[Chem. Pharm. Bull. 24(1) 97—101 (1976)] UDC 547.831.7.08 : 543.544.25.05.061

A Gas Chromatographic Determination Method of 5-Chloro-7-iodo-8-quinolinol and Its Conjugates in Biological Fluids

CHING-TAN CHEN, KEIJIRO SAMEJIMA, and ZENZO TAMURA

Faculty of Pharmaceutical Sciences, University of Tokyo[1]

(Received May 15, 1975)

A gas chromatographic determination method of 5-chloro-7-iodo-8-quinolinol (CF), its glucuronide (CF-G) and sulfate (CF-S) in serum, urine and milk was developed.

5,7-Dichloro-8-quinolinol and/or 5-chloro-7-bromo-8-quinolinol, and their glucuronides and sulfates were added to the sample as the internal standards. Successive extractions with pyridine–benzene (1 : 9) without treatment, after hydrolysis with β-glucuronidase and that with 1 N HCl at 40°, gave the fractions of free quinolinols, derived from the unconjugated, the glucuronides and sulfates, respectively. The free quinolinols were acetylated and determined by gas chromatography.

The present method was a demonstration of an effective use of structurally similar internal standards for determination of a glucuronide and sulfate.

A lot of papers dealing with the metabolism and function of 5-chloro-7-iodo-8-quinolinol (clioquinol or chinoform) (CF) including the drug intoxication experiments have been reported since CF and its ferric chelate were isolated from the urine of a SMON (subacute myelo-optico neuropathy) patient.[2] It is believed that the elucidation of intoxication mechanism of CF is one of the subjects assigned to the SMON research, and that the quantitative study of the metabolic fate of this drug may be an important approach to this problem.

In 1951, Haskins and Luttermoser[3] developed a spectrophotometric method for the determination of 8-quinolinols and estimated CF conjugates in the urine of rabbits after administration of Vioform.[4] Thereafter, their method was also employed with some modifications for the determination of CF glucuronide in the human urine.[5] Owing to the low sensitivity, however, the method was not applied to the blood samples. Liewendahle, *et al.*[6] searched the metabolites in urine and plasma of man administered with ^{125}I-labeled CF by radiochromatographic analysis, and reported the amounts of metabolites only in relative values.

In our laboratory, a sensitive method for the determination of CF was developed by gas chromatography (GC) using an electron capture detector (ECD).[7] This paper deals with a method of separatory determination of CF and its main metabolites, the glucuronide and sulfate, in serum, urine and milk. Data obtained by the method in animal experiments will be reported in the following paper.

Experimental

Materials and Internal Standards——CF, 5-chloro-8-quinolinol and 5,7-dichloro-8-quinolinol (DC) were kindly supplied by TANABE SEIYAKU Co., Ltd. 5-Chloro-7-bromo-8-quinolinol (BC) was synthesized by bromination of 5-chloro-8-quinolinol in CCl_4. DC and BC were dissolved in benzene and used as internal stand-

1) Location: *Hongo-7-3-1, Bunkyo-ku, Tokyo, 113, Japan.*
2) M. Yoshioka and Z. Tamura, *Igaku no Ayumi*, **74**, 320 (1970).
3) W.T. Haskins and G.W. Luttermoser, *Anal. Chem.*, **23**, 456 (1951).
4) W.T. Haskins and G.W. Luttermoser, *J. Pharmacol. & Exp. Therap.*, **109**, 201 (1953).
5) L. Berggren and O. Hansson, *Clin. Pharmacol. Therap.*, **9**, 67 (1968).
6) K. Liewendahle, V. Kivikangas, and B-A. Lamberg, *Nucl. Med. (Stttg.)*, **6**, 32 (1967).
7) Z. Tamura, M. Yoshioka, T. Imanari, J. Fukaya, J. Kusaka, and K. Samejima, *Clin. Chim. Acta*, **47**, 13 (1973).

ards. CF-glucuronide (CF-G)[8] and CF-sulfate (CF-S)[9] have already been synthesized in this laboratory, and these synthetic methods have been employed for the preparation of the conjugates of DC and BC (DC-G, DC-S, BC-G and BC-S) which were used as internal standards. Chemical and physical parameters were measured to confirm the structures of these newly synthesized conjugates as follows:

DC-G——Colorless needles from acetone, mp 158—160° (decomp.), IR ν_{max}^{KBr} cm^{-1}: 1741 (–COOH), *Anal.* Calcd. for $C_{15}H_{13}O_7NCl_2 \cdot H_2O$: C, 44.13; H, 3.46; N, 3.43. Found: C, 43.73; H, 3.32; N, 3.54.

