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Short communication

Simultaneous determination of chlorzoxazone, indicator of CYP2E1, and its metabolite in human serum using a new reversed-phase chromatographic column of 2-μm porous microspherical silica-gel

Einosuke Tanaka [1]

Department of Legal Medicine, Institute of Community Medicine, University of Tsukuba, Tsukuba-shi, Ibaraki-ken 305, Japan

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1. Introduction

Chlorzoxazone (CX; 5-chloro-2-benzoxazolinone) is a widely used centrally-acting skeletal muscle relaxant drug. Recently, Peter et al. [1] demonstrated that CYP2E1 was involved in the in vitro hydroxylation of CX to 6-hydroxychlorzoxazone (HCX) in human liver microsome samples and this compound has been suggested for use as a indicator to assess the activity of CYP2E1 in vivo [2–8]. Several high-performance liquid chromatographic (HPLC) methods have already been reported for the determination of CX and its major metabolite [1,9–12]. However, these methods are not very convenient to carry out in a routine laboratory. Those that have been published use a reversed-phase column with packing 5-μm in size (carbon content 10–15%) [1,9–12].

Recently, a new reversed-phase chromatographic column, TSK gel Super-ODS, based on 2-μm silica-gel (carbon content 8%), became commercially available from Tosoh (Tokyo, Japan) [13]. This column showed less ionic and metal interactions than previous columns. In addition, the use of the smaller particle size gives a higher column efficiency (over 200 000 theoretical plates (TP) m^{-1}) than other conventional reversed-phase columns (ca. 60 000–150 000 TP m^{-1}) for many compounds. Therefore, faster separation and better resolution can be achieved on Super-ODS. A few factors must be considered, however, when using this column: the retention capacity is lower than that of other conventional ODS columns so that the content of organic modifier in the mobile phase should be lowered, and the void volumes in the operating system must be reduced to a minimum.

[1] Tel./fax: +81 298 533057; e-mail: einosuke@md.tsukuba.ac.jp

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We have already reported the forensic analysis of benzodiazepine [14] and antiepileptic drugs [15] by using the Super-ODS in human biological samples. In this paper, we report the simultaneous determination of CX and its metabolite in human serum using this reversed-phase column in comparison with a conventional column.

2. Materials and methods

2.1. Regents and materials

CX and HCX were kindly provided by Fuso Yakuhin (Tokyo, Japan) and Dr T. Horie of Esai (Tokyo, Japan), respectively. 5-Chloro-2-methylbenzoxazole, used as an internal standard (I.S.), were purchased from Tokyo Kasei (Tokyo, Japan). β-Glucuronidase (Type G0751) was obtained from Sigma (St. Louis, MO, USA). The mobile phase was prepared by mixing deionized water obtained using a Milli-Q system (Millipore, Bedford, MA, USA) and HPLC-grade organic solvent.

2.2. Human study

Two healthy male volunteers, mean age 50 years, participated in this study. None had been prescribed long-term medications or suffered from impaired hepatic function; they also abstained from ethanol use for at least 3 days before the study. After overnight fasting, 200 mg CZ (Framenco®; Fuso Yakuhin Inc.) was orally administered and fasting was maintained for at least an additional 2 h. Blood samples were obtained from an arm vein at 0.5, 1, 1.5,2, 3, 4, 5 and 6 h after oral administration of CX.

2.3. Extraction procedure

To 0.5 ml serum (spiked standards) were added 1 ml of 0.2 M sodium acetate buffer (pH 4.75) and 1000 U of β-glucuronidase dissolved in 0.5 ml of 0.2% sodium chloride. Samples were vortex-mixed and incubated at 37°C for 3 h. After incubation, we added 100 µl I.S. (20 µg ml^{-1}), 400 µl 10% perchloric acid and 4 ml ethyl acetate in 15-ml culture tubes. After vortex mixing for 5 min, the tubes were centrifuged at $1200 \times g$ for 5 min. The organic phase was transferred to a clean conical tube and evaporated in a water bath at about 40°C under a gentle stream of nitrogen. The residue was dissolved in 50 µl mobile phase and aliquots of 10 µl were injected into the HPLC system.

