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Note

Steroids

V. Rapid thin-layer chromatographic assay for dehydroepiandrosterone in urine

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Dehydroepiandrosterone (DHEA, 3β-hydroxyandrost-5-en-17-one) is an important potential precursor for androgens and oestrogens, and inhibitory effects of DHEA on glucose-6-phosphate dehydrogenase, NADH-oxidase and other less important mechanisms have been found.

The DHEA is excreted in urine mainly as sulphate and glucuronide, whereas the unconjugated steroids occur only in small amounts. Studies by Lieberman et al. [1] showed that most of DHEA is excreted as sulphate. DHEA occurring as the 3β-glucuronide amounts to about 7.5% of the total [2].

Based on the fact that buffer hydrolysis described by Fotherby [3] is specific for the 3β-sulphates of 5-unsaturated steroids, and that neither sulphates nor glucuronides of androsterone and etiocholanolone hydrolyze [4], a rapid gas chromatographic assay for DHEA has been proposed [5]. The small amount of DHEA excreted as glucuronide is not determined by this method. The purpose of this paper is to present a method that can be applied in serial work in clinical laboratories in which a gas chromatograph is not available. The applicability, accuracy, precision, sensitivity, and specificity are comparable to those obtained with gas chromatographic assays for DHEA in urine [5].

REAGENTS AND EQUIPMENT

DHEA sulphate was obtained from Sigma (St. Louis, Mo., U.S.A.). The solvents, except for methanol and ethanol, were redistilled, and the other chemicals used were of reagent grade. Ready-made Silufol silica gel foils (Kavalier, Sklárny, Czechoslovakia) of dimensions 15 × 15 cm were used for thin-layer

chromatography. Absorbance readings were made with a Unicam SP 1800 spectrophotometer using glass cells of 1 cm light path.

Solutions

The following solutions were prepared: 2 *M* acetate buffer, pH 4.5 (88.5 ml of acetic acid + 136 g of sodium acetate and water to 500 ml); 2,4-dinitrophenylhydrazine reagent (15 mg of 2,4-dinitrophenylhydrazine in 50 ml of absolute benzene; stored at 4°); and 0.3% trichloroacetic acid in absolute benzene.

PROCEDURE

The procedure consists in the following steps.

(a) Hydrolysis and DHEA extraction

Conical ground-glass stoppered tubes (120 × 22 mm I.D.) were used for the hydrolysis. Urine (10 ml) is adjusted with 1 ml of 2 *M* acetate buffer to a pH of approximately 4.5 and heated for 2½ h at 100° to hydrolyze DHEA sulphate.

The well cooled mixture is shaken for 5 min with 10 ml of cyclohexane—diethyl ether (1 : 1) on a horizontal shaker at approximately 200 strokes/min. Urine samples do not form emulsions if they are cooled well before shaking.

The bottom phase is always removed by suction with the help of a capillary tube. The extract is then shaken for 1 min with 5 ml of 10% sodium hydroxide solution, then for 1 min with 10 ml of water, and centrifuged; then the aqueous phase is removed. The organic phase is evaporated to dryness.

(b) Reaction of DHEA with 2,4-dinitrophenylhydrazine

The preparation of the 2,4-dinitrophenylhydrazine was carried out by a slightly modified version of the method of Treiber and Oertel [6]. To the above residue, 0.5 ml of 2,4-dinitrophenylhydrazine reagent and 0.2 ml of trichloroacetic acid are added, then the samples are incubated at 70—80° in the dark for 10 min and subsequently evaporated slowly to dryness.

(c) Thin-layer chromatography and quantitation

The above residue is dissolved in a few drops of benzene containing 10% of ethanol, then the solution is applied on plates of silica gel divided into 2-cm strips with lines 1 mm broad. Excellent separation is achieved in chloroform—acetone (95 : 5) by the ascending technique in two runs; very often one run is sufficient for the separation. The time of development is 25 min. The R_F values of DHEA 2,4-dinitrophenylhydrazone after the first and second developments are 0.26 and 0.46, respectively. The spots on the thin layer of silica gel are cut out and placed in glass tubes.

