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High-performance liquid chromatographic determination of chlorzoxazone and 6-hydroxychlorzoxazone in serum: a tool for indirect evaluation of cytochrome P4502E1 activity in humans

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ABSTRACT

Chronic alcohol consumption is known to induce the enzyme cytochrome P4502E1 (CYP2E1), which is involved in the toxicity and carcinogenicity of a number of solvents and xenobiotics. It was recently suggested that *in vivo* chlorzoxazone metabolism could be a potential tool as a non-invasive probe for measuring CYP2E1 activity in humans. Therefore, a simple and sensitive method was developed for the determination of chlorzoxazone and its major metabolite 6-hydroxychlorzoxazone in both serum and urine. Biological samples were hydrolysed by *Helix pomatia* juice, deproteinized with perchloric acid, and then extracted using ethyl acetate. The compounds were separated by high-performance liquid chromatography on an octadecylsilane column with a mobile phase of acetonitrile–0.5% acetic acid in water (30:70, v/v) and detected at 287 nm. The linearity of the method was tested in the concentration range 0.5–20 μg/ml, and the limit of detection in biological samples was found to be 0.5 μg/ml. Within- and between-run precision was below 5% and 10%, respectively, for both compounds at three concentrations (0.5, 10 and 20 μg/ml). The accuracy of the procedure was in the range 0.3–6%. Serum levels and urinary excretion of chlorzoxazone and its metabolite were studied in five healthy controls and five alcoholic patients, following oral administration of 500 mg of chlorzoxazone. The concentration ratio 6-hydroxychlorzoxazone/chlorzoxazone in blood was shown to be a valuable tool for the evaluation of CYP2E1 activity in humans.

INTRODUCTION

The human enzyme cytochrome P4502E1 (CYP2E1) can catalyse the oxidation of many xenobiotic compounds, such as halogenated hydrocarbons, vinyl monomers or ethyl carbamate, to reactive forms [1]. It is of clinical relevance to have a non-invasive assay of CYP2E1, because large interindividual variations in the levels of this enzyme have been reported [2]. Some of this variability could be due to the effects of inducers, such as ethanol [3]. The assessment of CYP2E1 in humans has been limited because

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biopsy or surgical liver samples were required for this determination [4]. Recently, Peter *et al.* [5] demonstrated that CYP2E1 was involved in the *in vitro* hydroxylation of chlorzoxazone (5-chloro-2(3*H*)-benzoxazolone) to 6-hydroxychlorzoxazone (5-chloro-6-hydroxy-2(3*H*)-benzoxazolone) in human liver microsomal samples. *In vivo*, the major metabolic pathway consists of 6-hydroxylation of chlorzoxazone in the liver to 6-hydroxychlorzoxazone, which is excreted in the urine primarily as a glucuronide conjugate [6,7]. Therefore, as chlorzoxazone, a centrally acting skeletal muscle relaxant, is a relatively safe drug, the use of its 6-hydroxylation reaction has been suggested as a tool for measuring CYP2E1 *in vivo*. Accordingly, the ratio 6-hydroxychlorzoxazone/chlorzoxazone, determined in serum when drug concentrations are at a maximum, could reflect the induction level of CYP2E1.

Analytical methods previously reported for the measurement of chlorzoxazone and its hydroxy derivative in biological fluids are scarce. They are based on UV spectrophotometry [6], fluorimetry [8], gas chromatography [7] and high-performance liquid chromatography (HPLC) [7,9–11] after solvent or solid-phase extraction from biological samples. However, these methods are not very convenient to carry out in a routine laboratory, and no data are available concerning chlorzoxazone and its metabolite measured simultaneously in biological samples after drug administration. Therefore, we have developed an easy and reliable HPLC method that allows simultaneous determination of chlorzoxazone and its metabolite in blood or in urine. This method was applied to kinetic analysis of the metabolism of 6-hydroxychlorzoxazone and chlorzoxazone in five healthy controls and five alcoholic patients after oral administration of 500 mg of chlorzoxazone.

EXPERIMENTAL

Instrumentation

The HPLC system was a Spectra-Physics Analytical (San Jose, CA, USA) composed of a Model P2000 pump, an AS 2000 autosampler equipped with a Rheodyne automatic 20-μl injection valve, and a UV 2000 spectrophotometric detector attached to an SP 4400 data jet integrator.

