Journal of Chromatography. 116 (1976) 163–167
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 8641

METHOD FOR THE MEASUREMENT OF PLASMA DEHYDROEPIANDROSTERONE BY GAS–LIQUID CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION

ELIZABETH K. SYMES and B. S. THOMAS

Departments of Androgen Physiology and Clinical Endocrinology, Imperial Cancer Research Fund Laboratories, Lincoln's Inn Fields, London, WC2A 3PX (Great Britain)

(Received July 17th, 1975)

SUMMARY

A method is described for the measurement of plasma dehydroepiandrosterone, as the iodomethyldimethylsilyl ether derivative, by gas–liquid chromatography and electron capture detection using the relatively new and highly stable stationary phase Dexsil 300. Preliminary purification of the plasma extract was required and alumina column chromatography was utilised, both before and after derivatization of the steroid extract. Specificity, precision, sensitivity and accuracy were all satisfactory. The method was used to study the relationship between age and the level of plasma dehydroepiandrosterone in a group of normal women. A significant negative correlation was observed.

INTRODUCTION

A method for the measurement of testosterone in human plasma was described by Thomas[1] in which the practicability of the use of gas–liquid chromatography (GLC) techniques for the measurement of the very small quantities of steroid hormones in plasma was discussed. A particular advantage, namely the potential of the method for the analysis of other steroids within the same plasma extract, was also cited. It has been possible, with slight modification of the chromatography of the iodomethyldimethylsilyl ether derivatives on alumina, to measure free dehydroepiandrosterone (DHEA) also. The working time of the method has been reduced by the use of smaller alumina columns.

EXPERIMENTAL

Materials

Blood was obtained from normal women between 16.00 and 20.00 h. The heparinized plasma was separated and stored at −20°.

Neutral alumina (Woelm, Eschwege, G.F.R.) was partially deactivated by

equilibration with water (9%, w/w). [^{3}H]DHEA was obtained from the Radiochemical Centre (Amersham, Great Britain) (specific activity: 10–25 Ci/mmole).

Reagents were as described by Thomas[1]. All glassware was washed successively with hot methanolic Decon, water and ethanol. Chromic acid was used instead of the Decon mixture for every fifth wash.

GLC was carried out on a Pye 104, Model 74 gas chromatograph fitted with a 7.5 mCi [^{63}Ni]electron capture detector. The detector was used in the pulse mode (500-μsec interval) and the detector oven was set at 300°. The column (1.5 m $\times$ 4 mm) was all glass and fitted to the detector with a standard glass-to-metal connector. The column was packed with Gas-Chrom Q (100–120 mesh) and coated with 1.5% Dexsil 300 (Analabs, North Haven, Conn., U.S.A.). Prior to packing, any coated support smaller than 100 mesh was removed by sieving. Before use the column was conditioned for a minimum of 24 h at 300° with a slow flow of nitrogen at approximately 25 ml/min. The operating temperature of the column was 250–260° with a gas flow-rate of 100 ml/min. When not in use the column was kept at 150° and the gas flow-rate reduced to 25 ml/min. Under these conditions the useful life of the column was at least six months.

Radioactive counting was performed on a Nuclear Chicago, Mark II, liquid scintillation counter. The counting fluid (10 ml) was 2.5 l toluene containing 11 g Permablend 111 (Packard, Downers Grove, Ill., U.S.A.).

Method

[^{3}H]DHEA (30,000 dpm in toluene, 10 μl) was added to plasma (3 ml), followed by 1 *N* NaOH (three drops). The mixture was left at room temperature for 15 min. The steroids were extracted with ether (2 $\times$ 5 ml). The ether extracts were combined and washed with water (3 $\times$ 1 ml) and taken to dryness under a stream of nitrogen at 60°. (This was the standard technique for drying used throughout the method.)

Hexane (2 ml) and 70% aqueous methanol (2 ml) were added to the dried extract. After vigorous shaking the two layers were separated by centrifugation (1000 *g*, 5 min). The upper hexane layer was discarded. The partition was repeated using more hexane (1 ml). This step removed less polar compounds including progesterone. The remaining methanolic extract was dried.

This extract was redissolved in benzene (0.5 ml) and subjected to alumina chromatography. A slurry of alumina (1.5 g) was prepared in benzene. The column was formed in a pasteur pipette with a drawn-out end lightly plugged with silanised glass wool. The extract was applied to the column: 0.8% ethanol in benzene (5 ml) was used for a pre-wash and was discarded. DHEA was eluted in a further 5 ml of 0.8% ethanol in benzene.

