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Short communication

Rapid and sensitive high-performance liquid chromatographic assay for 6-hydroxychlorzoxazone and chlorzoxazone in liver microsomes

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Abstract

A high-performance liquid chromatographic assay was developed for the quantitation of chlorzoxazone and its major metabolite 6-hydroxychlorzoxazone. These compounds along with phenacetin, the internal standard, were extracted from incubation mixtures using ether extraction. The extracts were analyzed on a Brownlee Spheri-5 C_8 column with a mobile-phase of acetonitrile–0.5% phosphoric acid (30:70, v/v). The assay utilized UV detection at 287 nm which provided sensitivity and specificity to simultaneously quantify chlorzoxazone and 6-hydroxychlorzoxazone from liver microsomal samples at amounts of 10 ng and greater. The mean correlation coefficient of the standard curves for 6-hydroxychlorzoxazone and chlorzoxazone was 0.998 and 0.993, respectively, over the range of 25–400 ng, and the regression curves were found to be linear at least through 1600 ng. All components eluted within 7 min, resulting in a total analysis time of 8 min. The inter-day and intra-day coefficients of variation were <7 and $<3\%$, respectively. This method provides a rapid, sensitive and cost-effective assay for 6-hydroxychlorzoxazone and chlorzoxazone in liver microsomal incubations.

Keywords: 6-Hydroxychlorzoxazone; Chlorzoxazone

1. Introduction

Chlorzoxazone (CX) is a skeletal muscle relaxant used in the treatment of spasticity disorders. The major metabolite produced by hepatic microsomal metabolism of chlorzoxazone is 6-hydroxychlorzoxazone (HCX) (Fig. 1) [1]. The production of HCX appears to be predominantly mediated by cytochrome P450 2E1 and thus this process has been used as a metabolic probe to assess the activity of cytochrome P-450 2E1 both in vivo and in vitro [2–4].

Several groups have described column liquid chromatographic methods, using either UV or electrochemical detection, for the analysis of CX and HCX [5–9]. The major limitation of these methods is the degree of sensitivity achieved, based on their reported limits of quantitation and calibration curve ranges. Recently, a method employing solid-phase

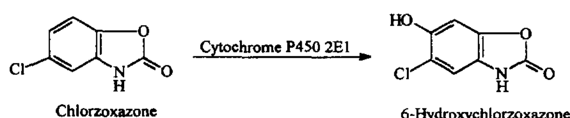

Fig. 1. Structures of chlorzoxazone and the 6-hydroxychlorzoxazone metabolite.

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extraction was reported which attempted to overcome these limitations [9]. However, the cost associated with use of solid-phase extraction columns decreases the overall usability of this method.

This paper describes a simple, cost effective, sensitive and specific HPLC method for the simultaneous quantitation of CX and HCX using UV detection. This method employs one liquid–liquid extraction step and subsequent chromatography on a Cs reverse-phase column with a total run time of 8 min. The method is sufficiently sensitive to detect CX and HCX at amounts as low as 10 ng.

2. Experimental

2.1. Reagents and chemicals

Diethyl ether, acetonitrile and phosphoric acid were obtained from the Fisher (Pittsburgh, PA, USA). Chlorzoxazone and phenacetin were purchased from Sigma (St. Louis, MO, USA). 6-Hydroxychlorzoxazone was purchased from Research Biochemicals International (Natick, MA, USA). All other chemicals were obtained from commercial sources and were of the highest purity available. Rat liver microsomes from male Sprague–Dawley rats were prepared by differential centrifugation according to methods approved by the Animal Care and Use Committee of West Virginia University.

2.2. Instrumentation

The HPLC system consisted of a Waters 501^R HPLC pump, a Waters 717^R autosampler and a Waters 486^R ultraviolet detector set at 287 nm. The mobile phase consisted of acetonitrile–0.5% phosphoric acid (30:70, v/v) pumped at 1 ml/min through a Brownlee Spheri-5 C_8 4.6×100 mm reversed phase column.

2.3. Preparation of stock solutions

6-Hydroxychlorzoxazone stock solutions were prepared in absolute ethanol and diluted to the appropriate concentrations for preparation of the standard curves. Phenacetin was initially dissolved in absolute ethanol to a concentration of 5 mg/ml, then further diluted to a final concentration of 1.5 μg/ml with acetonitrile. Chlorzoxazone was prepared by adding 150 μl of 3 *M* KOH to 8.48 mg of chlorzoxazone and then further diluting with 4850 μl of 100 m*M* K_2HPO_4–5 m*M* $MgCl_2$, pH 7.4 to make a final concentration of 10 m*M*. This solution was then further diluted with the phosphate buffer to make 1 m*M* and 0.1 m*M* solutions.