Reagents

Chlorzoxazone was obtained from Sigma (St. Quentin Fallavier, France) and 6-hydroxychlorzoxazone was a gift from Dr. R. Peter (University of Erlangen, Germany). Perchloric acid, ethyl acetate, sodium acetate, acetic acid and methanol (analytical grade) were from Merck (Darmstadt, Germany), *Helix pomatia* juice containing 100 000 Fishman units of β-glucuronidase and 1 000 000 Roy units of sulphatase per millilitre, was from IBF Biotechnics (Paris, France). Acetonitrile for HPLC was from Labs Scan (Dublin, Eire). Chlorzoxazone tablets were obtained from Goldline Labs. (Miami, FL, USA).

Standards

Stock solutions of chlorzoxazone (1 mg/ml) and 6-hydroxychlorzoxazone (1 mg/ml) were prepared in methanol and stored at 4°C. Standard solutions were prepared from stock solutions by appropriate dilution with water.

Sample preparation

Samples of 0.5 ml of serum or 0.5 ml of 1/100 diluted urine were added to 10-ml centrifuge tubes containing 0.5 ml of 2 *M* acetate buffer (pH 4.5) and 20 μl of *H. pomatia* juice. They were hydrolysed overnight at 37°C to liberate 6-hydroxychlorzoxazone from their conjugates. Proteins were then precipitated with 4 ml of 0.6 *M* perchloric acid. After centrifugation for 10 min at 3500 *g*, chlorzoxazone and 6-hydroxychlorzoxazone were extracted from the supernatant with two 4-ml volumes of ethyl acetate by shaking for 10 min. Following centrifugation for 10 min at 4°C, the organic phases were evaporated to dryness under a stream of nitrogen.

Chromatography

The HPLC column (250 × 4.6 mm I.D.) was packed with reversed-phase 5 μm Nucleosil ODS (Interchim, Montluçon, France). The mobile phase was acetonitrile–0.5% acetic acid (30:70, v/v), and the flow-rate was 1.0 ml/min. The residues were dissolved in 250 μl of mobile

phase, and 20-μl samples were injected. Chlorzoxazone and 6-hydroxychlorzoxazone were detected at 287 nm. Peak-area measurements were used for quantitation and compared with standard solutions (0.5–20 μg/ml) of chlorzoxazone and 6-hydroxychlorzoxazone.

Calibration curves

Chlorzoxazone and 6-hydroxychlorzoxazone dissolved in methanol were added to 0.5 ml of serum or a 1/100 dilution of urine to give concentrations of 0.5, 1, 2.5, 5, 10 and 20 μg/ml. The samples were then processed as described above and run in duplicate.

Limit of detection

A level of five times the signal-to-noise ratio was used to estimate the limit of detection in UV spectrophotometry. The limit of detection in biological samples was established by adding increasing amounts of chlorzoxazone and its metabolite to give concentrations of 0.1, 0.2, 0.5, 1, 2, 5 and 10 μg/ml. The limit of quantitation was defined as the limit at which the procedure will be sufficiently precise (within-run coefficient of variation <5%) to yield a satisfactory quantitative estimate of the concentration.

Precision

Within- and between-run precisions were determined by analysing ten times serum and urine samples at three concentrations of chlorzoxazone and 6-hydroxychlorzoxazone (0.5, 10 and 20 μg/ml).

Accuracy and recovery

Accuracy and recovery of chlorzoxazone and 6-hydroxychlorzoxazone from serum or urine were studied by adding increasing amounts of a standard dilution in methanol to 0.5 ml of serum or diluted urine (final concentrations 1, 2.5, 5, 10 and 20 μg/ml). Each sample was run in duplicate.

Specificity

The UV absorbance and fluorescence spectra of chlorzoxazone and 6-hydroxychlorzoxazone of biological samples dissolved in the elution solvent were compared with those of the pure compounds.

