Determination of unconjugated etiocholanolone in human plasma using gas-liquid chromatography

INTRODUCTION

Determination of unconjugated etiocholanolone in human plasma is clinically used for the diagnosis of the so called "etiocholanolone fever". Two methods have been described for this determination. One, using a dinitrophenylhydrazine colour reaction for quantitation[1,2] lacks sensitivity and is not very specific; the other method, a double isotope dilution technique[3], is not simple enough for clinical routine use.

In this paper a gas-chromatographic method is described, which is sufficiently specific and sensitive for detecting elevated etiocholanolone levels in patients with etiocholanolone fever.

Materials

Etiocholanolone and androsterone were obtained from Steraloids Inc. (Pawling, N.Y., U.S.A.), dehydroepiandrosterone from Organon (Oss, The Netherlands).

Alumina (CAMAG), 99% ethanol (BDH, Analar) and chloroform containing 2% ethanol (BDH, Analar) were used without purification. Diethylether (BDH, Analar) was distilled just before use. Benzene (BDH, Analar) was washed with concentrated sulphuric acid until washings were colourless, then with saturated $NaHCO_3$ solution and with water. After drying over anhydrous Na_2SO_4 the benzene was distilled twice.

Methods

Thin-layer chromatography (TLC) was done on glass plates (20×20 cm) covered with a 0.25-mm layer of alumina. Plates were activated before chromatography by heating at 110° for at least one hour. Chromatography was performed with complete saturation in the system chloroform–ethanol 98:2 (v/v).

For gas–liquid chromatography (GLC) an F & M (Avondale, U.S.A.) gas chromatograph, model 400, equipped with a flame ionisation detector, was used. U-shaped glass columns (100×0.4 cm) filled with 1% XE-60 on GasChrom Q were employed. Temperatures of column, detector and flash heater were 190°, 220° and 250° respectively. Nitrogen was used as carrier gas.

Preparation of plasma extracts

After addition of 0.25 ml 5 *N* NaOH to 10 ml of plasma, the plasma is extracted with ether, two times 30 ml and two times 15 ml. The combined extracts are washed twice with 5 ml of water, and taken to dryness. The residue is transferred to the glass plates for TLC with a few drops of chloroform–methanol 1:1 (v/v). Standards of etiocholanolone are run on both sides of the plates. After chromatography the standards are coloured with iodine in ethanol, and the area between the standards is scraped off. The alumina is collected in a test tube and 1 ml of water is added. Steroids are extracted from this mixture with 3×3 ml of benzene. The combined benzene extracts are evaporated under nitrogen at 40° and the residue is dissolved in 50 μl of

99% ethanol. This solution is used for GLC with etiocholanolone as reference. Amounts of 5 μl are injected into the column. The chromatograms obtained in this way contain no substances interfering with etiocholanolone.

RESULTS

1. The recovery of amounts of 0.1, 0.5 and 1.0 μg of etiocholanolone added to 10 ml of plasma was studied. The results were:

Amount added (μg)	*Number of experiments*	*Recovery in % mean ± s.d.*
0.1	6	60 ± 10
0.5	6	76 ± 2
1.0	6	74 ± 8

2. Sensitivity: Amounts of 0.004 μg of etiocholanolone can be detected with certainty with a flame ionisation detector. When starting with 10 ml of plasma and a recovery of 75%, the sensitivity of this method is 0.5 μg of etiocholanolone in 100 ml of plasma.

3. The specificity of our method is guaranteed by:

(a) identical R_F values during TLC of the substance obtained from the plasma and an authentic sample of etiocholanolone (R_F 0.58), which is separated from androsterone and dehydroepiandrosterone (R_F 0.40) in the system used;

(b) identical retention times during GLC. Again a separation between etiocholanolone, androsterone and dehydroepiandrosterone is found under the conditions used (retention times 1.0; 0.95 and 1.08 resp.). Further proof of the specificity was obtained with etiocholanolone isolated from the plasma of a patient. From the isolated substance the acetate-[4] and the trimethylsilylether-derivatives[5] were prepared and compared with etiocholanolone-acetate and -3-trimethylsilyl ether. No differences in retention time were observed.

4. In plasma of 7 normal subjects the values found for unconjugated etiocholanolone were always lower than 0.5 μg per 100 ml. Although the values found with our method—0.08–0.17 μg —are not very accurate, they are in agreement with those found by Gandy and Peterson[3] with a double isotope dilution technique, *viz.* 0.06–0.42 μg; but they are lower than those obtained with the dinitrophenylhydrazine reaction (refs. 1, 2, 6).

5. Our practical experience with the described method is still limited. Only in one patient suspected of "etiocholanolone fever" an elevated value of 5 μg/100 ml was found during spontaneous hyperpyrexia. A value of 4 μg/100 ml was found 3 h after intramuscular injection of 50 mg of etiocholanolone, which caused fever. A similar value was found after an infusion of etiocholanolone (40 mg during 30 min), which caused no reaction[7]. In the same patient, during an interval between periods of fever, a series of determinations was made after stimulation of the adrenals with ACTH (60 U daily for 4 days), stimulation of the testes with human chorionic gonadotrophin (1500 IU daily for 4 days), after an intramuscular injection of testosterone-propionate (100 mg) and of 50 mg of dehydroepiandrosterone. In all these instances values below 0.5 μg/100 ml of plasma were found.

6. The clinical applicability of the method described is illustrated by the fact that it is possible to measure the levels of unconjugated etiocholanolone in 10–12 plasma samples within 2–3 days.

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