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DETERMINATION OF DEHYDROEPIANDROSTERONE AND DEHYDROEPIANDROSTERONE SULPHATE IN HUMAN PLASMA USING ELECTRON CAPTURE DETECTION OF 4-ANDROSTENE-3,6,17-TRIONE AFTER GAS–LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the measurement of dehydroepiandrosterone (DHA) and of its sulphate (DHAS) in human peripheral plasma is described and evaluated. After isolation of DHA from the sample the steroid is oxidized to 4-androstene-3,6,17-trione, which is measured with an electron capture detector after gas–liquid chromatography. It is possible to detect 100 pg 4-androstene-3,6,17-trione. The smallest amount of DHA per sample that can be distinguished from zero is approximately 4 ng, when recovery ($27{\cdot}9 \pm 8{\cdot}8$ %) and method blank ($0{\cdot}23 \pm 0{\cdot}38$ ng) are taken into account. The oxidation to 4-ene-3,6-diones is specific for steroidal 5-en-3-ols. Specificity for DHA is ensured by several chromatographic steps. Repeated estimation of 10 ng DHA gave a mean value of $9{\cdot}6 \pm 1{\cdot}45$ (S.D.) ng ($n = 35$). Mean concentrations and their standard deviations for DHA and DHAS in peripheral plasma from 18 individuals were $0{\cdot}50 \pm 0{\cdot}25$ and 78 ± 40 μg/100 ml, respectively, at 08.30 h and $0{\cdot}32 \pm 0{\cdot}17$ and 84 ± 34 μg/100 ml, respectively, at 17.00 h of the same day. Levels of plasma cortisol in the same plasma samples estimated with a competitive protein-binding method were $16{\cdot}7 \pm 1{\cdot}8$ and $11{\cdot}9 \pm 3{\cdot}8$ μg/100 ml, respectively. No significant differences between the sexes were observed by any of the three assays. The mean values of the plasma concentrations of cortisol and DHA in the morning were significantly higher than those in the evening ($P < 0{\cdot}001$ and $P < 0{\cdot}005$, respectively). In contrast, the mean value of the plasma levels of DHAS in the morning was significantly lower than that in the evening ($P < 0{\cdot}025$).

INTRODUCTION

A large number of methods has been described for the estimation of dehydroepiandrosterone sulphate (DHAS) in human peripheral plasma (see reviews by Eberlein, Winter & Rosenfield, 1967; Eik-Nes, 1968), but there are only a few methods available for the estimation of unconjugated dehydroepiandrosterone (DHA) in peripheral

plasma. This may be partly due to the difficulties encountered in the estimation of the low concentration of DHA in plasma, which is of the order of 0·20–1·0 μg/100 ml. Methods using flame ionization detection after gas–liquid chromatography (g.l.c.) (Goldfien, Jones, Yannone & White, 1965; Aakvaag & Fylling, 1968) require rather large volumes of plasma, whereas double isotope dilution techniques (Rivarola, Saez, Meyer, Kenny & Migeon, 1967; Gandy & Peterson, 1968) are laborious and time-consuming. Recently Heyns & de Moor (1970) and Rosenfield (1971) described competitive protein-binding methods for DHA, which involve the initial conversion of DHA into 5-androstene-3β,17β-diol. The limit of detection which can be reached using these methods is comparable to the sensitivity which can be obtained with g.l.c. with electron capture detection of suitable derivatives. The only method using the latter technique described so far is that of Kumari, Collins & Sommerville (1969), who used the electron capturing ability of DHA heptafluorobutyrate. Samples containing 10 ng DHA could be estimated with reasonable accuracy with this method. The use of the (iodomethyl)-dimethylsilyl ether of DHA as suggested by Eaborn, Holder, Walton & Thomas (1969) might lead to a method which could detect less than 1 ng per sample (Thomas, 1971).

A third electron capturing derivative of DHA, 4-androstene-3,6,17-trione, was suggested by van der Molen, Groen & van der Maas (1965), who estimated that the electron capturing ability of this compound is five times as high as that of DHA heptafluorobutyrate. The present investigation was undertaken to assess the practical possibilities of the latter observation for the estimation of DHA and its sulphate in human peripheral plasma. The concentration of cortisol was measured in the same plasma samples to ensure that adrenal cortical function was normal in the subjects from whom the plasma was taken. The method used to estimate cortisol was a modification of the competitive protein-binding method described by Murphy (1967), which, although not entirely specific, appeared to give a reasonable estimate of this steroid.

Brief accounts of some of the results described in this paper have been reported previously (de Jong & van der Molen, 1970, 1971).

