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Specific assay for unconjugated dehydroepiandrosterone in human plasma by capillary gas chromatography with electron-capture detection

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ABSTRACT

A specific and sensitive method for the determination of unconjugated dehydroepiandrosterone in plasma is described. After extraction and purification of the extracts on a Celite column, the iodomethyldimethylsilyl ether derivative of dehydroepiandrosterone was isolated on an aluminium oxide column and assayed by gas chromatography with electron-capture detection. The method is sensitive: sample volumes of 0.5–1 ml are sufficient for the determination of dehydroepiandrosterone in plasma of normal male and female subjects aged 1–80 years. The assay is highly specific and has the potential to be used as a reference method for the determination of unconjugated dehydroepiandrosterone in biological samples.

INTRODUCTION

Unconjugated plasma dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulphate (DHEAS) are considered to be primarily of adrenal origin. It has been shown that DHEA and DHEAS levels in plasma are significantly increased in adrenal hirsutism [1,2]. The origin of these two androgens is partially common and their metabolism is closely related because of their peripheral interconversion, although the mechanism of secretion regulation appears to be different for the two compounds [3–5]. More information can be obtained on their regulation by the quantitative measurement of both DHEA and DHEAS.

Difficulties in the measurement of DHEA result essentially from its low concentration in plasma. Radioimmunoassay (RIA) is generally used [6–13]. Quantitative data vary widely with the specificity of each technique, which depends on the quality of the antisera and chromatographic separation steps. The range of published normal values varies from 16.65 ± 5.4 [7] to 30.38 ± 3.5 nmol/l [11].

Techniques using gas chromatography (GC) with electron-capture detection (ECD) are more specific [14–16]. However, methods reported so far used packed GC columns and required extensive purification, with the consequent poor recovery of steroids and a loss of sensitivity.

In this paper a capillary GC method is described, which uses the iodomethyldi-

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methylsilyl ether derivatives (IDMSE) employed by Symes and Thomas [16] and Thomas [17]. This method offers a good specificity and sensitivity because of the resolution and efficiency of the purification steps.

EXPERIMENTAL

Chemicals

Standard steroids were obtained from Steraloids (Wilton, NH, USA) and [1,2,6,7-^{3}H]dehydroepiandrosterone from Amersham International (Cardiff, UK). Aluminium oxide (standardised activity II–III according to Brockman) was purchased from Merck (Darmstadt, Germany) and used without preparation. Bromomethyldimethylchlorosilane (BDMCS) was obtained from Supelco (Bellefonte, PA, USA). A [^{3}H]DHEA-RIA kit was obtained from BioMerieux (Marcy l'Etoile, France). All other reagents were of analytical-reagent grade.

Apparatus

Gas chromatography. GC was carried out using a Girdel 30 gas chromatograph (Delsi-Nermag Instruments, Argenteuil, France) equipped with a 23-m-long open-tubular glass capillary column coated with OV-73. The capillary column (I.D. 0.3 mm, film thickness 0.2 μm) was prepared as described by Bouche and Verzele [18]. Injection was achieved with a glass needle injection system. Detection used an electron-capture detector. Signals from the detector were integrated using a data processor (Hewlett-Packard 3353). The operating conditions were as follows: oven temperature, 225°C; injector temperature, 235°C; detector temperature, 250°C. Argon with 5% methane was used as the carrier gas at a flow-rate of about 1–2 ml/min. The same gas was added as the make-up gas at the column exit, at a flow-rate of approximately 20 ml/min. The electron-capture detector was operated with a 10 V, 200 μs pulse range and a 1 μs pulse width.

Gas chromatography–mass spectrometry. Gas chromatography–mass spectrometry (GC–MS) was performed on an R1010-B quadrupole mass spectrometer (Delsi-Nermag Instruments), coupled to a Girdel 31 gas chromatograph. The chromatograph was equipped with a glass needle injection system and a 25 m $\times$ 0.30 mm I.D. glass capillary column coated with OV-73 (film thickness 0.2 μm). The carrier gas was helium at a pressure of approximately 0.7 bar (1 bar = 10^5 N/m^2). The mass spectrometer was operated in the electron-impact (EI) mode at 70 eV.

