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STUDIES ON STEROIDS

CLXXIX. DETERMINATION OF ESTRIOL 16- AND 17-GLUCURONIDE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A high-performance liquid chromatographic method for the determination of estriol 16-glucuronide and 17-glucuronide in bile and urine has been developed. The electrochemical detector was found to be more superior than the UV detector with respect to selectivity and sensitivity and therefore more suitable for the determination of estrogen conjugates in biological fluids. The use of the present method revealed that both estriol 16-glucuronide and 17-glucuronide were excreted in rat bile, while only the former was present in human pregnancy urine.

INTRODUCTION

In recent years considerable interest has been focused on the physiological significance of estrogen conjugates in the feto-placental unit. These conjugates are determined by a variety of methods, i.e. spectrophotometry [1], gas chromatography—mass spectrometry [2, 3] and radioimmunoassay [4]. In addition, several papers describe the methods for the determination of estrogens in biological fluids by high-performance liquid chromatography (HPLC) which involve prior hydrolysis and/or solvolysis of the conjugates [5, 6]. Deconjugation, however, has disadvantages with respect to simplicity of the procedure and reliability of the analytical result. In 1978, Van der Wal and Huber reported the HPLC separation of estrogen conjugates employing the authentic samples [7]. In the previous paper we also described the separation of monoglucuronides of estrone, estradiol, estriol and 16-epiestriol on a reversed-phase column by HPLC [8]. This present paper deals with a method for the simultaneous determination of estriol 16-glucuronide and 17-glucuronide in biological fluids by HPLC with electrochemical detection and its application for rat bile [9] and human pregnancy urine.

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EXPERIMENTAL

High-performance liquid chromatography

The apparatus used for this work was a Toyo Soda HLC-803A high-performance liquid chromatograph (Toyo Soda Co., Tokyo, Japan) equipped with a Model SF-770 ultraviolet (UV) detector monitoring the absorbance at 280 nm and a Yanagimoto Model VMD 101 electrochemical detector (ECD) (Yanagimoto Co., Kyoto, Japan). The potential of the ECD was set at +1.0 V vs. a Ag/AgCl reference electrode. A TSK GEL LS-410 ODS-SIL (5 μm) (Toyo Soda Co.) column (30 cm × 0.4 cm I.D.) was employed under ambient conditions. The pH of the mobile phase was adjusted using phosphoric acid.

Materials

Estrogen glucuronides were synthesized in these laboratories by known methods [10]. All the reagents used were of analytical reagent grade. Solvents were purified by distillation prior to use.

Bile samples from rats

Male Wistar rats weighing ca. 200 g were used. Rats were anestherized with ether, cannulated to the bile duct with polyethylene tube (PE 10) (Clay Adams, Parsippany, NJ, U.S.A.) by surgical operation and housed in a Bollman cage for collection of bile. All animals were starved overnight prior to administration of estriol. A suspension of estriol (50 mg) in dimethylsulfoxide (0.1 ml) with saline (0.7 ml) and Tween 80 (0.2 ml) was given orally to each rat, and bile was collected every 2 h over a period of 26 h following administration [9].

Urine samples from human pregnancy

Twenty-four-hour urine samples were collected without preservative from pregnant women (30, 34 and 38 weeks of gestation). Each aliquot was taken and stored at −20°C until analysis.

Procedure for determination of conjugated metabolites in biological fluids

To an aliquot of bile or urine sample were added 5 μg of 16-epiestriol 17-glucuronide as internal standard, and the solution was percolated through a column (5 cm × 0.6 cm I.D.) packed with Amberlite XAD-2 resin. After thorough washing with distilled water (5 ml), the conjugate fraction was eluted with 5 ml of methanol (pH 10.0 adjusted by NH_4OH). Evaporation of the solvent under reduced pressure gave a residue which in turn was redissolved in methanol and subjected to a piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) column (2 cm × 0.6 cm I.D.) [11]. The column was washed with 90% methanol (5 ml) and the estrogen conjugate fraction was then eluted with 5 ml of 2.5% ammonium carbonate in 70% methanol. After evaporation of the solvent under reduced pressure below 45°C, the residue obtained was dissolved in methanol of which an aliquot was subjected to HPLC.