The strips are eluted by gentle shaking on a horizontal shaker for 10 min with 3 ml of methanol. Absorbances were read at 366 nm.

RESULTS AND DISCUSSION

Evaluation of the method

Specificity. The specificity of the method is ensured by the combination of selective hydrolysis under the conditions described above, solvent extraction,

utilization of a colour reaction that is specific only for some oxosteroids and chromatographic separation. The exception in the selective hydrolysis, viz., epiandrosterone, was discussed earlier [5] and is of little practical importance.

Other sulphates of 3β-hydroxysteroids without an oxo group which might possibly be present in urine in amounts approximately comparable to that of DHEA, and which would be expected to hydrolyze, e.g., 5-pregnenediol (5-pregnene-3β, 20α-diol) and 5-pregnenolone (3β-hydroxy-5-pregnen-20-one), do not react with 2,4-dinitrophenylhydrazine. The R_F values of related oxosteroids in the system chloroform—acetone (96 : 4) have been given previously [7].

Precision. To determine the precision, 18 samples containing 1, 2 and 4 mg/l of DHEA sulphate were analyzed. The coefficient of variation was 5.8-8.0%.

Sensitivity. The sensitivity for the substance to be determined, defined as the least amount significantly differing from zero, can be calculated theoretically [8,9] from replicate analyses of the blank extracted from the plate of the non-hydrolyzed urine. This gave an arithmetic mean of absorbance 0.015 and a standard deviation of ±0.0039. Based on these values, the theoretical sensitivity of the method is about 0.1 mg of DHEA in 1000 ml of urine with a probability of 95%.

Accuracy and recovery. The standard deviations of duplicate urine assays were 0.07 and 0.08 mg/l for DHEA concentrations of 1 and 4 mg/l, respectively (24 samples in each instance). The recovery was measured by determining DHEA sulphate in urine containing 1.4 mg of conjugate per 1000 ml. For different urine samples run in triplicate the recovery was 90—97%. By assaying urine at two dilutions (urine to water ratios of 1 : 0 and 1 : 1), the recovery after dilution was 104%.

Capacity. Using this method, one technician can perform 18 determinations in one day.

Normal values. It has been reported that DHEA sulphate is secreted by the human adrenal gland in amounts comparable with cortisol [10]. The level of DHEA in the urine of an adult human undergoing constant physiological changes depends on many factors, such as age, sex and physical activity. The daily excretions of DHEA for 10 normal men and 10 normal women aged 20—40 years were 2.08 ± 1.54 (0.4—6.0) and 1.62 ± 1.09 (0.3—3.5) mg per 24 h, respectively. Comparisons of values reported in various papers for the excretion of DHEA by normal adults [5] and children [11] have been published. It is possible to conclude that the determination of urinary DHEA could be used as an index of abnormal adrenocortical function.

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REFERENCES

1 S. Lieberman, B. Mond and E. Smyles, Recent Progr. Horm. Res., 9 (1954) 113.
2 W. Nowczynski, F. Fragachan, J. Silah, B. Millette and J. Genest, Can. J. Biochem., 46 (1968) 1029.

3 K. Fotherby, Biochem. J., 73 (1959) 339.
4 J. Bitman and S.L. Cohen, J. Biol. Chem., 191 (1951) 351.
5 M.G. Metcalf, Clin. Biochem., 4 (1971) 241.
6 L. Treiber and G.W. Oertel, Z. Klin. Chem. Klin. Biochem., 5 (1967) 102.
7 J. Hakl, J. Chromatogr., 71 (1972) 319.
8 H. Kaiser, Z. Anal. Chem., 209 (1965) 1.
9 H. Kaiser, Z. Anal. Chem., 216 (1966) 80.
10 E.E. Baulie, Recent Progr. Horm. Res., 19 (1963) 306.
11 S.B. Pal and W.M. Teller, Clin. Chim. Acta, 38 (1972) 371.