15. Steroid saponins from *Balanitis roxburghii* Planch

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The fruit pulp of *Balanitis roxburghii* Planch yielded five new steroid saponins of diosgenin named as Balanitisin A, B, C, D and E. The seed kernels, the roots and the stemwood contain respectively Balanitisin F and G, Balanitisin H, and Balanitisin I. Balanitisin A contains 2 moles of glucose and 1 mole of rhamnose and has the structure, diosgenin-3-O-α-D-glucopyranosyl(1 → 3)-O-α-D-glucopyranosyl(1 → 4)-O-α-L-rhamnopyranoside. Balanitisin C contains glucose and rhamnose (1:3) while Balanitisin E contains glucose, arabinose, xylose and rhamnose (4:1:2:2), the carbohydrate moiety being attached to the steroid sapogenin diosgenin, a starting material for the synthesis of sex hormones and oral contraceptives. Balanitisin D, F and G contain glucose and rhamnose in molar ratios of 2:1, 4:1 respectively.

2. ADVANCES IN ANALYTICAL METHODS

16. Application of antibodies against 12α-derivative of progesterone to a direct radioimmunoassay of progesterone in plasma

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Antibodies against the succinyl-BSA derivative of 12α-hydroxyprogesterone were obtained from 2 out of 3 immunized rabbits. One of those antisera was used to develop a sensitive and reproducible direct assay (without extraction), the practical range being 0.5–20 ng/ml plasma, when taking 20 μl of plasma per assay tube. Transcortin present in human plasma had to be saturated with cortisol. The antiserum tested under assay conditions was homogenous and had a $K_A = 3 \times 10^9$ l/mol. With the exception of 5α-dihydroprogesterone there was no significant interference from other progesterone metabolites or steroid hormones. Calibration and plasma dilution curves were parallel to each other. The results obtained for human menstrual cycle and pregnancy were fully compatible with those of other authors.

17. Universal reagent immunoassay. Labelled second antibody assay for norethisterone

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The theoretical advantages of using radioiodine labels for steroids in radioimmunoassay have not been fully realized. This preliminary communication describes a novel solid-phase system which employs radioiodine-labelled second antibodies. The method consists of two steps, (i) the competitive interaction between added norethisterone, specific antibody and norethisterone–bovine albumin conjugate linked to polystyrene-tube surfaces and (ii) the assessment of bound antibody by its interaction with radioiodine-labelled anti-rabbit-immunoglobulin antibodies (universal reagent). Standard curves could be generated over the range 7.8–1000 pg per tube. The coefficient of variation at each point ranged from 50–1.6%, respectively. The least dose detectable was 2 pg and a 50% reduction in binding was elicited by 60–100 pg of norethisterone. The recovery of norethisterone added to pooled human plasma was quantitative. Cross-reaction studies revealed non-parallelism between standards and related steroids. At low concentrations of metabolites the discrimination was less than observed in conventional assay systems.

18. Methods for GC/MS analysis of steroids based on exchange of oxime functions

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Simple and O-substituted oxime derivatives of steroids may be interconverted by an acid-catalyzed reaction of the oxime with the appropriate substituted hydroxylamine hydrochloride in pyridine. The reaction involves cleavage of the carbon–nitrogen bond and follows pseudo-first order kinetics in the presence of excess reagent. The rate of conversion depends on the position of the oxime function and decreases in the order: C-3 > C-20 > C-17 > C-11. The proximity and configuration of other functional groups also influence the rates. Thus, determination of reaction rates with the aid of gas chromatography–mass spectrometry may be used to determine positions of functional groups. The rapid exchange of oximes at C-3 permits selective "labeling" of saturated 3-ketosteroids in analyses of complex mixtures. The exchange reaction has been used to convert unsubstituted oximes of 3-ketosteroids, which can be isolated from biological materials by ion exchange, into methoximes which are more suitable for GC/MS analysis.

19. A sensitive enzyme immunoassay for testosterone

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We report here the first sensitive enzyme immunoassay of testosterone. A testosterone–penicillinase conjugate was prepared using the carbodiimide method. Antiserum to testosterone was raised using testosterone-3-carboxymethyl-BSA. The separation of bound from free fraction of the label was achieved by the double antibody technique. The enzyme activity in the bound fraction was measured by the method of Novick (1962). The sensitivity of the assay was 15 pg/tube. The within and between assay errors, measured as a coefficient of variation, were within acceptable limits (< 12%). A high degree of correlation was obtained ($r = 0.97$) between the assay reported here and RIA. This substantiates the validity of the assay.

20. Computerised gas chromatography–mass spectrometry (GC–MS) applied to the investigation of steroids in human seminal fluid

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Gas chromatography–mass spectrometry (GC–MS) has not been previously employed to analyze steroids in seminal fluid. For this purpose, ethereal extracts and ethyl acetate extracts from the solvolyzed aqueous phase (sulphate fraction) of human seminal fluid were purified by column chromatography on Sephadex LH-20 and thin layer chromatography and subjected to analysis by computerised GC–MS using a 25 m capillary column (OV-101). The steroid fractions were derivatised as their methoxime-trimethylsilyl and *t*-butyldimethylsilyl (TBDMS) ethers. The most important steroid found in both free and sul-

phate fractions was dehydroepiandrosterone, occurring in much greater amounts in the conjugated fraction. The TBDMS ethers showed distinct advantages for the detection of trace steroids, e.g. testosterone using mass chromatography and mass fragmentography. The results are in agreement with those previously obtained by others using radioimmunoassays.