Quantitative analysis

Extraction. Plasma samples (1 ml) were mixed with a known amount of tritium-labelled DHEA (approximately 1000 dpm) and vortex-mixed twice with 5 ml of diethyl ether. The aqueous layer was frozen and the ether solution was decanted and dried by evaporation.

Celite column chromatography. Columns (200 mm × 6 mm I.D.) were prepared by packing with a mixture of 0.5 g of Celite and 0.3 ml of ethylene glycol. A 5% ethyl acetate solution in isooctane was used as the mobile phase. The dried extracts were dissolved in 0.5 ml of the mobile phase and applied to the column. The first elution fraction of 3.5 ml was discarded: the second fraction of 3.5 ml contained DHEA. A mass of 16 ng of the internal standard, 5α-pregna-9,16-diene-3β-ol-20-one in 200 μl of ethanol, was then added to the dried eluates. Radioactivity was measured in 40-μl aliquots for the estimation of recovery and 160 μl were evaporated for the preparation of derivatives.

Preparation of IDMSE derivatives. The silylating reagent was a mixture of 2 ml of hexane, 200 μl of BDMCS and 100 μl of diethylamine. The reagent (100 μl) was added to the residue and left for 45 min at room temperature. After removal of the excess of reagent under a stream of nitrogen, 100 μl of a saturated solution of sodium iodide in acetone were added and the tube was stored at 37°C for 30 min.

Purification of IDMSE derivatives. The IDMSE derivatives were isolated on an aluminium oxide column. A mixture of hexane–dichloromethane–ethanol–acetone (55:44:0.5:0.5, v/v) was used as the eluent. The column (200 mm × 6.0 mm I.D.) was prepared by packing 3 g of aluminium oxide in the solvent. The dried residues were dissolved in 0.5 ml of the solvent and were applied to the column. The elution was carried out after discarding 3.5 ml of the solvent. The following 3.5 ml contained the IDMSE derivatives.

Gas chromatography. The dried residues were dissolved in 100 μl of hexane: 1–3 μl samples were injected into the chromatograph.

RESULTS

Formation of derivatives

The mass spectra of the derivatives of DHEA and the internal standard are given in Fig. 1. The GC–MS analysis showed only the iodomethyldimethylsilyl ether derivative. Derivatization was complete and no other derivative was detected.

Specificity

Figs. 2 and 3 show gas chromatograms obtained from the standard mixture and plasma extracts, respectively. The Kovats indices were 2975, 2989, 3007, 3080, 3092 and 3194 for dehydroandrosterone, androsterone, etiocholanolone, dehydroepiandrosterone, epiandrosterone and the internal standard, respectively. These steroids were also resolved on the capillary GC column and no interference from endogenous steroids was observed in the determination of DHEA. The reproducibility of the retention times is good; for DHEA and the internal standard, the variation in retention times was always less than 5%.