BC-G——Colorless needles, mp 181—182° (decomp.), IR ν_{max}^{KBr} cm^{-1}: 1755 (–COOH). The Na-salt was recrystallized from MeOH–H_2O. colorless needles, *Anal.* Calcd. for $C_{15}H_{12}O_7NClBrNa \cdot 4H_2O$: C, 34.08; H, 3.81; N, 2.65. Found: C, 34.42; H, 3.65; N, 2.67.

Na-salt of DC-S——It was recrystallized from H_2O. Colorless needles, IR ν_{max}^{KBr} cm^{-1}: 1290—1250 (νas SO_2), 1050 (νs SO_2). *Anal.* Calcd. for $C_9H_4O_4NSCl_2Na$: C, 34.20; H, 1.28; N, 4.48; S, 10.14. Found: C, 34.06; H, 1.20; N, 4.44; S, 10.39.

Na-salt of BC-S——It was recrystallized from H_2O. Colorless needles, IR ν_{max}^{KBr} cm^{-1}: 1290—1250 (νas SO_2), 1055 (νs SO_2). *Anal.* Calcd. for $C_9H_4O_4NSClBrNa$: C, 29.98; H, 1.12; N, 3.89. Found: C, 29.91; H, 1.10; N, 4.18.

All other organic solvents and inorganic chemicals used were commercially available highly purified reagents.

β-Glucuronidase was obtained from TOKYO ZOKI Co., Ltd. (13,000 Fishman units/ml).

Florisil (a magnesium silicate) was purchased from FLORIDIN Co., Ltd. Before use, it was washed successively with 1% pyridine in methanol, 1% acetic acid in methanol, methanol and acetone, and dried at 100° for 2—3 hr.

Preparation of Samples for GC Analysis of CF——The procedure was similar to that described previously.[7] Pyridine–benzene (1: 9 v/v) was used as a solvent for extraction of free CF and internal standards (DC and BC). The extract collected after centrifugation was evaporated to dryness *in vacuo* at 40°. The residue was dissolved in 0.5 ml of benzene and adsorbed on a Florisil column (3×15 mm). The column was washed with benzene (0.5 ml) and acetone (0.5 ml) successively, then eluted with 1 ml of MeOH–AcOH (100: 1). The eluate was evaporated to dryness *in vacuo*, and the residue was acetylated with 0.2 ml of pyridine–acetic anhydride (1: 1) at 60° for 30 min. After removal of the reagent *in vacuo*, the residue was dissolved in 0.1 ml of *n*–hexane, and an aliquot (1—3 μl) of the solution was injected into the following gas chromatograph.

Gas Chromatographic Analysis[7]——A SHIMADZU gas chromatograph type GC–1C, equipped with an electron capture detector (ECD) was used for the analysis. The column (a 3 mm×91 cm siliconized U-shaped glass tube packed with 2% OV-17 coated on siliconized Chromosorb-W, 60—80 mesh) was conditioned as follows with the column outlet disconnected from ECD; 10 μl of hexamethyldisilazane was injected five times at an interval of 1 hr at 180° in a stream of N_2, and the column was kept overnight under the same condition. Then 10 μl of hexane solution nearly saturated with acetylated CF was injected about ten times at an interval of 1 hr.

The sample solution was injected on the column which was operated at 180° with N_2 for the carrier gas (flow rate: 50 ml/min) keeping ECD at 190° (Fig. 2).

Results

Preliminary Studies

CF-G and CF-S were fairly stable in a neutral or a weakly alkaline solvent as observed a few percent hydrolysis after a month at room temperature. Both CF-G and CF-S in aqueous solution or biological fluids were not extracted with pyridine–benzene (1: 9), and quite stable under vigorous shaking with this solvent system, while CF was easily extracted. For systematic determination of free CF, CF-G and CF-S in biological fluids, therefore, a simple fractionation was advisable such as successive extractions of free CF liberated after separative hydrolyses of CF-G and CF-S.

Acid hydrolyses of CF-G and CF-S were examined as shown in Fig. 1. The hydrolysis rate of CF-S was influenced by both acidity and temperature while that of CF-G was small and mainly influenced by temperature. The recognized hydrolysis of CF-G in acidic media suggested that the acid hydrolysis of CF-S should be done after complete hydrolysis of CF-G by β-glucuronidase.

