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Short communication

Estimation of chlorzoxazone hydroxylase activity in liver microsomes and of the plasma pharmacokinetics of chlorzoxazone by the same high-performance liquid chromatographic method

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Abstract

We have developed a HPLC method which allows the determination of chlorzoxazone and its hydroxy metabolite in rat liver microsomes and in human plasma. We found that dehalogenated chlorzoxazone or 2-benzoxazolinone was a convenient and stable internal standard. Proteins were precipitated with diluted perchloric acid and the supernatant was extracted with ethyl acetate. Complete resolution of the peaks was achieved within 20 min with a Spherisorb ODS-1 column. The inter-day R.S.D.s were 6.5% at 0.5 μg/ml of hydroxychlorzoxazone and 5.8% at 1 μg/ml of chlorzoxazone in human plasma. The reproducibility of the method has been demonstrated for a large number of samples over a long period.

Keywords: Chlorzoxazone; Hydroxychlorzoxazone; Benzoxazolinone

1. Introduction

Chlorzoxazone (CZX) has been used for many years as a potent long-acting central muscle relaxant [1]. The drug is rapidly absorbed from the gastrointestinal tract and 6-hydroxychlorzoxazone (OH-CZX) is the main metabolite present in plasma as a conjugate.

Metabolic investigations of this compound were undertaken after some cases of hepatotoxicity were reported [2]. Human cytochrome P-4502 E1 (CYP2E1) was found to be the primary catalyst of chlorzoxazone 6-hydroxylation in human liver [3].

Considerable variations in the rates for this hydroxylation reaction have been measured in man and a low enzyme activity could be responsible for a hepatotoxic reaction but this remains to be determined [4,5]. Nevertheless, the use of this drug as a non-invasive probe for human CYP2E1 activity is now recognized and widely used [6,7].

In parallel with these investigations, new high-performance liquid chromatography (HPLC) methods have been developed to measure CZX and OH-CZX concentrations in biological fluids and in microsomes [8–13]. All the published methods are based on the same preparation scheme: deconjugation of OH-CZX and liquid–liquid extraction. In most of these methods, an internal standard has been used and the compounds were separated by isocratic elution and quantified by UV detection. It must be pointed out that most of the compounds used as an

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internal standard are not chemically related to CZX or are synthesized by the investigators.

We find that dehalogenated CZX or 2-benzoxazolinone which is commercially available could be a convenient and stable internal standard. This paper describes a sensitive HPLC assay for CZX and OH-CZX in rat hepatic microsomes and in human plasma.

2. Experimental

2.1. Chemicals and standards

CZX and 2-benzoxazolinone (internal standard) were purchased from Aldrich (Milwaukee, WI, USA). OH-CZX was obtained from Ultrafine (Manchester, UK). Acetonitrile HPLC ultra gradient grade, methanol and ethyl acetate HPLC grade were from Labscan (Dublin, Ireland). *Helix Pomatia* juice and nicotinamide–adenine dinucleotide phosphate, reduced (NADPH) tetrasodium salt were purchased from Boehringer (Mannheim, Germany). All other reagents were of the highest purity available.

2.2. Preparation of analytical standards

Stock solutions of CZX (1 mg/ml), OH-CZX (1 mg/ml) and 2-benzoxazolinone (0.1 mg/ml) were prepared in methanol and stored at 4°C in the dark. Working solutions were made freshly from these stock solutions in 0.1 *M* Tris buffer (pH 7.6) for microsomes and in 0.4 *M* acetate buffer (pH 4.5) for plasma. Standards for CZX hydroxylase activity in microsomes were prepared by transferring an aliquot of the buffered working solution of OH-CZX to a clean tube containing rat liver microsomes that were not incubated but immediately treated with zinc sulphate. The following series of microsomes standards were thus prepared: 0, 1, 2, 3, 4, 5, 7.5, 10 and 17.5 μg of OH-CZX. Plasma standards were prepared by transferring an aliquot of the buffered working solutions to a clean tube and by adding blank plasma. The following series of plasma standards were thus prepared: 0, 1, 2, 3, 4, 5, 6, 8, 12 μg/ml of CZX and 0, 0.5, 1, 1.5, 2, 2.5, 3, 4 μg/ml of OH-CZX.

