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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CLIOQUINOL AND ITS CONJUGATES IN BIOLOGICAL MATERIALS

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SUMMARY

A method has been established for the determination of clioquinol (C) and its glucuronide (CG) and sulfate (CS) in biological materials. C and its internal standard were extracted with benzene—pyridine from samples. CG and CS were also hydrolyzed to C and extracted by the same method. The extracts were evaporated to dryness and redissolved in methanol. The methanol solution was subjected to HPLC using a column packed with Iatrobeads 6cp-2010 and a UV monitor (254 nm). The mobile phase was 0.1 *M* citric acid—methanol—*n*-hexane (8:86:6). The detection limit of C was 1 nmole and its recovery was above 92%.

INTRODUCTION

After the isolation of the ferric chelate of 5-chloro-7-iodo-8-hydroxyquinoline (clioquinol or chinoform, C) from the urine of a subacute myelo-optico neuropathy (SMON) [1] patient, the elucidation of the intoxication mechanism of C was one of the important goals of SMON research [2].

Some analytical methods were developed to study the metabolism and intoxication of the drug. In 1951, Haskins and Luttermoser [3] reported a spectrophotometric method for 8-hydroxyquinolines and estimated C conjugates in the urine of rabbits [4]. This method was also applied to the determination of C glucuronide in human urine [5]. However, the method could not be applied to blood samples because of its low sensitivity. Liewendahl et al. [6] investigated the metabolites in urine and plasma of man administered ^{125}I-labelled C, by radiochromatographic analysis. Tamura et al. [7] developed a sensitive method for the determination of C by gas chromatography (GC) using an electron-capture detector (ECD). Chen et al. [8] established a method of separatory determination of C and its glucuronide (CG) and sulfate (CS) in serum, urine and milk. Some modified methods for analysis of C by GC

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were also reported [9—11], and many studies on metabolism of C were reported using those methods. Although the GC method was highly sensitive to C, the procedure involved time-consuming manipulations.

Chen et al. [12] reported a method for the determination of CG and CS by high-performance liquid chromatography (HPLC) using an ion-exchange column, and applied the method to human urine. But this method could not be applied to plasma or tissues because the separation of CG and CS from large amounts of coexisting biological constituents absorbing at 254 nm was incomplete. Miura et al. [13] used HPLC in the assay of multiple enzyme activities with 8-hydroxyquinoline derivatives as substrates. However, this method could not be used to determine CG and CS in biological materials and the sensitivity was not enough to analyze micro amounts of C in blood.

We have therefore established a sensitive and simple HPLC method to determine C, CG and CS in animals.

EXPERIMENTAL

Materials

C, CG, CS (sodium salt), 5-chloro-8-hydroxyquinoline, 5,7-dichloro-8-hydroxyquinoline, 5-chloro-7-bromo-8-hydroxyquinoline and 5,7-dibromo-8-hydroxyquinoline were all kindly provided by Professor Tamura of the University of Tokyo. 8-Hydroxyquinoline and 2-methyl-8-hydroxyquinoline were purchased from Tokyo Kasei (Tokyo, Japan). 8-Hydroxyquinolines were used after recrystallization. β-Glucuronidase (13,000 Fishman units per ml) was obtained from Tokyo Zoki Co. (Tokyo, Japan). All other reagents used were of analytical grade.

Instruments for HPLC

The HPLC system used in this study comprised a Kyowa minimicro pump KSU-16H (Kyowa Seimitsu Co.), a Pyrex column (30 cm × 3 mm I.D.; Kyowa Seimitsu Co), a Mitsumi monitor SF-1205 (Mitsumi Scientific Industry Co.), equipped with a low-pressure mercury vapor lamp (254 nm), and a Toa recorder EPR-10B (Toa Electronics Ltd.). The column was packed with Iatrobeads 6cp-2010 (10 μm, polystyrene-type porous polymer; Iatron Chemical Products).

Sample preparation

To a biological sample (0.2 ml) in a glass-stoppered centrifuge tube were added a known amount of internal standard in 20 μl of methanol, 0.2 ml of 0.2 *M* EDTA (disodium salt) and 0.8 ml of distilled water. Then 4 ml of benzene—pyridine (9:1, v/v) were added to the tube, and the mixture was shaken vigorously for about a minute. After centrifugation (3500 *g*, 5 min) the organic phase collected was evaporated to dryness in vacuo and the residue was dissolved in 0.2 ml of methanol for HPLC analysis (C fraction). To remove a trace amount of C, internal standard and a fairly large amount of pyridine, the aqueous phase was washed twice with 6 ml of benzene, and the benzene was discarded. Then a known amount of internal standard in methanol, β-glucuronidase (final concentration 200 units per ml) and 0.15 ml of 1 *M* acetate

buffer (pH 5) were added to the aqueous phase, and incubation was performed with shaking at 37°C for 2 h. The liberated C and internal standard were similarly extracted and determined (CG fraction). The aqueous phase containing sulfate was similarly washed with benzene, and its acidity was adjusted to 1-*N* with 6 *N* HCl. Then a known amount of internal standard was added to the aqueous phase, and hydrolysis was carried out at 40°C for 2 h. The acid hydrolyzate, prior to extraction with benzene—pyridine, was almost neutralized with 3 *N* NaOH. The resultant extract was submitted to HPLC analysis (CS fraction). An aliquot (2—20 μl) of the methanol solution was injected into the column.