MATERIALS AND METHODS

Solvents were obtained from BDH (Analar) or Merck, and purified before use as described by van der Molen & de Jong (1972).

Glassware was cleaned by soaking overnight in a detergent solution (Extran, Merck) and washing in a laboratory glassware washing machine, which rinses the glassware with distilled water after washing. Glass tubes in which steroids were taken to dryness were silanized before cleaning, using a 5% solution of dimethyldichlorosilane in toluene.

Paper chromatography was carried out on unwashed Whatman No. 20 chromastrips (55 × 2 cm). Descending chromatography was used after overnight equilibration.

Thin-layer chromatography (t.l.c.) was performed on pre-coated silica gel plates (Merck F_{254}, 20 × 20 cm) for the first chromatography. For the second chromatography glass plates (20 × 20 cm) were coated with a 0·25 mm thick layer of silica gel (Merck, GF_{254}), which was purified by boiling with methanol.

Gas–liquid chromatography was performed on a Hewlett-Packard model 402

Biomedical analyser, fitted with a 100 mCi tritium electron capture detector. The detector was operated in the pulsed mode with pulse intervals of 150 μs. U-shaped glass columns (90 cm, i.d. 6 mm) were packed with 1 % QF-1 coated on Gas–Chrom Q (80–100 mesh, Applied Science). The column temperature was kept at 210 °C. The temperature of the flash heater was 250 °C and the detector temperature was 200 °C. A mixture of 95 % argon and 5 % methane was used both as carrier gas and as purging gas. Carrier gas flow as measured at the detector outlet was 60 ml/min. The flow of the purging gas was also 60 ml/min.

Detection of radioactivity on paper chromatograms was performed with a Packard Radiochromatogram scanner (model 7200).

Estimation of radioactivity was performed using a Packard liquid scintillation counter (model 3375) or a Nuclear Chicago liquid scintillation counter (Mark I). Samples were counted in a scintillation fluid prepared by dissolving 4 g PPO (2,5-diphenyloxazole) and 40 mg POPOP (1,4-bis-2-(5-phenyloxazolyl) benzene) in 1 l toluene. Samples from the competitive protein-binding assay for cortisol were counted in a scintillation fluid which contained 100 g naphthalene, 300 mg POPOP and 7 g PPO made up to 1 l with dioxane.

Steroids. [15-^{3}H]DHA (19 Ci/mM) as prepared by Emiliozzi, Condom, Audinot & Pichat (1971) was obtained through the courtesy of Professor E.-E. Baulieu. The compound was used as an internal standard because the 7α-^{3}H label of DHA is lost during oxidation (H. J. van der Molen, unpublished observation), probably due to the enolization of the 4-ene-3,6-dione system. The ammonium salt of [4-^{14}C]DHAS (58·8 mCi/mM) was obtained from New England Nuclear Corporation, Boston, and [1,2-^{3}H]corticosterone (39·1 Ci/mM) from the Radiochemical Centre, Amersham. Radioactive steroids were purified by paper chromatography and t.l.c. until no radioactive impurity could be detected when the radioactivity of the main component caused full deflexion of the scanner over a short period of time. Radiochemical purity was checked every 2 months. Non-radioactive steroids were obtained from Steraloids Inc. and used without purification.

4-Androstene-3,6,17-trione was prepared from DHA as follows. A solution of 100 mg CrO_3 in 20 ml 90 % acetic acid was added to 100 mg DHA. After 18 h in the dark at room temperature, water (20 ml) was added to the reaction mixture. The water layer was saturated with sodium chloride and then extracted with diethyl ether (2 × 30 ml, 2 × 15 ml). The combined ether extracts were washed with water (3 × 15 ml), dried with anhydrous sodium sulphate and taken to dryness under a stream of nitrogen. The residue was applied at the starting line of a 20 × 20 cm thin-layer plate, coated with a 2 mm layer of silica gel F_{254} (Merck), which was pre-cleaned by running in the solvent system benzene:ethanol (9:1, v/v). After development of the chromatogram in the same solvent system the 4-androstene-3,6,17-trione zone was located under u.v. light (254 nm). The compound was eluted by mixing the silica gel with an equal volume of water and extraction of the mixture with equal volumes of benzene (4 times). The combined benzene extracts were washed with water (2 × 2 ml). After filtration, part of the benzene was evaporated under nitrogen and the resulting yellow crystals (m.p. 222–225 °C) were collected by filtration. The yield of this conversion, used to obtain milligram amounts of 4-androstene-3,6,17-trione, was approximately 25 %.