As illustrated in Fig. 2, DC and BC were suitable internal standards for CF.

8) I. Matsunaga and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **19**, 1056 (1971).
9) C.T. Chen, K. Samejima, and Z. Tamura, *Chem. Pharm. Bull.* (Tokyo), **21**, 911 (1973).

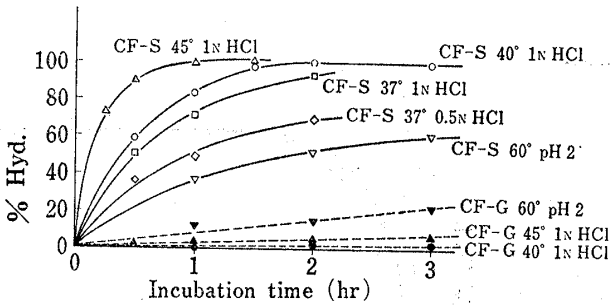

Fig. 1. Acid Hydrolyses of CF-G and CF-S at Various Acidities and Temperatures

A clioquinol conjugate solution (0.2 mg of CF-S or 0.3 mg of CF-G in1 ml) of pH 2 (NaOAc-HCl buffer), 0.5N or 1N HCl was allowed to stand at appropriate temeperature. The CF liberated was extracted with 5 ml of pyridine-benzene (1: 9) and the absorbance was measured at 350 nm.

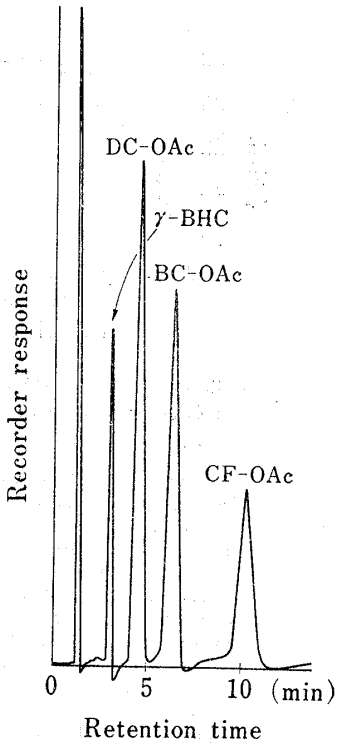

Fig. 2. A Gas Chromatogram of DC-OAc, BC-OAc and CF-OAc together with γ-BHC

Conditions are described in experimental section.

Hydrolyses of glucuronides of DC, BC and CF were examined as shown in Fig. 3. DC-G and BC-G were more stable than CF-G, but all the glucuronides were hydrolyzed with 2.6 units of β-glucuronidase/ml of final solution to attain the complete hydrolyses within 2 hr. In serum, however, the amount of enzyme had to be increased up to 130 units/ml to attain complete hydrolysis within 2 hr. This might be caused by some inhibitors in serum, and for safety 200 units/ml was used for biological samples. Under the conditions, hydrolyses of the sulfates were not observed.

Hydrolyses of the sulfates of DC, BC and CF by aryl sulfatase were extremely difficult to proceed, so that acid hydrolysis in 1N HCl at 40° was studied following the result of Fig. 1. Though there were slight differences at early stage in the rate of hydrolysis among the sulfates, the hydrolyses were completed within 80 min as shown in Fig. 4. This pattern of acid hydrolysis was reproducible in serum, and to ensure the complete hydrolysis in other biological fluids, an incubation time of 2 hr was selected.

Assay Procedure of CF and Its Conjugates in Biological Fluids

Separation procedure is summarized in Chart 1. To a sample (0.1—1.0 ml) in a 10 ml glass-stoppered centrifuge tube were added known amounts of internal standards and 1M acetate buffer, pH 5 (0.1—0.15 ml), and the total volume was made up to 1—1.5 ml with distilled water. Four ml of pyridine-benzene (1: 9) was added to the tube, and the mixture was shaken vigorously for about a minute, and centrifuged to separate into two phases. The organic phase was evaporated to dryness *in vacuo* and submitted to the GC analysis (unconjugated fraction). In the aqueous phase, there still remained a trace amount of free quinolinols and fairly large amounts of pyridine. To remove them, the aqueous phase was washed twice with 6 ml of benzene, and the benzene was discarded. Then β-glucuronidase was added to the aqueous phase to attain the concentration of 200 units/ml, and incubation was performed under shaking at 37° for 2 hr. The liberated quinolinols were similarly extracted and determined (glucuronide fraction). The aqueous phase containing sulfates was similarly washed with benzene, and its acidity was adjusted to 1N with 6N HCl. Hydrolysis was performed at 40° for 2 hr. The acid hydrolyzate, prior to the extraction with pyridine-benzene, was roughly neutralized with 3N NaOH. It was preferable to keep slightly acidic, since the acidity was bufferized by pyridine in the extraction solvent, and the solubility of the quinolinols in aqueous

