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Short communication

Determination of chlorzoxazone and 6-hydroxychlorzoxazone in plasma by gas chromatography–mass spectrometry

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Abstract

A gas chromatographic–mass spectrometric method is presented which allows the determination of chlorzoxazone and 6-hydroxychlorzoxazone after derivatization with the reagent N-*tert*.-butyldimethylsilyl-N-methyltrifluoroacetamide. No interference was observed from endogenous compounds following the extraction of plasma samples from six different human subjects. The standard curves were linear over a working range of 20 to 4000 ng/ml and of 20 to 1000 ng/ml for chlorzoxazone and 6-hydroxychlorzoxazone, respectively. Recoveries ranged from 65 to 97% for the two compounds and intra- and inter-day coefficients of variation were always less than 9%. The limit of quantitation of the method was found to be 5 ng/ml for the two compounds, hence allowing its use for single low dose pharmacokinetics. © 1998 Elsevier Science B.V.

Keywords: Chlorzoxazone; 6-Hydroxychlorzoxazone

1. Introduction

Chlorzoxazone (CLZ) is a skeletal muscle relaxant used for the treatment of painful muscle spasms. Its main metabolite is 6-hydroxychlorzoxazone (CLZOH, see Fig. 1) and it has been proposed that this metabolic pathway could be a useful marker for the activity of cytochrome P450IIE1 (CYP2E1) [1]. CYP2E1 is a member of the P-450 superfamily of enzymes and is involved in the oxidation of numerous low-molecular-mass compounds, several of them being potent procarcinogens which require this enzyme to be reactive [2]. Thus, CYP2E1 is induced in alcoholic patients [3], and it has been proposed that CYP2E1 may be involved in the development of

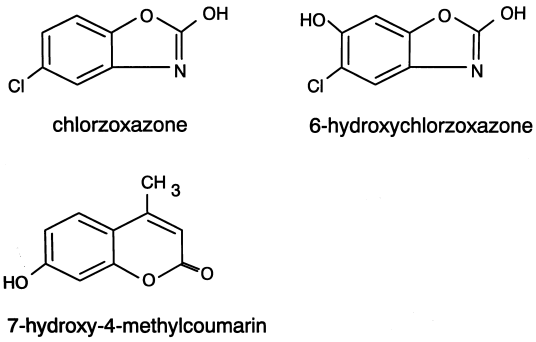

Fig. 1. Chemical structures of chlorzoxazone, 6-hydroxychlorzoxazone and 7-hydroxy-4-methyl coumarin (internal standard).

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alcohol-induced liver disease, possibly through increasing liver peroxidation [4].

The involvement of CYP2E1 in CLZ metabolism is supported by the increase of CLZ metabolism which has been demonstrated in alcoholic patients as compared to control nonalcoholic subjects [5]. It has to be mentioned that other enzymes – namely cytochrome P4501A1 [6], cytochrome P4501A2 [7] and cytochrome P4503A4 [8] – are also involved in this biotransformation of CLZ. However, a negligible participation of cytochrome P4501A1 and cytochrome P4501A2 in the CLZ metabolism can be assumed, for CYP1A1 is not constitutively expressed in human liver, as no differences in CLZ metabolism were found between smokers and nonsmokers [5], and as furafylline, a selective CYP1A inhibitor, demonstrated little inhibition of CLZ 6-hydroxylase activity [8].

In humans, CLZ exists in the unconjugated form in plasma, while CLZOH is predominantly present (around 80%, own unpublished data) as a glucuronide conjugate [9]. In a study with six healthy male subjects receiving an oral dose of 250 mg of CLZ, a mean half-life of 1 h, a volume of distribution of 42 l, and a clearance of 28.8 l/h were calculated [10]. Previously published analytical methods for the measurements of the total concentrations of CLZ and CLZOH are based on fluorimetry [11], gas chromatography (GC) with flame ionisation detection [12] and high-performance liquid chromatography [9,13–17] after liquid–liquid or solid-phase extraction from biological samples. Some of these methods only separate and quantitate CLZ [11,15], and none are very sensitive. A sensitive (down to the low ng/ml range) and specific gas chromatography–mass spectrometry (GC–MS) method for the determination of the total concentrations of this drug and its metabolite is described.