Recovery test for estriol monoglucuronides

One milliliter of control bile or non-pregnancy urine was spiked with known

amounts of estriol 16-glucuronide and 17-glucuronide. The assay was then carried out according to the procedure described above.

Enzymatic hydrolysis of estriol monoglucuronides

The eluate corresponding to the peak on the chromatogram was dissolved in 0.1 *M* acetate buffer (pH 5.4) and incubated with the acetone powder of snail digestive juice at 37°C overnight.

RESULTS AND DISCUSSION

Initially, our effort was directed to the determination of estriol 16-glucuronide and 17-glucuronide in rat bile by using a UV detector. However, numerous interfering peaks appeared on the chromatogram even when several clean-up procedures were carried out (see Fig. 1b). In order to overcome this problem the use of ECD, which is responsive selectively for electrochemically active materials, was undertaken [12]. One-tenth or one-twentieth of a milliliter of rat bile was subjected to chromatography on Amberlite XAD-2 resin, followed by ion-exchange chromatography on PHP-LH-20. As illustrated in Fig. 1a, no interfering peaks were observed on the chromatogram when ECD was used.

Known amounts of estriol 16-glucuronide and 17-glucuronide were added to control bile samples, and their recovery rates were then determined. As listed in

TABLE I

RECOVERY OF ESTRIOL MONOGLUCURONIDES ADDED TO RAT BILE

Amount added (μg/ml)	Percentage recovery (mean ± S.D., n = 5)	
	Estriol 16-glucuronide	Estriol 17-glucuronide
20	78.6 ± 1.6	78.6 ± 2.1
40	78.8 ± 2.1	79.0 ± 2.4
80	81.0 ± 1.6	81.4 ± 1.2

TABLE II

ACCURACY AND PRECISION OF THE PROPOSED METHOD FOR DETERMINATION OF ESTRIOL MONOGLUCURONIDES IN RAT BILE

Amount added (μg/ml)	Amount found (μg/ml) (mean ± S.D., n = 10)	
	Estriol 16-glucuronide	Estriol 17-glucuronide
20	20.0 ± 0.5	20.0 ± 0.3
100	102.4 ± 3.1	100.5 ± 3.3

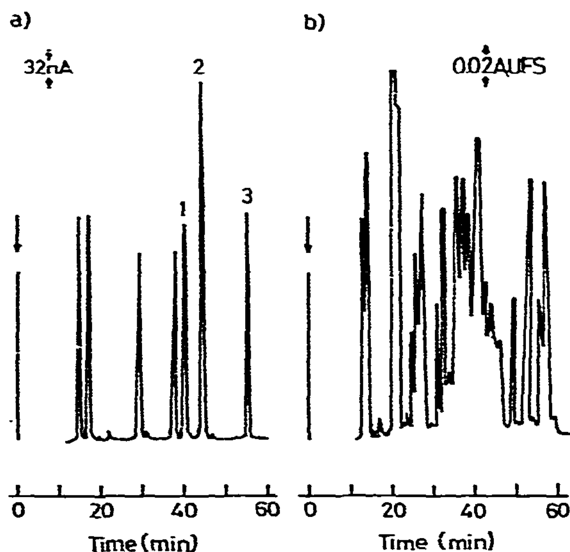

Fig. 1. Separation of estriol monoglucuronides in rat bile by HPLC. 1 = estriol 17-glucuronide, 2 = estriol 16-glucuronide, 3 = internal standard. Conditions: column, TSK GEL LS-410 ODS-SIL; mobile phase, 0.7% Na_2HPO_4 (pH 3.0)—tetrahydrofuran (6:1), 1 ml/min; detection, (a) electrochemical detector, (b) UV detector at 280 nm.

