline, which is used in microsomal assays, could interfere with the internal standard, phenacetin, but with the current gradient there is adequate separation.

Operating under linear conditions adapted from reported procedures [17,18], the formation of OH-CZX was easily detectable at 10 μ*M* as seen in Fig. 2C. Enzyme activity for the sample was 216 nmol/min/mg protein. This was calculated by dividing the amount of product formed by incubation time and microsomal protein content. In this particular sample of porcine liver microsomes, the estimated K_m for the process was 43 μ*M*.

4. Discussion

Enzyme metabolism studies require a method that is simple, quick, sensitive and reproducible. An HPLC assay, utilizing UV detection, has been developed to investigate the 6-hydroxylation of CZX

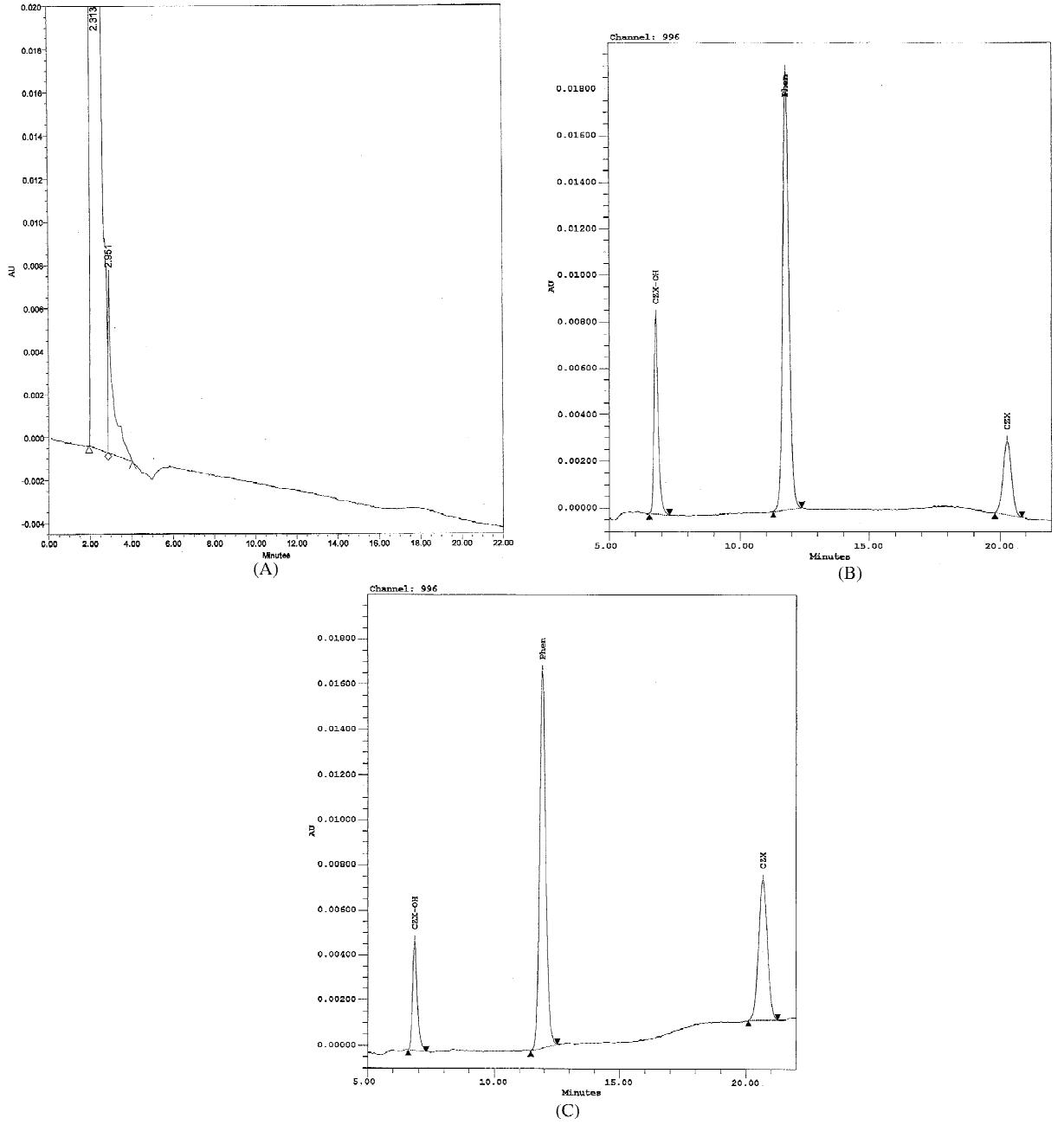

Fig. 2. (A) Blank microsome chromatogram with no drug added. Peaks 2.32 and 2.95 result from microsomal generating solution. (B) Chromatogram of a 500 ng/ml standard. Peaks: CZX-OH=OH-CZX; Phen=phenacetin; CZX=CZX. (C) Chromatogram of a porcine microsomal sample after incubation with 10 μM of CZX. Peaks: CZX-OH=OH-CZX; Phen=phenacetin; CZX=CZX.

by microsomal fractions of porcine liver. The assay is sensitive, specific and reproducible, with a high recovery of the metabolite.

