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DISPLACEMENT ANALYSIS OF ALDOSTERONE AND CORTICOSTERONE IN THE RAT ADRENAL VENOUS BLOOD

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SUMMARY

A method is described for the determination of aldosterone and corticosterone in the adrenal venous blood of the rat. The procedure includes extraction and separation of corticosteroids by thin layer chromatography. Aldosterone content of the t.l.c.-eluates is estimated by means of radioimmunoassay while that of corticosterone is determined by competitive protein binding assay. The accuracy of aldosterone (0·5–2·7 ng) and corticosterone analysis (10–150 ng) is about 100%. Other criteria of reliability are also discussed. The collection period of adrenal venous blood may be reduced to 1 min or less if the present method is used for analysis.

INTRODUCTION

THE DETERMINATION of aldosterone secretion rate in the rat has been possible by double isotope dilution techniques. However, only a few laboratories could afford the introduction of such techniques because of their expense and technical difficulties. The recently introduced radioimmunoassay (RIA) technique for the estimation of aldosterone concentration [1, 2] appears to be a more economic and less laborious way of analysing ultramicro amounts of aldosterone.

The present paper gives an account of a method designed specifically for the *simultaneous* assay of aldosterone and corticosterone (B) in adrenal venous blood of the rat. The application of thin layer chromatography for the separation of corticosteroids rendered chromatogram scanning avoidable. A competitive protein binding analysis of B in peripheral plasma had been described previously [3]. The introduction of aldosterone analysis, however, necessitated some slight modifications. For this reason the major steps of B analysis will be also described.

The present analytical procedure was used for the assessment of aldosterone and B secretion rate by normal, surgically-stressed rats. Two methods were used for the collection of adrenal venous blood. The results thus obtained are compared with previously published data.

EXPERIMENTAL

Surgical procedures

Adrenal venous blood was collected in two series of nembutal-anaesthetized male rats, kept on a semisynthetic diet (Na^+: 165 mEq/kg, K^+: 150 mEq/kg). In *experiment* 1 (6 rats of 205–230 g body weight) adrenal venous blood was collected by means of a method described previously [4]. Briefly it is as follows: after the administration of 250 units of heparin and the ligation of the renal end of the left adrenal vein, the vessel was opened carefully, (but *not* transected). Collection of blood flowing without resistance through the incision was carried out by means of previously cleaned cotton-wool pieces.

In *experiment* 2 (5 rats of 180–250 g b.w.) blood was collected by Vogt's classical method[5]. In both *expts.* 1 and 2 adrenal venous blood collected between the 30th and 150th sec of bleeding was used for analysis.

Materials

Methylene chloride, methanol, toluene, chloroform, hexane and ethanol, all analytical grade, were further purified[6]. Florisil, 60/100 mesh, was pretreated as described by Rittel *et al.*[7]. [1,2-^{3}H]-aldosterone (54 Ci/mmol, New England Nuclear, Boston, Mass.) and [1,2-^{3}H]-corticosterone (31 Ci/mmol, Radiochemical Centre, Amersham) were purified by t.l.c. in chloroform–acetone–acetic acid (100:40:2·5 by Vol) and tested for purity by paper chromatography with E_2B[8] and/or Bush B5[9] and/or formamide–benzene[10] systems.

Glassware was acid or methanol-washed.

Extraction

In *expt.* 1 11000 d.p.m. (34 pg) of ^{3}H-aldosterone and 42000 d.p.m. (240 pg) of ^{3}H-B were pipetted for recovery determination on the cotton-wood pieces used for the 2-min blood collection. The blood was washed from the cotton-wool with distilled water. The blood content of the washing fluid was estimated by analysing the haemoglobin concentration in it and in a blood-sample obtained with heart puncture[4]. Using a cyanomethaemoglobin method[11] for analysis, 96 ± 0·64% (S.E., $n = 30$) of known amounts of blood has been recovered in previous examinations[4]. In *experiment* 2, 5000 d.p.m. of both ^{3}H-aldosterone (15 pg) and ^{3}H–B (28 pg) were added to one-minute samples of adrenal venous blood. The samples were made up to 500 μl with distilled water. Extraction of half of the washing fluid (= 1 vol) in *experiment* 1 or the haemolyzed blood samples in *experiment* 2 was carried out with 20 vol of cold methylene chloride. The methylene chloride phase was washed with 0·8 vol of 0·1 N NaOH, 0·8 vol of 0·1 M acetic acid and 1·4 vol of distilled water. The upper surface of the organic phase was rinsed twice more with water. After drying with Na_2SO_4 the material was transferred to a siliconized tube and the solvent was evaporated under a stream of nitrogen. In *experiment* 1 non-polar lipids were removed after evaporation by partitioning between 70% methanol and toluene-hexane (1:9 v/v).

t.l.c.