The fraction thus obtained was dried and converted to the iodomethyldimethylsilyl ether (IDMSE) derivatives[1]. These derivatives were dissolved in cyclohexane (0.5 ml) and subjected to further alumina chromatography. Chromatography was performed as before with the following exceptions: The slurry was prepared in cyclohexane; the sample was loaded in cyclohexane; the pre-wash was with a 1% acetone in cyclohexane mixture (5 ml): the derivatives were eluted in 1% acetone in cyclohexane (5 ml).

5α-Androstene-3β,17β-diol-IDMSE (20 ng) was added to the column eluate as

internal standard for the GLC analysis. The mixture was dried and re-dissolved in hexane (0.1 ml). An aliquot (1/10) was taken for the determination of radioactivity. From the amount of radioactivity, correction was made for procedural losses. Further fractions (1/20) were taken for GLC analysis. The concentration of DHEA in plasma was calculated as follows:

$$\text{DHEA (ng/100 ml plasma)} = \frac{\text{ng in DHEA peak} \times 10^4}{\text{equivalent amount of plasma injected (ml)} \times \%\ \text{recovery } [^3\text{H}]\text{DHEA}}.$$

RESULTS

Calibration curves of the ratio of the peak height of DHEA-IDMSE to the peak height of the internal standard (1 ng) against the quantity of DHEA were both linear and reproducible over the range 0.1–0.5 ng of DHEA (Fig. 1). A standard curve was run with each batch of analyses. A typical chromatogram of a plasma extract from a normal woman is shown in Fig. 2.

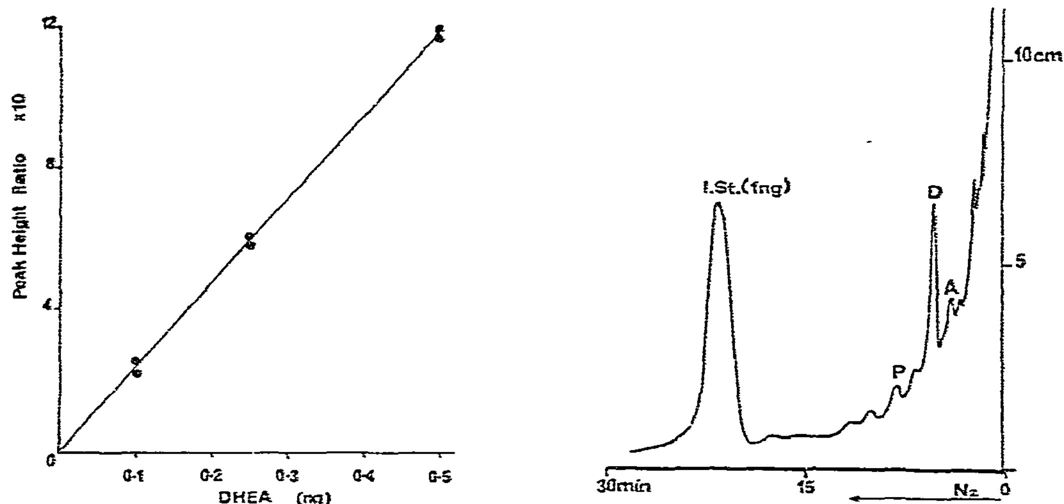

Fig. 1. Calibration curve of dehydroepiandrosterone-IDMSE using 5α-androstene-3β,17β-diol-IDMSE as internal standard.

Fig. 2. Gas chromatogram of the DHEA-IDMSE fraction from female plasma (3 ml). D = DHEA; A = androsterone; P = pregnenolone; I.St = internal standard. Relative retention times: A = 0.165, D = 0.242, P = 0.367, I St. = 1.00 (21.4 min).

Precision and sensitivity were calculated using the difference between duplicate analyses[2] and are shown in Table I.

Accuracy was determined by analysing normal saline samples to which a known amount of DHEA had been added, over the range 200–1000 ng/100 ml. The mean recovery from 24 analyses was 103.7% with a standard deviation of 16.7% (range 80.4–134.3%). Recoveries were also determined after the addition of DHEA (500–1000 ng/100 ml) to plasma samples. A pool of plasma was repeatedly assayed.

TABLE I

PRECISION AND SENSITIVITY OF THE DETERMINATION OF DHEA IN PLASMA

No. of duplicates	*DHEA (ng/100 ml) (mean ± S.D.)*	*Precision (%)*	*Sensitivity DHEA (ng/100 ml)*
19	531 ± 36.9	± 7	56

The mean and standard deviation of the DHEA concentration were 740 ± 70 ng/100 ml ($n = 6$). The mean and standard deviation of the recovery were 104.7 ± 20.2% ($n = 9$, range 75–129%).

The overall recovery of radioactivity through the method was 42.6 ± 9%. This figure was determined from the recoveries obtained in the assay of 23 female-plasma samples.