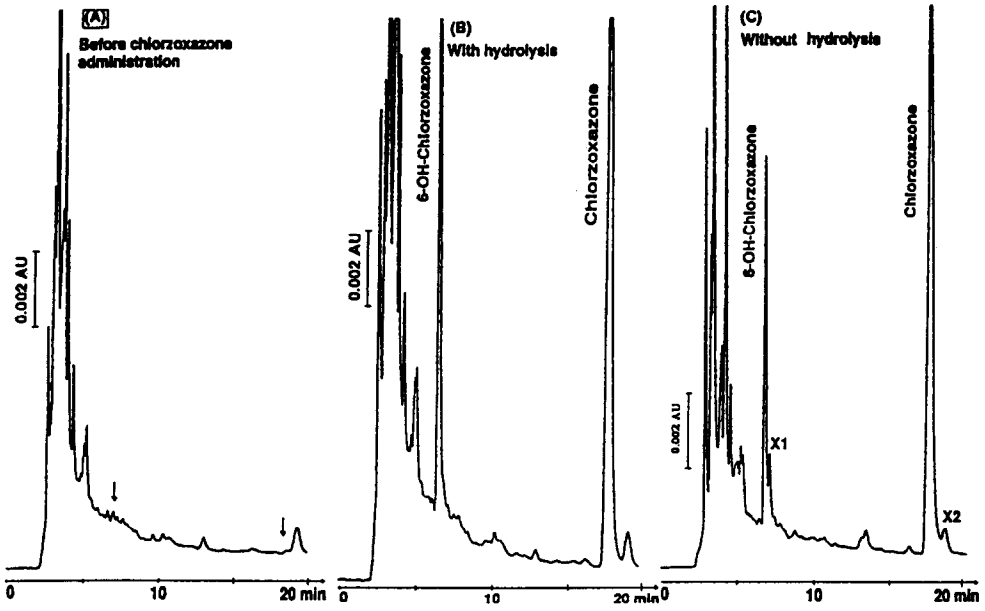

Fig. 1. Representative chromatograms of 6-hydroxychlorzoxazone and chlorzoxazone in serum: (A) before drug administration; (B) 2 h after administration of 500 mg of chlorzoxazone; (C) 2 h after administration of 500 mg of chlorzoxazone but without hydrolysis with *H. pomatia* juice. X1 and X2: non-identified peaks.

Patients

Five healthy control males (mean age 40.8; 35–49 years) and five alcoholic patients (40.8, 36–45 years), with no clinical or biological evidence of liver cirrhosis, participated in this study with the full agreement of the "Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Brest" (Morvan Hospital, Brest, France). The subjects, after fasting for a period of 12 h, received orally a tablet of 500 mg of chlorzoxazone. Blood samples (5 ml) were drawn at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6, 7, 8 and 24 h after administration. The samples were collected in dry tubes, stored at 4°C and centrifuged within 2 h. Urine was collected every 2 h for 8 h, then from 8 to 24; The samples were stored at −40°C until analysis (less than 1 month).

RESULTS

Chlorzoxazone and 6-hydroxychlorzoxazone were well separated in biological samples. Fig. 1 shows typical chromatograms of blood samples obtained before (A) and 2 h after (B) administration of a 500-mg chlorzoxazone tablet. 6-Hydroxychlorzoxazone was eluted in 6.5 min and chlorzoxazone within 17 min, and the relative retention time of 6-hydroxychlorzoxazone was 0.385 ± 0.006 ($n = 20$). The contribution of the extracted serum blank was minimal (Fig. 1A) as well as that from urine (results not shown). However, enzymic hydrolysis by *H. pomatia* juice was necessary to determine the total 6-hydroxychlorzoxazone, not only in urine as already described, but also in serum (Fig. 1B and C), because the amount measured was increased *ca.* three-fold after hydrolysis. β-Glucuronidase was also assayed for enzymic hydrolysis, but yielded results 12% lower than *H. pomatia* juice.

Calibration curves

A linear relationship between peak area and concentration (0.5, 1, 2.5, 5, 10 and 20 μg/ml) for both compounds was found (Table I).

Limit of detection

The limit of detection for 6-hydroxychlorzoxazone and chlorzoxazone solutions was calculated as five times the signal-to-noise ration, and was found to be 1 ng under the analytical HPLC conditions (concentration 0.05 μg/ml, C.V. 9.3%, $n = 10$). The lowest quantifiable concentration of these compounds in biological samples was estimated to be 0.5 μg/ml, a level at which the intra-assay C.V. was below 5% (Table II).

