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ESTIMATION OF 18-HYDROXYCORTICOSTERONE CONCENTRATION IN HUMAN PERIPHERAL PLASMA BY GAS–LIQUID CHROMATOGRAPHY WITH ELECTRON CAPTURE DETECTION

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SUMMARY

A gas–liquid chromatography method for determining the plasma concentration of plasma 18-hydroxycorticosterone based on the formation of the heptafluorobutyrate is described and evaluated. Detector contamination was avoided by using high column and detector temperatures and a high quench gas flow rate. The concentration in a group of normal subjects varied from 17.26 ± 7.66 (SD) ng 100 ml^{-1} at 08.00 h to 11.27 ± 6.16 at 12.00 h and 5.97 ± 2.42 at 23.00 h, indicating some dependence on ACTH secretion. Coefficient of variation at a mean concentration of 10.39 ng 100 ml^{-1} was 15.7% and there was no detectable reagent blank.

INTRODUCTION

18-Hydroxycorticosterone (18-OH-B) is secreted by the adrenal cortex and metabolised in the liver to tetrahydro-18-hydroxy-11-dehydrocorticosterone in which form it is excreted in the urine [1]. If the behaviour of other 18-oxygenated steroids, with the exception of aldosterone, is any guide, at physiological concentrations 18-OH-B probably has little direct effect on sodium and potassium metabolism (e.g., [2]) although recent work suggests that it may be involved in acid base balance in animals [3]. However, 18-OH-B may be an important precursor of the potent mineralocorticoid aldosterone [1, 4, 5], although whether it is an immediate precursor has been disputed [6, 7]. As a possible immediate and rate-limiting precursor of aldosterone, 18-OH-B may be the locus in the biosynthetic pathway at which factors in the control of aldosterone formation act and studies of its secretion and concentration in the peripheral circulation may therefore be of fundamental interest. Few attempts have been made to measure plasma concentrations of 18-OH-B. Although one method using radioimmunoassay has been published [8], no values for plasma concentration have yet been published. This paper describes the application of gas–liquid chromatography (g.l.c.) with electron capture detection, previously used in the analysis of plasma for other corticosteroids [9, 10], to the estimation of human peripheral plasma 18-OH-B concentrations.

MATERIALS AND METHODS

Reagents. 18-Hydroxy-[1,2(n)-^{3}H] corticosterone was obtained from The Radiochemical Centre, Amersham at a S.A. of 32 Ci. $mmol^{-1}$ and stored at 4° in benzene–methanol (9:1 v/v) solution containing triethylamine (0.02%). Approximately 100 pg (20,000 d.p.m.) was added to samples before extraction. Unlabelled 18-OH-B was obtained from Ikapharm. Other materials were as described by Mason and Fraser[10].

Gas–liquid chromatography. G.l.c. was carried out using a Pye 104 (model 84) chromatograph equipped with ^{63}Ni electron capture detector. Siliconised Pyrex glass columns (3.4 m × 2 mm i.d.) were packed with Supelcon AW-DMCS (80–100 mesh) coated with a stationary phase of 3% SP2250 (Supelco Inc.). The quench gas (20 ml/min) and carrier gas (100 ml/min) were both methane:argon 1:9 (Air Products Ltd.). Chromatography was carried out at 310° with detector and flash heater temperatures of 350 and 330° respectively. The detector was used in the pulsed mode (pulse space – 150 μs, pulse width – 0.75 ± 0.25 μs, pulse amplitude – 47.60 V positive). Where required, a peak trapping apparatus (Manual Preparative Kit, Pye Unicam) consisting of a siliconised glass U tube containing Chromosorb (80–100 mesh, Supelco) soaked in toluene was fitted to the gas chromatograph *via* a 100:1 effluent splitter. When in use, the trap was cooled by means of an acetone:solid carbon dioxide mixture.

