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SENSITIVE AND SELECTIVE METHOD FOR THE DETERMINATION OF CHLORMEZANONE IN PLASMA BY ELECTRON-CAPTURE GAS CHROMATOGRAPHY

KAZUMI OHYA, SHOZO SHINTANI, WATARU SUZUKI and MITSUJI SANO*

Drug Metabolism Research Center, Research Institute, Daiichi Seiyaku Co. Ltd., 1-16-13 Kitakasai, Edogawa-ku, Tokyo 132 (Japan)

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SUMMARY

A sensitive and selective determination method of chlormezanone in plasma has been divised. Chlormezanone in plasma was extracted with toluene at pH 4.5, and converted into *p*-chlorobenzaldehyde in 0.1 *N* NaOH. Using *p*-bromobenzaldehyde as an internal standard, the hydrolysis product and the internal standard were extracted with *n*-hexane, and the extract was concentrated in vacuo in the presence of isoamyl alcohol to prepare the sample solution. The sample solution was submitted to electron-capture gas chromatography. Chlormezanone was determined by use of the peak height ratio of *p*-chlorobenzaldehyde against the internal standard. The method was utilized successfully for pharmacokinetic studies of chlormezanone in plasma.

INTRODUCTION

The tranquilaxant chlormezanone (CM), 2-(4-chlorophenyl)-3-methyl-4-*m*-thiazanone-1,1-dioxide, has been widely used in the treatment of spastic paralysis, low back pain and neurosis, etc., whereas only one paper [1] has described briefly the pharmacokinetics of the drug in man. One report [2] has clarified the metabolism and pharmacokinetics of CM in mice and rats using ^{14}C-labelled CM, and indicated that CM exerts its medicinal effect in unchanged form. Therapeutic and toxic responses to CM appear to be related to its plasma concentration. Therefore it may be important to monitor the plasma levels of CM in patients treated with the drug.

Only a colorimetric method [1] has been presented for the determination of CM in plasma; however, the method was not sufficiently sensitive and

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selective to measure therapeutic levels of CM. A more sensitive and selective method was required for reliable pharmacokinetic studies of CM in man.

Gas chromatography with electron-capture detection (ECD-GC) is a powerful tool for highly sensitive analyses of electrophilic compounds. In applications of ECD-GC to the determination of CM, it was found that CM hydrolyzed readily in alkaline solutions to form volatile *p*-chlorobenzaldehyde (*p*-CBA). By applying the hydrolysis product, *p*-CBA, to ECD-GC, a highly sensitive and selective method for the determination of CM in plasma was developed. This method was sufficiently suitable for the routine analysis of CM at plasma levels of 0.1—2 μg/ml.

This paper describes the method devised and the pharmacokinetics of CM in man.

EXPERIMENTAL

Chemicals and reagents

CM was of J.P.IX grade (Sterling-Winthrop, New York, NY, U.S.A.). All other chemicals were of reagent grade (Tokyo-kasei Kogyo, Tokyo, Japan, or Kishida Chemicals, Tokyo, Japan). A stock solution of CM was prepared by dissolving 5.0 mg of CM in 100 ml of acetate buffer solution (pH 4.5), and stored at 5°C protected from the light. Standard samples were prepared by spiking blank plasma with the stock solution at concentrations of 0.1—2 μg/ml, and stored at —20°C. An internal standard solution was prepared by dissolving *p*-bromobenzaldehyde (*p*-BBA) in *n*-hexane at a concentration of 0.5 μg/ml. An acetate buffer solution (pH 4.5) was prepared with 0.1 *M* acetic acid and 0.1 *M* sodium acetate.

Plasma samples

Plasma samples were obtained at six time intervals up to 48 h after single doses of 200 mg of CM in the form of a commercial tablet. Two tablets were administered to five healthy male subjects together with 200 ml of water. Each subject was fasted for 3 h both before and after drug administration. Blood was collected in heparinized syringes by venipuncture, and centrifuged in the usual manner to separate the plasma. The plasma samples were stored at —20°C until they were analyzed.