TABLE I

CALIBRATION DATA FOR STANDARD CURVES CARRIED OUT IN SERUM OR IN URINE

Sample	Concentration (μg/ml)	Recalculated concentration[a]	Relative error (%)
6-Hydroxychlorzoxazone			
Serum (1)	0.5	0.42	16
	1	0.94	12
	2.5	2.51	0.2
	5	5.14	2.8
	10	10.22	2.2
	20	19.86	0.7
Urine (2)	0.5	0.45	10
	1	0.95	5
	2.5	2.45	2
	5	5.11	0.5
	10	10.11	1.1
	20	19.92	0.4
Chlorzoxazone			
Serum (3)	0.5	0.57	13.6
	1	0.93	7.2
	2.5	2.42	7.2
	5	5.24	4.8
	10	10.14	1.4
	20	19.88	0.6
Urine (4)	0.5	0.47	6
	1	0.96	4
	2.5	2.48	0.8
	5	5.12	2.4
	10	10.05	0.5
	20	19.95	0.2

[a] Each concentration was recalculated using the equation of the appropriate regression line, and the relative error was expressed as a percentage. Samples were run in duplicate.
(1) $y = 107.7\ (\pm 1.6)x + 10.4\ (\pm 7.8)$, $r = 0.9998$, $p < 0.0001$.
(2) $y = 137.9\ (\pm 0.7)x + 1.5\ (\pm 5.8)$, $r = 0.9999$, $p < 0.0001$.
(3) $y = 75\ (\pm 0.7)x + 13.4\ (\pm 6.1)$, $r = 0.9998$, $p < 0.0001$.
(4) $y = 103\ (\pm 0.4)x + 4.5\ (\pm 3.4)$, $r = 0.9999$, $p < 0.0001$.

Precision

Within-run and between-run precision was determined by analysis of serum and urine to

which 0.5, 10 and 20 μg/ml (final concentration) of both 6-hydroxychlorzoxazone and chlorzoxazone were added. Results are shown in Table II.

Accuracy and recovery

The accuracy and recovery using the described procedure are reported in Tables III and IV, respectively.

Specificity

The UV absorbance and fluorescence spectra of chlorzoxazone and 6-hydroxychlorzoxazone in biological samples dissolved in the elution solvent were found to be similar to those of the pure compounds. Chlorzoxazone showed an absorption maximum at 280 nm, and 6-hydroxychlorzoxazone at 295 nm. Therefore, the UV detection for chlorzoxazone and its metabolite

TABLE II

WITHIN-RUN AND BETWEEN-RUN PRECISIONS

Concentration (mg/l)	Coefficient of variation (%)			
	6-Hydroxychlorzoxazone		Chlorzoxazone	
	Serum	Urine	Serum	Urine
Within-run ($n = 10$)				
0.5	4.4	4.0	4.7	4.2
10	4.2	3.0	4.5	4.1
20	4	2.6	4.2	3.9
Between-run ($n = 10$)				
0.5	8.6	7.7	11	7
10	7.3	6.2	8.6	5.8
20	6.5	5.3	7	5.2

TABLE III

ACCURACY OF 6-HYDROXYCHLORZOXAZONE AND CHLORZOXAZONE DETERMINATION IN SERUM AND IN URINE

Concentration (μg/ml)	Serum		Urine	
	Concentration found[a] (μg/ml)	Accuracy (%)	Concentration found[a] (μg/ml)	Accuracy (%)
6-Hydroxychlorzoxazone				
1	1.05	5	0.97	3
2.5	2.56	2	2.53	1.2
5	5.13	2	5.06	1.2
10	10.34	3.4	9.97	0.3
20	19.82	0.9	20.3	1.5
Chlorzoxazone				
1	1.06	6	0.98	2
2.5	2.52	0.8	2.55	2
5	5.15	3	5.09	1.8
10	10.18	1.8	9.95	0.5
20	20.13	0.6	20.05	0.3

[a] Based on duplicate determination.

TABLE IV

RECOVERIES OF 6-HYDROXYCHLORZOXAZONE AND CHLORZOXAZONE FROM SERUM AND URINE

Concentration (μg/ml)	Serum		Urine	
	Concentration found[a] (μg/ml)	Recovery (%)	Concentration found[a] (μg/ml)	Recovery (%)
6-Hydroxychlorzoxazone				
1	0.94	94	0.76	76
2.5	2.10	83.9	1.96	78.4
5	4.28	85.6	3.92	78.4
10	8.55	85.5	7.71	77.1
20	16.31	81.5	15.69	78.4
Chlorzoxazone				
1	0.84	83.7	0.89	86
2.5	1.82	72.8	2.19	87.6
5	3.60	72	4.34	86.8
10	7.00	70	8.45	84.5
20	13.7	68.5	16.97	85

[a] Based on duplicate determination.

was carried out at 287 nm. For 6-hydroxychlorzoxazone fluorescence, excitation and emission wavelengths were set at 290 and 326 nm, respectively.