Preparation of samples. Neutral extracts of plasma (5 ml) containing [^{3}H] 18-OH-B were prepared using dichloromethane (1 × 10 vol.) and washing the extract with alkali (1 vol. 0.1 M NaOH), acid 1 vol. 0.1 M acetic acid) and water (1 vol.). These were evaporated to dryness at 34° under nitrogen and the residue oxidised, first with periodic acid solution and then with chromium trioxide solution. Both procedures were carried out as described by Mason and

Fraser[10]. The resulting residue was then chromatographed in paper chromatography system B3 [11] for 8 h. The 18-OH-B derivative was located by scanning the chromatogram for ^3H, eluted with methanol and one tenth removed to assess ^3H yield. The remaining eluate was evaporated to dryness as described above and the residue redissolved in benzene (30 μl). Heptafluorobutyric anhydride (30 μl) was then added and the resulting solution heated at 60° for 30 mins. Excess reagents were then removed by evaporation under nitrogen and the residue dissolved in benzene (50 μl) containing the internal standard 18-hydroxy-11-deoxycorticosterone-γ-lactone HFB (100 pg/μl). Depending on the amount of 18-OH-B present, one tenth to one fiftieth of this was injected into the gas chromatograph.

RESULTS

Chromatographic separation. Chromatography of 18-OH-B or [^{3}H]-18-OH-B on the paper chromatography system B5 [11] gave two spots with R aldosterone values of 0.4 and 1.3. 90–96% of the compound ran at the slower rate. Only a single spot was obtained when the oxidised 18-OH-B was run in system B3 and this was well separated from the lactones of aldosterone and 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) (R aldosterone-γ-lactone values; 18-OH-B derivative: 0.79, 18-OH-DOC-γ-lactone: 1.43). Good separation of the heptafluorobutyrate (HFB) derivatives of the oxidation products of the three compounds was also obtained during g.l.c. (relative retention times: aldosterone-γ-lactone HFB: 1.00, 18.OH.DOC-γ-lactone HFB: 0.61, 18.OH.B derivative HFB: 0.92). The retention time of the aldosterone derivative under these conditions was approximately 7 min.

Since 18-OH-DOC-γ-lactone HFB was used as an internal standard, it was necessary to ensure that all endogenous 18-OH-DOC was removed from the samples before g.l.c. On no occasion when random samples were tested without addition of internal standard was there a perceptible peak in the 18-OH-DOC-γ-lactone HFB region. In addition, 5 analyses of normal plasma samples to which 10 μg of 18-OH-DOC had been added, showed no evidence of 18-OH-DOC contamination of the final residue.

Efficiency of esterification. The oxidation product of 18-OH-B (1 μg) containing [^{3}H] labelled compound (200,000 d.p.m.) was esterified to form the HFB derivative as described above. Aliquots were analysed by g.l.c. and the peaks corresponding to the 18-OH-B derivative were collected in the trap. The contents of the trap were transferred quantitatively to a vial for liquid scintillation spectrometry. Mean yield was 95.5% (range 93.7–96.8%).

Yield, accuracy and sensitivity of detection. The mean yield of [^{3}H] at the point in the process before esterification was 44.5 $\pm$ 10.1% S.D. (n = 143). Calculated from the S.A. of the [^{3}H] compound, the quantity of steroid added as a recovery marker was 2.16 $\pm$ 0.12 (S.D.) ng 100 ml^{-1} (n = 19) and blank values using water (2.07 $\pm$ 0.3 (S.D.) ng 100 ml^{-1}, (n = 15) or plasma from an adrenalectomised subject (2.66 $\pm$ 0.14 (S.D.) ng 100 ml^{-1}, n = 5) were not significantly different from this. Plasma concentrations were corrected by subtracting the calculated mass of [^{3}H] 18-OH-B. Recovery of 18-OH-B added in triplicate to plasma at concentrations equivalent to 5, 10 and 20 ng 100 ml^{-1} were 97.7% (range 92–106%), 107.1% (range 98–114%) and 92.7% (range 89–98%) respectively. At full detector sensitivity, 0.7 pg of 18.OH.B, converted to the derivative, gave a peak 1 cm high.

