J. Steroid Biochem. Molec. Biol. Vol. 38, No. 3, pp. 389–398, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

THE DETERMINATION OF 11β-HYDROXYANDROSTENEDIONE IN HUMAN FOLLICULAR FLUID AND PLASMA

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(Received 9 November 1990)

Summary—The development of a chromatographic/immunoassay method is presented for the measurement of 11β-hydroxyandrostenedione (11β-OH-A4) in ovarian follicular fluid (FFL) and plasma from women undergoing embryo transfer for *in vitro* fertilization. This method incorporates high-performance liquid chromatography (HPLC) and permits the simultaneous measurement of other steroids from a single sample in order to assess the intraovarian environment. Authenticity of 11β-OH-A4 in follicular fluid was confirmed using selected ion monitoring (SIM) gas chromatography/mass spectrometry (GC/MS). Our results demonstrate a mean concentration of 18.6 nmol/l in follicular fluid compared with 3.2 nmol/l in plasma. The origin of 11β-OH-A4 in follicular fluid requires further investigation but these findings supports the hypothesis of ovarian 11β-hydroxylase activity on C_{19} steroids.

INTRODUCTION

11β-Hydroxylase activity is essential for the biosynthesis of glucocorticoid (C_{21}), mineralocorticoid (C_{21}) steroids [1]. 11β-Hydroxylation of the androgen androstenedione (A4), leading to the production of 11β-hydroxy-androstenedione (11β-OH-A4), has been considered to occur exclusively in the adrenal gland [2–6] although 11β-OH-A4 may also arise by side-chain cleavage of cortisol [7–9] (Fig. 1). The plasma concentration of 11β-OH-A4 is usually regarded as an indicator of adrenal androgenic function [3, 4, 10]. High concentrations of 11β-OH-A4 have been reported in the ovarian veins of adrenalectomized women, suggesting that this steroid is also made in the ovary [1].

Ovarian production of sex steroids (oestrogens, androgens) and progesterone occurs in granulosa and theca cells, depending on the stage of the menstrual cycle. 11β-Hydroxylase activity has been well documented in the ovary acting however only on C_{21} steroids [12–14]. High concentrations of both possible precursors, cortisol and androstenedione, have been described in follicular fluid [14, 15]. 11β-OH-A4 is a weak androgen [4, 16] which may be important in the regulation of active androgen production [16–20]. It was therefore considered important to prove whether 11β-OH-A4 is present within the ovary.

Previous studies have used direct/extracted radioimmunoassay methods which can be unreliable due to the presence of high concentrations of other cross reacting steroids [4, 5, 21]. This may be particularly unsatisfactory in the steroid rich environment of the ovary [22–24]. A procedure for solvent extraction and high-performance liquid chromatography (HPLC) separation was evaluated prior to measurement of steroids by radioimmunoassay to minimize potential cross reactants as well as providing the means for simultaneous measurement of steroids from a single sample [25, 26].

EXPERIMENTAL

Instrumentation

A Waters HPLC system was used (Waters Associates, Milford, Mass., U.S.A.) with a solvent degasser, dual pumps (6000 A), solvent programmer (660), autoinjector (WISP) and data module (M730). A Lichrosorb diol cartridge column (25 cm × 4.6 mm and 10 μm pore size) incorporating a guard column (Cat. No. 8749, obtained from Alltech Associates/Applied Science, Carnforth, Lancs., England) was maintained at 40°C by means of a column thermostat

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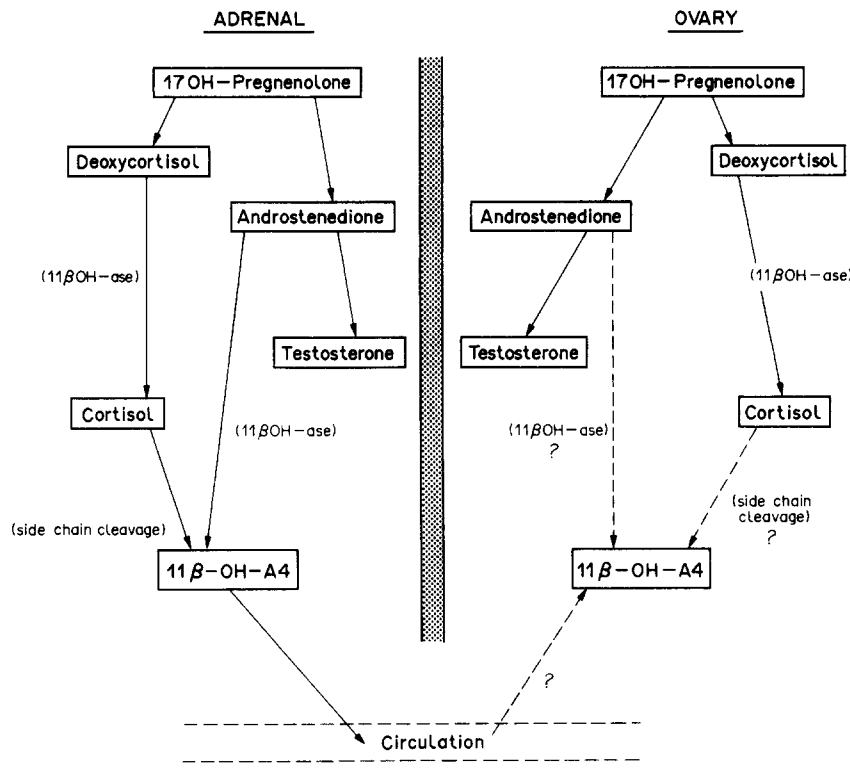