Because we did not use an extraction for our samples, the NADP generating solution produced a very large peak initially. In order to prevent any interference with OH-CZX, the mobile phase was adjusted to 60% A and 40% B. This percentage was

Table 1
Intra-assay precision for chlorzoxazone and 6-hydroxychlorzoxazone ($n=4$)

Concentration added (ng/ml)	CZX concentration measured (ng/ml)	Coefficient of variation (%)	OH-CZX concentration measured (ng/ml)	Coefficient of variation (%)
60	60	4.6	63	3.2
800	766	2.9	761	0.3
1700	1761	1.8	1749	0.9

Table 2
Inter-assay precision for chlorzoxazone and 6-hydroxychlorzoxazone ($n=4$)

Concentration added (ng/ml)	CZX concentration measured (ng/ml)	Coefficient of variation (%)	OH-CZX concentration measured (ng/ml)	Coefficient of variation (%)
25	24	2.1	26	11
50	51	7.8	48	2.1
100	102	8.3	94	5.3
250	243	1.9	244	6.6
500	491	2.6	477	5.1
1000	989	1.5	975	6.0
2000	1897	6.0	1989	2.0

maintained until the elution of the internal standard in order to prevent any interference from furafylline when it was used in microsomal preparations. We did try to optimize the chromatography after the elution of the internal standard; however, an increase in the percent methanol in the mobile phase higher than what we have listed caused a large shift in the baseline, which was not acceptable. If the quantitation of CZX is not necessary or important for the experiment of interest, the assay time can be shortened to eliminate CZX.

Table 3
Chemicals tested for assay interference

Chemicals	Retention times (min)
Quinidine	7.82
Furafylline	10.37
7,8-Benzoflavone	ND
Diethyldithiocarbamate	ND
Ketoconazole	ND
Ciprofloxacin	4.96
Bufuralol	ND
Itraconazole	ND
Phenacetin	11.92
NADP	2.32
Potassium phosphate	2.45
Magnesium chloride	ND
Glucose-6-phosphate	ND
Glucose-6-phosphate dehydrogenase	ND

ND=no peaks were detected.

Most published procedures do not list validation parameters such as limit of detection or recoveries for OH-CZX and CZX which makes it difficult to compare our method with others. We do feel that our limit of detection and recovery for both compounds is more than adequate for microsomal studies. In cases where validation parameters are listed, ours are equal to or better than existing methods. Use of phenacetin as the internal standard corrects for intra- and inter-assay variability in the method.

HPLC procedures involving extraction of OH-CZX and CZX described by Thummel et al. [5] used 5 ml of methylene chloride for extraction and have a quantitation range of 250–5000 ng/ml. While Peter et al. [7] and Taavitsainen et al. [15] require microsome samples to be extracted two times with methylene chloride before evaporation and injection, with a limit of detection of 90 ng/ml. Lillibridge et al. [12] also used methylene chloride and indicates that standard curves were linear over their respective ranges and inter-day and intra-day coefficients of variation were less than 10%. Chittur and Tracey [13] used an ether extraction, could quantify samples at amounts of 10 ng or greater and had a recovery between 40 and 45%. Cummings et al. [14] used ethyl acetate and flash freezing in a dry-ice acetone bath. Leclercq et al. [10] used a zinc sulfate homogenization procedure followed by filtering of the sample. Our procedure eliminates the use of time

consuming liquid–liquid extractions involving toxic and expensive organic solvents and does not require the use of dry ice, homogenizers, nitrogen evaporation or sample filters. It is a rugged procedure with the column still in use after over 2000 injections and the guard column replaced roughly every 300 injections. We chose this particular column because of its longevity and ruggedness as well as its ability to tolerate the sample type used in the assay without effecting the resolution of the compounds of interest. Even after 2000 injections the resolution and peak shape are exceptional with very little increase in pressure.

The present study was conducted in order to develop a method to determine metabolism of CZX in porcine microsome samples from liver. The method has been applied to xenobiotic effects on porcine microsomes involving CZX in this laboratory. In conclusion, a simple, quick and sensitive HPLC procedure has been developed for analysis of OH-CZX and CZX in porcine hepatic microsome samples.

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