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STUDIES ON STEROIDS. CC. DETERMINATION OF 17-KETOSTEROID SULPHATES IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION USING PRE-COLUMN DERIVATIZATION

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SUMMARY

A new sensitive method is described for the determination of 17-ketosteroid sulphates, particularly dehydroepiandrosterone sulphate, in human serum by high-performance liquid chromatography with electrochemical detection. The 17-ketosteroid sulphates in serum were extracted with acetonitrile and derivatized with *p*-nitrophenylhydrazine in trichloroacetic acid—benzene solution. The *p*-nitrophenylhydrazones were separated by high-performance liquid chromatography on a μBondapak C_{18} column using methanol—0.5% ammonium dihydrogen phosphate (8:3) as a mobile phase. The proposed method proved to be applicable to the quantitation of 17-ketosteroid sulphates with satisfactory sensitivity and reliability, providing a quantitation limit of 80 ng/ml and coefficient of variation of 4%. A good correlation was observed between the values obtained by the present method and radioimmunoassay for dehydroepiandrosterone sulphate in serum.

INTRODUCTION

The measurement of dehydroepiandrosterone sulphate, one of the principal adrenal secretory products in man, has proved to be valuable for assessing adrenocortical disorders [1, 2]. Numerous methods have been developed for the analysis of dehydroepiandrosterone sulphate in biological fluids, including gas chromatography [3, 4], gas chromatography—mass spectrometry [5], high-performance liquid chromatography (HPLC) [6, 7] and radioimmunoassay (RIA) [8—10]. In almost all cases, the conjugated steroids are determined indirectly after solvolysis and/or hydrolysis. However, the incomplete deconjugation and formation of artifacts may disturb the analytical results. In addition, deconjugation has a disadvantage that no information about the conjugated position can be obtained. Recently, Kawasaki et al. [11] reported a sensitive

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HPLC method for the determination of conjugated 17-ketosteroids in biological fluids using dansylhydrazine as a fluorescence labelling reagent.

HPLC with electrochemical detection (ED) is well recognized to be as sensitive as HPLC with fluorescence detection and extremely useful for the determination of trace components in biological fluids [12, 13]. A highly sensitive method has previously been developed for the quantitation of 17-ketosteroids in human serum after deconjugation, using *p*-nitrophenylhydrazine as a derivatization reagent for HPLC—ED [14]. This paper describes a simple and sensitive method for the determination of 17-ketosteroid sulphates in human serum by means of HPLC—ED.

EXPERIMENTAL

Instruments

The apparatus used for this work was a Waters Model ALC/GPC 202 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a Yanagimoto Model VMD 101 electrochemical detector (Yanagimoto Co., Kyoto, Japan). The applied potential was set at 0.8 V versus an Ag/AgCl reference electrode. A test sample was introduced by a Waters Model U6K sample loop injector with an effective volume of 2 ml. HPLC was carried out on a μBondapak C_{18} (5 μm) column (30.5 × 0.4 cm I.D.) (Waters Assoc.) using methanol—0.5% $NH_4H_2PO_4$ (pH 3.0, adjusted with phosphoric acid) (8:3, v/v) as a mobile phase at a flow-rate of 1 ml/min.

Materials

Androsterone, *p*-nitrophenylhydrazine and trichloroacetic acid were purchased from Tokyo Kasei Co. (Tokyo, Japan). Dehydroepiandrosterone, etiocholanolone and epiandrosterone were kindly donated by Teikoku Hormone Mfg. Co. (Tokyo, Japan). The 17-ketosteroid sulphates were prepared by the conventional method in these laboratoires. 2-Hydroxyestrone 3-methyl ether was prepared from estrone according to the method of Honma and Nambara [15].

Reagent solutions

p-Nitrophenylhydrazine solution. *p*-Nitrophenylhydrazine was purified by repeated recrystallization from methanol. Ten milligrams of *p*-nitrophenylhydrazine were dissolved in 0.2 ml of ethyl acetate. The reagent solution was freshly prepared prior to use.

Trichloroacetic acid—benzene solution. Thirty milligrams of trichloroacetic acid were dissolved in 10 ml of benzene.

Steroid stock solutions. Each solution was prepared by dissolving each 2 mg of dehydroepiandrosterone, androsterone, epiandrosterone and etiocholanolone sulphates in 10 ml of methanol and stored at −20° C until use.