Fig. 1. Adrenal and ovarian biosynthesis of 11β-OH-A4.

(Model 7960, Jones Chromatograph Ltd, Mid-Glamorgan, Wales). Column eluates were collected using a fraction collector (LKB 7000 Ultra Rac) and a home made column switching device [27].

A quadrupole mass spectrometer (Hewlett–Packard 5970 benchtop MSD) of ionization energy 70 eV coupled to a gas chromatograph (HP 5890 GC) was used. The voltage at the electron multiplier was 2800 V and digital signals were processed and stored on a 40 Mbyte hard disc HP300 data system. The GC was equipped with a fused silica column (Chrompack CP-Sil-5, London, England), 25 m long and internal diameter of 0.32 mm). The liquid scintillation counter was a three channel Packard 3330.

Chemicals and reagents

All steroids were obtained from the MRC Steroid Reference collection. Tritium labelled steroids [1,2-^{3}H]cortisol (45 Ci/mmol), [1,2,6,7-^{3}H]androstenedione (90 Ci/mmol) and [1,2-^{3}H]-testosterone (47 Ci/mmol) were obtained from Amersham International (Aylesbury, Bucks., England).

The antisera for the 11β-OH-A4 assay was raised against a 11β-hydroxyandrostenedione-6β-hemisuccinyl-BSA conjugate [28]. Donkey anti-rabbit serum (DARS) and normal rabbit serum (NRS) were bought from Immunodiagnostics (Washington, England). The separation reagent consisted of a mixture of second antibody (DARS at a 1:100 dilution) and normal rabbit serum (at 1:1000 dilution) made up in 8% Polyethylene Glycol 7000 (PEG) assay buffer. Quality control samples (Lyphochek) were purchased from Biorad Laboratories (Watford, Herts., England). A commercial kit for 11β-OH-A4 was purchased from Intersci Diagnostics (Los Angeles, Calif., U.S.A.) Testosterone kits were purchased from St Thomas's Hospital (STRIA, Department of Chemical Pathology, London, England), Bioclinical Services Ltd (Cardiff, Wales) supplied cortisol kits and Guildhay Antisera Ltd (University of Surrey, Guildford, England) provided antisera for androstenedione.

Solvents and buffer reagents (pyridine, cyclohexane, dichloromethane, isopropanol, hexane, ethanol, methanol, toluene, citric acid, sodium dihydrogen/hydrogen phosphate, sodium azide, PEG, gelatin, sodium metabisulphite and sodium chloride) were bought from BDH Ltd (Dagenham, Essex, England; analar grade or where applicable HPLC grade). Sodium bismuthate and bovine serum albumin (BSA) were obtained

from Sigma Chemical Co. Ltd (Poole, Dorset, England). Lipidex-Tm-5000 was supplied by United Technologies Packard (Reading, Berks., England). Trimethylsilylimidazole (TMSI), methoxyamine hydrochloride (MO-HCl) and hexamethyldisilalzane (HMDS) were obtained from Pierce (Luton, Beds., England).

Sep-Pak cartridges (C_{18}) were purchased from Waters. Thin-layer chromatography (TLC) glass plates used were of dimensions 20 × 20 cm square and 0.25 mm (Merck, Kieselgel 60/ Kieselgur F-254) supplied by BDH. Scintillation fluid used was the biodegradable "Ecoscint" obtained from National Diagnostics (Manville, N.J., U.S.A.).

Synthesis of tritium labelled 11β-OH-A4 from cortisol

Tritium labelled 11β-OH-A4 was synthesized from commercially available cortisol label ([^{3}H]-cortisol) using the sodium bismuthate oxidation method [29]. The synthesis was performed on 120 μCi neat [^{3}H]cortisol label (specific activity 45 Ci/mmol). The final reaction products were purified by TLC separation on Kieselgel F-254 silica plate using a mobile phase of dichloromethane and methanol (97:3 v/v). Standards of cortisol and 11β-OH-A4 (100 ng) were run alongside, facilitating the detection by u.v. light of the required fraction. The area corresponding to 11β-OH-A4 ($R_f = 0.25$) was eluted into ethanol overnight at 4°C then further purified by HPLC. Fractions corresponding to the retention time of 11β-OH-A4 were collected. These pooled fractions were dried down and resuspended in toluene:ethanol (9:1) and stored at −25°C ready for use in subsequent radioimmunoassay. When required, an aliquot (250 μl) of this solution was removed, dried down and resuspended in assay buffer ready for each 100 tube radioimmunoassay giving approximately 10,000 counts/10 min/tube.