2. Experimental

2.1. Reagents

Chlorzoxazone was obtained from Streuli (Uznach, Switzerland) and 6-hydroxychlorzoxazone was kindly provided by the R.W. Johnson Pharmaceutical Research Institute (Spring House, USA). 7-Hydroxy-4-methyl coumarin (COU, internal standard) was purchased from Aldrich (Steinheim, Germany). *Helix pomatia* β-glucuronidase+sulfatase was from Sigma (type HP-2S, catalogue No. G-7770, St. Louis, MO, USA). N-*tert*.-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) was from Fluka (Buchs, Switzerland). Stock solutions of CLZ, CLZOH and COU were prepared in methanol at a concentration of 1 mg/ml. Working solutions of CLZ and CLZOH were made at a concentration of 100 ng/μl in 0.001 *M* NaOH, and that of COU at a concentration of 10 ng/μl in 0.001 *M* NaOH. All other reagents were of analytical or HPLC grade. The cartridge holder was from Supelco (Visiprep Supelco, Buchs, Switzerland).

2.2. Instrumentation and chromatographic conditions

Analyses were performed on a Hewlett-Packard HP 5890 series II gas chromatograph equipped with a splitless capillary system and linked to a quadrupol HP 5988 A mass spectrometer system operating in the electron impact (EI) mode. The MS conditions were: ionization potential 70 eV, emission 300 μA, ion source temperature 200°C, and GC–MS capillary direct interface 250°C. Splitless injections of 2 μl were made into a fused-silica OV1 capillary column (Macherey-Nagel, Oensingen, Switzerland), 25 m× 0.25 mm l.D., 0.25 μm film thickness, with helium as the carrier gas. The column head-pressure was set to 20 KPa, total flow to 60 ml/min, and septum purge to 3 ml/min. GC conditions were: initial temperature 150°C, heating rate 20°C/min, final temperature 250°C, and injector temperature 320°C. Analyses were performed in the selected-ion monitoring (SIM) mode for the ions of *m/z* 226 (CLZ), 233 (COU) and 356 (CLZOH) (see Fig. 2).

2.3. Extraction and derivatization conditions

A 1-ml sample of heparinized plasma was spiked with 500 ng of COU and was incubated with 1 ml of 0.2 *M* acetate buffer pH 5.0, 50 μl 4% sodium azide (to prevent bacterial growth), and 10 μl *H. pomatia* β-glucuronidase at 37°C overnight with gentle shaking. In a preliminary experiment, it was found that the use of up to 50 μl of β-glucuronidase did not