Analytical procedure

Sample preparation. To 0.1—1-ml plasma samples corresponding to 0.1—2 μg of CM in a 12-ml glass-stoppered centrifuge tube were added 1 ml of acetate buffer solution (pH 4.5) and 6 ml of toluene. The tube was shaken vigorously for 15 min and centrifuged at 2000 *g* for 5 min. A 5-ml volume of the toluene layer was pipetted into a 12-ml glass-stoppered centrifuge tube and evaporated in vacuo to dryness. The residue was dissolved in 1 ml of 0.1 *N* NaOH and allowed to stand for 15 min at room temperature. After addition of 2.0 ml of internal standard solution, the reaction mixture was extracted in a similar manner as the plasma. The aqueous layer was frozen in dry-ice—acetone then all of the *n*-hexane layer was transferred to a glass tube. After 1 ml of isoamyl alcohol had been added to the *n*-hexane extract,

the extract was concentrated in vacuo to a volume of about 1 ml.

Gas-chromatographic conditions. A Shimadzu gas chromatograph, Model 5A, equipped with a ^{63}Ni-ECD (Shimadzu Model-ECD-5), was used. The column was a 1 m × 3 mm I.D. glass tube packed with 5% EGS polyester on 80—100 mesh Gas-Chrom Q. The column was kept at 100°C isothermally, and the injection port and the ^{63}Ni-ECD were held at 150°C and 120°C, respectively. Highly purified nitrogen was used as carrier gas at a flow-rate of 40 ml/min. The sensitivity and the range were set at 10^2 MΩ and 16 mV, respectively. The sample size was 1—2 μl.

Calculations. The concentrations of CM in the plasma samples were determined from a calibration curve prepared by plotting peak height ratio of *p*-CBA against the internal standard (*p*-BBA). The calibration curve was obtained by using the standard samples in the same manner as described under *Plasma samples*.

Conversion rates of CM into p-CBA

The stock solution of CM was diluted five times with water of different pH values (0.5 *N* HCl—0.2 *N* NaOH) to prepare test solutions (10 μg/ml). The test solutions were allowed to stand at room temperature, and the ultraviolet (UV) absorption curves were then measured at a certain time interval with a Hitachi 323 recording spectrophotometer. The conversion rates were calculated from the absorbance at 260 nm due to *p*-CBA.

Identification of the hydrolysis product of CM

The hydrolysis product *p*-CBA was identified by means of gas chromatography—mass spectrometry using an Hitachi gas chromatograph—mass spectrometer, Model RMU-6MG. The mass spectrum was measured at 70 eV with an ion source temperature of 160°C.

Temperature dependence of p-CBA and p-BBA on ^{63}Ni-ECD response

An injection solution containing about 100 ng of both *p*-CBA and *p*-BBA in 1 ml of *n*-hexane was used. The gas chromatograms were obtained using 5.0 μl of the solution under similar GC conditions to those described under *Analytical procedure.* The ^{63}Ni-ECD temperature was adjusted from 80°C to 240°C at intervals of 20°C. The relative sensitivity was calculated from peak height.

Recovery test

The stock CM solution was diluted with water to prepare reference CM solutions at concentrations of 0.1—2 μg/ml. Both the standard samples described in *Chemicals and reagents* and the reference CM solutions were analyzed by means of the analytical procedure. The values of CM for the standard samples were compared with those for the reference CM solutions; the difference was calculated as the recovery of CM from plasma.