Electron-capture detector response was linear over the range 0–100 pg (r = 1.0, y = 0.22, x − 0.04).

Precision and normal ranges. Replicate analyses of a normal human plasma pool contained 10.39 $\pm$ 1.64 (S.D.) ng 100 ml^{-1} (n = 22). Venous plasma samples were taken from normal subjects (5 male, 4 female) at 08.00 h after overnight recumbency and at 12.00 and 23.00 h ambulatory. Plasma 18-OH-B concentrations were 17.26 $\pm$ 7.66 (S.D.) ng 100 ml^{-1}, 11.27 $\pm$ 6.16 (S.D.) ng 100 ml^{-1} and 5.97 $\pm$ 2.42 (S.D.) ng 100 ml^{-1} respectively. Thus, concentrations rose between night and morning ($P < 0.001$) and then fell between morning and midday ($P < 0.001$) and again between midday and late evening ($P < 0.02$).

DISCUSSION

18-OH-B exists in solution in at least two different forms which are readily separated by chromatography [1]. It is possible that the more polar form is a monomer and the less polar compound a dimer [12]. The relative proportions of these forms are variable although in mildly alkaline solution the monomer predominates. To avoid the obvious variable loss in yield of 18-OH-B which would result from the use of heterogenous[^{3}H] 18-OH-B as a recovery monitor, the steroid was oxidised, first to the γ-lactone and then to the 11-oxoderivative before any type of chromatographic procedure was undertaken. In addition to converting 18-OH-B to a single molecular species, the severe oxidation procedure had the effect of destroying many contaminants in the biological extract and of converting other corticosteroids such as cortisol to derivatives of such altered polarity as to be more easily removed by a single chromatography stage. The corticosteroids most likely to interfere with the estimation of plasma 18-OH-B concentrations, those with an oxygen function at C-18, are converted to γ-lactones which separate both on paper chromatography and on g.l.c.

The assessment of yield before the esterification step is made necessary by the relative instability of heptafluorobutyrates during chromatography [13]. However, formation of the derivative is quantitative—more than 95% of the [^{3}H] label was recovered from

the g.l.c. detector and this is likely to be an underestimate of esterification yield because of difficulties in quantitative peak trapping.

G.l.c. with electron capture detection is a technique of high potential sensitivity but the difficulties of maintaining high and reproducible detector performance have prevented its employment in routine analyses of biological material. Contaminants from extracts of tissues such as plasma and from materials such as solvents used in analyses frequently coat the detector surfaces, at best reducing sensitivity and at worst producing a massive 'solvent' peak which obscures the compounds under investigation. One solution to this problem is to reroute the volatile compounds away from the detector by means of a bypass valve [9, 10] and to allow only the fraction of the column eluate containing the steroid derivative through the detector. Unfortunately, the longer column used in these experiments made it necessary to use much higher temperatures to keep retention times conveniently short and the valves available at present contain materials which deteriorate above 250°. However, the oxidation procedure, the use of high column and detector temperatures and rapid quench gas flow appear to have prevented detector contamination. The improved characteristics at high temperature of the stationary phases and supports now available probably also contributed to the elimination of detector 'noise'. Consequently sensitivity of detection was at least comparable with techniques such as radioimmunoassay used to analyse plasma for corticosteroids such as aldosterone. The precision of estimation (coefficient of variation 15.7%) is of the same order as that found in methods based on other techniques and variability is small compared with the range of concentration of 18-OH-B.

Ulick *et al.*[1] found that 18-OH-B secretion rates, although changing in proportion to those of aldosterone, were considerably higher. The normal range of plasma concentration found here, however, is of the same order as that widely quoted for aldosterone. Unlike aldosterone, the diurnal variation is similar in pattern to that found in plasma cortisol concentration, possibly indicating that the secretion of 18-OH-B is more dependent on ACTH than that of aldosterone.

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