HPLC conditions

The mobile phase was 0.1 *M* citric acid—methanol—*n*-hexane (8:86:6, v/v). The flow-rate was 0.75 ml/min. The column was maintained at 37 ± 0.5°C.

RESULTS

Derivatives of 8-hydroxyquinoline gave intense absorbance at around 254 nm, which was advantageous for the microanalysis of C by HPLC with a UV detector at 254 nm. A combination of a non-polar resin with an acidic alcohol as the mobile phase was expected to give satisfactory resolution of 8-hydroxyquinolines by HPLC [12].

Therefore, resin types such as styrene—divinylbenzene and octadecylsilane-treated silica were examined for resolution of 8-hydroxyquinolines with acid—methanol solution as the mobile phase. The sample solution of 8-hydroxyquinoline, 5-chloro-8-hydroxyquinoline, 5,7-dichloro-8-hydroxyquinoline and C in methanol was used in this experiment. Both the styrene—divinylbenzene types, Iatrobeads 6cp-2010 and Hitachi 3010, could separate the four compounds described above, the former giving a better resolution and higher peaks than the latter. Iatrobeads 6cp-2010 were therefore used in the further

TABLE I

MOLAR ABSORPTIVITIES AND RETENTION TIMES OF 8-HYDROXYQUINOLINES

Compound	Absorptivity*		Retention time on HPLC** (min)
	λ_{max} (nm)	ϵ (× 10^4)	
8-Hydroxyquinoline (8HQ)	255	2.53	4.6
2-Methyl-8HQ	257	3.98	4.5
5-Chloro-8HQ	245	3.05	5.6
5,7-Dichloro-8HQ	248	3.81	6.8
5-Chloro-7-bromo-8HQ	248	3.75	7.8
5,7-Dibromo-8HQ	248	3.64	9.0
5-Chloro-7-iodo-8HQ (clioquinol)	255	3.94	9.6

*The solution was a mixture of 0.1 *M* citric acid—methanol—*n*-hexane (8:86:6).
**The conditions were as described under HPLC conditions.

study. Among the many mobile phases used, a mixture of 0.1 *M* citric acid—methanol—*n*-hexane (8:86:6) gave the best resolution of 8-hydroxyquinolines. Addition of *n*-hexane to the mobile phase at 6%, which was almost the solubility limit, reduced the retention time and made the peaks higher without affecting the resolution. The pressure in the column was lower at 37°C than at room temperature, and the resolution of the compounds was found to be reproducible at the constant temperature. The molar absorptivities and retention times of several 8-hydroxyquinolines are listed in Table I.

It was desirable to choose an internal standard for the method from among the 8-hydroxyquinolines, because it was expected to have a similar behaviour to C in the procedure. As is seen in Table I, 5,7-dichloro-8-hydroxyquinoline was separated completely from C, whereas the separation of 5-chloro-7-bromo-8-hydroxyquinoline or 5,7-dibromo-8-hydroxyquinoline from C was incomplete. The biological samples that did not contain any 8-hydroxyquinoline showed no peak at the retention time of 5,7-dichloro-8-hydroxyquinoline in the chromatogram. Thus 5,7-dichloro-8-hydroxyquinoline was chosen as internal standard. A chromatogram of C and the internal standard using Iatrobeads 6cp-2010 is shown in Fig. 1.

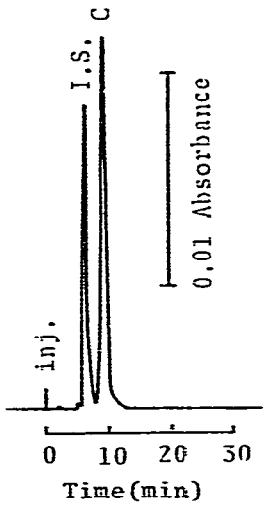

Fig. 1. Chromatogram of clioquinol (C) and 5,7-dichloro-8-hydroxyquinoline (I.S.).

C and the internal standard in biological samples showed clear peaks, whereas extracts of control biological samples free of C and internal standard, showed no peaks at their corresponding retention times in the chromatograms. CG and CS contained in biological samples were hydrolyzed to C by β-glucuronidase and HCl, according to the method by Chen et al. [8]. The extracts of these fractions also showed sharp peaks. Typical chromatograms for C in plasma, urine, bile and kidney are shown in Fig. 2.