RESULTS AND DISCUSSION

Gas chromatography

Attempts to submit CM to GC without any chemical modifications failed because of the thermal decomposition of CM during chromatography. A volatile derivative derived from CM was required for GC analysis of the drug. In some examinations for converting CM into volatile compounds, it was found that CM hydrolyzed readily in alkaline solution to give *p*-CBA. This finding lead to the idea that *p*-CBA be used for the GC analysis of CM. Fig. 1 shows the typical gas chromatograms resulting from blank plasma

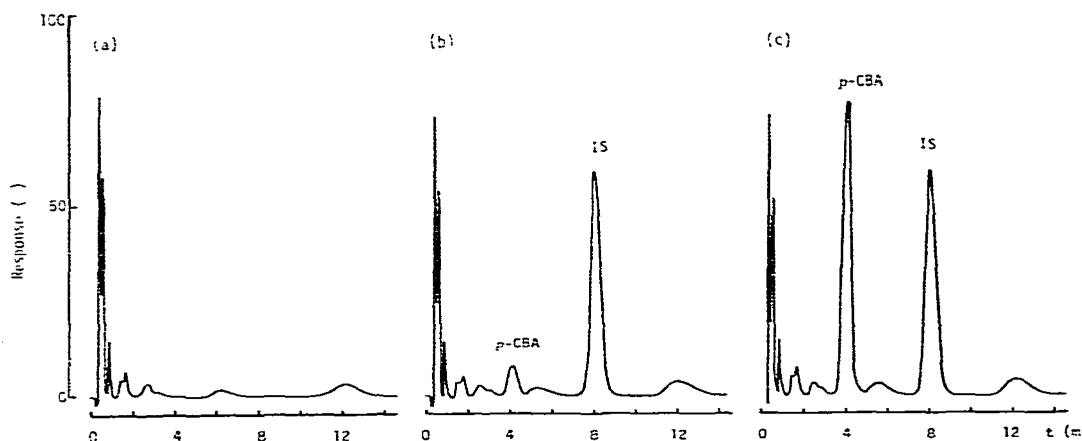

Fig. 1. Gas chromatograms of blank plasma (a), and standard samples prepared by spiking blank plasma with chlormezanone at concentrations of 0.2 μg/ml (b) and 2 μg/ml (c). *p*-CBA = *p*-chlorobenzaldehyde; IS = internal standard (*p*-bromobenzaldehyde).

and standard samples prepared by spiking blank plasma with CM. *p*-CBA and the internal standard were eluted at 4.2 min and 8.1 min, respectively, and were resolved satisfactorily from the peaks due to endogenous plasma components and impurities in the reagents.

Conversion of CM to p-CBA

The UV absorption curve of CM suggested that CM hydrolyzed readily in alkaline solutions at room temperature. The absorbance at 228 nm due to CM disappeared within 15 min in 0.1 *N* NaOH coincident with the appearance of absorbance at 260 nm due to its hydrolysis product. The product *p*-CBA was identified by comparing both the retention time and mass spectrum with those of authentic *p*-CBA, whose mass spectrum is characterized by peaks at *m/z* 140, 139 (base peak) and 111 corresponding to $[M]^{+\cdot}$, $[M-H]^{+}$ and $[M-CHO]^{+}$, respectively. Fig. 2 summarizes the conversion rates of CM into *p*-CBA under several conditions.

On the other hand, CM was stable in acidic solutions as seen in Fig. 2. No changes were observed in acetate buffer solution at pH 4.5 even after 24 h at room temperature, and little CM changed in 0.5 *N* HCl under similar aging conditions. These results suggested that weak acidic conditions were