The synthesized label was verified after repeating the TLC separation using a small amount of the reaction mixture remaining after the sodium bismuthate oxidation step. After TLC separation the product was located by its radioactivity. After elution from the plate the product was also checked for immunoreactivity. This was accomplished by using a crude and unoptimized radioimmunoassay, measuring the percentage displacement between zero standard and a standard with 11β-OH-A4 present in excess (40 nmol/l).

Sample preparation

Steroids were extracted from a 200 μl aliquot of plasma or follicular fluid into 2 ml of dichloromethane. The upper layer was removed by aspiration and a fixed volume (1.8 ml) of the solvent was transferred into a tube and then dried down under oxygen free nitrogen (OFN). The dried residue from a follicular fluid sample was reconstituted in 300 μl of initial HPLC mobile phase of which 200 μl were injected onto the diol column. The initial mobile phase consisted of 5% isopropanol in hexane followed by a linear gradient to 20% IPA over 50 min at a flow rate of 1 ml/min. Equilibration time between runs was 30 min and the back pressure during operation never exceeded 1500 psi.

Recoveries were assessed after spiking parallel samples with the radioactively labelled (tritiated) steroids in question and repeating the extraction/ HPLC procedure as described above. Radioactivity was determined after extraction and HPLC separation. To ensure equivalence of quenching in subsequent liquid scintillation counting, a constant aqueous matrix was used prior to counting before and after extraction/ HPLC. 5 ml of "Ecoscint" were added after thorough mixing, vials were counted for 20 min.

Radioimmunoassay

For each sample, in addition to fraction collection for radioimmunoassay and recoveries, a blank run was performed in parallel. Here an injection of initial mobile phase was made followed by collection of fractions with retention times corresponding to the steroids in question. These fractions were used to assess non-specific interference in each of the steroid radioimmunoassay methods by any unknown material in the column eluates.

For the radioimmunoassay, each of the steroid fractions were resuspended in assay buffer by vortexing, sonication followed by a 30 min incubation at room temperature. To the 11β-OH-A4 fraction was added 700 μl of 50 mM sodium phosphate/saline buffer (pH = 7.4) containing 0.1% BSA and 0.1% sodium azide. A 300 μl aliquot was used for each radioimmunoassay duplicate. The 11β-OH-A4 fraction was assayed using a modification of our in-house radioimmunoassay method [26]. Samples (300 μl) or standards (in the range 0.2–15.0 nmol/l) were mixed with 11β-OH-A4 label (100 μl) and antisera (100 μl) at a working dilution of 1:15,000.

The reaction mixture was vortexed thoroughly and left to incubate overnight at room temperature. 500 μl of separation reagent was added followed by vortexing and a further incubation for at least 30 min. The incubation mixture was centrifuged at 1500 ***g*** for 30 min at 4°C after which 0.5 ml aliquots of supernatant were removed into scintillation vials followed by the addition of 5 ml of "Ecoscint" scintillant and vigorous mixing by vortex before counting. Sample results were calculated by using a four-parameter logistic curve fitting procedure incorporated in the computer programme (Mr P. Edwards, Middlesex Hospital). Final results were adjusted for known volume losses and measured recoveries.

Derivatization of follicular steroids

Follicular fluid (25 ml) was pooled from several ovarian follicles. Steroids were extracted with 250 ml of dichloromethane. The solvent was dried down in stages under OFN in a 100 ml round bottomed glass flask. This ensured a concentration of extracted material into the bottom of the flask. All subsequent reactions were carried out in silanized glassware.

The dried residues were subjected to solid phase extraction by reconstitution in 25 ml 11β-OH-A4 assay buffer and passage through a (C_{18}) Sep-Pak cartridge which had been previously primed with 2 ml ethanol followed by 5 ml water. The steroids retained on the cartridge were then eluted with 5 ml ethanol and dried in stages under OFN. A mixture of internal standards (androstanediol, stigmasterol and cholesteryl butyrate) at concentrations of 100 μg/ml were added to the dried residue and also dried down as before, This dried mixture was suspended in 200 μl 2% methoxyamine hydrochloride in pyridine. This was incubated at 60°C for 1 hr followed by an addition of 100 μl of trimethylsilyl imidazole and an overnight incubation at 100°C. Derivatized steroids were recovered after Lipidex chromatography as previously described [30]. A similar procedure was adopted using the 11β-OH-A4 fraction after HPLC separation of 25 ml follicular fluid samples.

