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Determination of Chinoform and Its Metabolites in Plasma by Gas Chromatography–Mass Spectrometry

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A gas chromatographic–mass spectrometric (GC–MS) procedure using selected ion monitoring is described for the determination of chinoform (CF) and its two metabolites, glucuronide and sulfate, in plasma. The method consists of extraction of CF from plasma with pyridine–benzene (1:9, v/v) mixture containing 5-chloro-7-bromo-8-quinolinol as an internal standard, selective hydrolysis and extractions of the two conjugates, acetyl derivatizations and GC–MS analysis. The lower limit of CF quantitation by the present method was 3.3 pmol (1 ng) in 0.1 ml plasma volume. The method has sufficient sensitivity to permit pharmacokinetic studies with rats after single oral administration of CF.

Keywords——chinoform; chinoform glucuronide; chinoform sulfate; rat plasma; GC–MS; selected ion monitoring; chinoform-oral administration

Since chinoform (clioquinol, 5-chloro-7-iodo-8-quinolinol) (CF) and its ferric chelate were isolated and identified from the green urine and feces of SMON (subacute myelo-opticoneuropathy) patients,[1] CF has been studied by many investigators as regards intoxication,[2] metabolism,[3] distribution,[4] epidemiology,[5] *etc.* However, for detailed studies on the pharmacokinetics and metabolic disposition of CF in laboratory small animals, in particular the rat, it is desirable to have available a microanalytical method to determine plasma concentrations of CF and its main metabolites.

Several analytical procedures for the determination of CF and/or its conjugated metabolites have been described. Although an earlier colorimetric procedure based on the formation of CF-iron complex[6] and its modification[7] are simple and rapid, they suffer from a lack of sensitivity and specificity. An analytical procedure using thin-layer chromatographic separation followed by ultraviolet spectrophotometric analysis has also been reported for determining CF and its conjugate in plasma.[8] This method allows the determination of CF concentrations as low as 40 ng per ml, but the plasma sample volume required is 1—5 ml. More recently, a high-performance liquid chromatographic (HPLC) method for determining CF and its two conjugates in biological samples has been developed.[9] Although the sensitivity of this HPLC method is higher than that of another HPLC method,[10] the quantitation limit is only 1 nmol for CF, which is not adequate for pharmacokinetic studies in small animals following a single low dose of CF. On the other hand, several gas chromatographic (GC) methods using an electron-capture detector have been reported for the determination of CF alone[11] or CF and its two conjugates.[12] The GC methods[11a,12] based on derivatization of CF to its acetyl ester are time-consuming, since a clean-up procedure on a magnesium silicate column prior to derivatization is essential for the removal of interfering substances. The methods[11c,d] based on extractive methylation suffer from the instability of the product.[11e] In view of the requirements for higher specificity and sensitivity (down to 1 ng per 0.1 ml of plasma) a gas chromatographic–mass spectrometric (GC–MS) method appears to be the technique of choice. However, no such method has yet been reported.

In this paper, we describe a GC–MS method which has high sensitivity, specificity and precision for the determination of CF and its main conjugates, CF-glucuronide and CF-sulfate,

in plasma, and which may be suitable for pharmacokinetic and metabolic studies of CF in small animals.

Experimental

Materials and Reagents——CF, kindly supplied by Tanabe Seiyaku Co.(Osaka, Japan), was recrystallized from ethanol. 5-Chloro-7-bromo-8-quinolinol, used as an internal standard (I.S.), was synthesized by bromination of 5-chloro-8-quinolinol (Tokyo Kasei Kogyo Co., Tokyo, Japan) in carbon tetrachloride.[13] CF-glucuronide was synthesized by the method of Matsunaga and Tamura.[14] CF-sulfate was kindly provided by the Division of Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo. Acetic anhydried and sodium carboxymethylcellulose were obtained from Wako Pure Chemical Industries (Tokyo). Pyridine (max. 0.01% H_2O) was obtained from E. Merck (Darmstadt, G.F.R.). β-Glucuronidase (13000 Fishman units/ml) was obtained from Tokyo Zouki Co.(Tokyo). All other solvents and reagents used were of reagent grade.

Apparatus and Operating Conditions——The GC–MS system (JEOL, Tokyo) used in this study was composed of a JGC-20K gas chromatograph, a JMS D-300 mass spectrometer equipped with a multiple ion detector, and a JMS-2000 data processing system including a JEC-980B computer. The chromatographic column was a silanized and coiled glass tubing (1 m×2 mm i.d.)packed with 1% OV-17 on Chromosorb W AW DMCS, 80—100 mesh (Wako Pure Chemical Industries). Prior to use, the column was conditioned by maintaining for 48 h at 290°C with a carrier gas (helium) flow rate of 50 ml/min while the column was not connected to the mass spectrometer, and 10 μl of hexamethyldisilazane was then injected into the column five times at about 1 h intervals, at column and inject-port temperatures of 215°C.