21. Enzymic determination of plasma and urine oestrogens

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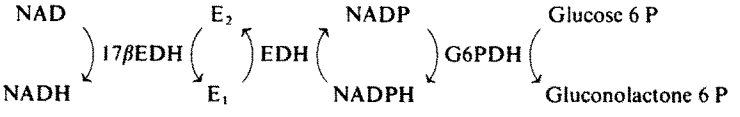
The method uses the transhydrogenase function of the 17β oestradiol dehydrogenase (EDH). We have determined the conditions to obtain a direct relationship between transhydrogenase activity and oestrogen concentrations. The NADPH is produced from a small amount of NADP by the system glucose 6 phosphate + glucose 6 phosphate dehydrogenase (G6 PDH). Hydrogen of NADPH is transfered to an excess of NAD by the oestradiol dehydrogenase free of endogenous oestrogens.



This method is specific for oestrone (E_1) and oestradiol (E_2) which can be determined together or separately if oestrone is previously reacted with hydrazine. In the plasma, this method allows the determination of 10 pg of oestrogen per tube. It seems to be more advantageous than radioimmunologic and immunoenzymatic methods. In urine the determination is performed without extraction on 10 to 25 μl of hydrolyzed urine. This specific and handy method presents advantages on the conventional technics of urinary oestrogens determination.

22. The influence of plasma-extract on the separation of antibody bound and unbound oestrogens by dextran coated charcoal (DCC)

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In radioimmunoassay, the adsorption of the unbound ligand to dextran coated charcoal (DCC) depends on the incubation time in the presence of DCC and on the amount of plasma-extract. Indeed, the latter decreases the effectiveness of DCC to adsorb the unbound ligand. As a result, the presence of excess radioactivity in the supernatant involves an overestimation of the apparent antibody-bound fraction of the tracer and an important underestimation of the amount of oestrogens in the extract. The fact is evidenced by a non-linear relationship between the plasma volumes extracted and the estrogen values. Also, the recovery of added steroid decreases significantly with increasing amounts of plasma-extract. Increasing the concentration of DCC reduces the underestimation of steroid present in the extract; one thus obtains a linear relationship between plasma volumes and amount of oestrogen measured, comparable to the results observed with the more elaborate and time consuming chromatographic method. However, the incubation time with DCC is important and the dissociation velocity of the steroid antibody complex becomes critical. Several examples of the above mentioned aspects will be shown. The nature of the antibody is important, and in routine analysis, the use of different "specific" kits implies the necessity of individual adaptation in so far as DCC concentration and incubation time are concerned.

23. A direct magnetic solid-phase radioimmunoassay for plasma aldosterone

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A simple and direct radioimmunoassay for plasma aldosterone which can be easily automated is described. The assay uses a highly specific aldosterone antiserum coupled covalently to a magnetic cellulose solid-phase and ^{125}I-labelled aldosterone ligands. Aldosterone antisera were produced in sheep. The magnetic cellulose solid-phase antibodies and various ^{125}I-labelled aldosterone ligands were prepared using modifications of previously described methods (aldosterone-3-mono-oxime) [^{125}I]-iodohistamine, aldosterone-3-(*p*-hydroxybenzoyl)hydrazone-[^{125}I], and aldosterone-3-(*p*-hydroxyphenylpropionyl) hydrazone-[^{125}I]). The assay was carried out by adding a 100 μl aliquot of plasma or aldosterone standard to a 100 μl of solid-phase antibody and 10,000 c.p.m. of [^{125}I]-aldosterone ligand in 100 μl phosphate buffer: the tubes were mixed and incubated at room temperature for 4h, placed on a permanent magnet to separate the antibody-bound from free fraction and the supernatant aspirated. The bound fraction was counted. The solid-phase assay was slightly less sensitive but had greater specificity than the liquid-phase system. The sensitivity of the assay was 10 pg/ml with zero blank values. The direct solid-phase radioimmunoassay was evaluated by comparing results with those obtained by a previously validated direct assay using liquid-phase antiserum. This radioimmunoassay for plasma aldosterone is easy to perform, rapid, cheap and uses magnetic solid-phase antiserum which has the major advantage over the liquid form of ease of separation of antibody-bound from free steroid. The use of magnetic solid-phase particles obviates the need for centrifugation. The assay described here and the reagents produced now form the basis of a fully automated plasma aldosterone radioimmunoassay.

24. New analytical methods for steroids, including some comparisons of methods with regard to specificity

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With the aim of carrying out large-scale clinical metabolic studies on estrogens, radioimmunoassay (RIA) methods for urinary estrone, estradiol, estriol, estriol-16α-glucuronide, estriol-3-glucuronide and a mass fragmentographic procedure for a number of estrogens in urine were developed. In addition the first analyses of estrogens in faeces of men, and non-pregnant women during the menstrual cycle have been carried out. With these methods it has been possible to study the influence of diet and drugs on estrogen metabolism and the physiology of the menstrual cycle in detail. Further work on enzymatic fluorometric procedures has resulted in the first method for a synthetic steroid, medroxyprogesterone acetate (MPA). The method can detect 3×10^{-13} mol of standard. Comparisons with a "specific" RIA of MPA revealed that the new method gives almost 50% lower values, which were in the same range as those obtained by mass fragmentography. Thus the use of specific steroid enzymes (in this case 3α,20β-hydroxysteroid dehydrogenase) combined with adequate purification procedures can yield highly specific and sensitive methods