For the separation of corticosteroids 0·3 mm thick Kieselgel G (Merck) layers containing 0·5% Leuchtpigment (Riedel-de-Haen) were used. The plates were activated at 110°C for 40 min. The evaporated extracts were transferred to the plate in methylene chloride containing 0·05% acetic acid. The chromatogram was run for 12·5 cm by means of sandwich technique with chloroform–acetone–acetic acid (100:40:2·5 by vol). Mean R_F values of aldosterone, B, deoxycorticosterone (DOC) and progesterone were 0·2, 0·4 0·6 and 0·7, respectively. Steroids were located by running standards (about 1 μg each) in parallel lanes and examining the plates in U.V. light at 254 nm. The spots corresponding to aldosterone and B were scrapped off and eluted in 100 μl of 50% ethanol and 2 × 1 ml of chloroform. The combined organic phase was evaporated in siliconized tubes under nitrogen and stored, if necessary, under a few drops of toluene at −20°C.

Prior to assay, aldosterone was dissolved in 1·1 ml of ethanol. One 0·25 ml

aliquot of the eluate was taken into 10 ml of a POPO-POPOP-toluene–dioxane scintillation solution for recovery determination, while an aliquot of 0·25 ml and one of 0·50 ml were taken for the assay. B was eluted in methylene chloride–methanol (1:1 by vol). An aliquot was used for recovery determination and two unequal aliquots were used for the assay. In order to work in the reliable range of the assay the volume of these aliquots was adjusted according to the anticipated B content of the sample.

Aldosterone assay

Antibody to aldosterone was a generous gift from Dr. Sylvia A. S. Tait (The Middlesex Hospital Medical School, London) whose help is gratefully acknowledged. The original sample (1:100 dilution in 4% bovine serum albumin in 0·15 M potassium phosphate buffer pH 7 containing 0·1% (w/v) sodium azide) was divided and stored in ampoules under nitrogen at −20°C. Stock solutions (1:500) were kept at +4°C.

For each run a 1:100,000 dilution of antiserum was prepared in 0·05 M borate buffer (pH 8·0), containing 0·1% bovine serum albumin (Fraction V, Calbiochem), 0·5% gamma globulin (Humán Serum Institute, Budapest) and 0·1% sodium azide. The duplicate sample extracts and duplicate standard samples (0–800 pg) together with 5000 d.p.m. (15 pg) of ^{3}H-aldosterone were transferred into small centrifuge tubes. Ethanol was evaporated under nitrogen and 250 μl of the binding solution was added. The tubes were carefully vibrated for 30 min at room temperature and incubated at 4°C overnight. Cold, saturated ammonium sulphate, 250 μl, was then added to each tube. The tubes were swirled on a vortex mixer and centrifuged at 5000 *g* for 2 min at 1°C. Three hundred μl of supernatant was transferred into another centrifuge tube, albumin and ammonium sulfate were precipitated with $2 \times 1{\cdot}5$ ml of ethanol and the ethanol supernatant was transferred into a PPO-POPOP-toluene-dioxane scintillation solution for tritium counting.

B assay

The details of B binding assay were previously described [3]. The only modification was as follows: after adding Florisil the tubes were centrifuged at 3000 *g* for 30 sec at 1°C and 0·5 ml of the supernatant was directly transferred into the scintillation solution.

Radioactivity measurements and calculations

Tritium activity was counted for 2×20 min (or 10,000 counts) either in a Packard Tricarb 3375 or in a Packard Tricarb 3380 liquid scintillation spectrometer (the latter completed with a Model 544 Absolute Activity Analyser). Counting efficiency of each sample was estimated by means of automatic external standardization. The calculation of the free fraction of ^{3}H-aldosterone involved correction for the residual activity (d.p.m.) of tritium used for recovery estimation. The computation of protein-bound B involved correction for the experimentally measured inefficiency of Florisil to absorb B [3]. The calculations were carried out with a Hewlett–Packard 9100 B computer.