Analysis by gas chromatography/mass spectrometry (GC/MS)

A total ion chromatogram (TIC) was obtained from the GC/MS analysis of derivatized steroids from follicular fluid. The gas chromatograph was run at a temperature ramped programme as follows. After sample injection the column was held for 2 min at 60°C. Thereafter the column was heated at 60°C per min to 200°C and then at 3°C per min to 280°C and held at 280°C for 9 min. The mass spectrometer was set to monitor and record all fragment and molecular ions produced after electron impact and quadrupole analysis of the gaseous gas chromatograph column effluent. An ion current chromatogram was constructed by computer which enabled the mass spectrum for each peak to be plotted. Derivatized steroids were identified by comparison with mass spectra of reference compounds. The main purpose of the TIC in this study was to approximate the concentrations of the major steroids in follicular fluid by response relative to the internal standards.

Selected ion monitoring (SIM) analysis by GC/MS

The acquisition parameters on the mass spectrometer data system were set up for the specific monitoring of the characteristic molecular ion of derivatized 11β-OH-A4 using a short GC temperature ramp programme of maintaining the column at 160°C for 2 min after sample injection then heating at 30°C per min to 260°C after which the column was held at 260°C for 10 min. The GC run was optimized using a 11β-OH-A4 standard derivative.

Patient samples

Plasma and follicular fluid was obtained from 40 women undergoing super-ovulation for *in vitro* fertilization/embryo transfer. Blood was taken at the time of oocyte collection and clear follicular fluid was obtained by ultrasound directed percutaneous needle aspiration [31, 32]. The samples were spun and the serum and clear follicular fluid were stored at −20°C for later analysis.

RESULTS

Preparative chromatography of steroids

Dichloromethane was found to be the most suitable solvent for the extraction of the steroids under study. Due to the wide range in polarities of the steroids, recoveries with this solvent ranged between 49% for androstenedione and 84% for 11β-OH-A4 (Table 1).

The normal phase HPLC system gave insignificant background binding in the subsequent radioimmunoassay of steroids separated from extracts of follicular fluid. A mixture of these steroids (each at a concentration of 2 ng/μl)

Table 1. Recoveries of steroids after extraction and HPLC (Mean (%) ± SD)

11β-Hydroxyandrostenedione	84% ± 20% (n = 27)
Testosterone	64% ± 23% (n = 22)
Androstenedione	49% ± 23% (n = 28)
Cortisol	68% ± 20% (n = 23)

(Fig. 2) was separated into constituent components and this enabled the collection, for subsequent radioimmunoassay, of multiple eluent fractions from a single sample. The recoveries of steroids (Table 1) were measured in parallel for every sample and were considered to be acceptable.

Synthesis of tritium labelled 11β-OH-A4 from cortisol

The synthesis of labelled 11β-OH-A4 was verified in two ways. First, the positions on the TLC plate corresponding to the cold 11β-OH-A4 standard gave peaks of radioactivity and immunoreactivity. Furthermore when the reaction products were subjected to HPLC separation, the majority of radioactivity (53%) was found to reside in the fraction corresponding to 11β-OH-A4 as previously determined using an unlabelled standard. For all the subsequent radioimmunoassay analyses, the aforementioned HPLC fraction, collected between 18.5–20.5 min, constituted the purified preparation of label.

Radioimmunoassay

Optimization. The optimum concentration of antibody in the radioimmunoassay was determined from an antibody dilution curve. A working dilution of 1:15,000 of antibody gave 50% binding when the total counts used per tube were approximately 20,000 counts/20 min. Separation

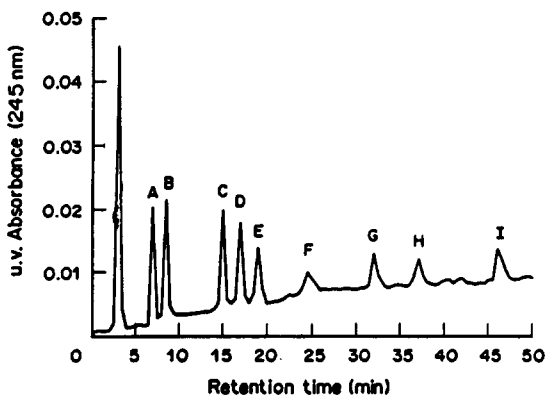

Fig. 2. HPLC chromatogram of steroids. Key to steroid elution (retention times in min): (A) progesterone (7.22), (B) androstenedione (9.35), (C) testosterone (15.45), (D) 17OH-progesterone (17.47), (E) 11β-OH-androstenedione (19.87), (F) 17β-oestradiol (25.12), (G) corticosterone (32.72), (H) cortisone (37.45), (I) cortisol (47.05).