The gas chromatograph was operated at column and injection-port temperatures of 215°C with a helium flow-rate of 37 ml/min (inlet pressure of 1 kg/cm^2). The mass spectrometer was operated in the electron impact ionization mode under the following conditions. For measurement of mass spectra in the scanning mode: ionization energy, 70 eV; emission current, 0.3 mA; scan speed, m/e 0—800/5 s. For quantitation in the selected ion monitoring mode: ionization energy, 24 eV, emission current, 0.3 mA. The separator and ion-source temperatures were maintained at 210 and 200°C, respectively.

Prior to GC–MS analysis, a concentrated solution of acetyl derivatives of CF and the I.S.(300 μg of each in 1 ml of *n*-hexane) was always injected several times at the beginning of each run to minimize loss by adsorption onto the column materials.

Data obtained were stored directly on a disk and displayed on a cathode ray tube or printed out on a graphic printer.

Sample Preparation for GC–MS Analysis——Separative extraction of CF, CF-glucuronide and CF-sulfate from plasma was performed according to a minor modification of the method of Chen *et al.*[12]

Unchanged CF: To 0.1 ml of plasma in a 10-ml glass-stoppered centrifuge tube were added 1.5 ml of 0.1 M acetate buffer at pH 5 and 4 ml of benzene–pyridine (9:1,v/v) mixture containing 58.1 pmol (15 ng) of the I.S. per ml. The mixture was vigorously shaken with a mechanical shaker for 2 min then centrifuged with a KN-70 centrifuge (Kubota Seisakusho Co., Tokyo) at 3000 rpm for 5 min. The upper organic phase was transferred to another tube and evaporated to dryness on a rotary vacuum evaporator with the tube immersed in a water bath at 35°C. To the residue were added 0.1 ml of acetic anhydride and 0.1 ml of pyridine. The tube was stoppered tightly, agitated and immersed in a water bath at 65°C for 30 min. After acetylation, the excess reagent was largely evaporated off under a stream of nitrogen. To completely remove trace amounts of acetic anhydride, the tube was kept in a desiccator containing silica gel under reduced pressure at ambient temperature for over 30 min. The dried residue was dissolved in 100 μl of *n*-hexane and 1—3 μl of this solution was injected into the chromatographic column.

The aqueous phase was used for the analysis of CF-glucuronide and CF-sulfate.

CF-glucuronide: The aqueous phase obtained by centrifugation was washed twice with 6 ml of benzene each time to remove trace amounts of CF, the I.S. and fairly large amounts of pyridine that remained in the aqueous phase. After removal of the benzene phase, 1 ml of the aqueous phase was transferred to a new centrifuge tube, and then 750 Fishman units of β-glucuronidase was added. The mixture was incubated under gentle shaking in a water bath at 37°C for 2 h. The CF liberated by enzymatic hydrolysis was analyzed in the same manner as described for unchanged CF above.

CF-sulfate: The residual aqueous phase containing CF-sulfate was washed twice with benzene as before, 0.5 ml of the aqueous phase was transferred to a glass-stoppered centrifuge tube, and the solution was acidified with 0.1 ml of 6N HCl. Hydrolysis of CF-sulfate was performed by gently shaking the tube in a water bath at 40°C for 2 h. The solution was cooled to ambient temperature, and the aqueous phase was adjusted to pH 5 by adding 0.2 ml of 3N NaOH. The liberated CF was analyzed in the same manner as described for unchanged CF above.

Calibration Curves——A series of samples containing various quantities of CF, CF-glucuronide and CF-sulfate were prepared by dissolving each compound in 0.04N NaOH and by adding 0.1 ml of each solution to 0.1 ml of rat blank plasma (pmol of CF/pmol of CF-glucuronide/pmol of CF-sulfate: 655/0/0, 327/655/655,

164/327/327, 82/164/164, 33/82/82, 16.4/33/33, 8.2/16.4/16.4, 3.3/8.2/8.2). The plasma samples were then assayed by the method described above. The peak height ratios were calculated by dividing the peak height due to the acetyl derivative of CF (*m/e* 305) by that due to the acetyl derivative of the I.S. (*m/e* 259) and were plotted against the amount of each compound. Calibration curves were constructed on the same day as the analysis of unknown samples, and the plasma concentrations were calculated by comparison of the peak height ratios with the calibration curves obtained on the same day.