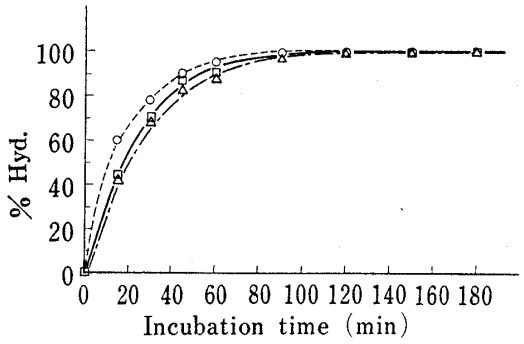

Fig. 3. Time Course of Hydrolyses of DC-G, BC-G and CF-G with β-Glucuronidase

An aqueous solution (17.6 ml) of DC-G (31.42 μg), BC-G (43.68 μg) and CF-G (52.83 μg) was mixed with 2.0 m of 1M acetate buffer (pH 5) and warmed at 37°. To the warmed solution was added 0.4 ml of enzyme solution (52 units), and the mixture was incubated at 37°. After time intervals, duplicate aliquots were pipetted and extracted with 5 ml of pyridine–benzene (1: 9), 4 ml of which were evaporated and acetylated for GC analyses. This time, the residues were dissolved in 100 μl of *n*-hexane containing 0.13 μg of γ-BHC/ml. The graph was depicted after calculating % hydrolysis based on peak height ratios of liberated DC, BC and CF with respect to γ-BHC.

---○---○---: DC-G —□—□—: BC-G
--△--△---: CF-G

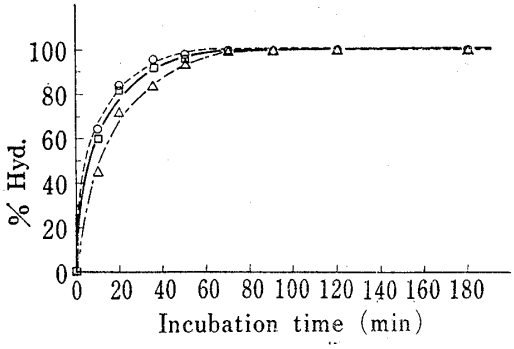

Fig. 4. Time Course of Acid Hydrolyses of DC-S, BC-S and CF-S

An aqueous solution (16.6 ml) of DC-S (23.41 μg), BC-S (33.17 μg) and CF-S (42.78 μg) was warmed at 40°. To the warmed solution was added 3.4 ml of 6N HCl to make the final acidity to 1N, and incubation was continued at 37°. The subsequent treatment were the same as described in the legend of Fig. 3, except extraction was done after neutralization.