2.4. Incubations

Liver microsomes (0.4 mg/ml final protein concentration) and chlorzoxazone (over a 5 μ*M* to 1 m*M* range of concentrations) were incubated in the presence of 1 m*M* β-NADP, 10 m*M* glucose 6-phosphate, 0.2 units glucose 6-phosphate dehydrogenase and 100 m*M* K_2HPO_4, pH 7.4 containing 5 m*M* $MgCl_2$ in a total volume of 400 μl and allowed to react for 20 min at 37°C. The samples were then quenched with 200 μl of acetonitrile containing phenacetin (300 ng) as internal standard.

2.5. Extraction

After incubation, the samples were vortexed for 30 s and then centrifuged for 4 min at 10 000 *g*. The supernatant was transferred to a fresh tube, 2 ml of diethyl ether added, the samples vortexed for 30 s and then centrifuged at 2000 *g* for 1 min to separate the layers. The ether layer was then transferred to fresh tubes and dried under vacuum. The residues were reconstituted with 150 μl of mobile phase, vortex-mixed and transferred to Wisp microinserts and 100 μl aliquots injected into the HPLC column.

3. Results

Chromatograms depicting the analysis of HCX and CX in human liver microsomes and a blank chromatogram are shown in Fig. 2A–C. HCX, CX and the internal standard, phenacetin, were separated within the total 8 min of the chromatographic run. The retention times for HCX, phenacetin and CX were approximately 2.7, 4.3 and 6.4 min, respectively.

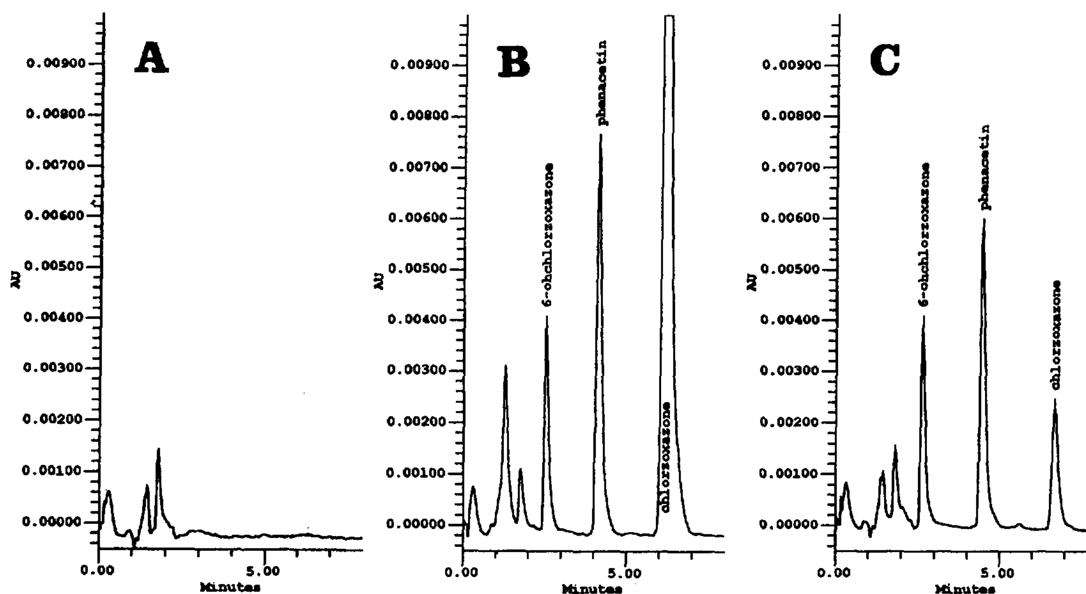

Fig. 2. Chromatographic analysis of chlorzoxazone and 6-hydroxychlorzoxazone in liver microsomes. Panel A – blank chromatogram. Panel B – chromatogram demonstrating the formation of 6-hydroxychlorzoxazone from chlorzoxazone by rat liver microsomes in the presence of a NADPH-regenerating system. Panel C – chromatogram of calibration curve standard containing 50 ng each of 6-hydrochlorzoxazone and chlorzoxazone in rat liver microsomes.

3.1. Calibration and linearity

Calibration curves were obtained daily for three days using six different amounts of CX and HCX, ranging from 25 to 400 ng, in microsomal incubation mixtures by calculating the peak-area ratios of these compounds to that of phenacetin, the internal standard. The amounts of the standards evaluated were 0, 25, 50, 100, 200 and 400 ng. A $1/y$ weighting scheme was applied to the calibration curves during the linear regression analysis. The mean correlation coefficients of the standard curves for 6-hydroxychlorzoxazone and chlorzoxazone were 0.998 and 0.993, respectively. The calibration curves were linear through at least 1600 ng (data not shown).