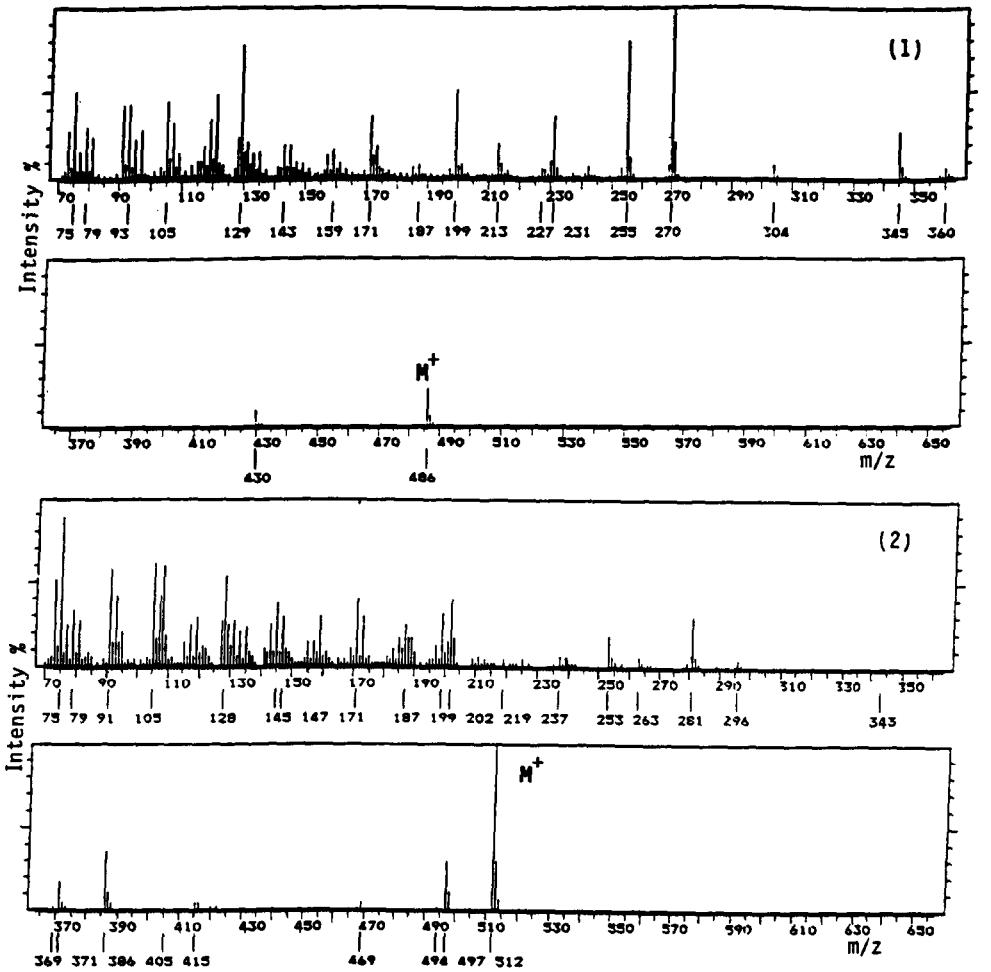

Fig. 1. Mass spectra of IDMSE of (1) DHA and (2) 5α-pregna-9,16-diene-3β-ol-20-one. Electron-impact mode, 70 eV.

Recovery

The overall recovery of DHEA was determined using tritium-labelled steroids. The mean recovery was 90 ± 4.3% ($n = 12$), as determined from the plasma of normal adult women.

Standard curve: internal standard

The 5α-pregna-9,16-diene-3β-ol-20-one was chosen as the internal standard. Its IDMSE derivative was eluted on the aluminium oxide column under the same conditions as used for DHEA. This derivative was separated from other steroids on the GC column and its retention time was reasonable (27 min). The calibration plot was linear for ratios of DHEA to internal standard > 0.8.