---○---○---: DC-S —□—□—: BC-S
--△--△---: CF-S

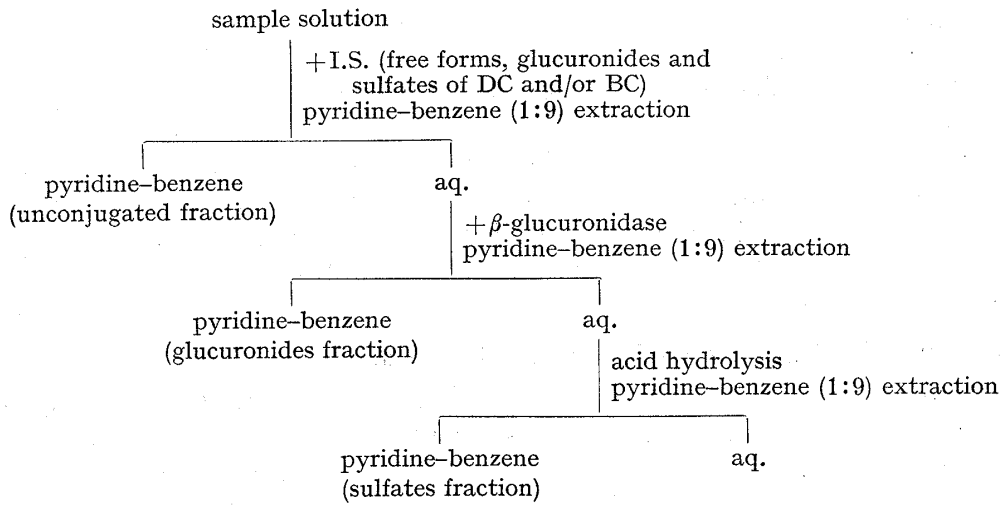

Chart 1. Separation of Clioquinol and Its Conjugates

DC: 5,7-dichloro-8-quinolinol, BC: 5-chloro-7-bromo-8-quinolinol

phase was remarkably increased by acidity or basicity. The resultant extract was submitted to the GC analysis (sulfate fraction).

CF in each fraction was determined based on a working curve (peak height ratio *vs.* molar ratio) prepared with the aqueous standard solutions, the amount covering 0—1.6 μg of CF added with 0.84 μg of BC and 0.20 μg of DC as internal standards.

The precision of the method was determined by repeated analyses of serum and buffer solution added with a standard mixture containing CF, BC, DC and their glucuronides and sulfates. The results are presented in Table I. The values suggest that BC and its conjugates are generally better internal standards than DC and its conjugates.

Table I. Precision of the Determination of Clioquinol and Its Conjugates in Buffer and Serum Solution

Sample[a]	Relative standard deviation (s/x)					
	CF		CF-G		CF-S	
I.S.	DC	BC	DC-G	BC-G	DC-S	BC-S
In buffer	0.040	0.016	0.053	0.024	0.086	0.095
In serum	0.047	0.025	0.043	0.022	0.046	0.042

a) $2.3-3.4\times10^{-9}$ mole/ml
$n=10$

Addition recovery test using 1.0 ml of normal serum containing CF (1.38—6.90 nmole), CF-G (1.37—6.85 nmole), CF-S (1.31—6.55 nmole) and the internal standards (4.1 nmole of BC, 4.0 nmole of BC-G and 3.8 nmole of BC-S) in a tube, demonstrated that the errors of the measurements were 2—8% for CF and CF-G, 2—10% for CF-S. Experiment using urine gave the errors of 3—5% for CF, 1—10% of CF-G, and 3—10% of CF-S.

The present method was then applied to measure CF and its conjugates in milk samples of a mongrel dog administered CF for 73 days. Both the standard addition extrapolation method and the procedure using calibration graphs gave results well agreed within experimental error as follows: CF, CF-G and CF-S were 3.7, 7.1, and 11.3 μg/ml of milk respectively by the former, and 3.9, 6.9 and 10.6 μg/ml by the latter.

Discussion

The biological sample is preferable to be analyzed as soon as possible or stored in a freezer, since dehalogenated product (5-chloro-8-quinolinol) which was not detected in a fresh urine sample, was found in the urine kept in a refrigerator (4°) for several days. In addition to the factors mentioned above, the stability of CF conjugates is greatly affected by metal-catalyzed hydrolysis similar to that of 8-quinolinol conjugate.[10-13] Both the sulfate and the glucuronide were hydrolyzed rapidly in the presence of Cu^{2+}, Ni^{2+} and Pb^{2+}; and other biometals such as Zn^{2+}, Mg^{2+} and Ca^{2+} also showed a mild catalytic action.[13] Therefore, contamination of the metal ions should be prevented in each assay procedure.

An overnight hydrolysis with β-glucuronidase is undesirable because the detectable amount of CF would be liberated from the sulfate in the sample.

The introduction of the structurally similar internal standards increased the reliability of the present method. It was possible to use two series of internal standards (BC, BC-G, BC-S, and DC, DC-G, DC-S) simultaneously for a sample, thus the data would be doubly checked, and a broad range of unknown sample would be covered by using two series of internal standards (BC and DC) of widely different amounts of them.

Acknowledgement This work was supported by a Special Research Grant from the Ministry of Health and Welfare. We are also grateful to Tanabe Pharmaceutical Co., Ltd. for the generous gift of chinoform.

10) C.R. Clark and R.W. Hay, *Chem. Commun.*, **1970**, 794.
11) G. Wagner and H. Frenzel, *Arch. Pharm.*, **300**, 591 (1967).
12) K. Nagasawa and H. Yoshidome, *Chem. Pharm. Bull.* (Tokyo), **21**, 903 (1973).
13) K. Hayakawa, C.T. Chen, T. Imanari, and Z. Tamura, *Annual Report of SMON Research Commission*, **1974**, 229.