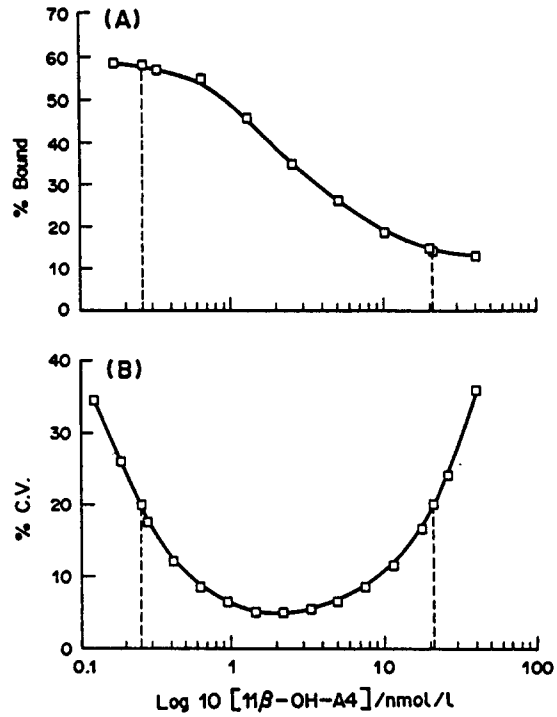

Fig. 3. (A) Standard curve of 11β-OH-A4 in-house RIA. (B) Precision profile of 11β-OH-A4 in-house RIA.

using a second antibody (DARS)-NRS-PEG assisted reagent was preferred as it was found to be the quickest, the most stable and the most accurate technique when compared with charcoal and saturated salt precipitation. A typical standard curve for the 11β-OH-A4 radioimmunoassay is shown in Fig. 3.

Characterization. The precision profile (Fig. 3) was used to assess the detection limit of the assay [33]. At 20% coefficient of variation the sensitivity of the assay was found to be 0.25 nmol/l. The affinity of the antibody was determined from a Scatchard plot, including an NSB correction factor [34]. The affinity of our in-house assay was calculated as $K = 8.13 \times 10^{-8}$ l/M. The intra- and inter-assay precision was assessed by the coefficient of variation of three plasma quality controls. The results are presented in Table 2.

Accuracy of the radioimmunoassay was tested by a series of dilution and spiking experiments. Three plasma samples, containing a known, low concentration of 11β-OH-A4, were spiked with increasing amounts of 11β-OH-A4 standard. A

Table 2. Precision of in-house 11β-OH-A4 RIA (in %-CV)

	Intra-assay variation (n = 14 duplicates)	Inter-assay variation (n = 7 radioiommunoassays)
1.13 (nmol/l)	10.6%	25.3%
4.72 (nmol/l)	8.3%	14.0%
10.55 (nmol/l)	7.9%	9.4%

Table 3. Analytical recoveries of 11β-OH-A4 RIA

	11β-OH-A4 added	11β-OH-A4 measured	% Recovery
Sample 1	3.0	2.7	90
	5.0	5.7	114
	9.0	10.3	114
Sample 2	3.4	3.6	106
	5.4	4.8	89
	9.4	9.0	96
Sample 3	3.5	4.2	120
	5.5	6.0	109
	9.5	10.7	112
Sample 4	1.4	1.2	88
	2.7	1.7	63
	5.5	5.1	93
Sample 5	1.5	1.5	100
	3.0	2.8	93
	5.9	6.5	110
Sample 6	1.8	1.2	68
	3.6	3.0	83
	7.1	7.9	111

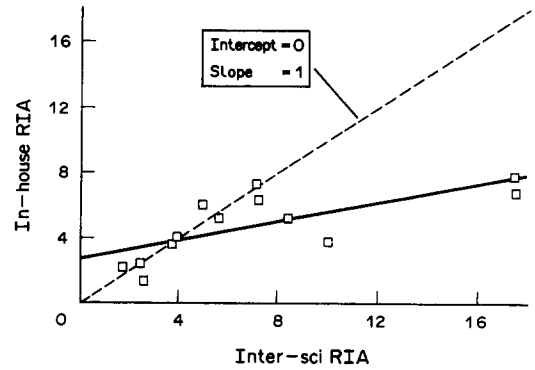

Fig. 4. Regression analysis between inter-sci and in-house 11β-OH-A4 RIA ($n = 13$ samples).