Procedure

To human serum (0.1 ml) in a centrifuge tube was added acetonitrile (2 ml), and the whole was allowed to stand at room temperature for 5 min and then centrifuged at 1000 *g* for 5 min. The supernatant was transferred to another

tube. After addition of 2-hydroxyestrone 3-methyl ether (internal standard) (100 ng), the mixture was evaporated to dryness below 40°C. To the residue were added *p*-nitrophenylhydrazine solution (10 μl) and trichloroacetic acid—benzene solution (100 μl), successively. The solution was heated at 60°C for 20 min, and then evaporated to dryness under a stream of nitrogen gas. The residue was redissolved in methanol (200 μl) and an aliquot of the solution was applied to HPLC.

RESULTS AND DISCUSSION

In the preliminary report [14] we described a highly sensitive method for the determination of 17-ketosteroids in human serum using *p*-nitrophenylhydrazine as a derivatization reagent for HPLC—ED. The conventional method involving prior solvolysis and/or hydrolysis of the conjugated steroids is tedious and time-consuming. In addition, the information about the conjugated form would be lost by deconjugation. Therefore, we have attempted to develop a new method for the direct determination of 17-ketosteroid sulphates without solvolysis. Initially, a suitable procedure for derivatization was investigated using dehydroepiandrosterone sulphate as a model compound. Condensation of dehydroepiandrosterone sulphate with *p*-nitrophenylhydrazine proceeded quantitatively without fission of the sulphate bond, when these two were heated at 60°C for 20 min in 0.3% trichloroacetic acid—benzene solution. The favourable condition was found to be similar to that for free 17-ketosteroids described in the previous paper [14]. 2-Hydroxyestrone 3-methyl ether, an internal standard, was similarly transformed into the *p*-nitrophenylhydrazone. Among several columns tested, μBondapak C_{18} was most suitable for efficient separation of conjugated steroids and the internal standard. A typical hydrodynamic voltammogram is illustrated in Fig. 1. The current (peak height) at each applied potential was divided by the current at the most positive potential to obtain the relative current ratio. This value was plotted against the applied

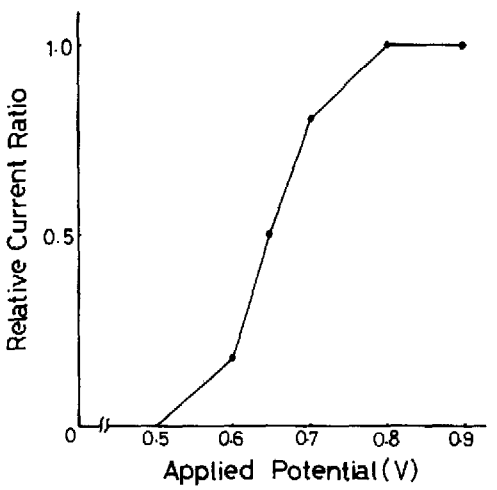

Fig. 1. A hydrodynamic voltammogram of the *p*-nitrophenylhydrazone formed from dehydroepiandrosterone sulphate.

potential. The detector gave a linear response up to +0.8 V versus an Ag/AgCl reference electrode. The *p*-nitrophenylhydrazones formed from dehydroepiandrosterone, epiandrosterone, etiocholanolone and androsterone sulphates and internal standard were satisfactorily resolved on a μBondapak C_{18} column (Fig. 2a). Each derivatized steroid showed a single peak of the theoretical shape. The detection limit of the *p*-nitrophenylhydrazones was 360 pg at 4 nA full scale (signal-to-noise ratio = 2).