2.4. Standard solutions and calibration

A standard stock solution containing CX and its metabolite was prepared in methanol at a concentration of 1 mg ml^{-1} of each compound and this remained stable for at least two months at -20°C. Plasma standards were prepared containing 0.05, 0.5, 1, and 5 µg ml^{-1} of each compound by diluting appropriate aliquots of stock solution with drug-free serum. A calibration curve was obtained by linear regression of the peak-height ratio versus concentration.

2.5. Apparatus

The HPLC equipment consisted of a pump (Model CCPS, Tosoh, Tokyo, Japan) and a variable-wavelength UV detector (Model UV-8020, Tosoh, Tokyo, Japan). The separation was achieved using a C_{18} reversed-phase column (column 1: 100×4.6 mm I.D., particle size 2 µm, TSK gel Super-ODS; column 2: 100×4.6 mm I.D., particle size 5 µm, Hypersil ODS, Yokogawa, Tokyo, Japan). The mobile phase was composed of ammonium acetate (pH 5; 0.15%)/acetonitrile (90:10, v/v) and the flow rate was 0.3 ml min^{-1} for both columns. The absorbance of the eluent was monitored at 287 nm. All instruments and the two columns were operated at ambient laboratory temperature (ca. 23°C).

2.6. Accuracy and recovery

The accuracy and recovery were calculated by comparing the peak heights of CX-spiked samples (0.01, 0.1 and 1 µg ml^{-1}) after extraction from serum with the peak heights of a series of unextracted reference standards.