4-Pregnene-3,6,20-trione was prepared using the same method, starting from 3β-hydroxy-5-pregnen-20-one. The melting point of the crystals was 192–196 °C. This steroid is now commercially available (Steraloids Inc.).

The purity of 4-androstene-3,6,17-trione and 4-pregnene-3,6,20-trione was assessed using g.l.c., infrared spectrophotometry and mass spectrometry. Each compound gave rise to a single g.l.c. peak on QF-1, SE-30 and XE-60 columns using both electron capture and flame ionization detection.

The infrared spectrum of 4-pregnene-3,6,20-trione was identical with the spectrum of this compound published by Neudert & Röpke (1965). The infrared spectrum of 4-androstene-3,6,17-trione showed the characteristic carbonyl absorptions with ν_{max} at 1750 and 1700 cm^{-1}, as reported by Menini & Norymberski (1962).

Mass spectra of both compounds showed peaks with mass:charge ratios (*m:e*) of 136 and 137, which are characteristic for steroidal 4-ene-3,6-diones (Djerassi, Karliner & Aplin, 1965). Peaks with *m:e* 288 (molecular ion of DHA) or 316 (molecular ion of 3β-hydroxy-5-pregnen-20-one) could not be detected. From the mass spectra it appeared that the amount of impurities with molecular weights higher than those of the molecular ions of 4-androstene-3,6,17-trione (300) or 4-pregnene-3,6,20-trione (328) was smaller than 2 %.

Estimation of DHA and DHAS

Extraction and solvolysis of steroids. Plasma was separated by centrifugation of heparinized blood and stored at −18 °C. For the estimation of unconjugated DHA 5 ml plasma were placed in a ground-glass-stoppered conical 50 ml centrifuge tube. A solution of 40000 d.p.m. [15-^{3}H]DHA in 10 μl benzene:ethanol (9:1, v/v) was added and the solvent was allowed to evaporate. Following addition of 0·25 ml 5 M-NaOH the plasma was extracted with ether (6 × 10 ml). The combined ether extracts were washed with water (2 × 5 ml) and taken to dryness at 45 °C under a stream of nitrogen. The residue was then treated as outlined below. For the estimation of DHAS 0·25 ml plasma was used. After addition of a solution of 3000 d.p.m. [4-^{14}C]DHAS in 10 μl ethanol, steroid sulphates were solvolysed as described by Burstein & Lieberman (1958).

Initial chromatography. The material obtained by extraction with ether or after solvolysis was chromatographed on paper in the Bush A_2 system – light petroleum (b.p. 60–90 °C):methanol:water (10:7:3, by vol.) – until the solvent front reached the end of the chromatogram.

After scanning for radioactivity the zone corresponding in mobility to authentic DHA was eluted with 10 ml methanol. The methanol was evaporated and the residue was submitted to t.l.c. on pre-coated silica gel plates in the solvent system benzene:ethanol (9:1, v/v). Authentic DHA (10 μg), which had been chromatographed in a parallel lane, was located with concentrated sulphuric acid. The corresponding zone from the analysed sample was eluted by mixing with 1 ml benzene, followed by addition of 0·2 ml water and repeated mixing. The mixture was centrifuged and the benzene layer was transferred to another tube. The water layer was extracted with benzene (2 × 1 ml). The combined benzene extracts were taken to dryness.

Oxidation and purification. The t.l.c. fraction was oxidized by addition of 0·1 ml CrO_3 in 90 % acetic acid (5 mg/ml). After 10 min, 1 ml water was added and the

mixture extracted with ethyl acetate (2 × 2 ml, 1 × 1 ml). The combined ethyl acetate layers were washed twice with 1 ml water and taken to dryness. The residue was submitted to t.l.c. on silica gel (0·25 mm on glass plates) in the system benzene: ethanol (9:1, v/v). Authentic 4-androstene-3,6,17-trione (10 μg) in reference lanes was located under u.v. light (254 nm). The corresponding zones of the sample lanes were eluted as described above and the combined benzene extracts were taken to dryness.

Gas–liquid chromatography and measurement. The material from the second t.l.c. was dissolved in 50 μl benzene, and 5 μl of this solution was submitted to g.l.c. An approximate estimate of the amount of 4-androstene-3,6,17-trione in the sample was made by comparing the peak height with that obtained after injection of a known amount of the authentic material. A more accurate estimate was then made by mixing the remaining material with an approximately equal amount of 4-pregnene-3,6,20-trione. Part of this mixture was taken for measurement of radioactivity and another part was submitted to g.l.c. The area under each peak was determined as the product of peak height and width at half height. The amount of DHA present in the original sample was then calculated using the formula

$$\text{ng DHA} = 100\,AUCf/R$$

where: A = ng 4-pregnene-3,6,20-trione added; U = ratio of peak areas 4-androstene-3,6,17-trione:4-pregnene-3,6,20-trione in sample; C = ratio of peak areas 4-pregnene-3,6,20-trione:4-androstene-3,6,17-trione for equal weight amounts of both steroids; f = ratio of molecular weights DHA: 4-androstene-3,6,17-trione = 0·96; R = % recovery of radioactive DHA.

