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THE IDENTIFICATION, QUANTIFICATION AND POSSIBLE ORIGIN OF NON-POLAR CONJUGATES IN HUMAN PLASMA

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Summary—The existence and quantification of non-polar conjugates of pregnenolone, dehydroepiandrosterone (DHA) and androstenediol in human plasma is described. The plasma level of non-polar pregnenolone conjugate is 200% higher than that of pregnenolone but the conjugates of DHA and androstenediol are 10 and 5–10% respectively of the plasma levels of the unconjugated steroid. Non-polar pregnenolone conjugate concentrations were found to be highly elevated in the plasma of one pregnant subject, and elevated in the plasma of patients with acne and breast cancer. Non-polar DHA conjugate levels were significantly elevated in hirsute patients and were approaching significance for patients with acne. A subject taking the combined oral contraceptive pill had very low plasma DHA conjugate levels. No significant alterations in the plasma levels of the androstenediol conjugates were found. A role for the non-polar conjugates in the aetiology of hirsutism and acne is proposed.

INTRODUCTION

The existence of non-polar conjugates of steroids was first described by Hochberg *et al.*[1] with the demonstration of a non-polar derivative of pregnenolone in extracts of bovine adrenal cortex. Since this initial report there have been further papers on the presence of non-polar derivatives of both 5α-pregnan-3β-ol-20-one and dehydroepiandrosterone (DHA) in extracts of bovine corpora lutea and adrenal [2, 3]. The non-polar characteristics of these steroids are derived from conjugation of the steroid to a fatty acid [2].

In the literature there are reports on other non-polar conjugates formed during *in vitro* incubations, non-polar corticosteroid formed by incubation of corticosteroid with acini from lactating rat mammary gland [4], and non-polar derivatives of oestradiol and androsterone by incubation with human breast tumour tissue [5, 6]. Non-polar conjugates of dehydroepiandrosterone sulphate (DHAS) have been reported by Oertel and Benes[7], which were formed by incubation of DHAS with human erythrocytes.

To date there has only been one report of the measurement of a non-polar conjugate in human plasma [8], in which plasma oestradiol concentrations in normal women and women on human chorionic gonadotrophin therapy were shown to be increased when measured before and after saponification of a non-polar fraction of an ether extract of plasma.

During the course of our work we noticed the formation of non-polar metabolites after incubation of steroid with whole blood. Saponification of the non-polar metabolite resulted in the recovery of the incubated steroid. Due to the possible biological significance of these compounds we have attempted to prepare and isolate the non-polar conjugates of a number of steroids present in human plasma, and to investigate the plasma level of these conjugates in normal subjects and patients with acne, hirsutism or breast cancer.

EXPERIMENTAL

[7-^{3}H]Pregnenolone, [1,2,6,7-^{3}H]DHA, [2,4,6,7-^{3}H]oestradiol, [1,2,6,7-^{3}H]androstenedione, [1,2,6,7-^{3}H]testosterone, [2,4,6,7-^{3}H]oestrone and [1,2,4,5,6,7-^{3}H]dihydrotestosterone were obtained from Amersham International PLC. (Amersham, England). [1,2-^{3}H]5-Androstene-3β,17β-diol and [7-^{3}H]DHAS were obtained from New England Nuclear (Dreieich, W. Germany). Labelled steroids were regularly tested for purity using thin layer or paper chromatography and purified if necessary. Stock solutions of labelled steroid were stored in ethanol at 4°C. Silica gel thin layer chromatography (TLC) plates (No. 5333) were obtained from Merck (Darmstadt, Germany). Scintillator was prepared from 12 g *p*-terphenyl, 0.16 g dimethyl POPOP and 80 ml methanol made to 4 l with toluene. All other solvents and chemicals were obtained from Fisons Ltd, Loughborough, England.

Steroid radioimmunoassays

DHA was measured with a specific antiserum raised in rabbits against a DHA-7-carboxymethyloxime–bovine serum albumin (CMO–BSA) derivative using a Dextran coated charcoal (DCC) separation using a standard curve covering the range 10–1280 pg/assay tube. Assay sensitivity was 9 pg per assay tube, which is equiv-

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alent to 2.2 ng/100 ml when corrected for assay recovery.