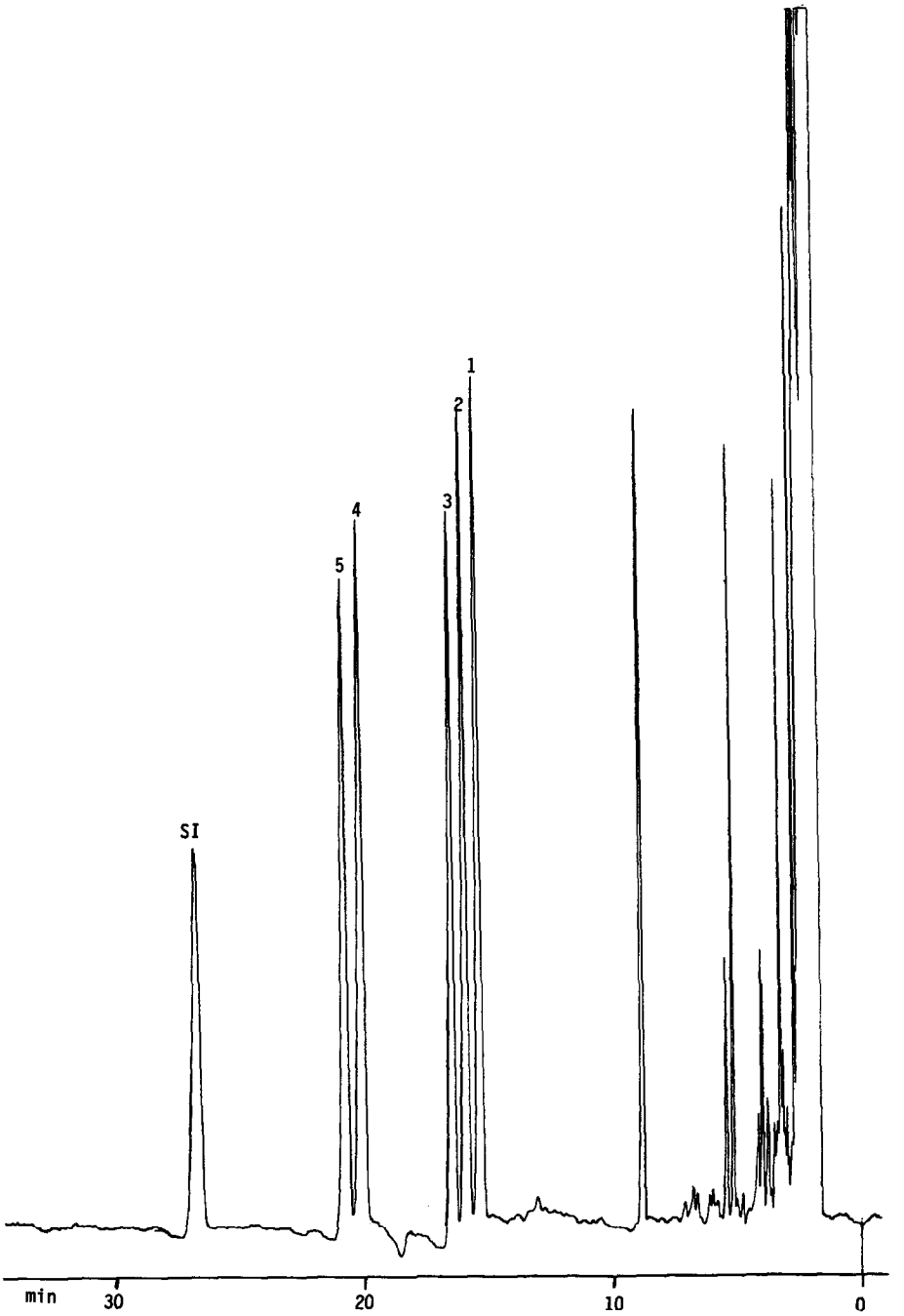

Fig. 2. Gas chromatogram of derivatives of standard steroids. Peaks: 1 = dehydroandrosterone; 2 = androsterone; 3 = etiocholanolone; 4 = dehydroepiandrosterone; 5 = epiandrosterone; SI = internal standard. The conditions used are described under Experimental.