2.3. Sample preparation

2.3.1. Rat hepatic microsomes

To 500 μl of diluted rat hepatic microsomes containing 0.3 mg of proteins were added 50 μl of 0.1 *M* magnesium chloride, 0.6 mg of NADPH tetrasodium salt dissolved in 200 μl of 0.1 *M* Tris buffer (pH 7.6) and 200 μl Tris buffer (pH 7.6) containing 20 μg of CZX. Samples were incubated for 20 min at 37°C in a shaking water bath. The reaction was stopped by addition of 200 μl zinc sulphate (15%, w/v) and vigorously homogenized for 15 s. After the addition of the internal standard (1 μg in 100 μl Tris buffer) and further homogenization, the tubes were centrifuged for 10 min at 3500 *g*. The supernatant was filtered with a 0.45-μm filter and 200 μl were injected onto the HPLC column.

For microsomes standards, 450 μl of Tris buffer containing various amounts of OH-CZX were mixed with 500 μl of diluted rat microsomes. After the addition of 200 μl of zinc sulphate (15%, w/v), the samples were processed as described here above.

2.3.2. Human plasma

To a 0.5-ml aliquot of plasma standard (see Section 2.2) or of plasma sample (obtained after a single 500 mg CZX oral dose) were added: 0.5 ml acetate buffer (0.4 *M*, pH 4.25) and 20 μl *Helix Pomatia* juice (containing 5.5 U/ml of β-glucuronidase and 2.8 U/ml arylsulfatase). The mixture was incubated overnight at 37°C. After addition of the internal standard (1 μg in 100 μl acetate buffer) and precipitation of the proteins with 4 ml of 0.6 *M* perchloric acid, the sample was centrifuged for 10 min at 3500 *g*. The supernatant was transferred to a glass tube (125×16 mm, screw cap style) containing 0.5 g of sodium chloride. After the dissolution of the salt, 4 ml of ethyl acetate were added and the compounds extracted by slow rotation for 15 min.

The mixture was again centrifuged (10 min; 4°C; 2500 *g*) and the upper organic phase was removed and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 600 μl of mobile phase, filtered with a 0.22-μm filter and 200 μl were injected onto the column.

2.4. Instrumentation

The HPLC system consisted of a 7125 Rheodyne injector with a 200-μl loop (Cotati, CA, USA), a Gilson Model 307 pump (Villers-le Bel, France) and a Dynamax Model UV-1 detector from Rainin (Woburn, MA, USA) with a Z-shaped flow cell operated at 287 nm. Data processing was performed by using the Chromatopak CR3A integrator from Shimadzu (Kyoto, Japan).

2.5. Operating conditions

A guard column filled with CN or ODS packing (15 mm×3.2 mm I.D., Applied Biosystems, Foster City, CA, USA) was used. The separation was performed on a Spherisorb ODS-1 5 μm column fitted with the continuously adjustable piston (CAP) system (150 mm×4.6 mm I.D., Alltech Associates, Deerfield, IL, USA) at ambient temperature. The mobile phase composition was ammonium acetate buffer (0.05 *M*, pH 4.25)–acetonitrile (80:20) at a flow-rate of 0.55 ml/min.

2.6. Assay validation

For microsomes, calibration curves in the range of 1 to 17.5 μg of OH-CZX were constructed as the peak height ratios of OH-CZX to internal standard. Least-squares linear regression analysis was used to determine the slope, intercept and correlation coefficient. Inter-day reproducibility was determined by calculating the relative standard deviations (R.S.D.s) of the slopes and intercepts of five calibration curves. The accuracy of the assay was determined by calculating the relative error (RE) on the standards of the calibration curves.

Intra-day precision of the chlorzoxazone hydroxylase assays was determined with two preparations of rat liver microsomes, one with a low activity (control) and one with a high activity (induced by 4-methylpyrazole).

For plasma, calibration curves in the range of 1 to 12 μg/ml for CZX and 0.5 to 6 μg/ml for OH-CZX were constructed as the peak height ratios of CZX and OH-CZX to internal standard. Least-squares linear regression analysis was used to determine the slope, intercept and correlation coefficient.

Inter-day reproducibility was determined by calculating the R.S.D.s of the slopes and intercepts of four calibration curves. The accuracy of the assay was determined by calculating the RE on the standards of the calibration curves.