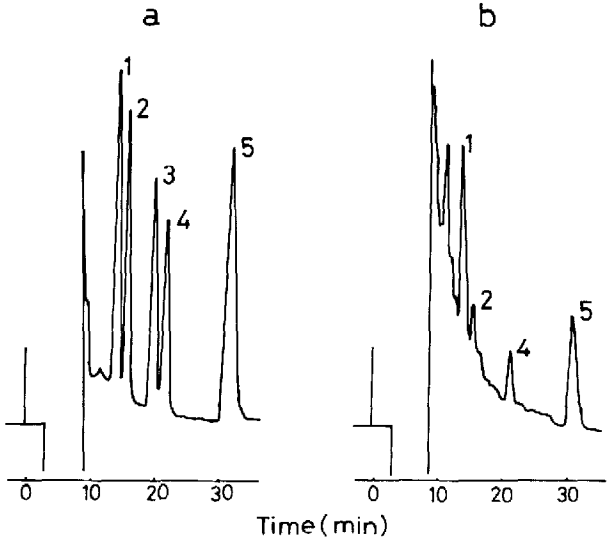

Fig. 2. High-performance liquid chromatograms obtained with (a) standard samples and (b) normal human serum sample. Peaks: 1 = dehydroepiandrosterone sulphate, 2 = epiandrosterone sulphate, 3 = etiocholanolone sulphate, 4 = androsterone sulphate, 5 = internal standard. Interference from unused reagent and endogenous polar substances due to saturation of the output of the detector was overcome by removing the connector for about 5 min after injection.

The next effort was focused on establishing a clean-up procedure for conjugated 17-ketosteroids in serum. After deproteinization of a serum sample with acetonitrile, 17-ketosteroids in the supernatant were derivatized with *p*-nitrophenylhydrazine in trichloroacetic acid—benzene solution. A typical chromatogram of 17-ketosteroid sulphates in human serum is illustrated in Fig. 2b. Three peaks corresponding to dehydroepiandrosterone, epiandrosterone and androsterone sulphates appeared on the chromatogram. No detectable amount of etiocholanolone sulphate, however, was observed with serum specimens analyzed in this study. The internal standard peak was not disturbed by coexisting substances in serum. This assay procedure was rather simple and suitable for the routine work.

A known amount of dehydroepiandrosterone sulphate was added to human serum and the overall recovery was estimated by the standard procedure. As listed in Table I, the spiked steroid was recovered at the rate of about 80% with a coefficient of variation (C.V.) of 4%. The calibration curve for each 17-ketosteroid sulphate was constructed by plotting the peak height ratio against the amount of 17-ketosteroid sulphate where a satisfactory linearity was observed

TABLE I

RECOVERY OF DEHYDROEPIANDROSTERONE SULPHATE ADDED TO NORMAL HUMAN SERUM

Added (μg/ml)	Expected (μg/ml)	Found (μg/ml)	Recovery ± S.D.* (%)
0	—	2.07	—
0.94	3.01	2.82	79.3 ± 3.3
1.88	3.95	3.59	80.9 ± 3.0

*$n = 6$.

in the range of 2.5–15 ng. When 0.1 ml of serum sample was used for the assay, the detection limit of dehydroepiandrosterone sulphate was approximately 8 μg/dl. The detection limit obtainable by the present method is comparable to that by the fluorescence HPLC method [11].

For the purpose of assessing the reliability of the proposed method, dehydroepiandrosterone sulphate levels in serum samples taken from fourteen healthy male volunteers (24–38 years' old) were determined by both HPLC and direct RIA without hydrolysis [16]. The values obtained by the two methods showed a good correlation ($r = 0.952$; $n = 14$), the regression equation being $Y = 0.98X + 0.011$ (Fig. 3).

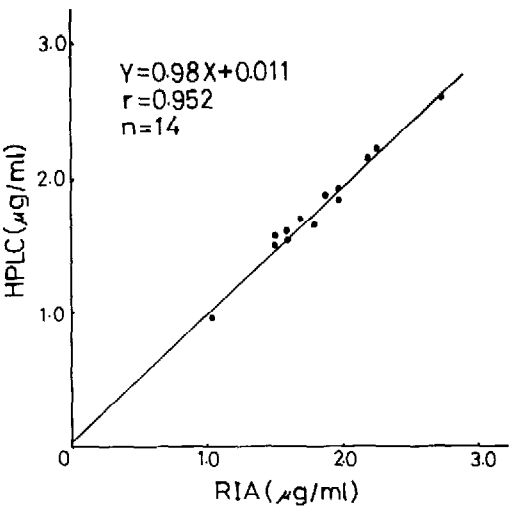

Fig. 3. Comparison of serum dehydroepiandrosterone sulphate levels determined by HPLC and RIA.

In conclusion, the newly developed HPLC method is satisfactory with respect to sensitivity, accuracy and precision. The assay procedure is simple and convenient, and therefore clinically applicable to the routine analysis of serum 17-ketosteroid sulphates.

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