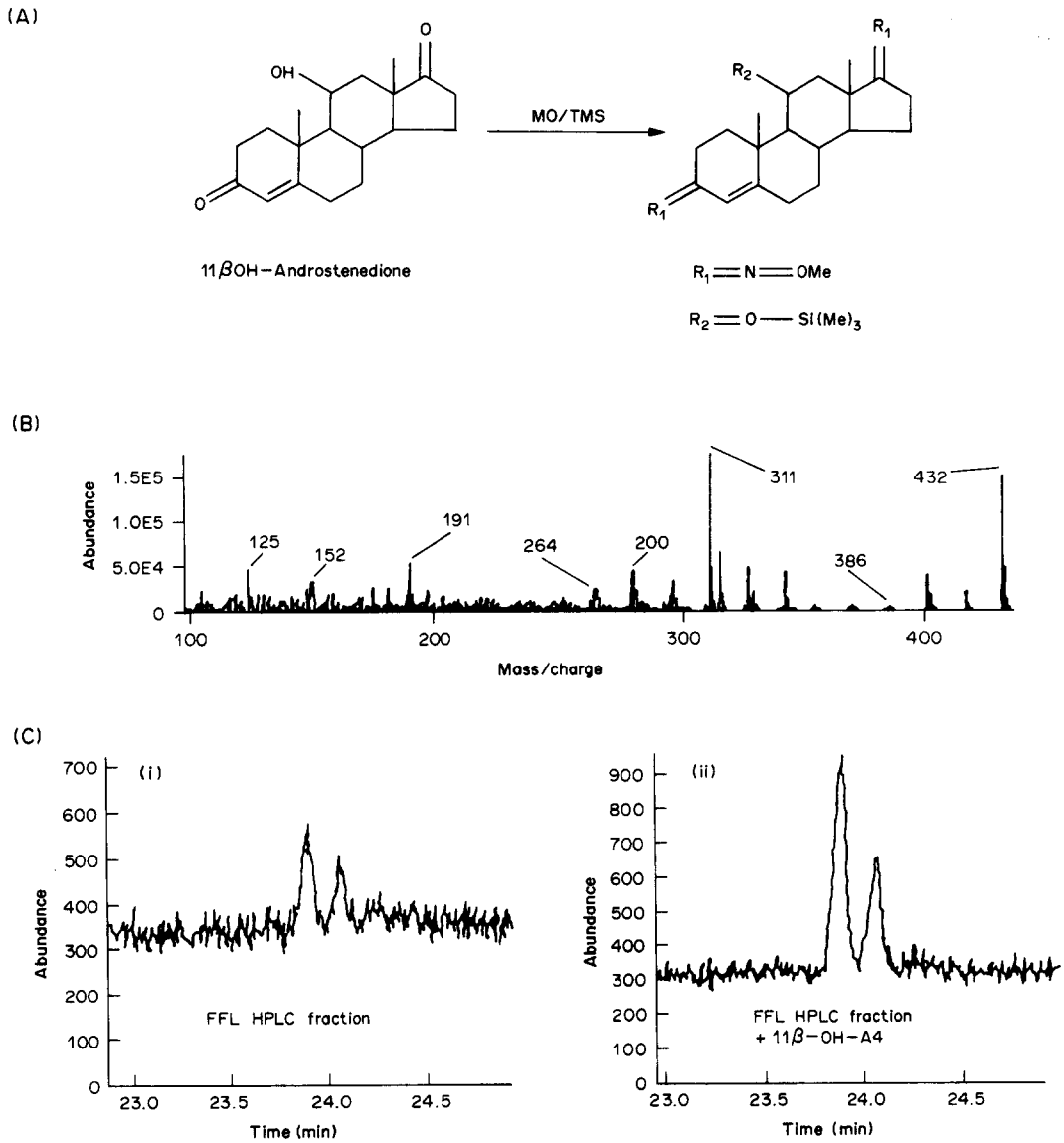

Fig. 5. (A) Derivatization of 11β-OH-A4 for GC/MS analysis. (B) Mass spectrum of 11β-OH-A4-Mo-TIMS derivative. (C) Selected ion monitoring of 11β-OH-A4 in follicular fluid (i) before, (ii) after addition of 1 ng 11β-OH-A4 standard.

further three samples containing known high concentrations of 11β-OH-A4 were diluted. In all cases a radioimmunoassay was performed to check for parallelism. There was no significant difference ($P > 0.3$) between observed and expected values (Table 3).

Specificity of the antisera has been reported elsewhere [28]. Most of the common steroids would not be present in the HPLC eluates found in the present study. The results with our procedures were compared with the only commercially available method; the Intersci kit. The regression analysis demonstrates significant differences between the methods due to higher results with the Intersci kit. This explains why the intercept and slope lie outside the 95% confidence limits of 0 and 1.0 respectively (Fig. 4). The affinity constant of the Interscience radioimmunoassay was calculated to be 3.7×10^{-8} l/M.

GC/MS of follicular fluid steroids

Authenticity of the 11β-OH-A4 in the relevant follicular fluid HPLC fraction was established using the technique of selected ion monitoring–gas chromatography–mass spectrometry. The characteristic fragmentation pattern of derivatized 11β-OH-A4 is shown in Fig. 5 with a molecular ion (m/z) of 432. Using selected ion monitoring (SIM), 11β-OH-A4 was defined by the response for the molecular ion with a characteristic double peak (due to syn- and anti-isomers of the oxime derivatives) and the GC retention times. SIM analysis of follicular fluid samples demonstrated the presence of this 432 double peak at the identical time to that of the standard. On re-running the follicular fluid sample, spiked with approximately 1 ng of derivatized 11β-OH-A4 standard, a reinforcement of this 432 double peak was obtained (Fig. 5).

