2. Analytical methods

2A. Gas liquid chromatography and g.l.c. mass spectrometry

27. Gas–liquid chromatographic studies of cholecalciferol and related compounds

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The preparation of derivatives for g.l.c. analysis of cholecalciferol and related compounds was investigated for reproducible conversion of the hydroxyl group(s) to the less polar heptafluorobutyryl ester function(s) by use of heptafluorobutyric anhydride and heptafluorobutyryl imidazole ester as derivatizing agents. Separation efficiency was checked on 15 different analytical columns of which 1% OV-25, DEGA, STAP and FFAP as liquid phases showed better performances. Detector systems used were FID and ECD (^{63}Ni) giving detection limits of 0·1 μg and 1 ng—absolute amounts injected on top of columns—respectively. Qualitive and quantitative analysis of ergocalciferol, cholecalciferol and its 25-hydroxylated metabolite was carried out in the presence of cholestanol and dihydrotachysterol as internal standards which were derivatized identically and simultaneously with compounds to be determined. Mass fragmentography by monitoring specific ions at m/e 378 for ergocalciferol and 366 for cholecalciferol heptafluorobutyryl ester is undoubtedly most specific. Such a system furthermore permits quantitative analysis at the subnanogram level which looks most promising for determination in human blood samples of the compounds mentioned.

28. Detection and isolation of unknown steroids in the urine of patients with hypertension and adrenal disorders

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The γ-lactone of aldosterone and 18-OH-DOC were estimated by gas chromatography in our laboratory in the urine of 168 patients, most of them with various forms of hypertension (labile hypertension, hypertension with vascular and adrenal disorders, malignant hypertension, renal hypertension) and adrenal disorders (cyclic oedema with hypokalemia and without hypertension). In the urine of 48 patients elevated amounts of aldosterone and/or variable amounts of 18-OH-DOC and/or of two unknown compounds called x and y (both more polar than aldosterone) were found. The latter compounds were detected in 22 patients. Generally, compounds x and y were not found at the onset but after some progression of the disease. The plasma potassium levels and the plasma renin activity are not necessary disturbed. Compounds x and y were extracted from the urine and the extract chromatographed on celite and Sephadex columns and on paper in the system benzene–acetone–water. The specificity of the binding of the isolated products to the soluble nuclear proteins of the rat kidney was estimated.

29. Application of high-resolution capillary gas chromatography to the evaluation of urinary steroid-spectra in different endocrine disorders of childhood

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The pathologic disturbances in secretion and metabolism of steroid hormones were studied in different endocrine disorders of childhood by fractionated determination of 29 urinary C_{19}- and C_{21}-steroids (adrenal carcinoma (n = 1), congenital adrenal hyperplasia of undetermined type (n = 1), AG-syndrome (n = 5), precocious puberty (n = 3), Addison's Disease (n = 1). The steroid patterns of 34 normal children of various ages served as controls. Trimethylsilylether derivates of 9 C_{19}- and 20 C_{21}-steroids were separated on a glass capillary column coated with methylsilicone and detected by FID. In adrenal carcinoma an excessive amount of pregnanetriol and pregnanetriolone was excreted together with a slightly increased excretion of C_{21}-steroids. After exstirpation, the metastases again produced high excretion of DHA and etiocholanolone. In a case of congenital adrenal hyperplasia one month after birth an excessive amount of pregnanetriol and pregnanetriolone was excreted together with a slightly increased excretion of corticosteroid metabolites (TH-DOC, THA, THS, THE, several cortols). A pathognomonically high excretion of pregnanetriol, pregnanetriolone, allo-pregnanediol, androsterone, and etiocholanolone and decreased amounts of tetra- and hexahydrocorticosteroid metabolites were observed in congenital 21-hydroxylase deficiency. In precocious puberty a consistently elevated excretion of C_{19}- and C_{21}-steroid fractions was found. The results showed that the fractionation of 29 different urinary C_{19}- and C_{21}-steroids by a method like capillary gas-chromatography with high sensitivity, specificity, precision, and practicability is a valuable means for rapid identification of various adrenal and/or gonadal disorders in childhood. (Supported by DFG, SFB 87, Project L/M/P).

30. Steroid hormone assays of human tumour tissue by high resolution mass fragmentography

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The technique of high resolution molecular ion detection during combined gas chromatography-mass spectrometry has been employed to measure steroid concentration in human diseased tissue. A simple solvent extraction procedure was used to minimise losses and the crude extracts containing the steroids were treated with bis (trimethylsilyl) acetamide prior to analysis. Standard solutions of the steroids of particular interest (as trimethylsilyl ethers) were prepared in the concentration range of 0·1–10 ng/μl and their mass fragmentograms used to calibrate the system. Similar fragmentograms obtained from the tissue extracts allowed determination of oestradiol-17β, oestriol and oestrone at levels greater than 1 ng/g wet weight tissue, while dehydroepiandrosterone, testosterone, androsterone, epiandrosterone and 5α-dihydrotestosterone were assayed when their levels exceeded 5 ng/g. Analysis of primary breast tumours from postmenopausal women has revealed oestradiol levels between 10 ng/g and 15 μg/g with oestrone

levels relatively considerably lower in the same extracts. The same steroids have also been assayed in tissue from males with gynaecomastia and with carcinoma of the breast. The endogenous steroid levels in the tumours have been related to other biochemical parameters such as "oestradiol-17β receptor" concentration.