Pregnenolone was measured using an antiserum raised against pregnenolone-3-CMO–BSA using DCC to separate the bound and free fraction, using a standard curve covering the range 10–1000 pg/assay tube. Water blanks put through the assay procedure gave values of between 15 and 20 pg/assay tube. This is equivalent to a plasma value of 14 ng/100 ml. The results quoted for the pregnenolone conjugates shown in Table 4 have had the blank value subtracted.

Both assays have shown quantitative recovery of added unlabelled steroid from plasma samples and demonstrated linearity over a range of plasma volumes.

With samples containing recovery counts, the assay standard curve was corrected with the equivalent amount of labelled steroid as that found for the mean recovery count. To samples with a recovery differing by more than 10% from the mean, a compensating quantity of tritiated steroid was added to bring the amount of radioactivity present to the same level as the mean recovery.

The androstenediol and oestradiol assays were as described by Bonney *et al.*[9] and Braunsberg *et al.*[10]. The assay sensitivity for the androstenediol assay was 2.7 pg/assay tube which is equivalent to a plasma level of 1 ng/100 ml.

Specificity of steroid assays

For each steroid assay, specificity is conferred by the use of two TLC purification stages prior to assay as described subsequently, and the use of specific antisera for each radioimmunoassay. For pregnenolone, the cross-reactions are: progesterone: <5%; 17-hydroxypregnenolone: <5%; DHA <0.02%; androstenedione: <0.005%. For DHA: 16-hydroxy DHA, 3%; 3β,17β-androstenediol, 1.3%, androstenedione, 0.7%.

Preparation of tritiated non-polar conjugates

Non-polar conjugates were prepared by incubation of plasma or whole blood (2–3 ml) with 1×10^6 or 2×10^6 dpm of pure tritiated steroid in 50 μl of distilled water for 2–60 h at 37°C.

After incubation an equivalent volume of distilled water was added to the whole blood samples to lyse the cells. The non-polar conjugates were extracted with 2 × 10 ml of ethyl acetate, the extracts pooled, evaporated to dryness under nitrogen and the residue applied to silica gel TLC plates. The plates were developed in hexane–diethyl ether–glacial acetic acid (80:20:1, by vol) and the radioactive areas located with a Panax chromatogram scanner. The mobility of the steroids and the non-polar conjugates using this system are shown in Table 1.

The areas of radioactivity which ran in front of the parent steroid were excised and eluted with 10 ml of diethyl ether for 16 h. After elution the ether was decanted, evaporated to dryness under nitrogen and the residue reconstituted in 1 ml of chloroform. Control incubations with saline were performed for each steroid investigated.

Table 1

Compound	Mobility R_f
Pregnenolone	0.03
N-P-pregnenolone	0.35
DHA	0.03
N-P-DHA	0.25
A-5-diol	0.01
N-P-A-5-diol	0.14
E_2	0.03
N-P-E_2	0.18

N-P = non-polar conjugate.
A-5-diol = androstenediol.
Mobility of the conjugates and parent steroids in hexane–diethyl ether–acetic acid (80:20:1, by vol), run on silica gel TLC plates.

To determine which component of blood produces the non-polar conjugates, a fresh lithium heparin blood sample was taken and divided into four 3 ml aliquots. Two aliquots were centrifuged and the plasma decanted. The cells were washed with 3 × 2 ml of sterile normal saline, the saline being decanted and discarded after each wash and centrifugation. The volume of the washed cells and the decanted plasma were made to 3 ml with sterile normal saline. The whole blood, washed cells and plasma were incubated with either [^{3}H]pregnenolone or [^{3}H]DHA at 37°C for 3 h.

The non-polar conjugates prepared by these methods have been stored in chloroform for periods of up to 2 months with no detectable decomposition to the parent steroid. These compounds are stable when incubated with either lithium heparin or EDTA plasma for 16 h at 37°C.