Estimation of cortisol

Plasma cortisol was estimated using a modification of the method of Murphy (1967). All samples were assayed in duplicate with different amounts of plasma (10 and 20 μl). To each sample 1 ml ethanol was added. After centrifugation the ethanolic layer was decanted and the protein residue washed with 0·5 ml ethanol. The combined ethanolic extracts were taken to dryness under a stream of nitrogen. Then, 200 μl of an approximately 50 times diluted pregnancy plasma containing 40000 d.p.m. [1,2-^{3}H]corticosterone, were added. After mixing with a Vortex mixer, the mixture was shaken for 1 h at 4 °C. Florisil (3 mg) was then added and the mixture shaken at the same temperature for another 20 min. A sample (100 μl) of the supernatant was taken for the measurement of protein-bound radioactivity. The amount of cortisol was estimated from a calibration curve which was simultaneously prepared with amounts of cortisol varying between 0 and 5 ng. The smallest amount of cortisol detectable was 1 ng. Recoveries for standards containing 1–5 ng cortisol were 95 ± 6·8 (S.D.) % (n = 34). The standard deviation calculated from 47 pairs of duplicate results (range 6–20 μg cortisol/100 ml plasma) was 1·6 μg/100 ml plasma. The specificity of this method is not high, as shown by Murphy (1967). Nevertheless, we found a good correlation (correlation coefficient is 0·83; n = 69) between results obtained with this method and those obtained by fluorimetric measurement of cortisol after column chromatography (Eechaute, 1966).

RESULTS

Reliability of DHA estimation

Specificity

The specificity of the present method for the estimation of DHA depends on its specific oxidation to 4-androstene-3,6,17-trione and on the chromatographic steps before and after oxidation. Only steroidal 5-en-3-ols are oxidized to electron capturing compounds. Neither 4-ene-steroids nor steroids saturated at position 5 are converted to derivatives which can be detected sensitively with the electron capture detector.

DHA is separated from other 5-en-3-ols during the chromatographic steps preceding oxidation; R_F values for the relevant compounds are given in Table 1. After oxidation, 4-androstene-3,6,17-trione is separated from other oxidation products and unreacted DHA during the second t.l.c. as shown in Table 1. After purification and oxidation of plasma extracts, the g.l.c. tracings never showed peaks other than those attributable to 4-androstene-3,6,17-trione.

Table 1. *R_F values of steroidal 5-en-3-ols and of the corresponding 4-ene-3,6-diones in the chromatographic systems used*

Steroid	R_F values: p.c. Bush A_2	R_F values: t.l.c.* Benzene: ethanol (9:1, v/v)
DHA	0·26	0·39
5-Androstene-3β,17β-diol	0·00	0·33
5-Androstene-3β,17α-diol	0·00	0·34
3α-Hydroxy-5-androsten-17-one	—	0·49
3β-Hydroxy-5-pregnen-20-one	0·49	0·42
5-Pregnene-3β,20β-diol	—	0·34
5-Pregnene-3β,20α-diol	—	0·33
3β,17α-Dihydroxy-5-pregnen-20-one	0·00	0·35
4-Androstene-3,6,17-trione	—	0·53
4-Pregnene-3,6,20-trione	—	0·56

* Partly from Lisboa (1965, 1969).
DHA = dehydroepiandrosterone; p.c. = paper chromatography; t.l.c. = thin-layer chromatography.

In order to ensure that the detection of the small amounts of unconjugated DHA did not result from hydrolysis of DHAS during the extraction of unconjugated steroids, repeated ether extraction of a plasma sample containing [4-^{14}C]DHAS after freezing and thawing of the sample was performed. Radioactivity could not be detected in the ether extracts.