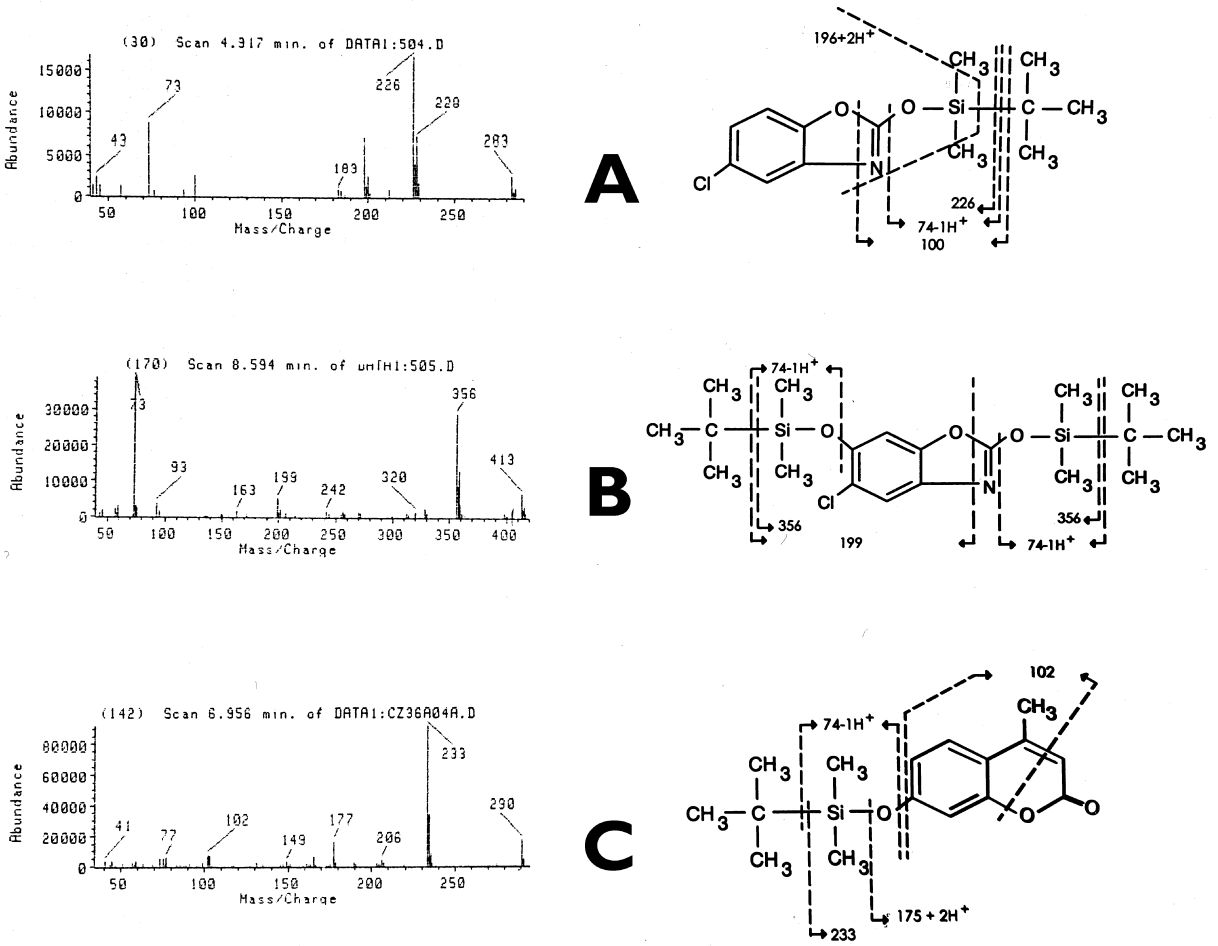

Fig. 2. Electron-impact mass spectra of chlorzoxazone (A, M^+=283; SIM: 226), 6-hydroxychlorzoxazone (B, M^+=413; SIM: 356) and 7-hydroxy-4-methyl coumarin (C, M^+=290; SIM: 233) after derivatization with N-*tert*.-butyldimethylsilyl-N-methyltrifluoroacetamide. Examples of the probable fragmentation modes are given.

result in the formation of higher concentrations of total CLZOH (data not shown). Extraction cartridges were firstly conditioned with 2 ml of methanol and 2 ml of 1% acetic acid solution by means of a weak vacuum (110 mm Hg; 1 mm Hg=133.322 Pa). To the hydrolysed plasma sample was added 1 ml of 0.5 *M* phosphate buffer pH 7.4 (0.5 *M* KH_2PO_4 titrated to pH 7.4 with 0.5 *M* Na_2HPO_4). The mixture was vortexed and slowly passed (220 mm Hg) through the cartridge. The cartridge was washed, firstly with 2 ml of a methanol–acetic acid (1%, v/v) (20:80) and the eluent discarded, and then washed with 2 ml of a methanol–acetic acid (1%, v/v) (70:30). The eluent was dried under nitrogen at 50°C to obtain a final volume of approximately 500 μl, which allows a complete evaporation of methanol (this can also be visualised by a very rapid decrease of the volume at the beginning of the evaporation step due to preferential evaporation of methanol, followed by a very slow decline of the volume when only water is present). To the acidic solution were added 1 ml of 1% acetic acid and 1.5 ml ethyl acetate. After shaking (15 min) and centrifugation (4 min, 2300 *g*), the solvent was transferred to small vials and completely dried under nitrogen at 50°C. 70 μl of MTBSTFA was added to the dry residue and the

capped vial was left for 1 h at 64°C. The reagent was then transferred to injection vials and 2 μl injected into the GC–MS system.

3. Results and discussion

Due to the presence of the hydroxy groups on CLZ and CLZOH, a derivatization step was necessary to improve their chromatographic properties (data not shown). After several trials, MTBSTFA was found to be a good derivatizing reagent for these two compounds. However, the preliminary trials to evaporate MTBSTFA at the end of the derivatization step and to replace it with another solvent were unsuccessful, due to instability of the derivatized molecules (data not shown) On the other hand, these *tert*.-butyldimethylsilyl derivatives were very stable when left in the derivatization reagent, and were thus injected directly. It was found that the disadvantage of such a direct injection was that a small contamination from the previous injection may be observed if the injection is made from a concentrated solution, a contamination which can be prevented by raising the temperature of the injection port from the usual 280°C to 320°C (data not shown).