Table 4. Concentrations of steroids in plasma and follicular fluid (Mean (%) ± SD, nmol/l)

	Plasma	Follicular fluid	FFL/plasma ratio
11β-OH-A4	3.21 ± 1.64 ($n = 27$)	18.6 ± 17.2 ($n = 27$)	5.8×
Cortisol	816 ± 378 ($n = 23$)	958 ± 1111 ($n = 23$)	1.2×
Androstenedione	8.46 ± 4.42 ($n = 28$)	568 ± 827 ($n = 28$)	67.1×
Testosterone	4.49 ± 2.89 ($n = 23$)	32.7 ± 23.2 ($n = 23$)	7.3×

In order to demonstrate which steroids were in the highest concentrations in follicular fluid, a straightforward total ion chromatogram (TIC) was obtained by analyzing 25 ml of derivatized follicular fluid through the GC/MS. The resultant TIC trace is shown in Fig. 6 with a list of identification steroids together with an estimate of their concentrations. As can be seen 11β-OH-A4 was undetectable by this analysis and its presence could only be confirmed by the very sensitive SIM method.

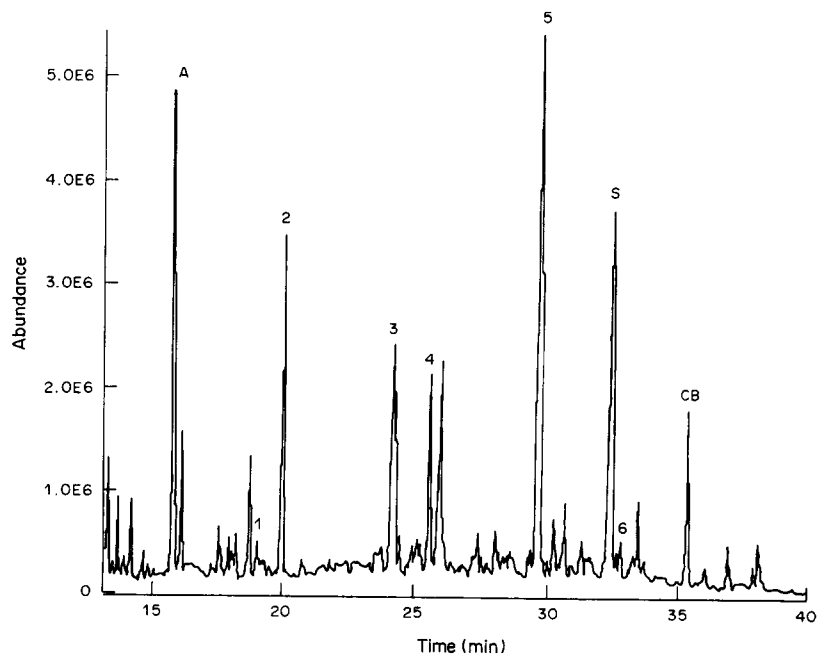

Fig. 6. Total ion chromatogram of follicular fluid. Key to steroids and concentrations (μmol/l): A = Androstanediol (internal standard), 1 = androstenedione (1.3), 2 = 17β-oestradiol (13.9), 3 = progesterone (9.7), 4 = 17OH-progesterone (8.6), 5 = cholesterol (24.7), S = stigmasterol (internal standard), 6 = cortisol (2.2), CB = cholesteryl butyrate (internal standard).

Patient samples

11β-OH-A4, androstenedione, testosterone and cortisol concentrations in follicular fluid were measured by the HPLC and radioimmunoassay procedure (Table 4). All results were corrected for background binding, recoveries and known procedural losses. Some samples were assayed for 11β-OH-A4 by radioimmunoassay alone without HPLC (mean = 38.9 + 30 nmol/l, $n = 19$). These results were higher than those when preceded by HPLC (18.6 ± 17.2 nmol/l, $n = 27$). The plasma samples were measured by the specific in-house radioimmunoassay after extraction alone.

DISCUSSION

A radioimmunoassay method is presented for the measurement of 11β-OH-A4 in follicular fluid in which a preparative HPLC separation is a prerequisite. This initial step is necessary in order to eliminate potentially cross reacting steroids [15], which are present in high concentrations in follicular fluid [35–37]; for example progesterone and oestradiol are present in micromolar amounts, as confirmed in our own GC/MS analysis of a pooled sample of follicular fluid (Fig. 6).