Sensitivity

The relative responses of the electron capture detector to equimolar amounts of 4-androstene-3,6,17-trione, 4-pregnene-3,6,20-trione and some other steroid derivatives containing chloro or fluoro atoms are shown in Table 2. As shown in Fig. 1, 100 pg of either 4-androstene-3,6,17-trione or 4-pregnene-3,6,20-trione can easily be detected. The mean blank value for bovine serum albumin solution was $0{\cdot}23 \pm 0{\cdot}38$ ng ($n = 37$). The overall recovery of radioactive DHA for the method was $27{\cdot}9 \pm 8{\cdot}8$ %

Table 2. *Relative molar responses (RMR) of different electron capturing derivatives of some steroids*

(A tritium electron capture detector was used)

Steroid	RMR
Testosterone 17β-chloroacetate	≡ 100
Testosterone 17β-heptafluorobutyrate	500
DHA 3β-chloroacetate	37
DHA 3β-heptafluorobutyrate	100
4-Androstene-3,6,17-trione	500
4-Pregnene-3,6,20-trione	550

DHA = dehydroepiandrosterone.

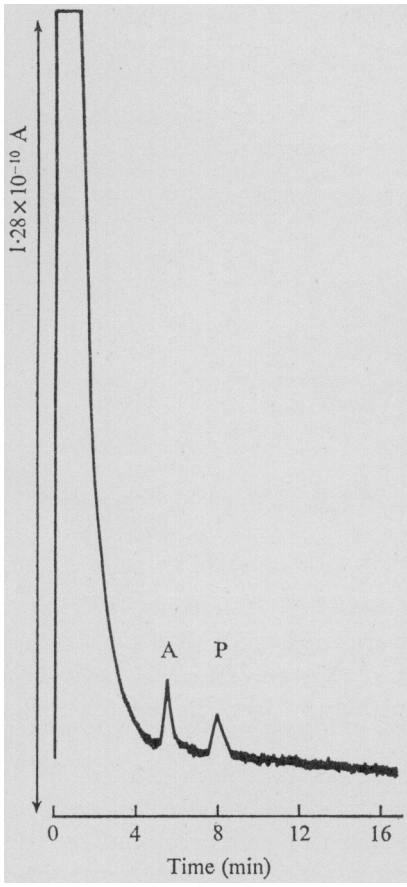

Fig. 1

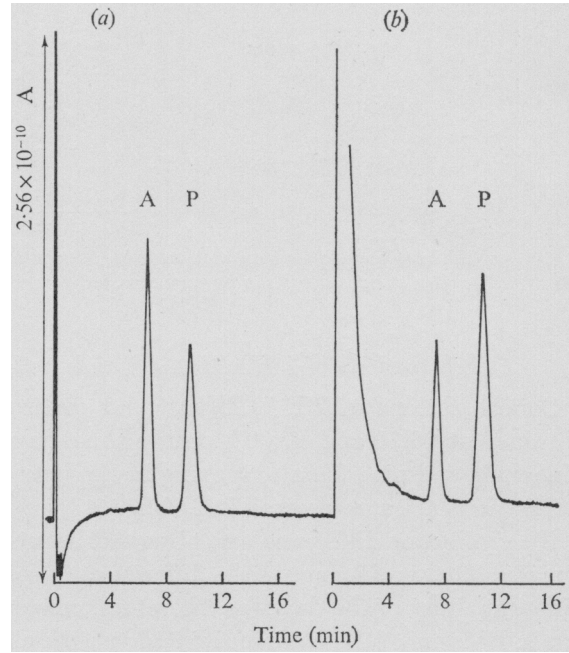

Fig. 2

Fig. 1. Gas–liquid chromatography of 100 pg 4-androstene-3,6,17-trione (A) and 100 pg 4-pregnene 3,6,20-trione (P). Retention times, relative to 5α-cholestane, are 17 for androstenetrione and 25 for pregnenetrione.

Fig. 2. Gas–liquid chromatography (g.l.c.) of: (*a*) a standard mixture of 1·6 ng 4-androstene-3,6,17-trione (A) and 1·6 ng 4-pregnene-3,6,20-trione (P); and (*b*) 4-androstene-3,6,17-trione (A), obtained after oxidation of unconjugated dehydroepiandrosterone isolated from 4 ml human peripheral plasma. 4-Pregnene-3,6,20-trione (P) (10 ng) was added as an internal standard and one-third of the sample was used for g.l.c.

(n = 56). Therefore, the lowest amount of DHA, which can significantly be distinguished from zero, was calculated to be 4 ng per sample. This sensitivity allows estimation of DHA in 1 ml plasma samples, whilst DHAS can be measured in 10 μl plasma. Since accuracy and precision are relatively low at this level, unconjugated DHA was routinely estimated in 5 ml plasma and the sulphate in 250 μl plasma. A typical g.l.c. tracing of the material isolated from a plasma sample is shown in Fig. 2.