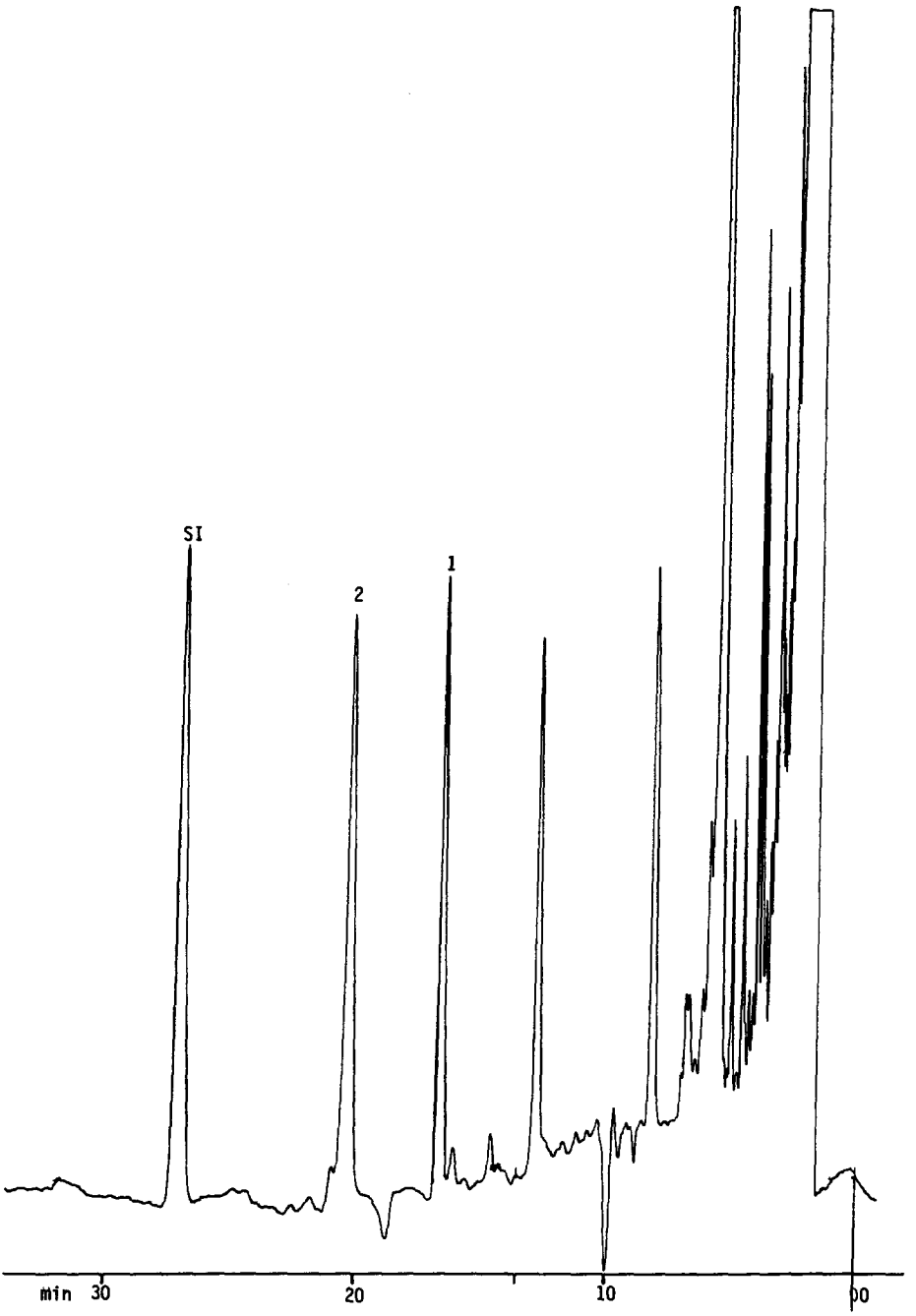

Fig. 3. Gas chromatogram obtained for a plasma extract. Peaks: 1 = etiocholanolone; 2 = DHA; SI = internal standard. The conditions used are described under Experimental.

TABLE I

REPRODUCIBILITY OF THE ASSAY

Reproducibility	Sample	*n*	DHEA level (mean ± S.D.) (nmol/l)	Coefficient of variation (%)
Within-run	Pool A	10	33.7 ± 1.44	4.3
	Pool B	10	10.1 ± 0.19	1.9
Between-run	Pool B	10	10.2 ± 0.34	3.3

Precision

The precision of the method was determined from repeated measurements of DHEA concentration in two pools of plasma. The coefficients of variation were always less than 5% (Table I).

Detection limit

Under the described conditions, the smallest amount which could be detected by GC was 30 pg (signal-to-noise ratio >3), which corresponds to a plasma concentration of 2.1 nmol/l.