3.2. Precision and accuracy

The precision and accuracy of the assays were determined through a single-blind analysis of CX and HCX solutions. The amounts of the quality control samples analyzed were 37.5 and 187.5 ng. Three samples at each concentration were analyzed daily for four days after which the inter- and intra-day means, standard deviations and coefficients of variation (C.V.) were calculated (Table 1). Coeffcients of variation were less than 7% in all cases.

3.3. Recovery

Recovery of CX and HCX were determined by comparison of the peak-area ratios of the eluates

Table 1
Intra- and inter-day precision and accuracy for CX and HCX

Compound	Amount added (ng)	Amount found (mean±S.D., n=3) (ng)	C.V. (%)
Intra-day			
CX	50	53.66±0.88	1.64
	400	437.58±9.73	2.22
HCX	50	52.71±1.52	2.88
	400	408.75±5.44	1.33
Inter-day			
CX	50	51.31±3.50	6.82
	400	424.76±11.51	2.71
HCX	50	51.16±1.47	2.87
	400	411.78±1.68	0.41

obtained from samples containing the analytes as well as microsomal protein following the full extraction procedure to the peak-area ratio of the eluates following direct injection of the stock solutions of CX and HCX. The mean±S.D. recoveries ($n=3$) for CX at 25 and 400 ng were 45.1±1.7 and 40.8±1.2%, respectively.

3.4. Incubation of chlorzoxazone with rat liver microsomes

This assay methodology was then applied to an experiment in which CX was incubated with rat liver microsomes and the production of HCX measured, as an estimate of cytochrome P450 2E1 activity. A plot depicting the formation velocity of this reaction is shown in Fig. 3.

Operating under linear conditions, the formation of HCX was readily detectable at substrate concentrations of 5 μM. These values were fit to the Michaelis–Menten equation using non-linear regression techniques to obtain estimates of K_m and V_{max}. In this particular sample of rat liver microsomes, the estimated K_m for this process was 128 μM and the estimated V_{max} was 1071 pmol/min/mg protein.

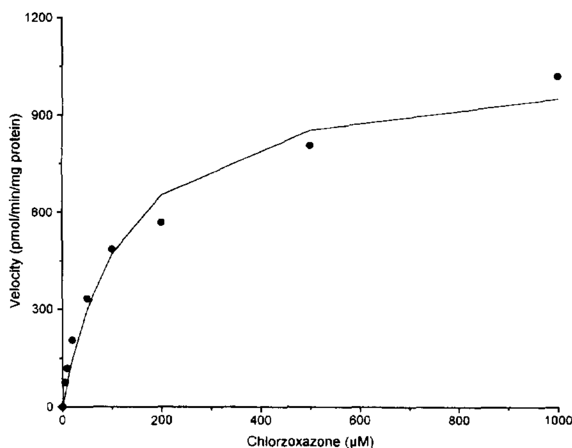

Fig. 3. Plot of 6-hydroxychlorzoxazone formation velocity following incubation of chlorzoxazone with rat liver microsomes and an NADPH regenerating system. Line represents the best non-linear regression fit of the experimental data. Kinetic parameter estimates are listed in text.

4. Discussion

The study described herein demonstrates a rapid and sensitive HPLC assay for 6-hydraxychlorzoxazone and chlorzoxazone in rat liver microsomal incubations. This method was applied to incubations of rat liver microsomes to measure the formation of 6-hydroxychlorzoxazone from chlorzoxazone as a probe for cytochrome P-450 2E1 activity. This method proved to be more than adequate for the determination of the K_m and V_{max} parameter estimates for the 6-hydroxylation of chlorzoxazone in rat liver tissue. Furthermore, this method compared to previous assays, shows an increased sensitivity of about 10-fold over the solid-phase extraction method reported by Stiff et al. [9] and a 2-fold increase over the liquid–liquid extraction protocol reported by Peter et al. [2]. The use of ether extraction instead of methylene chloride affords the organic layer containing the extracted components on the top, thereby eliminating inclusion of contaminants from the aqueous phase and simplifying transfer of the organic layer to another tube for evaporation. Also, a single extraction step is suffcient to obtain the compounds in good yields. The recovery is slightly lower than the solid-phase method but is very consistent and the assay shows greater sensitivity. The fact that the HPLC run time for the assay is less than 8 min provides additional support towards the ease and cost effectiveness of this method.

Acknowledgments

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