Kinetic analysis

The concentration–time profiles for 6-hydroxychlorzoxazone and chlorzoxazone in five control and five alcoholic patients are shown in Fig. 2. Maximum levels of chlorzoxazone and its metabolite were reached between 1 and 1.5 h after drug administration. The mean concentrations (±S.D.) were 8.2 (±1.7) μg/ml and 2.52 (±0.7) μg/ml in controls and 5.04 (±2.54) μg/ml and 3.26 (±0.47) μg/ml in alcoholic patients, respectively. These values were significantly different in controls and in alcoholics for both chlorzoxazone and 6-hydroxychlorzoxazone ($p < 0.05$, Mann–Whitney U test). Blood concentrations at the 8-h time-point were below 0.5 μg/ml, indicating that chlorzoxazone and its metabolite were rapidly eliminated from the body. The urinary excretion data (Table V) revealed that 70% of the dose was excreted as 6-hydroxychlorzoxazone in both controls and alcoholics. Chlorzoxazone levels in urine were below the detection limit. The ratio 6-hydroxychlorzoxazone/chlorzoxazone, determined in serum when the concentrations were at their maxima, *i.e.* at $t = 1.5$ h, may eliminate variations due to differences in drug absorption or in the distribution volume. This ratio could not be determined in urine as the chlorzoxazone levels were too low, but in serum it was found to be 0.31 (±0.07) and 1.03 (±0.38) for control and alcoholic patients, respectively, 1.5 h after drug intake ($p < 0.005$, Mann–Whitney U test). This ratio, which was increased three-fold in alcoholics compared with controls, reflects the increased level of CYP2E1 observed in alcoholics [3].

DISCUSSION

Previous studies [6,7] showed that, after oral administration, chlorzoxazone is rapidly absorbed and completely metabolized in humans to 6-hydroxychlorzoxazone, which is excreted in urine as the glucuronide conjugate. *In vitro* experiments have established the liver [6] as the principal site of metabolism of chlorzoxazone. More recently, CYP2E1 has been shown to be the principal enzyme involved in the 6-hydroxylation of chlorzoxazone [5]. In this perspective,

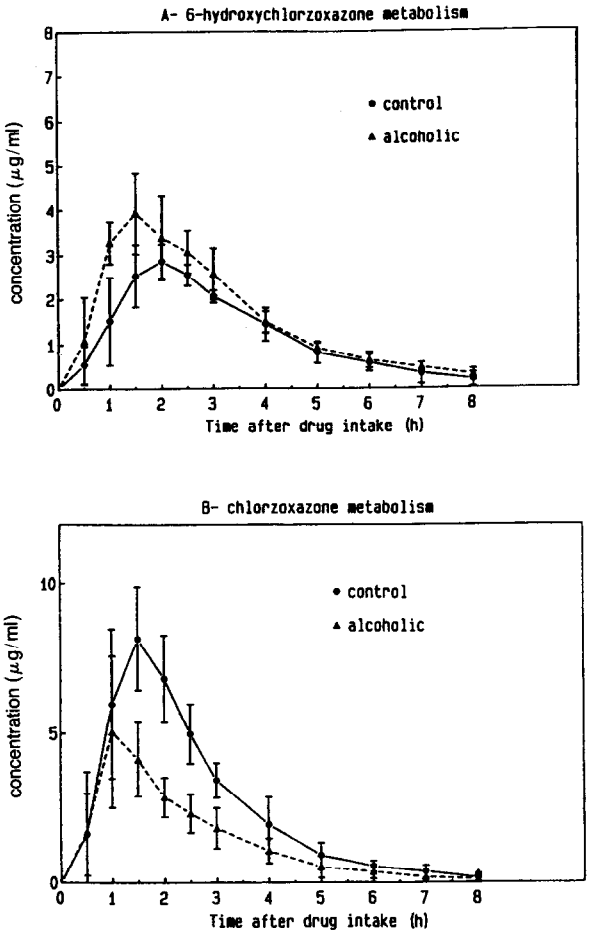

Fig. 2. Serum concentrations (mean ± S.D.) of 6-hydroxychlorzoxazone (A) and chlorzoxazone (B) in five controls and five alcoholic patients following oral administration of 500 mg of chlorzoxazone.

determination of chlorzoxazone and its hydroxy metabolite in blood and in urine was performed by HPLC.