Sample Collection——CF was administered intravenously or orally to male Wistar rats (230—250 g), and blood samples were collected from the femoral artery through a polyethylene cannula at appropriate time intervals up to 7 h after administration. Plasma samples were separated from blood by centrifugation at 3000 rpm for 15 min and stored at −20 °C until assay.

Results and Discussion

Mass Spectra and Selected Ion Monitoring

The mass spectra of the acetyl derivatives of authentic CF and 5-chloro-7-bromo-8-quinolinol recorded at 70 eV ionization energy are shown in Fig. 1(a) and (b). The acetyl derivative of CF gave a molecular ion (M^+) peak at *m/e* 347 and a base peak at *m/e* 305 (M^+-CH_2CO). The isotope peaks for the molecular ion peak and some fragment peaks also appeared at two mass number units up-scale with an intensity of about 32% relative to that of the molecular ion peak and to those of the parent fragment ion peaks (Fig. 1(a)), respectively. The mass spectrum of the acetyl derivative of 5-chloro-7-bromo-8-quinolinol gave a molecular ion (M^+) peak at *m/e* 299 and a base peak at *m/e* 259 (Fig. 1(b)). The ion peak at *m/e* 259 was an isotope peak related to the parent fragment ion at *m/e* 257 (M^+-CH_2CO). This compound was used as the I.S. for the analysis of CF and its metabolites, because its structure is similar to that of CF, and a suitable ion for the analysis in the electron impact ionization mode was observed. The fragment ions at *m/e* 305 for CF and at *m/e* 259 for the I.S. were used for selected ion monitoring (SIM) analysis. To confirm the purity of the gas-chromatographic peak of acetylated CF, the intensity of the isotope ion at *m/e* 307 was monitored simultaneously.

In order to select a suitable ionization energy in the SIM mode, the ion intensities at both *m/e* 305 and 259 were examined at 20, 22, 24, 26, 30, or 70 eV ionization energy. The most

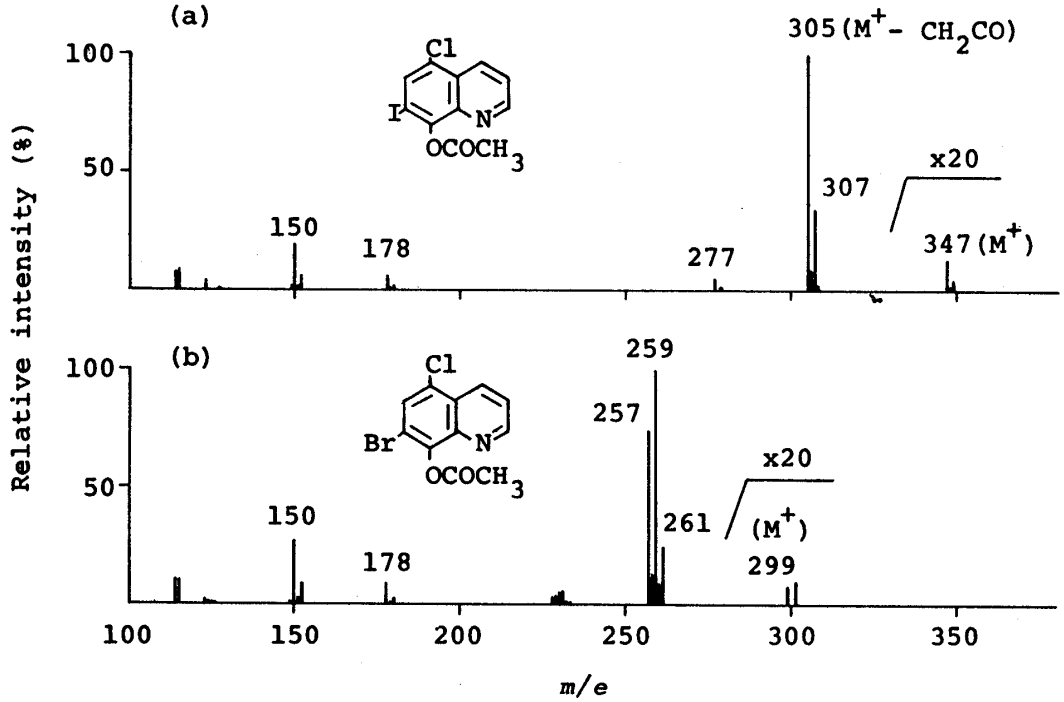

Fig. 1. Electron Impact Mass Spectra of Acetyl Derivatives of (a) Chinoform and (b) 5-Chloro-7-bromo-8-quinolinol (used as the Internal Standard)

intense peaks were obtained at 24 eV and these intensities were approximately twice those at 70 eV. Therefore, 24 eV ionization energy was used for the analysis in the SIM mode.