The standard curve was constructed by plotting the percentage of unbound aldosterone (Fig. 1) and bound B (Fig. 2), respectively, against the amount of unlabeled standard added. The amount of steroid in the single aliquots was read on the calibration curve and was corrected for the recovery in the respective ali-

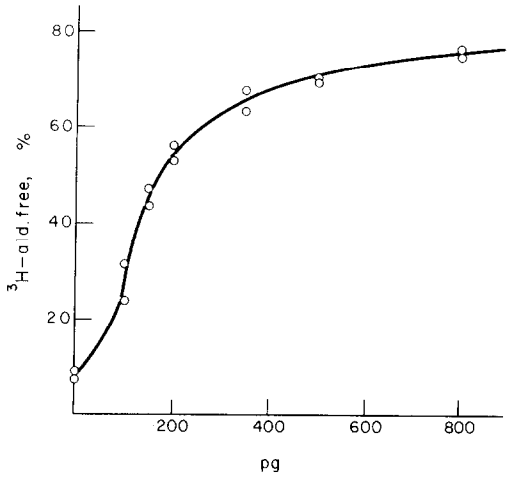

Fig. 1. A typical calibration curve: aldosterone added (pg) vs the percentage of free ^{3}H-aldosterone.

quot. The calculated values of the corresponding aliquots were averaged. No correction was made for blank.

RESULTS

Accuracy

Accuracy was determined by adding known amounts of aldosterone and B tc 0·5 ml of phosphate buffer (pH 7·0) containing 0·5% human serum albumin

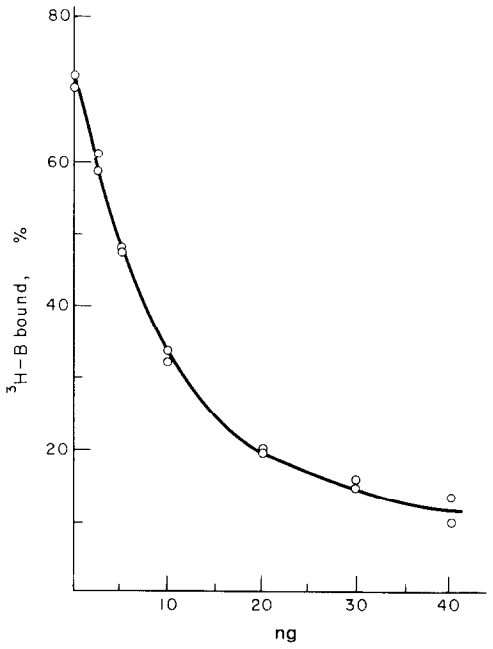

Fig. 2. A typical calibration curve: corticosterone added (ng) vs the percentage of bound ^{3}H-corticosterone.

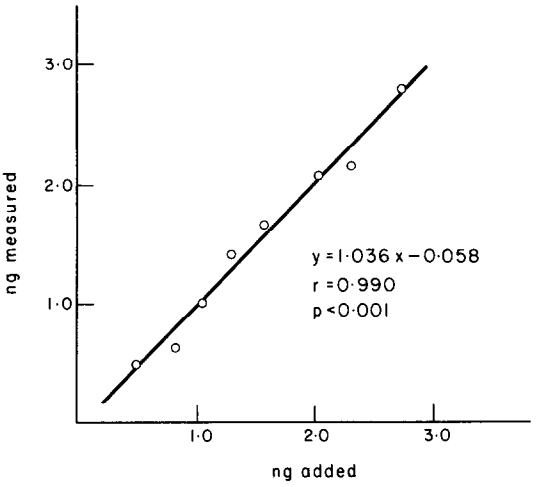

Fig. 3. Study of accuracy: aldosterone added vs aldosterone quantified.

(Humán Serum Institute, Budapest). A linear relationship was obtained between the amount added and quantified in the range of 0·5–2·7 ng for aldosterone (Fig. 3) and in the range of 10–150 ng for B (Fig. 4). The mean recovery was 98·24 ± 10·22 (S.D.) % ($n = 8$) and 109·40 ± 8·47 (S.D.)% ($n = 8$), respectively.