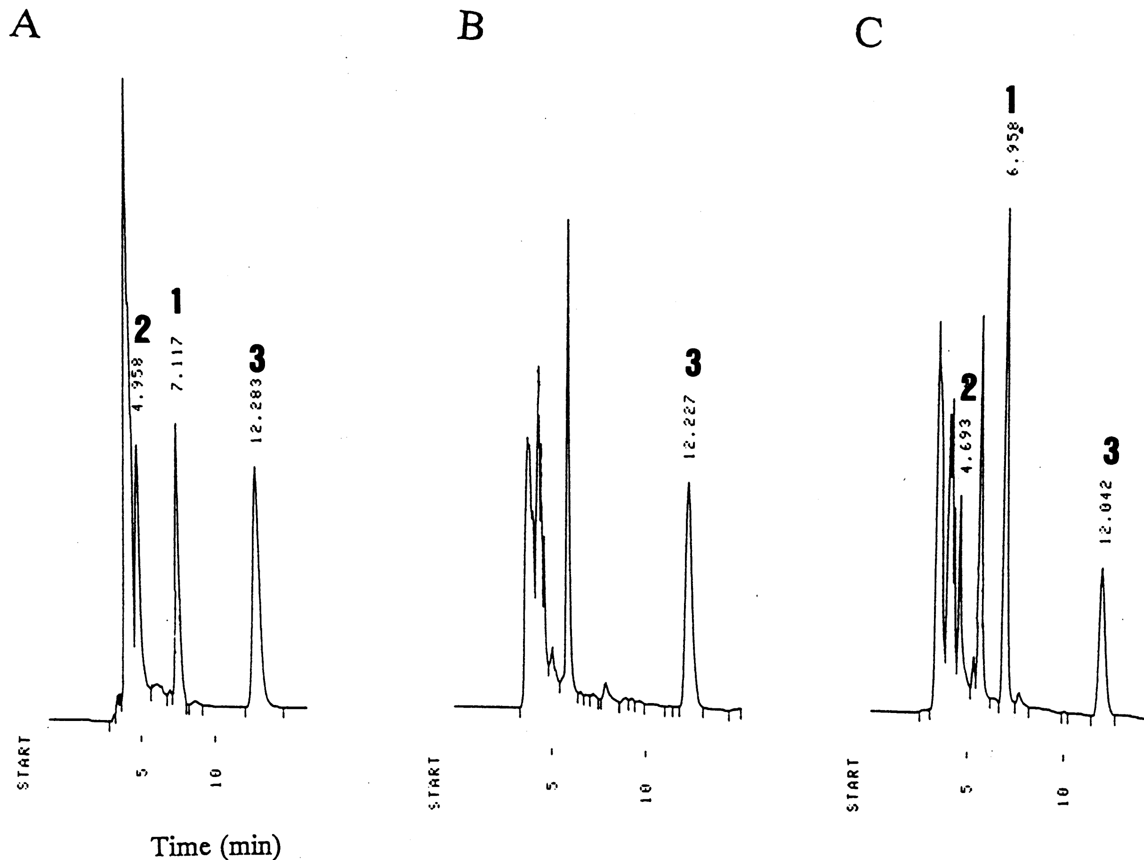

Fig. 1. Chromatograms of chlorzoxazone and its metabolite, 6- hydroxychlorzoxazone, in human serum. (A) Human serum spiked with CX and HCX. The concentrations are 1 μg ml^{-1} for CX and HCX. (B) Human blank serum. (C) Human serum obtained 4 h after oral administration of 200 mg CX to two subjects. Column, 100 × 4.6 mm I.D., particle size 2 μm, TSK gel Super-ODS; mobile phase, 0.15% ammonium acetate (pH 5)/acetonitrile (90:10, v/v); flow rate, 0.3 ml min^{-1}; detection wavelength, 287 nm. All instruments and the two columns were operated at ambient laboratory temperature (ca. 23°C). 1, chlorzoxazone (CX); 2, 6-hydroxychlorzoxazone (HCX); 3, internal standard (5-chloro-2-methylbenzoxazole).

2.7. Data analysis

The serum concentration versus time profiles of CX after oral administration were analyzed with use of linear regression of log-serum concentration versus time plots to estimate the serum elimination rate constant (k). The elimination half-life ($t_{1/2}$) was calculated from $t_{1/2} = 0.693/k$. The k is the elimination rate constant and estimated by applying logarithmic regression analysis to the terminal part of the serum concentration–time profile (AUC). The AUC was calculated using the linear trapezoidal rule up to 6 h and extrapolated to infinity with k.

3. Results and discussion

3.1. Retention time

Fig. 1 shows chromatograms of CX and its metabolite separated using column 1. The retention times (capacity factor) of HCX, CX and I.S. were approximately 5 (1.52), 7 (2.12) and 12 (3.64)

Table 1
Precision of the determination chlorzoxazone and its metabolite, 6-hydroxychlorzoxazone, in human serum by the present HPLC method using Column 1

Drug	Added (μg ml^{-1})	Mean (μg ml^{-1})	Within-day (R.S.D., %)	Between-day (R.S.D., %)
CX	0.01	0.011	2.5	3.9
	0.1	0.11	3.2	4.2
	1	1.1	1.9	3.8
	5	5.1	1.8	3.6
HCX	0.01	0.010	2.4	4.1
	0.1	0.11	1.7	3.6
	1	1.0	2.3	4.7
	5	4.9	1.9	3.6

$n = 6$. Column 1: 100 × 4.6mm I.D., particle size 2 μm (TSK gel Super-ODS); mobile phase, 0.15% ammonium acetate (pH 5)/acetonitrile (90:10, v/v); flow rate, 0.3 ml min^{-1}; detection wavelength 278 nm. All instruments and the column were operated at ambient laboratory temperature (ca. 23°C).
CX, chlorzoxazone; HCX, 6-hydroxychlorzoxazone; R.S.D., relative standard deviation.

min for column 1, and 8 (2.42), 11 (3.33) and 19 (5.76) min for column 2. All drugs were well separated. These results show that the retention times of CX and its metabolites depend mainly on the carbon content of the reversed-phase column. No interfering peaks appeared from endogenous material eluted after the drug.

3.2. Limits of quantification

The calibration curves (the ratio between the peak-height of the drugs analysed and that of the I.S. vs. amount of each drug) were obtained over the concentration range 0.01–5 μg ml^{-1} plasma. The equations and r values for the curves were: $r = 0.998$ for HCX; $r = 0.999$ for CX.