Saponification of non-polar conjugates

The saponification method of Smith[11] was used to recover the parent steroid from the non-polar conjugate. Aliquots of tritiated non-polar conjugate in chloroform were taken, evaporated to dryness under nitrogen and 200 μl of distilled water added to the residue. One ml of 2 M potassium hydroxide in ethanol–water (90:10, v/v) was added and the tube contents mixed prior to incubation at 60°C for 40 min. When cool 1 ml of distilled water was added to each tube, the contents mixed and the steroid extracted with 10 ml of diethyl ether. The aqueous phase was frozen in an ethanol–dry ice bath and the ether decanted. The ether was blown to dryness under nitrogen and the residue applied to silica gel TLC plates with 2 × 100 μl of diethyl ether. The plates were developed with chloroform–acetone (85:15, v/v). The area of tritiated steroid was located and eluted with 5 ml of diethyl ether for at least 2–3 h. After elution the ether was decanted into plastic 5 ml scintillation vials and evaporated to dryness in a vacuum oven. The residue was reconstituted in 3 ml of toluene based scintillator and counted for recovery.

Recovery of pregnenolone and DHA after saponification and TLC was 80% for both DHA and pregnenolone. Tritiated pregnenolone and DHA extracted after saponification of the labelled non-polar conjugates had identical Rf values to authentic tritiated steroid run on the same plate.

Measurement of non-polar conjugates in plasma

Plasma (1 or 2 ml) was added to stoppered glass tubes containing 10,000 cpm of the tritiated non-polar conjugates of DHA and pregnenolone prepared as described above. The tube contents were Vortex mixed and allowed to equilibrate for 30 min prior to extraction with 2 × 10 ml of ethyl acetate. Each extraction was for 10 min on a mechanical shaker, followed by centrifugation at 200 *g* for 10 min. The supernatants were decanted and pooled for each sample. A third extraction with 10 ml of diethyl ether was performed and the organic phase was decanted after freezing the aqueous phase and combined with the pooled aliquots.

The extracts were blown to dryness in a water bath at 45°C under nitrogen. The residue was applied to silica gel TLC plates with 100 μl of chloroform and 100 μl of diethyl ether. The plates were developed twice to improve separation of the non-polar conjugates from free steroid in hexane–diethyl ether–glacial acetic acid (80:20:1, by vol). The tritiated non-polar conjugate added for recovery estimation enabled the exact location of the conjugates on the TLC plate and these areas were excised and eluted with 10 ml of diethyl ether for 16 h.

After decanting, the ether was blown to dryness under nitrogen and the non-polar conjugate saponified and run on TLC as described above. The residue remaining after these stages contained the liberated steroid and was reconstituted in redistilled ethanol. The reconstitution volumes were pregnenolone 1 ml, DHA 600 μl and androstenediol 500 μl.

Aliquots of 100 μl were taken for recovery estimation, evaporated to dryness in a vacuum oven and 1.7 ml of toluene based scintillator added prior to counting. Recoveries of labelled DHA, pregnenolone and androstenediol taken through the above procedures were 60.5, 51.5 and 46.1% respectively.

Duplicate aliquots of 200 μl were taken for each steroid estimation and were assayed by RIA.

RESULTS

The percentages of each incubated steroid converted to a non-polar conjugate are shown in Table 2. The figures represent mean values obtained from five incubations using different extraction solvents and incubation protocols. The pregnenolone conversion figure is underestimated due to a low recovery of labelled steroid and conjugate (30%) from the incubation mixtures. The extraction of pregnenolone using these solvents would be 80–90%, the 50% difference between the two figures may therefore represent unextracted non-polar conjugate. No polar conjugates have been detected for any of the incubations. The quantity of non-polar conjugate formed was dependent on the incubation time and was the same for male and female plasma.

Table 2

Steroid	% Conversion
Pregnenolone	20.5
DHA	12.4
Androstenediol	4.7
Oestradiol	0.2
Androstenedione	Nil
Testosterone	
Oestrone	
Dihydrotestosterone	
DHAS	

Mean percentage conversion of [^{3}H]steroids to non polar conjugates after incubation with plasma at 37°C. The results are the means of five experiments, with mean incubation time of 48 h.