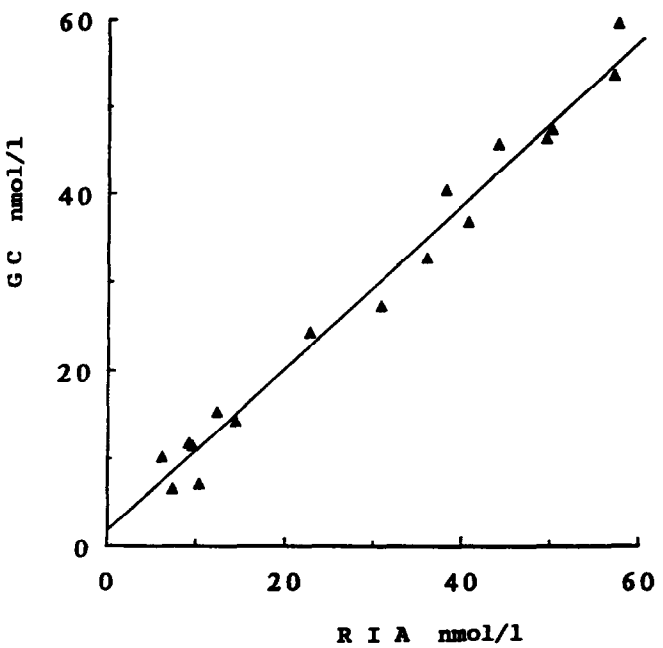

Fig. 4. Comparison of the GC and RIA assays of plasma DHEA ($n=20$).

TABLE II

CONCENTRATION OF UNCONJUGATED DHEA IN HEALTHY SUBJECTS

Age (years)	Sex	*n*	DHEA (nmol/l) (mean ± S.D.)
1–9	F	3	13 ± 5.2
	M	5	19.6 ± 4.3
10–19	F	3	21.7 ± 9.5
	M	3	28.1 ± 12.2
20–39	F	17	23.4 ± 6.0
	M	14	32.3 ± 8.9
40–59	F	12	20.3 ± 4.3
	M	7	20.2 ± 5.5
60–80	F	10	15.6 ± 8.2
	M	8	16.5 ± 8.7

Accuracy: recovery of DHEA added to plasma

A plasma sample was spiked with two concentrations of DHEA. The recoveries were 105 ± 2.6 and 102 ± 3.6% (mean ± S.D.) for added DHEA concentrations of 17.4 and 34.7 nmol/l, respectively.

Comparison with a radioimmunoassay

For the same samples, the results obtained by GC were compared with the results of an RIA (BioMerieux) using ether extraction and Celite column chromatography. The linear regression equation obtained was $y = 1.06x - 1.42$, with a correlation coefficient of 0.991 (Fig. 4).

Results obtained in healthy subjects

The method was used for the determination of DHEA concentrations in the plasma of male and female subjects aged from 1 to 80 years. The results are shown in Table II.

DISCUSSION

A specific and sensitive method has been developed which allows the determination of unconjugated DHEA in a small sample of plasma (0.5–1 ml). The combination of the purification step and capillary GC results in a very good specificity.

The previously published methods for the determination of DHEA were very time-consuming and required a large volume of plasma [14–16,19,20]. Several purification steps, with a great loss of steroid, were necessary, such as washing, thin-layer chromatography and column chromatography. Symes and Thomas

[16] used two adsorption chromatography columns and the overall recovery was about 43%. In the method reported here, only one adsorption chromatography column was used for separation of the IDMSE derivatives and the overall recovery of DHEA was 90%. The use of IDMSE derivatives gives a good sensitivity with an electron-capture detector and the use of a capillary GC column increases the sensitivity of detection.

The internal standards used for the GC in earlier work were testosterone [18] or 5α-androstane-3β,17β-diol [16]. A steroid not found in plasma, 5α-pregna-9,16-diene-3β-ol-20-one, was used as an internal standard in this work. The retention time of its derivative is different from that of steroid which may be found in plasma and not too long with respect to DHEA (27 and 20 min, respectively); its use therefore does not excessively increase the analysis time.

The findings reported here agree with the latest data [9,10,12,13], although higher [7] and lower [11] values have also been reported. The values obtained by this method are higher in men than in women between 20 and 40 years old. There is no significant difference between males and females older than 40 years.

The method described here is accurate and simple but is is more time-consuming than RIA. However, because of its good specificity, it may be a useful reference method for the determination of unconjugated DHEA in biological fluids.

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