The limit of quantification using column 1 is the lowest concentration on the standard curve which can be measured with acceptable accuracy (a relative standard deviation, R.S.D. $< 5\%$). The lowest practical limit of quantification was 0.01 μg ml^{-1} for CX and its metabolite (Table 1). The sensitivity for CX and its metabolite using the methods of Stiff et al. [9], Lucas et al. [10], Kharasch et al. [11] and Girre et al. [12] was much lower (about 10–50 times) than that of our method (column 1). The sensitivity using column 1 was about 10 times greater than that with column 2 (data not shown).

3.3. Precision and accuracy

The precision and accuracy obtained using column 1 are shown in Table 1. Within-day reproducibility was assessed by analysing six samples at three different concentrations on the same day. The R.S.D. ranged from 1.7–3.2%. Between-day reproducibility was determined six times using four different quality control samples over a period of 2 weeks. The R.S.D. ranged from 3.6–4.7%.

3.4. Recovery

A liquid–liquid extraction system was investigated, including ethyl acetate [10,12], dichloromethane/ethyl acetate (1:1) and dichloromethane. Ethyl acetate was chosen as the extraction solvent (indicated absolute recoveries 99–102% for CX and 97–103% for HCX because it afforded a better extraction than other solvents (92–94% for CX and 91–93% for HCX) [10,12].

Recovery was calculated from the values obtained using drug-spiked serum (Table 2). The R.S.D. ranged from 2.4–4.5%. The recovery with column 1 were approximately the same as that with column 2 (data not shown).

Table 2
Recovery of chlorzoxazone and its metabolites, 6-hydroxy-chlorzoxazone, from human serum by the present HPLC method using Column 1

Drug	Concentration (μg ml^{-1})	Recovery (%)	R.S.D. (%)
CX	0.01	99.3	2.4
	0.1	98.5	3.7
	1	102.3	4.1
	5	99.5	2.6
HCX	0.01	101.7	4.5
	0.1	103.2	3.7
	1	97.1	3.1
	5	98.9	3.2

The same as in Table 1.

3.5. Human study

The serum CX levels reached a peak at around 0.5–1 h in two subjects after oral administration of the drug and then gradually decreased. Serum level of HCX was also similar to those of the parent drug, although the metabolite's elimination $t_{1/2}$ was slightly longer (pharmacokinetic parameter:$t_{1/2}$ = CZ 1.8 h, HCX 2.1 h; AUC = CZ 16.78 μg ml^{-1} h^{-1}, HCX 11.07 μg ml^{-1} h^{-1} (Fig. 2).

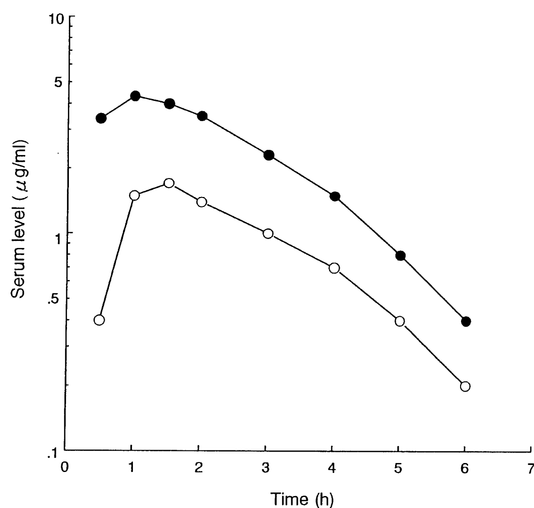

Fig. 2. Serum mean concentration–time profile of chlorzoxazone (solid circles) and its metabolite, 6-hydroxychlorzoxazone (open circles) after oral administration of 200 mg chlorzoxazone, in two subjects.

In addition, these parameters were similar to those of previous reports [2–8]. An ODS column packing with a particle size of 2 μm (TSKgel Super-ODS) and a pore volume and specific surface area about one third less than that of conventional ODS column packing has recently been developed [13]. This new ODS packing has the following advantages: (1) more rapid determination can be expected even at room temperature and without gradient elution, compared with conventional methods, and (2) the amount of organic solvent required is small.

4. Conclusion

These results show that the rapid turnaround time and accuracy of this method make it suitable for clinical medicine, allowing rapid detection, confirmation and quantification of CX and its metabolite using a single method.

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