Accuracy and precision

A linear relationship exists between the peak area and the injected amount of 4-androstene-3,6,17-trione or 4-pregnene-3,6,20-trione within the range 0–3 ng. Mean values of replicate assays of a 5 % bovine serum albumin solution to which 1, 10 and 100 ng DHA were added, and those of a plasma pool are shown in Table 3.

Table 3. *Accuracy and precision in the estimation of dehydroepiandrosterone (DHA) using gas–liquid chromatography of 4-androstene-3,6,17-trione*

(Known amounts of DHA were added to a 5 % bovine serum albumin solution and the mixture was processed as in the standard method)

ng DHA added	ng DHA found ± S.D.	Coefficient of variation (%)*
1† (12)	1·08 ± 0·22	19
10 (35)	9·6 ± 1·45	15
100 (13)	95 ± 11	12
10 ml plasma (8)	12·4 ± 0·8	6·5

* Coefficient of variation (%) $= \frac{\text{S.D. (in ng)}}{\text{ng DHA found}} \times 100.$

† Estimations without extraction from plasma and first chromatographic steps. Number of estimations in parentheses.

Concentrations of DHA, DHAS and cortisol in human peripheral plasma

Concentrations of DHA, DHAS and cortisol were measured in plasma samples obtained at 08.30 and 17.00 h on the same day from 18 normal healthy individuals, nine male and nine female, varying in age between 18 and 35 yr. The results of these estimations are summarized in Table 4.

No significant difference was observed between the results obtained for males and females. The significance of the differences in concentration between samples taken at different times of the day was, therefore, calculated for the whole group of 18 individuals. Because of the large individual variation of values obtained at the same time of day by each assay (Fig. 3.), it was not possible to apply a test of significance directly to the mean values of the concentrations that were measured. Hence, the ratio of the concentration at 17.00 h to that at 08.30 h was calculated in each case. Mean ratios and their standard errors are plotted in Fig. 4. All mean ratios are significantly different from unity using Student's t-test (Snedecor & Cochran, 1967). The levels of cortisol and DHA at 17.00 h were lower than at 08.30 h ($P < 0{\cdot}001$ and $P < 0{\cdot}005$, respectively) while the concentration of DHAS at 17.00 h was higher than at 08.30 h ($P < 0{\cdot}025$).

Table 4. *Concentrations (μg free steroid/100 ml plasma) of dehydroepiandrosterone (DHA), DHA sulphate (DHAS) and cortisol in human peripheral plasma at different times of the day (means ± S.D.)*

Steroid	Time (h)	Males (9)	Females (9)	Total (18)
DHA	08.30	0·51 ± 0·27	0·48 ± 0·25	0·50 ± 0·25
	17.00	0·35 ± 0·22	0·30 ± 0·10	0·32 ± 0·17
DHAS	08.30	78 ± 37	78 ± 45	78 ± 40
	17.00	84 ± 35	83 ± 34	84 ± 34
Cortisol	08.30	16·4 ± 2·1	16·9 ± 1·5	16·7 ± 1·8
	17.00	12·7 ± 4·3	11·2 ± 3·3	11·9 ± 3·8

Number of subjects in parentheses.

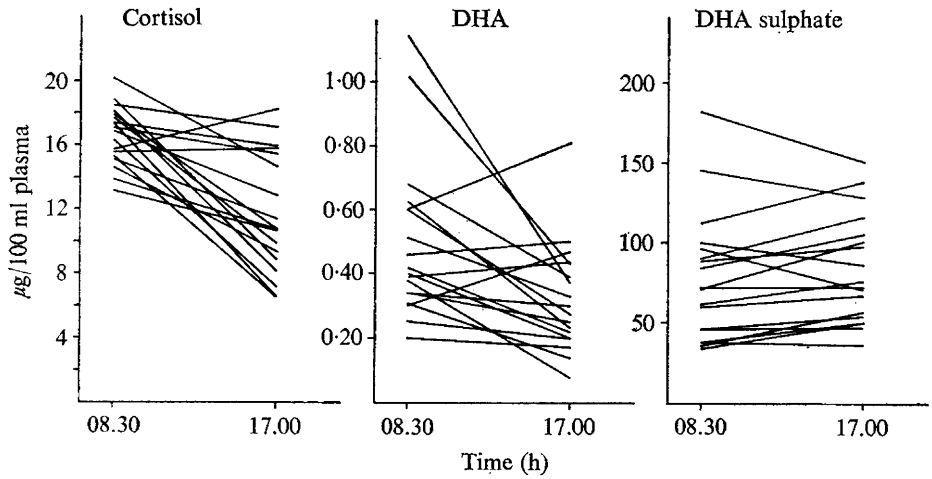

Fig. 3. Concentrations of cortisol, dehydroepiandrosterone (DHA) and DHA sulphate in peripheral plasma of normal men and women at 08.30 and 17.00 h.