Follicular fluid samples, which have been separated by HPLC prior to radioimmunoassay yield much lower results when compared with samples assayed after extraction alone. When comparing plasma sample results, using the commercially available method with the in-house method, the later resulted in a significantly lower 11β-OH-A4 concentration. This would suggest a greater specificity of the in-house method. There was also an improvement in sensitivity using the in-house method (0.25 nmol/l) over the value of 0.4 nmol/l using the Interscience Laboratories kit. Scatchard plots also confirmed a marginally greater affinity constant for our in-house assay. The HPLC/radioimmunoassay system was shown to be a reliable research technique as shown by the radioimmunoassay validation, high recoveries and consistent retention times. The non-specific binding was negligible when sample blanks were immunoassayed. Previous work in this laboratory had shown that the solvent eluent from other HPLC systems contained material that destroyed subsequent radioimmunoassay. The normal phase system, using a diol column as described in this study was seen to overcome this difficulty [25].

This method, by virtue of the good chromatographic separation of steroids, enables the simultaneous measurement of other steroids pertinent to this study, e.g. androstenedione, testosterone and cortisol from a single sample. The concentrations of steroids in follicular fluid are much higher than circulating plasma levels (see Table 4) and this includes steroids involved in the pathways of 11β-OH-A4 synthesis the ratio of steroid concentrations in follicular fluid compared with plasma was particularly high for androstenedione consistent with its synthesis in the ovary. High plasma cortisol concentrations are attributed to the stress of the oocyte collection procedure. The raised levels of testosterone in plasma are close to the limits reported elsewhere, e.g. 3.5 and 3.3 nmol/l for 24 h after HCG stimulation [38, 39] and women with polycystic ovaries 3.7–4.2 nmol/l [40]. GC/MS analysis of steroids from the pooled sample of follicular fluid confirmed these observations where the estimated steroid levels lie within limits reported in the literature [14, 15, 23, 35–37]. The presence of 11β-OH-A4 in follicular fluid was confirmed by the alternative analytical method of single ion monitoring GC/MS. The origin and implication of such high concentrations of 11β-OH-A4 in these circumstances is of great interest in relation to ovarian androgen metabolism.

Conventional wisdom holds that 11β-OH-A4 is exclusively adrenal in origin, unlike dehydroepiandrosterone [41], and predominantly arises from the 11β-hydroxylation of androstenedione [2, 6]. The production of this steroid in the ovary may divert steroids away from the synthesis of active androgens (such as testosterone and dihydrotestosterone) and into the production of the inactive androgen 11β-OH-A4 [9, 17]. This steroid then plays no further part in androgen production as it is subsequently metabolized by the liver and adrenal to 11β-hydroxyandrosterone and 11β-hydroxyaetiocholanone which are excreted in the urine. 11β-OH-A4 synthesis by side chain cleavage of cortisol has also been demonstrated in many studies [7–9]. Overall this constitutes a relatively minor contribution to overall 11β-OH-A4 synthesis.

11β-Hydroxylation of C_{21} steroids but not C_{19} steroids has been shown to occur in the ovary but more recently there have been reports of 11β-OH-A4 in ovarian veins of adrenalectomized patients [11]. This may reflect 11β-hydroxylase activity on C_{19} steroids in the ovary. Taken in conjunction with our results of higher concentrations of 11β-OH-A4 in follicular fluid (18.6

nmol/l on average) than in plasma this poses the intriguing question of whether 11β-OH-A4 production occurs in the ovary. Infertility, menstrual disturbances, hirsutism or acne may reflect abnormalities of adrenal or ovarian androgen metabolism. Increased levels of androstenedione and testosterone in the follicular fluid and plasma of such patients have been demonstrated [40, 42–44]. In accordance with the view that 11β-OH-A4 is strictly adrenal in origin, as shown by the evidence of dynamic tests and supporting clinical results [3, 4, 9, 10], previous reports on the plasma levels of 11β-OH-A4 have shown little difference between normal and hyperandrogenic women [3, 10].

It remains a possibility therefore that the large amounts of 11β-OH-A4 in follicular may reflect the abnormal expression of an enzyme involved in ovarian androgen metabolism [12, 45]. A likely candidate may be the 11-hydroxylase gene which has a central role in the control of androgen metabolism. The 11β-hydroxylase enzyme complex exhibits difference degrees of substrate specificity, for example a 11-deoxycorticosteroid (C_{21} is 11-hydroxylated at three times the rate of a C_{19} such as androstenedione [46]. Alternatively, the large amounts of 11β-OH-A4 in the follicular fluid may reflect the ability of the ovary to concentrate circulatory steroids into follicular fluid. This phenomenon may occur because of the large quantities of steroid binding proteins therein such as albumin, sex hormone binding globulin [47] and cortisol binding globulin.

The significance of 11β-OH-A4 in the ovary requires further investigation. Analysis of plasma and follicular fluid samples from women with normal and abnormal androgen metabolism in relation to ovarian dysfunction is currently in progress.

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