When C and the internal standard were added to 0.2 ml of rabbit plasma, their recoveries by the preparation prior to HPLC analysis were more than 92% (Table II). The minimum detectable amount of C in methanol by the method was 20 pmoles (signal-to-noise ratio = 3). The calibration curves of

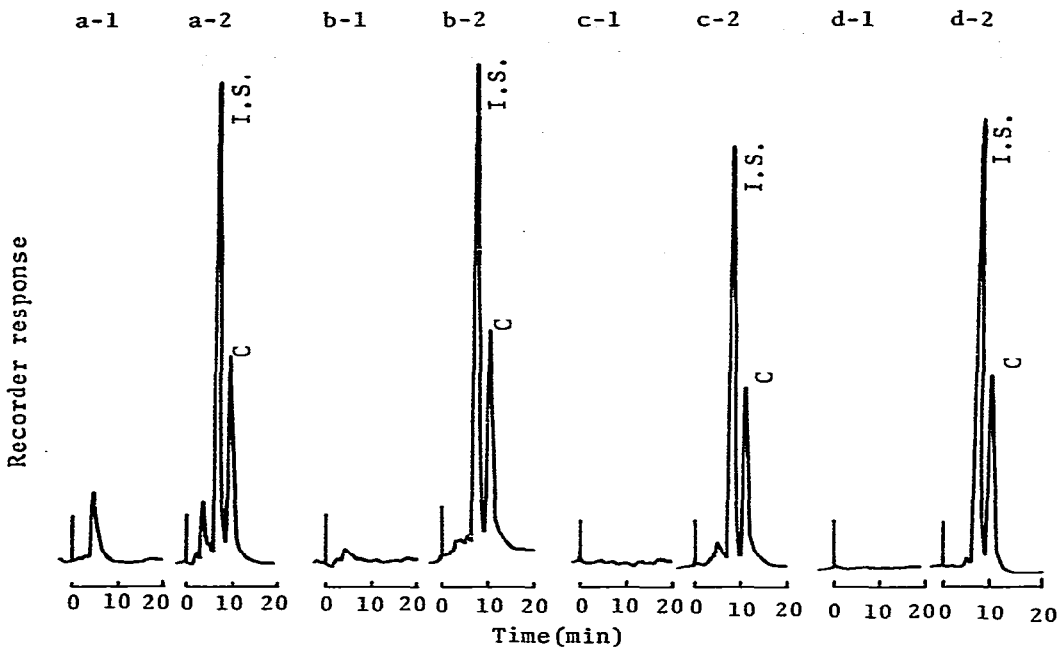

Fig. 2. Chromatograms of clioquinol (C) and 5,7-dichloro-8-hydroxyquinoline (I.S.) added in biological samples. (a-1) Extract from 0.2 ml of control plasma; (a-2) extract from 0.2 ml of plasma containing 4 nmoles of C and 12 nmoles of internal standard; (b-1) extract from 0.2 ml of control urine; (b-2) extract from 0.2 ml of urine containing 10 nmoles of C and 30 nmoles of internal standard; (c-1) extract from 0.2 ml of control bile; (c-2) extract from 0.2 ml of bile containing 10 nmoles of C and 30 nmoles of internal standard; (d-1) extract from 0.2 g of control kidney; (d-2) extract of 0.2 g of kidney containing 2 μmoles of C and 6 μmoles of internal standard.

TABLE II

RECOVERIES OF CLIOQUINOL AND 5,7-DICHLORO-8-HYDROXYQUINOLINE FROM PLASMA

Compound	Added (nmole/ml)	Recovered (nmole/ml) Mean ± S.D.	Recovery (%) Mean ± S.D.
Clioquinol	10	9.7 ± 0.13	97 ± 1.3
	100	99 ± 0.0	99 ± 0.0
5,7-Dichloro-8-hydroxy-quinoline	10	9.6 ± 0.57	96 ± 5.7
	100	92 ± 3.8	92 ± 3.8

C added to 0.2 ml of rabbit plasma showed excellent linearity in the range 2—40 nmoles/ml, when 8 nmoles of the internal standard were added.

DISCUSSION

It is well known that C is retained in the body for a long period after its administration. About 30 nmoles/ml of C were detected in the serum of a

SMON patient one month after stopping the administration of C [7], and more than 30 nmoles/g of C, CG and CS were detected in sera and several tissues of beagle dogs which showed neurological symptoms after the administration of C [14]. The proposed method was found to be effective in estimating these levels of C, CG and CS, although the GC method reported previously gave a slightly higher sensitivity than the HPLC method. Moreover, the HPLC method was found to be simpler and easier than the GC method, because the prerequisites of treatment by a Florisil column and acetylation of C in the GC method were unnecessary in the HPLC method. When both the proposed HPLC method and the GC method were applied to the analysis of C, CG and CS in plasma of a rabbit orally administered with 400 mg of C, the data obtained for the two methods were compatible (Fig. 3).