Plasma was assayed in a group of 23 normal women. The mean and standard deviation were 539.4 ± 161 ng/100 ml. The age range was 30–75 years (mean and S.D., 48.6 ± 12.4 years). The plasma DHEA concentration was negatively correlated with advancing age (Fig. 3). This correlation was significant ($P < 0.01$).

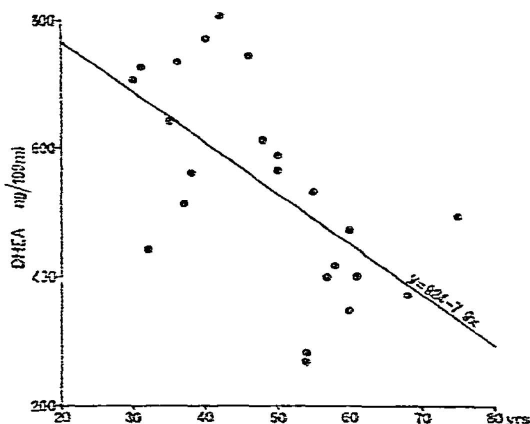

Fig. 3. Relationship of age and plasma DHEA in a group of normal women.

The specificity of the method was examined by the incorporation of a high-resolution paper chromatography step[3] into the method before the first alumina column stage. A series of nine plasma samples was processed in this manner and the results were compared with a duplicate series assayed in the normal way. The mean and standard deviation of the result were 439.0 ± 90.0 ng/100 ml, compared with that of 441.3 ± 90.7 ng/100 ml with the addition of paper chromatography to the method. No significant difference was found on application of a paired Student's t test to the comparisons ($t = -0.18$).

DISCUSSION

The precision and sensitivity of the method were found to be satisfactory. The accuracy as determined by analysis using normal saline as base was good but more

variability was found when plasma was used. However, this is to be expected since the latter procedure requires the calculation of the difference between two measurements, each with an associated error, while the former requires only one measurement. The reliability of the method is comparable to other published procedures[4–7].

The use of 5α-androstene-3β,17β-diol as internal standard was justified in that any endogenous steroid in the plasma extract would be removed by chromatography on the alumina columns. Also, no interference has been found in that position on the gas chromatograms.

The specificity of the method was satisfactory in that a paper partition chromatography step incorporated into the method did not alter the result. The purification procedures of the method do impart some additional evidence of specificity. Through the method the compound has to behave as its radioisotope both before and after derivatization. Whilst this does not provide complete evidence that the DHEA peak on the gas chromatogram is homogeneous, further evidence may be inferred from the results of other workers, who, by using different techniques, obtained similar results. For instance, Gandy and Peterson[6] obtained values of 480 ± 320 ng/100 ml (range 140–1250) in a group of normal women (age range 20–40 years), using a double isotope derivative technique; Nieschlag *et al.*[5] obtained values of 405 ± 216 ng/100 ml (range 153–730) using radioimmuno-assay, and Rosenfeld[7], by a competitive binding assay, obtained 421 ± 303 ng/100 ml (range 137–1261).

Dexsil 300 has proved an excellent phase both in terms of its low background noise and stability. To achieve this certain precautions were found necessary, namely the removal of oxygen and water from the carrier gas. This was done by incorporating an oxygen scrubber in the gas line (Oxisorb; Supelco, Bellefonte, Pa., U.S.A.). In some instances, when the ECD standing current was lower than required, the use of a nitrogen purge gas at 25 ml/min was found beneficial. Difficulties with high background noise have been encountered with the prolonged use of other phases. The high temperature required to analyse steroids is probably the major cause of this.

An advantage of GLC methods over others is the potential for measuring a number of steroids within the same sample. The method described here allows the estimation of DHEA but the fraction eluted from the second alumina column contains other steroids such as androsterone and pregnenolone (although measurements of these steroids have not been evaluated). Also, if the column is further eluted with a mixture of acetone–benzene–cyclohexane (1:50:49), testosterone may be estimated. However, if the measurement of DHEA alone is required, it is possible, with the great sensitivity of the electron capture detector to the IDMSE derivative, to use as little as 0.5 ml of plasma for the analysis.

REFERENCES

1 B. S. Thomas, *J. Chromatogr.*, 56 (1971) 37.
2 J. B. Brown, R. D. Bulbrook and F. C. Greenwood, *J. Endocrinol.*, 16 (1957) 41.
3 B. S. Thomas and R. D. Bulbrook, *J. Chromatogr.*, 14 (1966) 28.
4 C. M. Andre and V. H. T. James, *Clin. Chim. Acta*, 43 (1973) 295.
5 E. Nieschlag, D. H. Loriaux and M. B. Lipsett, *Steroids*, 19 (1972) 669.
6 H. M. Gandy and R. E. Peterson, *J. Clin. Endocrinol.*, 28 (1968) 949.
7 R. L. Rosenfield, *Steroids*, 17 (1971) 689.