Recovery of CZX and OH-CZX was measured by comparing the chromatographic peak heights of plasma standards following extraction to those obtained from direct injection of buffered (sodium acetate, pH 4.5) solutions of both compounds. Recoveries were determined at 1, 2.5, 5, 10 and 20 μg/ml plasma for CZX and at 0.5, 1, 2, 4 and 6 μg/ml plasma for OH-CZX. Intra-day accuracy and precision of the CZX and OH-CZX assays were determined at 2, 4 and 8 μg CZX/ml plasma and at 0.5, 1.5 and 3 μg OH-CZX/ml plasma (five samples per concentration). Inter-day accuracy and precision was determined at 1 and 5 μg CZX/ml plasma and at 0.5 and 3 μg OH-CZX/ml plasma (two samples per day per concentration for four days).

3. Results

3.1. Rat liver microsomes

CZX, its metabolite and the internal standard were well separated. Fig. 1 shows typical chromatograms obtained with rat liver microsomes before (A) and 20

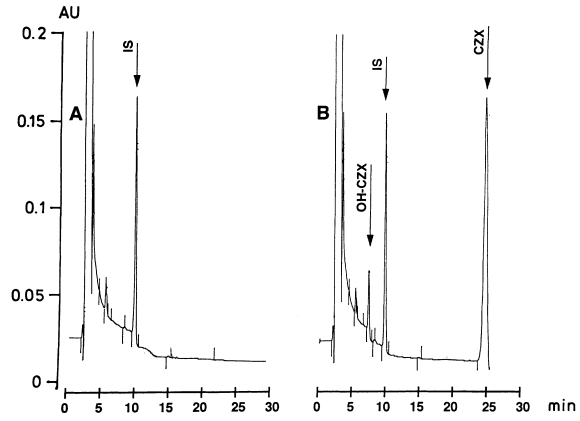

Fig. 1. Typical HPLC chromatograms of blank rat liver microsomes (A) and 20 min after incubation with CZX (B). The chlorzoxazone hydroxylase activity is 0.12 μg OH-CZX/mg protein/min.

min after (B) incubation with CZX. OH-CZX was eluted in 7.5 min, the I.S. in 9.5 min and CZX within 24.5 min.

3.1.1. Calibration curves

A linear relationship between peak heights and concentration of OH-CZX (1, 2, 3, 4, 5, 7.5, 10, 17.5 μg/ml) was found (Table 1). The R.S.D. for the slopes of the five standard curves was 8.2% and for the intercepts, 7.8%. The relative error on the standards (extremes) was found between +1.2 and +6.8% when above the expected value and between −2.9 and −5.1% when below the expected value.

3.1.2. Intra-day precision

The chlorzoxazone hydroxylase activity for 10 replicates of rat liver microsomes with a low CYP2E1 activity was 0.0766 μg of OH-CZX formed per mg of protein per min (S.D.: 0.0021; R.S.D.: 2.8%). After induction with 4-methylpyrazole, the activity for 10 replicates was: 0.407 μg/mg protein min (S.D.: 0.014; R.S.D.: 3.4%).

3.2. Human plasma

Fig. 2 shows typical chromatograms of human plasma before (A) and 2 h after (B) ingestion of one capsule containing 500 mg CZX.

3.2.1. Calibration curves

Calibration curves for CZX and OH-CZX were linear within the ranges studied (Table 2). The R.S.D.s for the slopes of the four standard curves were 4.4% and 6.7% for CZX and OH-CZX, respectively.

The RE on the standards (extremes) was found between +4.9 and +9.6% when above the target value and between −0.2 and −5.8% when below the expected value for CZX. Similarly, for OH-CZX the ranges were between +9.6 and 14.1% and between −2.6% and −6.3%, respectively.

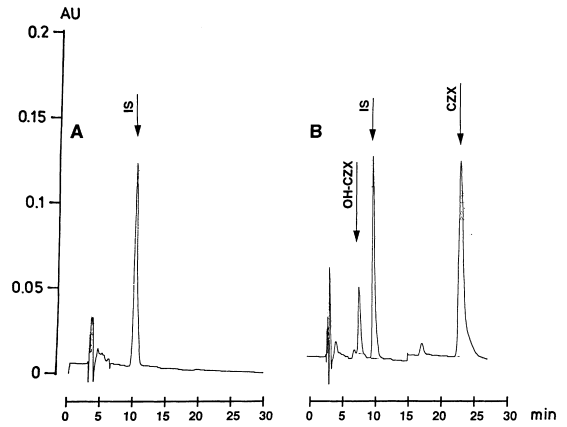

Fig. 2. Typical HPLC chromatograms of (A) blank human plasma and (B) patient sample, obtained 2 h after a 500-mg dose of CZX, containing 1.8 μg/ml of OH-CZX and 12 μg/ml of CZX.