Fig. 2 shows the El mass spectra of CLZ, CLZOH and COU after derivatization with MTBSTFA, with the probable fragmentation mode. Table 1 shows a summary of the statistical data on the analysis of CLZ and CLZOH. To summarize, the mean coefficients of correlation of the calibration curves (eight concentrations: 20, 50, 100, 200, 1000, 2000, 3000 and 4000 ng/ml, and 20, 50, 75, 100, 200, 500, 750 and 1000 ng/ml, for CLZ and CLZOH, respectively) obtained from six separate experiments were

Table 1
Statistical data concerning the analysis of chlorzoxazone (CLZ) and 6-hydroxychlorzoxazone (CLZOH)

Parameter	CLZ	CLZOH
Calibration ($n=6$)		
Range (ng/ml)	20–4000	20–1000
Slope: mean±S.D. (C.V.)	0.62±0.04 (6)	0.43±0.08 (19)
Intercept: mean (range)	−0.034 (0.070, 0.008)	−0.015 (−0.019, −0.008)
Coefficient of correlation: mean (range)	0.998 (0.996, 0.999)	0.999 (0.996, 0.999)
Recovery ($n=6$)		
Concentration used (ng/ml)	100	100
Recovery (%): mean±S.D. (C.V.)	93±26 (28)	97±22 (23)
Concentration used (ng/ml)	3000	1500
Recovery (%): mean±S.D. (C.V.)	68±10 (14)	65±11 (17)
Within-day variation ($n=7$)		
Theoretical values (ng/ml)	100	100
Measured values (ng/ml): mean±S.D. (C.V.)	106.5±2.5 (2)	106.5±4.1 (4)
Percentage of theory	106.5	106.5
Theoretical values (ng/ml)	3000	1500
Measured values (ng/ml): mean±S.D. (C.V.)	2878±122 (4)	1428±70 (5)
Percentage of theory	95.9	95.2
Day-to-day variation ($n=6$)		
Theoretical values (ng/ml)	100	100
Measured values (ng/ml): mean±S.D. (C.V.)	94.3±8.2 (9)	105.5±9.1 (9)
Percentage of theory	94.3	105.5
Theoretical values (ng/ml)	3000	1500
Measured values (ng/ml): mean±S.D. (C.V.)	2778±144 (5)	1475±76 (5)
Percentage of theory	92.6	98.3
Limit of quantitation ($n=8$)		
Theoretical values (ng/ml)	5	5
Measured values (ng/ml): mean±S.D. (C.V.)	5.3±0.4 (8)	4.8±0.4 (8)

S.D.=Standard deviation; C.V.=coefficient of variation (in %).

0.998 and 0.999 for CLZ and CLZOH, respectively. As pure standards of the derivatized compounds are not available, recovery was calculated by dividing mean areas ($n=6$) obtained after the complete extraction and derivatization procedure of plasmas containing 100 or 3000 ng/ml of CLZ and 100 or 1500 ng/ml of CLZOH by mean areas obtained after direct derivatization of the same quantities of pure standards. Recoveries were found to be satisfactory as they ranged from 65 to 97%. The variability of the assays, as assessed by the coefficients of variation (C.V.s) measured at two concentrations for each substance, was always less than 9%, both for the intra-day ($n=7$) and the inter-day ($n=6$) experiments. The deviations from the theoretical concentrations, which represent the accuracy of the method, were all within ±8%. The limits of quantitation, defined as the concentrations for which the mean value of replicate determination ($n=8$) is within 20% of the actual value, the C.V. is less than 20% and