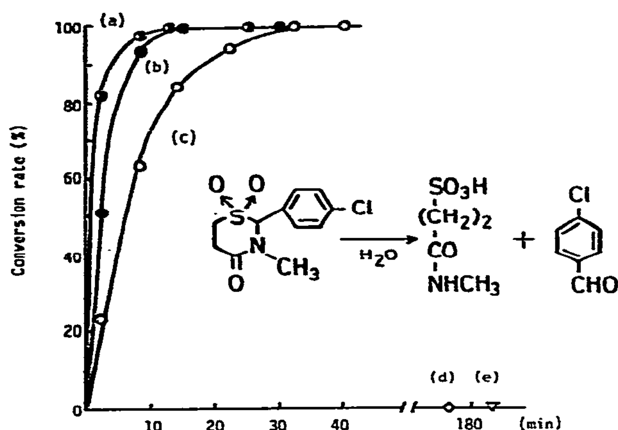

Fig. 2. Conversion rates of chlormezanone into *p*-chlorobenzaldehyde in 0.2 *N* NaOH (a), 0.1 *N* NaOH (b), 0.01 *N* NaOH (c), 0.5 *N* HCl (d) and acetate buffer (pH 4.5) (e), at room temperature.

desirable for the extraction and purification of CM in plasma samples to prevent the artifactual hydrolysis.

Detector temperature

It was presumed in the paper of Wentworth and Chen [3] that the ^{63}Ni-ECD response of *p*-CBA and *p*-BBA was dependent on the detector temperature; the response of the aromatic aldehydes would decrease with increasing detector temperature. Fig. 3 shows the temperature dependence of *p*-CBA and *p*-BBA with the ^{63}Ni-ECD used. The response of the aldehydes decreases with increasing detector temperature; furthermore, *p*-CBA is much more affected than *p*-BBA at about 140°C or above. This indicated that a lower detector temperature was desirable for sensitivity and precision. On

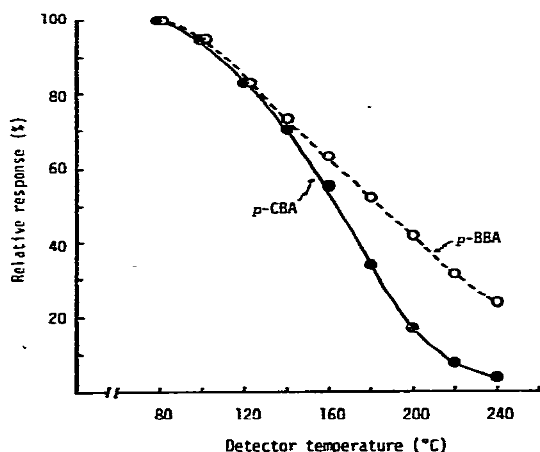

Fig. 3. Temperature dependence of *p*-chlorobenzaldehyde (*p*-CBA) and *p*-bromobenzaldehyde (*p*-BBA) with the ^{63}Ni-ECD used. The values are the mean of five measurements.

the other hand, a higher temperature would be desirable to avoid the accumulation of column bleed and/or plasma components in the detector. The detector temperature was set at 120°C by considering the conditions described above.

Selection of extraction solvents

Toluene was chosen for the extraction of CM from plasma in consideration of the extraction rate of CM and the removal of plasma components. CM in water was extracted completely by toluene, ethyl acetate and isoamyl alcohol; the toluene extract was less interfering than the others. *n*-Hexane was undesirable because of a low extraction rate for CM (about 30%) in spite of its being the least interfering of the solvents examined.

Benzene was as effective for both the extraction and clean-up as toluene; however, its use was avoided in view of it being a potential carcinogen.

However, *n*-hexane was chosen for the extraction of *p*-CBA from 0.1 *N* NaOH. *p*-CBA and internal standard (*p*-BBA) in 0.1 *N* NaOH were extracted completely by *n*-hexane, toluene, ethyl acetate and isoamyl alcohol. However, *n*-hexane was the most desirable of these solvents from the point of view of clean-up. The *n*-hexane extract contained fewer biological components than the others, and showed no interference at the retention times of *p*-CBA and *p*-BBA.