31. Assay of hormonal steroids by gas chromatography–mass spectrometry using stable isotope internal standards

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The mass spectrometer with a multiple ion detector can be used as a very sensitive detector in the gas chromatographic estimation of hormonal steroids. The intermittent focussing in the instrument on selected ions gives high specificity to the assay. The ideal internal standards for this type of assay are hormonal steroids labelled with stable isotopes. They allow correction for any losses in the initial procedures preceding the gas chromatography. Gas chromatography/mass spectrometry with stable isotope labelled internal standards produced by deuteration of appropriate steroids has been applied to the assay of hormonal steroids in body fluids. Practical assays for testosterone and estrogens in body fluids will be compared with radioimmunoassay methods for the same compounds.

2B. Steroid radioimmunoassay—I

32. Synthesis of 11α-C_{19}-steroid–protein conjugates for new RIA antisera

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The synthesis of specific steroid antigens for use in the development of radioimmunoassay (RIA) systems has been the subject of much recent investigation by several authors. Many different sites of conjugation of the steroid to protein have been tried, including the 3, 17, 6, 7 and 11 positions. The most specific steroid derivatives yet found utilize an 11α-hydroxyl group for conjugation to protein. Only in the case of progesterone is the 11α-hydroxy compound readily available. We have succeeded in synthesizing 11α-hydroxy-androstenedione from adrenosterone which is commercially available at low cost. The simple two step method may be run in any laboratory and gives a good yield of the 11α-hydroxy compound without contamination by the 11β-hydroxy isomer. Additionally, routes will be presented to give rise to other 11α-hydroxy androgens, such as, testosterone, dihydrotestosterone and androstanediol for use in developing new RIA systems. (Supported in part by The Robert A. Welch Foundation, Q-560 and St. Luke's Episcopal Hospital).

33. Antisera specific to corticosterone

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Five rabbits were immunized with a corticosterone-3-oxime bovine serum albumin (BSA) conjugate carrying 6 steroid molecules. Antibody sensitivity and specificity were tested 10–14 weeks after first immunization. Antibody dilutions that bound 50% of ^{3}H-corticosterone were between 500 and 1000. All antisera showed similar high sensitivity as indicated by the slopes of the standard curves. Three of the 5 sera showed highest cross-reaction with cortisol (70, 43, 26%) and low cross-reaction with deoxycorticosterone (DOC) (7, 5, 5%) and progesterone (3, 4, 5%). With these sera alterations in the chemical and steric configuration at C-11 resulted in a marked decrease in cross-reaction as indicated by displacement experiments with 11-deoxycortisol (0·9, 0·8, 0·7%), cortisone (5, 0·1, 0·06%) and 11-epi-cortisol (0·03, 0·02, 0·02%). Two of the 5 sera showed highest cross-reaction with progesterone (94, 67%) and DOC (82, 88%) and low cross-reaction with cortisol (6, 8%). With these sera, changes in the chemical configuration at C-17 resulted in a decrease in cross-reactivity as indicated by displacement experiments with 17α-OH-progesterone (8,2%). Our results indicate that with corticosterone-3-oxime BSA two different antibody populations can be raised in rabbits: (a) one predominantly directed against β-C-11, the other predominantly against α-C-17. Coupling through the double bond at C-3 preventing rotation of the hapten might have favoured these findings.

34. Specific antisera for estriol-16α-glucosiduronate

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Estrogen conjugates in plasma and urine have usually been determined indirectly after hydrolysis, followed by assay of the free estrogen. Such methods often do not distinguish between individual conjugates. We have developed an antiserum to estriol-16α-glucosiduronate for use in a direct radioimmunoassay of this conjugate. Estriol-16α-glucosiduronate-bovine serum albumin was prepared by coupling the carboxylic acid group of the glucosiduronate to ε-amino groups of lyside residues in bovine serum albumin by the mixed anhydride procedure. The protein conjugate was injected intradermally into rabbits with Freund's adjuvant. At a dilution of 1:2000 the antiserum gave a useful standard curve over the range of 10 pg to 200 pg. The antiserum cross-reacted 4% with estriol-17β-glucosiduronate, < 1% with estriol-3-glucosiduronate, 2% with estradiol-17β-17-glucosiduronate, and 1% with estrone glucosiduronate. As expected, the antiserum showed significant cross reactions with certain free estrogens: 16-epiestriol (12%), estriol (22%), estrone (32%), and estradiol-17β (20%). Thus preliminary routine separation of free estrogens is necessary before radioimmunoassay.

35. Highly specific antisera for solid-phase radioimmunoassay of 11β-hydroxy-4-androstene-3,17-dione

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The contribution of the adrenal cortex to the androgenic-anabolic interrelationship in the body has not been fully explored. The secretion of androgens by the adrenal, their change in response to environmental stress, and their effect on other organs like the testes are significant features of such concerns. From what is known of the production of C_{19}-steroids by the adrenal and from the analysis of the urinary metabolites of these compounds, it is evident that they are principally 11β-hydroxy secretory products. With a conversion of over 90% of preformed 4-androstene-3,17-dione to its 11β-hydroxy derivative within the adrenal, a measurement of the plasma levels of the latter compound should serve as a rational index of the biosynthesis of