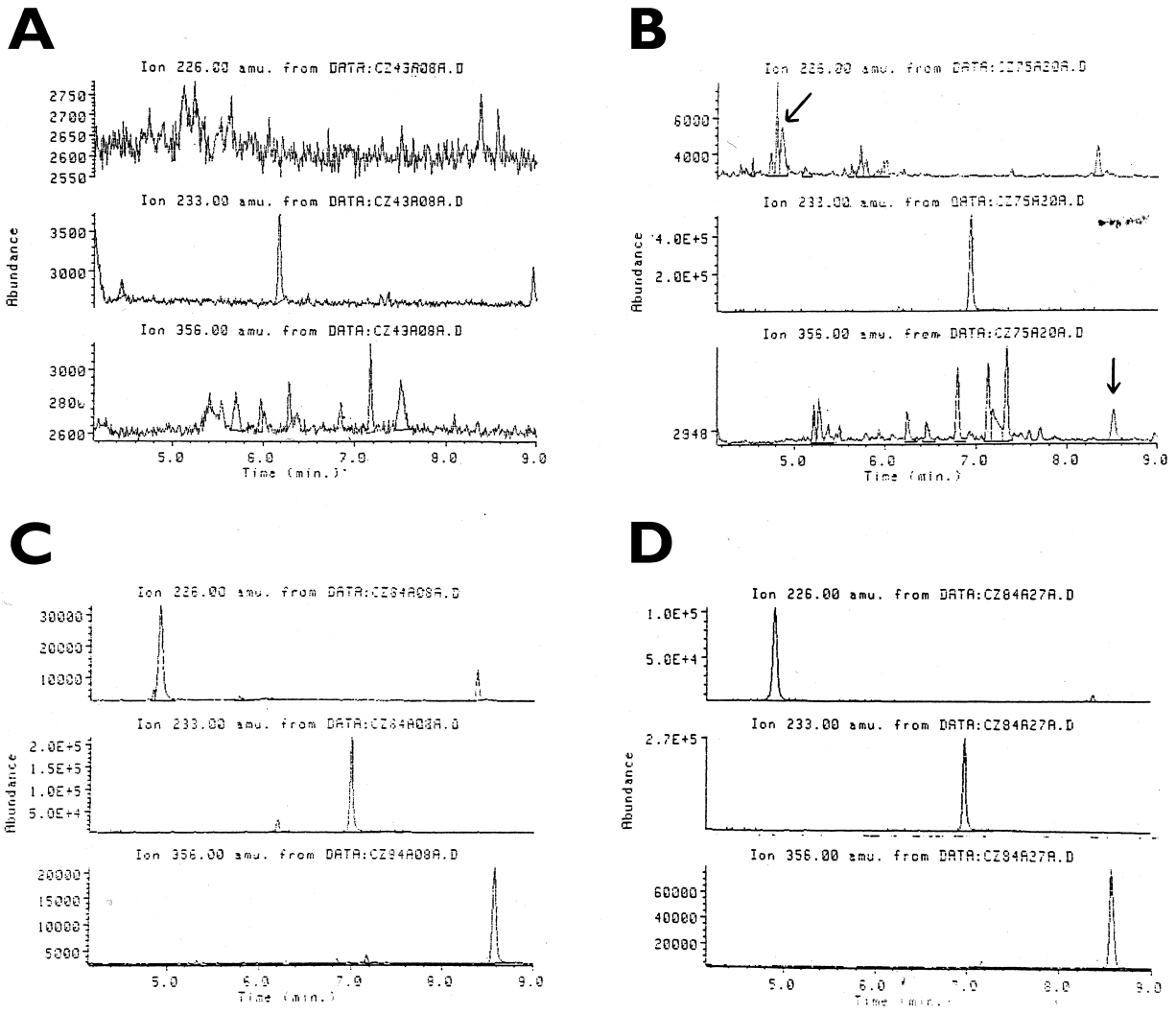

Fig. 3. SIM tracing of a 1 ml blank plasma (A), a blank plasma spiked with 5, 5 and 500 ng/ml (B), a blank plasma spiked with 200, 100 and 500 ng/ml of chlorzoxazone, 6-hydroxychlorzoxazone and 7-hydroxy-4-methyl coumarin, respectively (C), and of a plasma drawn 6 h after the oral ingestion of 250 mg of chlorzoxazone (D). Chlorzoxazone (ion 226, 4.92 min), 7-hydroxy-4-methyl coumarin (ion 233, 6.97 min) and 6-hydroxychlorzoxazone (ion 356, 8.55 min).

which gives a signal-to-noise ratio of at least 10 [18], were found to be of 5 ng/ml for the two substances. If necessary, this limit of quantitation can probably be lowered by raising the amount injected into the GC–MS from 2 to 3 μl. Although it was not precisely quantified, the limit of detection, defined as the concentration which gives a signal-to-noise ratio of at least 3, was found to be about 2 ng/ml for the two substances. No interference was observed from endogenous compounds following the extraction of plasma samples from six different human subjects. The stability of these two substances was also checked by analysing spiked plasmas stored at −20°C for different periods of time: no loss was noted after storage of up to three months. Finally, the stability of the derivatized molecules in the derivatization reagent was also checked: no loss was noted after storage of up to three days at room temperature (data not shown).

Fig. 3 shows the SIM tracing of a blank plasma, a blank plasma spiked with 5 ng/ml (limit of quantitation) of CLZ and CLZOH, a blank plasma spiked with 200 and 100 ng/ml of CLZ and CLZOH, respectively, and a plasma drawn from one subject 6 h after receiving a single dose of 250 mg of CLZ. The measured concentrations of CLZ and CLZOH in this subject at 6 h, that is, after approximately 6 elimination half-lives, were 925 and 611 ng/ml respectively. A full pharmacokinetic study of CLZ in five healthy volunteers and in 16 alcoholic patients receiving chlormethiazole (a CYP2E1 inhibitor) as comedication has been conducted (manuscript in preparation).

To summarize, this method, both sensitive and selective, allows the quantification of CLZ and CLZOH in plasma samples. Its sensitivity will allow the performance of pharmacokinetic experiments with a single low dose.

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