A good resolution of 6-hydroxychlorzoxazone and chlorzoxazone peaks was achieved by reversed-phase chromatography on an ODS column as reported by previous investigators [5,7,9,10] and using acetonitrile–0.5% acetic acid, 70:30 (v/v) as eluent under conditions close to those of Peter *et al.* [5], except that phosphoric acid was replaced by acetic acid in order to reduce the hydrolysis reaction of the alkyl chain bond of the stationary gel.

6-Hydroxychlorzoxazone and chlorzoxazone were extracted from biological fluids in acidic

TABLE V

URINARY EXCRETION OF 6-HYDROXYCHLORZOXAZONE IN FIVE CONTROLS AND FIVE ALCOHOLIC SUBJECTS AFTER ADMINISTRATION OF 500 mg OF CHLORZOXAZONE

Values are expressed as mean ± S.D. ($n = 5$).

Time (h)	6-Hydroxychlorzoxazone (mg)	
	Control subjects	Alcoholic patients
0–2	135 ± 43.7	148 ± 14.6
2–4	132 ± 26.8	137 ± 66.1
4–6	47.5 ± 23.2	57.5 ± 31.0
6–8	38.7 ± 10.1	18 ± 10.7
8–24	28.5 ± 19.8	17.1 ± 3.1
Total	381.7 ± 65.5	377.6 ± 99.0

medium with ethyl acetate [7] after precipitation of proteins with 0.6 *M* perchloric acid. This last step was useful to obtain clear samples that did not require further filtration before HPLC analysis. Other solvents, such as dichloromethane or chloroform–2-propanol (85:15, v/v) were also useful extraction solvents, but ethyl acetate yielded 20% higher recovery of 6-hydroxychlorzoxazone and chlorzoxazone under our pH conditions. Ether extraction [9] was also proposed, but did not appear very practical for safety reasons. More recently, solid-phase extractions have been reported [10,11]; these methods appear more expensive as they require special material.

Although several studies [6,7] have reported the levels of chlorzoxazone in human serum after administration of the drug, none has reported the levels of chlorzoxazone and its hydroxy metabolite together in a blood sample. In this paper, we have shown that the metabolite is present in the blood as a conjugate, at least in part, as hydrolysis with *H. pomatia* juice enhanced the amount of 6-hydroxychlorzoxazone but not that of chlorzoxazone determined in the serum. Because hydrolysis with β-glucuronidase yielded lower values than *H. pomatia* juice, it suggests that sulpho-conjugated derivatives may also exist. Conney and Burns [6] showed that there was no free 6-hydroxychlorzoxazone in urine, and that its urinary excretion was patient-

dependent. Between 50% and 90% of chlorzoxazone is excreted as the glucuronide of 6-hydroxychlorzoxazone. Our study corroborates these findings.

Alcoholics, whose CYP2E1 is induced by chronic ethanol consumption, should metabolize chlorzoxazone faster than non-alcoholics. Indeed, serum levels of chlorzoxazone were lower in alcoholic patients than in control patients, whereas the contrary was observed for the metabolite. The concentration ratio 6-hydroxychlorzoxazone/chlorzoxazone, determined in serum when maximum drug levels were reached ($t = 1.5$ h), constitutes a more sensitive index as it increases the difference between controls and alcoholics.

CONCLUSION

The HPLC method presented here for determining chlorzoxazone and its metabolite in blood or in urine is easy to carry out in a routine clinical laboratory. The accuracy and the lower limit of quantitation are compatible with blood levels after administration of the drug. This method allows the simultaneous measurement of chlorzoxazone and 6-hydroxychlorzoxazone in biological samples. The concentration ratio metabolite/drug, determined when the drug concentration is at a maximum, constitutes a useful tool for studying CYP2E1 status in humans.

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