From preliminary examinations of rat blank plasma, it was found that, probably as a result of reacetylation of CF and the I.S. produced and adsorbed on the column, two small "ghost peaks" appeared at the same retention times as those of the acetyl derivatives of CF and the I.S., when acetic anhydride remained in the tube after the acetylation of extraction residues. Therefore, prior to injection of a sample onto the column, excess reagent was evaporated off as far as possible under a stream of nitrogen and the residue was further dried in a vacuum desiccator. By these procedures the "ghost peaks" were completely eliminated from the chromatogram of blank plasma.

Typical chromatograms obtained from rat blank plasma and rat plasma supplemented with CF and the I.S. are shown in Fig. 2. No endogenous peak interfering with the determination of these compounds was observed on the chromatograms of blank plasma. The retention times of the acetyl derivatives of CF and the I.S. were approximately 1.5 and 2.3 min, respectively, and each peak was sharp and symmetric. CF-glucuronide and CF-sulfate in plasma samples were converted to CF by successive hydrolyses with β-glucuronidase and hydrochloric acid, respectively, according to the method of Chen *et al.*[12] Each of the peaks obtained from the analysis of these extracts was also sharp and symmetric.

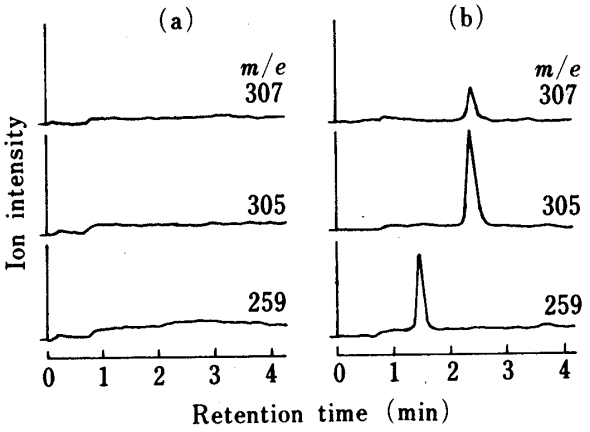

Fig. 2. Selected Ion Monitoring Chromatograms of Plasma

(a) blank plasma; (b) plasma containing chinoform (327 pmol) and the internal standard (232 pmol).

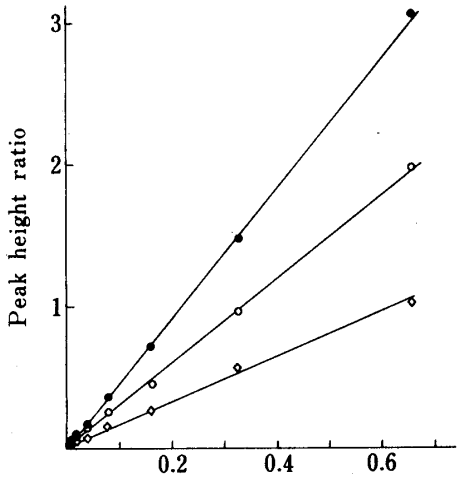

Fig. 3. Calibration Curves for the Determination of Chinoform, Chinoform Glucuronide and Sulfate in Rat Plasma

●, chinoform; ○, chinoform glucuronide; ◇, chinoform sulfate.

Calibration Curves

The calibration curves constructed by adding known amounts of CF, CF-glucuronide and CF-sulfate to rat blank plasma are shown in Fig. 3. Linear relationships between peak height ratio and the amount of each compound added to plasma were obtained in the range of 3.3—655 pmol for CF and 8.2—655 pmol for CF-glucuronide and CF-sulfate, and all of the lines approximately passed through the origin.

Extraction Recovery

The extraction recovery of CF from rat plasma was determined at four different concentrations (3.3, 16.4, 163.7, and 654.7 pmol/0.1 ml) by a slightly modified procedure in which the

TABLE I. Extraction Recoveries of Chinoform from Rat Plasma

Amount (pmol)		
Added	Found[a] Mean±S.D.	Recovery (%)[a] Mean±S.D.
3.3	3.2± 0.3	97.5±10.9
16.4	15.3± 0.9	93.3± 5.9
163.7	155.8± 7.7	95.0± 4.9
654.7	603.7±14.8	92.2± 2.5
	Mean	94.5

a) Mean±standard deviation (S.D.) of four determinations.