Table 3

Steroid	% Conversion to non-polar conjugates		
	Whole blood	Plasma	Washed cells
Pregnenolone	29.4	15.6	2.9
DHA	5.4	4.4	1.1

The production of non-polar conjugates by each component of blood during one experiment is shown in Table 3.

The plasma concentrations of the non-polar conjugates of pregnenolone, DHA and androstenediol for normal subjects and patients with a range of endocrine disorders are shown in Table 4. The comparison and significance levels between the groups of patients are also shown. The values for the conjugates of androstenediol in normal premenopausal subjects were less than the lowest standard as only 1 ml of plasma was assayed.

Plasma levels of the non-polar conjugates of oestradiol were not significantly different to the water blank values (10 pg/ml) for all samples. In experiments in which labelled oestradiol was added to the plasma, saponification, extraction and chromatography performed, the recovery of oestradiol was 46%.

In one male subject blood samples were taken at 1200 and 1600 h. Non-polar conjugates of pregnenolone and DHA measured in these samples demonstrated a possible diurnal rhythm. Plasma levels of pregnenolone and DHA conjugate were 168 ng/100 ml and 86 ng/100 ml at 1200 h, and 214 ng/100 ml and 40 ng/100 ml at 1600 h respectively.

The plasma levels of the non polar conjugates reported for the postmenopausal and breast cancer subjects were obtained on plasma pools prepared from samples taken at 0830, 1000, 1600 and 2200, thus minimising the effects of diurnal rhythm. The remainder of the samples were taken singly at random times.

Table 4

Subjects	n	NP-pregnenolone ng/100 ml	NP-DHA ng/100 ml	NP-adiol ng/100 ml
Male (23–45 yrs)	9	210.6 + 50.8	47.1 + 29.2	10.8 + 2.9
Females				
Premenopausal (20–30 yrs)	5	139.4 + 47.3	47.2 + 15.5	< 6.4
Postmenopausal (53–79 yrs)	5	69.8 + 25.5	29.5 + 13.8	4.5 + 0.8
Pregnant (12 weeks)	1	> 1220.0	57.0	—
Acne	5	243.8 + 92.0**	81.8 + 38.0**	6.2 + 1.2
Hirsutism (idiopathic)	3	150.3 + 16.5*	157.3 + 17.0***	7.7 + 2.3
(PCO)	2	120/132	60/75	3.2/4.5
Eugynon 30	1	74.0	10.0	—
Postmenopausal breast cancer	3	118.3 + 33.0**	39.7 + 15.9*	5.0 + 0.6*

*$P > 0.1$, ** $= 0.05 < P < 0.1$, *** $= P < 0.001$.

Plasma levels for the non-polar (NP) conjugates of pregnenolone, DHA and androstenediol in normal and abnormal subjects. The significance levels are those obtained by comparison with the group of premenopausal women.

Plasma levels of the pregnenolone and DHA non polar conjugates are significantly lower in postmenopausal compared to premenopausal women ($0.01 < P < 0.02$), while plasma pregnenolone and probably androstenediol conjugate levels are lower in premenopausal women than in men, ($0.02 < P < 0.05$).

DISCUSSION

In this paper we have reported the existence in human plasma of non-polar conjugates derived from pregnenolone, DHA and androstenediol. The plasma levels of the non-polar conjugates in normal subjects are 200, 10 and 5–10% of the plasma pregnenolone, DHA and androstenediol levels respectively. The proportion of non-polar DHA conjugate to DHA is increased to 33% in women with idiopathic hirsutism but is only 11% for patients with polycystic ovarian disease, while in the subject taking the oral contraceptive Eugynon 30 (ethinyl oestradiol, 30 μg, levonorgestrel 250 μg), it is decreased to 2%. The plasma DHA conjugate level for the five hirsute subjects was significantly raised compared to the control group ($0.01 < P < 0.02$). The greatest alteration in amount of conjugate to non conjugate, > 600%, is shown by the pregnant subject for pregnenolone conjugate to pregnenolone, but as this is only based on one value, more are needed to verify this finding.