Calibration curve and recovery

The calibration curve showed good linearity (r = 0.9998) between peak height ratio and CM concentration in plasma in the range 0.1—2 μg/ml. Higher amounts of CM in the samples did not give quantitative results for the saturation of response. This may be due to a narrower dynamic range of the linear response, which is generally well-known as a characteristic of ECD [4]. The precision and recovery of the method are shown in Table I. CM in plasma could be determined within ± 7% coefficient of variation over the range 0.1—2 μg/ml. No significant differences were found between the values

TABLE I

PRECISION AND RECOVERY OF THE GAS-CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF CHLORMEZANONE (CM) IN PLASMA

Standard samples and reference CM solutions were prepared by spiking blank plasma and water with CM as described in Experimental. The values were calculated from a calibration curve obtained using authenic CM.

CM added (μg/ml)	CM found (μg/ml)						Percentage recovery (A/B × 100)
	Standard samples (A)			Reference CM solutions (B)			
	Mean ± σ	n	C.V. (%)	Mean ± σ	n	C.V. (%)	
0.103	0.98 ± 0.0063	9	6.4	0.103 ± 0.0058	9	5.6	95.1
0.412	0.408 ± 0.0167	7	4.1	0.411 ± 0.0173	7	4.2	99.3
1.03	1.03 ± 0.052	6	5.0	1.02 ± 0.050	6	4.9	101.0
2.06	2.01 ± 0.111	6	5.5	2.03 ± 0.083	6	4.1	99.0

of CM from standard samples and those from reference CM solutions.

The addition of isoamyl alcohol to the *n*-hexane extract was essential to prevent the sublimation of *p*-CBA and *p*-BBA in the concentration step. If this precaution were not taken, no quantitative results could be obtained.

In addition, it was suggested that CM metabolizes to form *p*-CBA [1]; however, this metabolite was not identified in mice and rats [2]. The method presented here can distinguish CM from *p*-CBA. *p*-CBA would be removed in the evaporation process of toluene extract even if it were present.

Application to the pharmacokinetics of CM in man

The presented method was utilized for the pharmacokinetics of CM in man. Table II summarizes the plasma levels of CM in healthy male subjects

TABLE II

PLASMA LEVELS OF CHLORMEZANONE (CM) AFTER ORAL ADMINISTRATION OF 200 mg/MAN IN THE FORM OF A COMMERCIAL TABLET (100 mg/TABLET)

Subjects	Plasma levels of CM (μg/ml)					
	1 h	2 h	4 h	8 h	24 h	48 h
S.F.	0.85	2.03	2.92	2.59	1.85	1.20
S.T.	0.53	2.48	2.66	2.40	1.53	0.90
K.S.	0.82	1.91	2.50	2.21	1.54	0.72
A.S.	0.08	2.82	2.51	2.39	1.39	0.76
H.T.	2.93	3.35	3.18	2.39	1.53	0.72
Mean	1.04	2.52	2.75	2.40	1.57	0.86
S.E.	0.49	0.27	0.13	0.06	0.08	0.09

after a single oral administration of 200 mg of CM. The CM was absorbed rapidly from gastrointestinal tract with an absorption rate constant of $7.97 \cdot 10^{-1}\ h^{-1}$ reaching a maximum plasma level of about 2.7 μg/ml 4 h after administration. Elimination occurred very slowly according to first-order kinetics with an elimination rate constant of $3.45 \cdot 10^{-2}\ h^{-1}$, and CM remained in the plasma at a level of 0.86 μg/ml even after 48 h. The biological half-life of CM was calculated as 19.7 h. The elimination rate constant agreed well with that reported by McChesney et al. [1]. On the other hand, the absorption rate constant was a third of that reported by those authors [1]. This difference might be due to the CM formulation used, such as fine powder and tablet.

The method was also applicable to the determination of CM in whole blood. CM was extracted from hemolyzed whole blood as well as from plasma, and no effects of the red blood cells were found on either the extraction rate of CM or the gas chromatogram.

Several biomedical studies of CM are under investigation with successful results using the method. We believe that the method could be useful for the work of other laboratories.

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