DISCUSSION

The present results show that the oxidation of DHA to 4-androstene-3,6,17-trione followed by electron capture detection of the trione after g.l.c. can be utilized for the estimation of DHA. The sensitivity, accuracy and precision of this method are satisfactory for the estimation of both DHA and DHAS in peripheral human plasma. The specificity of the method for estimation of DHA in plasma is difficult to evaluate because of its very low concentration. Both 5-en-3α-ols and 5-en-3β-ols are oxidized to 4-ene-3,6-diones. The R_F values compiled by Lisboa (1965) make it unlikely, however, that the samples were contaminated with 3α-hydroxy-5-androsten-17-one before oxidation. This compound occurs in small amounts in plasma from some normal individuals as shown by Jänne, Laatikainen, Vainio & Vihko (1969). Therefore, efficient separation of DHA from other steroids containing a 5-en-3-ol fragment combined with its specific oxidation should exclude the possibility of interference by other steroids.

Recoveries of DHA are rather low. This is partly due to the low yield of 4-androstene-3,6,17-trione (60% for nanogram amounts). Variation of oxidation time or

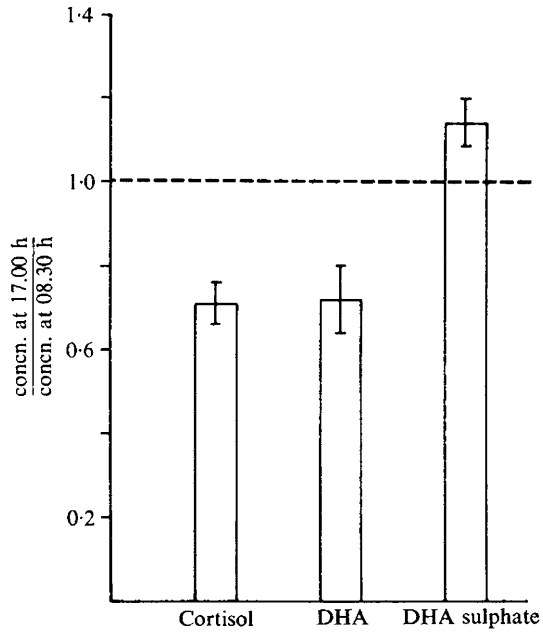

Fig. 4. Mean values of the ratios of the concentrations of dehydroepiandrosterone (DHA), DHA sulphate and cortisol in plasma samples from the same person taken at 08.30 and 17.00 h. The vertical lines indicate ± S.E.M.

Table 5. *Mean concentrations (μg free steroid/100 ml plasma) of dehydroepiandrosterone (DHA) and DHA sulphate (DHAS) in human peripheral plasma measured by different methods*

		Males		Females	
Reference	Method*	DHA	DHAS	DHA	DHAS
Goldfien *et al.* (1965)	g.l.c.–FID	—	—	0·73 (22)	—
Rivarola *et al.* (1967)	DID	0·46 (8)	—	0·53 (6)	—
Kumari *et al.* (1969)	g.l.c.–EC	—	—	0·66 (24)	32·6 (10)
Heyns & de Moor (1970)	CPB	0·49 (4)	—	0·76 (4)	—
Saxena & Gandy (1970)	DID	0·60 (11)†	167 (11)†	—	—
Migeon *et al.* (1957)	colorimetry	—	48 (24)	—	—
Yamaji & Ibayashi (1969)	g.l.c.–FID	—	165 (25)	—	109 (20)
Vihko (1966)	g.l.c.–FID	—	99·7 (62)	—	60·4 (54)
Wang, Bulbrook, Thomas & Friedman (1968)	g.l.c.–FID	—	122 (41)	—	107·5 (51)
Present investigation	g.l.c.–EC	0·51 (9)†	78 (9)†	0·48 (9)†	78 (9)†

* g.l.c.–FID = gas–liquid chromatography with flame ionization detection; g.l.c.–EC = gas–liquid chromatography with electron capture detection; DID = double isotope derivative technique; CPB = competitive protein binding.

† Concentrations at 08.00 or 08.30 h.