TABLE II. Reproducibility of the Analysis of Chinoform, Chinoform Glucuronide and Sulfate in Plasma after Intravenous Administration of Chinoform to a Rat

Sampling time	Plasma concentration (nmol/ml)[a]					
	Chinorm		Chinoform glucuronide		Chinoform sulfate	
	Mean±S.D.	C.V. %	Mean±S.D.	C.V. %	Mean±S.D.	C.V. %
5 min[b]	21.16±1.22	5.8	8.50±0.58	6.9	58.25±1.38	2.4
1 h	0.53±0.02	3.8	0.48±0.58	4.2	4.20±0.18	4.3
7 h	0.05±0.004	8.0	0.11±0.009	8.2	0.31±0.031	9.7

a) Mean±standard deviation (S.D.) and coefficient of variation (C.V.) of four determinations.
b) This sample was diluted four times with rat blank plasma.

I.S. (174.2 pmol) was added to the pyridine–benzene extract (3 ml) after completion of the extraction. The recovery percentages were calculated by dividing the peak height ratio (*m/e* 305 *versus* 259) of the acetyl derivative of CF to the acetyl derivative of I.S. obtained from the plasma samples by the peak height ratio obtained from equivalent amounts of standard without addition of plasma and extraction. As shown in Table I, the extraction recovery of CF from rat plasma was in the range of 92.2 to 97.5% and the mean value was 94.5%.

Reproducibility

To examine the precision of the present method, a single dose of 19.6 μmol of CF per kg of body weight was intravenously administered to a rat. About 1 ml of blood was drawn from the femoral artery through a polyethylene cannula at three different time periods after administration. Four 0.1-ml aliquots from the plasma sample at each sampling time were analyzed. The results are shown in Table II. The mean coefficients of variation for CF, CF-glucuronide and CF-sulfate were 5.9, 6.4 and 5.5%, respectively.

Monitoring of Plasma Concentration

CF suspended in 0.5% sodium carboxymethylcellulose aqueous solution was orally

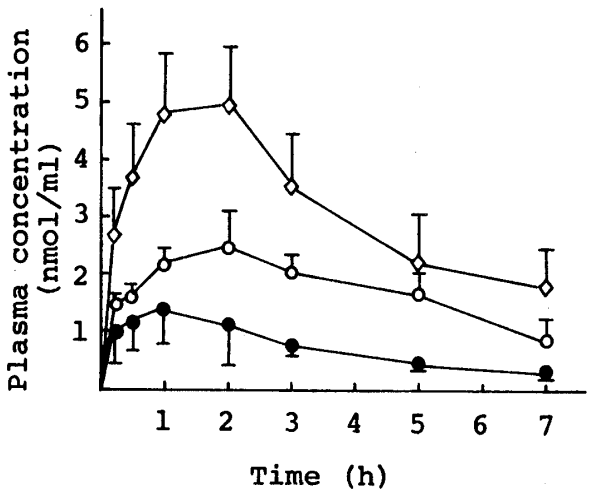

Fig. 4. Plasma Concentration–Time Courses of Chinoform and Its Glucuronide and Sulfate in Rats Following Oral Administration of 65.5 μmol/kg of Chinoform suspended in Carboxymethylcellulose Aqueous Solution

●, chinoform; ○, chinoform glucuronide; ◇, chinoform sulfate.
Each point represents the mean of three rats and the vertical bar indicates the standard deviation.

administered to three rats in a dose of 65.5 μmol per kg of body weight. Blood samples were drawn at 15 and 30 min, and 1, 2, 3, 5 and 7 h after administration. The method described in this study was applied to the determination of plasma concentrations of CF and its two conjugates. The mean concentration–time courses of these compounds in plasma are shown in Fig. 4. CF, CF-glucuronide and CF-sulfate were all present in the first blood sample withdrawn from each rat at 15 min after administration. Maximal plasma concentrations of CF, CF-glucuronide and CF-sulfate were observed at 1—2 h after administration and their mean concentrations were 1.40, 2.51 and 4.90 nmol per ml, respectively.

In conclusion, a highly sensitive and selective determination method for CF, CF-glucuronide and CF-sulfate in plasma by GC–MS analysis was established. It was found that CF in 0.1 ml of plasma could be precisely determined at a level as low as 3.3 pmol (1 ng) by the present GC–MS method. The method is simpler than the GC methods[11a,12] described previously, because the clean-up procedure using a magnesium silicate column is unnecessary in the present method. In addition, the GC–MS method is more sensitive and reliable than other previously described methods.[11,12] Consequently, the GC–MS method described here should be useful for pharmacokinetic and metabolic studies of CF in small animals. In our laboratory studies on the absorption and metabolic disposition of CF in rats are in progress using the method described here. These results will be reported elsewhere.

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