In contrast to the report by Janoko and Hochberg[8] we were unable to detect any non-polar conjugates of oestradiol in human plasma. As the plasma levels of the conjugate reported by Janoko should have been measurable using our method it is surprising that we did not detect any. Possible reasons for this may be that due to the lack of a tritiated conjugate tracer the area of the TLC plate containing the conjugate may have been missed.

The degree of specificity demonstrated by plasma for the production of the non-polar conjugates suggests the presence of a specific, or group of specific enzymes in human plasma. It is not known if the 21-hydroxy grouping of the corticosteroids will form a non-polar conjugate although the report by Hampel *et al.*[4] would suggest this possibility. Whether the alterations in the ratio of plasma conjugate to steroid level for specific steroids represents the actions of different enzymes or one enzyme with a different K_m value for each steroid is not known.

The possibility that plasma is the only production site for these conjugates in the human is unlikely. From the work of Hochberg *et al.*[1] and Albert *et al.*[2] on bovine adrenal and corpora lutea it is probable that these tissues could also produce non-polar conjugates in the human. Whether these tissues would demonstrate a similar degree of specificity for steroid conjugation to that seen in plasma is not known.

From the work of Albert *et al.*[2] and Mellon-Nussbaum *et al.*[12] it is anticipated that the non-polar conjugate is formed between the steroid and a fatty acid, although the work of Oertel and Benes[7] suggests the possibility of conjugation to a diglyceride. The observation that each non-polar conjugate migrates as a single spot in a system designed to separate the lipid subgroups on TLC suggests conjugation to a single type of lipid. During the investigation of solvents for conjugate extraction, a gradient of extractions from iso-octane through diethyl ether to ethyl acetate and ethyl acetate–*n*-propanol 13:1, v/v) was used on the same plasma. Using iso-octane only a small proportion of the conjugate was extracted, the majority coming out with the latter two solvents. The efficiency of the different solvent extractions may indicate a range of different conjugated fatty acids or very tight binding of the conjugate by plasma proteins.

Giorgi[13] has demonstrated that the permeability of the cell membrane to steroid is inversely proportional to the polarity of the steroid, and that for the majority of steroids passage through the cell membrane occurs by passive diffusion. Due to the very non-polar nature of these conjugates the possibility is raised that they will have a greater solubility in the cell membrane than free steroid. In view of the elevated plasma levels of the pregnenolone and DHA conjugates in hirsute and acne subjects it is interesting to hypothesise an increased uptake of these steroids

by the skin. Mellon-Nussbaum *et al.*[12] has demonstrated that the lipoidal conjugates of pregnenolone can be metabolised by incubation with a mitochondrial-microsomal fraction from adrenal cortical tissue to lipoidal derivatives of 17-hydroxy pregnenolone and DHA. Whether the further metabolism of the lipoidal DHA to testosterone or oestrogen conjugates is possible is not known. Thomas and Oake[14] have shown that human skin is capable of metabolising DHA to testosterone and DHT and that the degree of DHA utilisation and conversion to the active androgens is greater in the skin of hirsute women than normal controls. This suggests the possibility that an increased uptake of the non polar conjugates of DHA or pregnenolone, followed by direct metabolism or cleavage to the parent steroid followed by metabolism to the active androgens may have a role in the aetiology of hirsutism.

It is interesting to speculate that the formation of the non-polar conjugates and their increased tissue uptake and metabolism may be a link between the increased risk of cancer and other illnesses associated with obesity and dietary lipid.

In conclusion we have demonstrated the existence and plasma levels of a new group of non-polar conjugates formed from pregnenolone, DHA and androstenediol by human plasma. An alteration in the plasma levels of the non-polar conjugates when compared to age matched controls has been demonstrated in patients with hirsutism, acne, breast cancer and subjects taking the combined oral contraceptive pill.

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