Table I, the two compounds spiked to rat bile at three levels were recovered at a rate of more than 78%. Estriol monoglucuronides added to non-pregnancy urine were similarly recovered at a satisfactory rate. 16-Epiestriol 17-glucuronide was chosen as internal standard for the determination because it was absent in the bile of rats administered estriol. The recovery rate of the internal standard was determined to be 71.4 ± 2.1%. A calibration graph was constructed by plotting the ratio of the peak height of estriol 16-glucuronide or 17-glucuronide to that of the internal standard, dissolved in control bile or non-pregnancy urine, against the amount of the glucuronide, a linear response to each glucuronide being observed in the range 0—10 μg. It is evident from the data in Table II that the proposed method was satisfactory in accuracy and precision. The detection limit of these compounds was 5 ng per injection (signal-to-noise ratio = 2 at 2 nA full scale). A typical chromatogram illustrated in Fig. 1a demonstrates unequivocally the excretion of the two isomeric estriol monoglucuronides in rat bile.

Identification of the two peaks corresponding to estriol 16-glucuronide and 17-glucuronide was then carried out. These compounds showed chromatographic behaviors identical with those of the respective authentic samples, along with the change in pH of the mobile phase [8]. Upon methylation with diazomethane, the peaks of estriol monoglucuronides disappeared from the chromatogram. In addition, when treated with β-glucuronidase from snail digestive juice, both peaks were changed into a peak corresponding to estriol.

The excreted amounts of estriol 16-glucuronide and 17-glucuronide in rat bile following oral administration of estriol were determined. As shown in Fig. 2, the concentration of both conjugated metabolites in bile reached the maximum value at 22 h after administration.

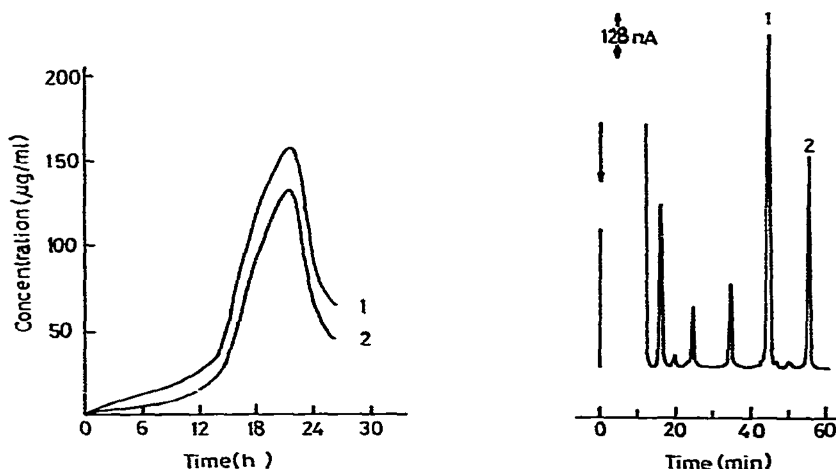

Fig. 2. Biliary excretion of estriol monoglucuronides in the rat after oral administration of estriol. 1 = estriol 16-glucuronide, 2 = estriol 17-glucuronide.

Fig. 3. Separation of estriol 16-glucuronide in pregnancy urine by HPLC. 1 = estriol 16-glucuronide, 2 = internal standard. Conditions as in Fig. 1a.

The application of the present method for the analysis of estriol monoglucuronides in pregnancy urine was undertaken. One-tenth of a milliliter of urine specimen was treated in a similar fashion as that described for rat bile. A typical chromatogram is shown in Fig. 3. Only one peak corresponding to estriol 16-glucuronide was observed on the chromatogram and its structure was unequivocally characterized in the manner described above.

The separation of isomeric monoglucuronides of estriol and 16-epiestriol has been previously attained on a reversed-phase column using an acidic mobile phase [8]. It has been demonstrated that ECD is much superior in selectivity and sensitivity than the UV detector for the determination of estrogen monoglucuronides in biological fluids. The excretion of estriol 17-glucuronide in pregnancy urine has been ambiguous since the reports by Hashimoto and Neeman [13] and Carpenter and Kellie [14]. The present study has revealed that both estriol 16-glucuronide and 17-glucuronide are excreted in rat bile while only the former is present in human pregnancy urine. The multiplicity of glucuronyltransferase, which catalyzes the formation of estrogen glucuronides, appears to be of interest.

The application of the present method to the quantitation of other estrogen conjugates in pregnancy urine and blood is being conducted in these laboratories and the details will be reported elsewhere in the near future.

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