Number of subjects in parentheses.

acetic acid concentration did not change the amount of 4-androstene-3,6,17-trione obtained (F. H. de Jong & H. J. van der Molen, unpublished observations). Fieser & Fieser (1959) and Menini & Norymberski (1962) report lower yields for the conversion of milligram amounts of steroidal 5-en-3-ols to 4-ene-3,6-diones using different oxidation procedures. Attempts to increase the recoveries through omission of some of the purification steps have been unsuccessful. If the first t.l.c. step is omitted, a number of g.l.c. peaks are found due to impurities eluted from the preceding paper chromatogram or to their oxidation products. Washing of the paper in a Soxhlet apparatus with methanol for 72 h overcomes this difficulty. However, omission of the t.l.c. step is not advisable, since paper chromatography generally gives poor separation of 3α- from 3β-hydroxy compounds (Neher, 1964). If, on the other hand, the paper chromatographic step is omitted, it is difficult to obtain a good separation of 3β-hydroxy-5-pregnen-20-one from DHA, which is required because pregnenolone would be oxidized to 4-pregnene-3,6,20-trione which is used as an internal standard during g.l.c. Partitioning between ether and alkali separates 4-androstene-3,6,17-trione from 4-pregnene-3,6,20-trione (Menini & Norymberski, 1962), but the recovery of the 4-androstene-3,6,17-trione in this step is lower than during paper chromatography. Using the present method for the determination of DHA, 16 samples including standards and blanks can be processed in 5 days by one technician.

It appears from the results in Table 5, that concentrations of DHA and DHAS estimated by the present method agree well with those found by a number of other authors. There was a good correlation between the variations of the concentrations of cortisol and unconjugated DHA in plasma throughout the day. Similar observations were reported by Saxena & Gandy (1970). Recently Rosenfeld, Hellman, Roffwarg, Weitzman, Fukushima & Gallagher (1971) reported that DHA was secreted episodically and synchronously with cortisol. These authors have measured the plasma steroids at 20-min intervals and emphasize the finding of a sequence of intermittent secretory episodes in the early morning as well as in the late afternoon. In agreement with our results, they also observed that peak values of DHA in the morning were higher than those in the afternoon.

The finding of a reversed diurnal rhythm for DHAS as compared with that of DHA and of cortisol was unexpected. It is in apparent disagreement with the findings of Migeon, Keller, Lawrence & Shepard (1957), Eik-Nes, Oertel, Nimer & Tyler (1959) and Gandy & Saxena (1970), who observed variations of DHAS concentrations during the day, with the highest levels at 08.00 h. De Moor & Heyns (1966) and Lamb, Dignam, Pion & Simmer (1964) could not detect a diurnal variation in plasma levels of DHAS. In agreement with our observations, Laatikainen & Vihko (1968) found, in six males, levels of DHAS that were lower at 08.00 h than at 12.00 or 16.00 h. They did not correlate these observations with the concentration of plasma cortisol.

The finding of highest plasma DHAS levels at 17.00 h may be explained by the fact that in the study of Laatikainen & Vihko (1968), as well as in the present investigation, blood was taken from persons who were carrying on with their daily work. These persons, being in the upright position, might have had a different hepatic blood flow and metabolic clearance rate from subjects in recumbent position (Tait & Burstein, 1964). Since in the present study plasma levels of cortisol and unconjugated DHA followed the expected pattern of diurnal adrenal function, a possible difference in

posture can hardly explain the different diurnal rhythm for DHAS in plasma. A variation of peripheral sulphation of DHA during the day also does not appear to offer an explanation: the results of Chapdelaine, MacDonald, Gonzalez, Gurpide, VandeWiele & Lieberman (1965) show that the peripheral conversion of the sulphate into the free steroid is larger than the reverse reaction. The most likely explanation for the difference in diurnal rhythm between plasma levels of unconjugated and conjugated DHA may be a difference in their secretion rates by the adrenal throughout the day. It has not been possible to demonstrate a pituitary hormone which specifically stimulates adrenal biosynthesis of DHA independent of the synthesis of DHAS, in spite of pertinent investigations (Milner & Mills, 1970). Therefore differences between the secretion rates of the two steroids might result either from a difference in adrenal sulphation of DHA during the day under the influence of corticotrophin (ACTH), as shown for the rat by Dominguez (1970), or from a difference in permeability of the adrenal cell membranes for DHA and DHAS. This difference in membrane permeability for free and sulphated steroids has been suggested to occur in the model system of the erythrocyte by Mulder, Lamers-Stahlhofen & van der Molen (1972). A slow transport of DHAS through the adrenal membrane could also account for the slow increase of the DHAS concentration in adrenal venous blood after administration of ACTH as shown by Vaitukaitis, Dale & Melby (1969).

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