Large amounts of coexisting biological constituents that absorb at 254 nm disturbed the direct determination of CG and CS in plasma or tissues [12] as mentioned above. Improvement of the preparation was carried out, and these compounds could be almost removed from methanolic solution of C and

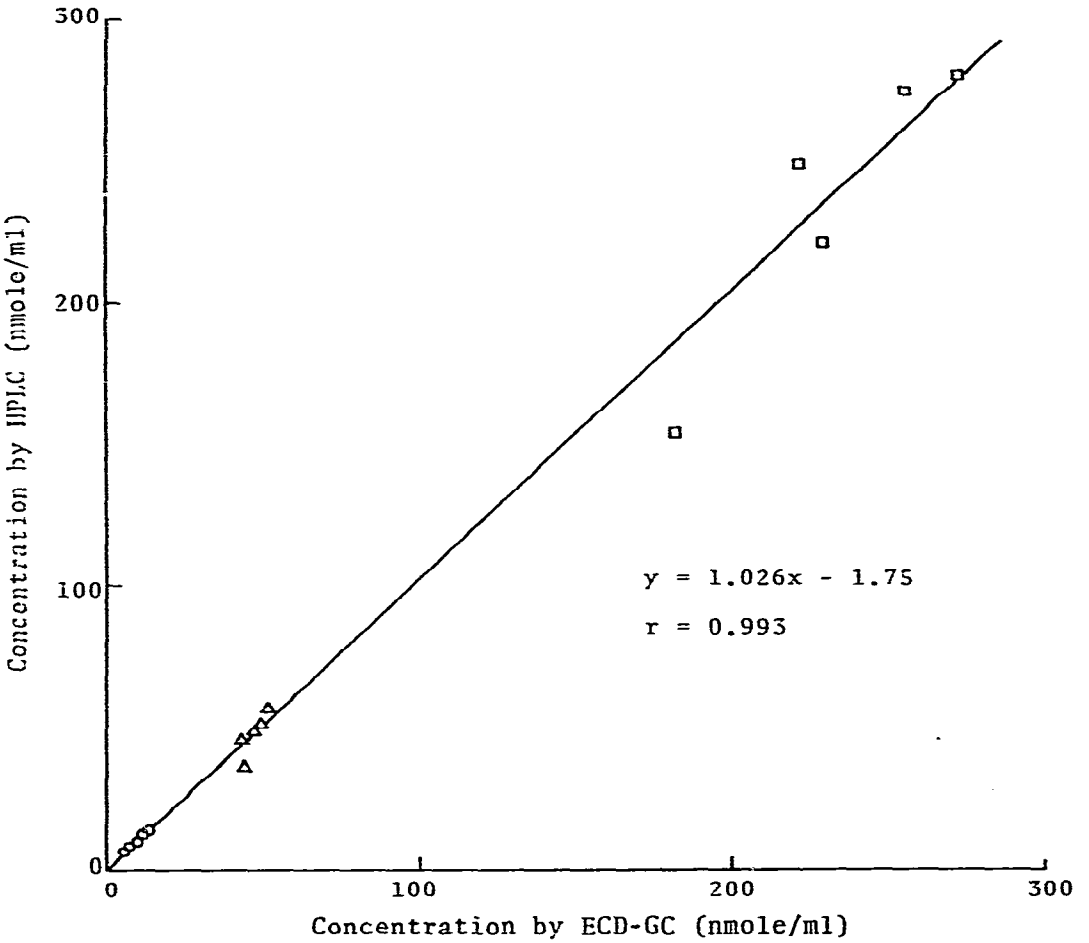

Fig. 3. Correlation between the values obtained by the HPLC method and the ECD-GC method in the analysis of C, CG and CS in plasma of a rabbit. (○), C; (□), CG; (△), CS.

internal standard by extraction with benzene—pyridine. On the other hand, the resolution of C and internal standard became worse when such metal ions as Cu^{2+}, Fe^{3+} and Zn^{2+}, which can chelate 8-hydroxyquinolines, were injected in the HPLC system. Addition of EDTA to the sample solution prevented these metal ions from being extracted into the benzene—pyridine phase and giving a sharp chromatogram of the two. Moreover, the peaks of C and internal standard were highest when *n*-hexane was added to the mobile phase near to saturation. By these improvements the sensitivity for C in the proposed method was higher than in the other HPLC method [13].

The present method should be useful in studying the metabolism and intoxication of C. Other 8-hydroxyquinoline derivatives [15] may also be detected easily by the modification of the HPLC conditions.

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