Table 1
Individual and mean values for the slopes, intercepts and correlation coefficients of five calibration curves for 6-hydroxychlorzoxazone in rat liver microsomes

Curve	Slope	Intercept	*r*
1	0.0781	0.3376	0.999
2	0.0803	0.3434	0.998
3	0.0657	0.2860	0.998
4	0.0726	0.3144	0.999
5	0.0697	0.2983	0.999
Mean	0.0733	0.3159	
±S.D.	0.0060	0.0247	
R.S.D. (%)	8.2	7.8	

Table 2
Individual and mean values for the slopes, intercepts and correlation coefficients for four calibration curves for CZX and OH-CZX in human plasma

Compound	Curve	Slope	Intercept	*r*
CZX	1	0.0419	−0.3950	0.999
	2	0.0433	−0.4210	0.997
	3	0.0461	−0.4460	0.998
	4	0.0441	−0.4167	0.997
	Mean	0.0439	−0.4200	
	±S.D.	±0.0018	±0.0021	
	R.S.D. (%)	4.4	5	
OH-CZX	1	0.0221	−0.0836	0.999
	2	0.0259	−0.0931	0.996
	3	0.0236	−0.0898	0.998
	4	0.0239	−0.0913	0.998
	Mean	0.0239	−0.0895	
	±S.D.	±0.0016	±0.0041	
	R.S.D. (%)	6.7	4.6	

Table 3
Intra- and inter-day precision and accuracy for CZX and OH-CZX in plasma

Compound	Concentration (μg/ml)		R.S.D.	RE
	Added	Found (mean±S.D.)	(%)	(%)
Intra-day[a]				
CZX	2	1.99±0.12	6.1	−0.5
	4	3.94±0.19	4.8	−1.5
	8	8.07±0.32	4.0	+0.9
OH-CZX	0.5	0.49±0.03	6.3	−2
	1.5	1.50±0.07	4.9	0
	3	2.95±0.08	2.7	−1.7
Inter-day[b]				
CZX	1	1.07±0.06	5.8	+7
	5	4.99±0.25	5	−0.2
OH-CZX	0.5	0.53±0.03	6.5	+7
	3	2.89±0.11	4	−3.6

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

3.2.2. Recovery

The recovery of CZX from plasma ranged from 95% (1 μg/ml) to 86% (20 μg/ml). The mean value was 90.8±3.4% with a R.S.D. of 3.8%. The recovery of OH-CZX from plasma ranged from 94% (0.5 μg/ml) to 88% (2 μg/ml). The mean value was 90.8±2.3% with a R.S.D. of 2.5%.

3.2.3. Intra- and inter-day precision and accuracy

The R.S.D.s and the REs for each concentration of CZX and OH-CZX are given in Table 3.

4. Discussion

In this paper we present a simplified method, based on the paper of Peter et al. [3], to measure chlorzoxazone hydroxylase activity in rat liver microsomes. The three main modifications introduced are: the use of pure NADPH instead of a generating system, the addition of an internal standard chemically related to CZX and the direct injection of the supernatant, obtained after acid precipitation, onto the HPLC column.

We verify the experimental conditions for what concerns linearity, duration of incubation, concentration of proteins and of substrate. Our results correspond to the conditions described elsewhere [3,8,9]. The use of pure NADPH has already been described [8] and a very sensitive method appeared recently [9]. Our goal was not to reach such sensitivity because the amount of OH-CZX produced during the reaction is always above 1 μg. The recovery has not been investigated but should be close to 100% since there is no liquid–liquid extraction step after the precipitation of the proteins. If we extract the supernatant with ethyl acetate, cleaner chromatograms are obtained with a better sensitivity but this additional step is time consuming.

The first HPLC method published for CZX and OH-CZX determination in plasma, uses acidic deconjugation in a hot waterbath [10]. This is an effective method of deconjugation for plasma that is currently used for the determination of OH-CZX in urine.

We modified the method of Lucas et al. [11] by introducing an internal standard and a single extraction with ethyl acetate is performed. Performances and recovery are similar to the original method. The deconjugation with β-glucuronidase/arylsulfatase is a mild method, which contributes little to the background of the chromatogram. A solid-phase extraction method [12] has been developed but the authors later switched to a liquid–liquid extraction method [13] as already used by Honigberg et al. [10].

In conclusion, we describe an easier method for rat liver microsomes which also incorporates a chemically related and available internal standard.

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