Precision

Precision was examined by replicate determinations of plasma-pools to which the synthetic steroids were added (Fig. 5). In the range examined, the coefficients of variations for aldosterone were 5·5, 21·1 and 9·6% (mean: 13·1%) while those for B were 6·0 and 7·6%.

Blank

0·5 ml of albumin containing phosphate buffer was used for the determination of blank. After correction for the loss of ^{3}H-steroid in course of the procedure, the

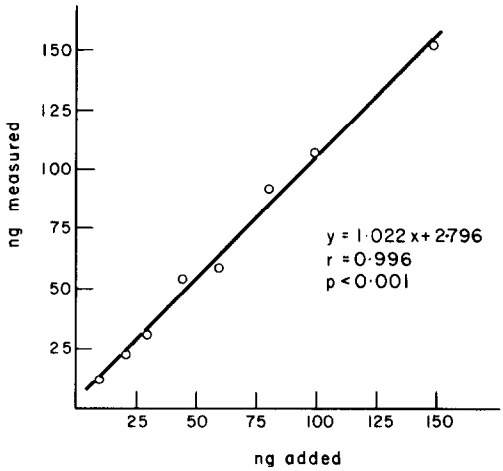

Fig. 4. Study of accuracy: corticosterone added vs corticosterone quantified.

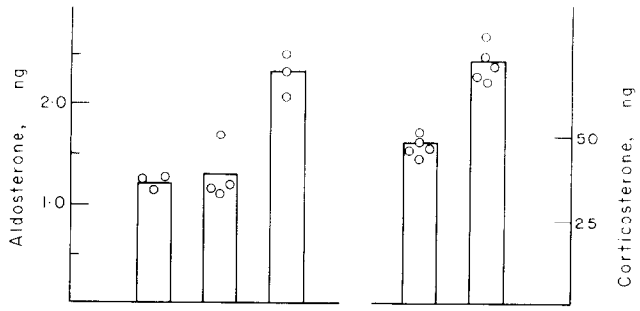

Fig. 5. Study of precision: replicate analysis of different plasma-pools.

aldosterone blank attained 91±61 pg (S.D., $n=6$). The mean blank for B was 0·54±0·65 ng (S.D., $n=8$). (A blank value of 11 ng, obtained at the same time as a blank of 1·1 ng within the same series, was omitted from the statistical calculations since some contamination was probable in this case.)

Sensitivity

Sensitivity of the overall assay (i.e. the smallest amount in the original sample significantly different from zero) was computed on the basis of the upper confidential limit of the regression curve in Figs. 3 and 4, respectively. Sensitivity may be set at 0·3 ng for aldosterone and 0·6 ng for B.

Specificity

Specificity of aldosterone antibody was tested by Dr. G. Williams (Peter Bent Brigham Hospital, Boston, Mass.). He found less than 1% cross-reaction for B, 18-OH-B and 18-OH-DOC. We examined the specificity of the overall assay by analyzing duplicate buffer samples containing 1 μg of B or progesterone or DOC or 5 μg of aldosterone for aldosterone and/or B.The results are summarized in Table 1.

Biological values

Adrenal venous blood was collected by the method of Spät *et al.*[4] in *experiment* 1. Aldosterone secretion rate from the left gland attained 2·10±0·68 (S.D.) ng/min or 1·00±0·34 ng/100 g/min ($n=5$). Simply taking the animals out of a common cage consistently increased B output (Table 2). A comparable mean

Table 1. Study of specificity

Steroid* added	Estimated as aldosterone (ng)	B (ng)
1 μg B	0·06	—
1 μg DOC	0·16	2·9
1 μg progesterone	0·37	0
5 μg aldosterone	—	1·7

*The steroid standards were not purified by the authors.

Table 2. Experiment 1: aldosterone and corticosterone secretion in normal, surgically stressed rats. Adrenal venous blood collected by the method of Spät *et al.*[4] (Single measurements).

Time of anaesthesia	9·20	10·00	11·25	12·13	12·45	13·20
Aldosterone (ng/100 g/min)	0·38	0·41	2·23	0·99	0·99	—
B (ng/100 g/min)	100	120	131	245	293	357

aldosterone secretion rate was measured in *experiment* 2 where blood was collected by Vogt's method[5]: $1{\cdot}78 \pm 0{\cdot}23$ ng/min or $0{\cdot}86 \pm 0{\cdot}13$ ng/100 g/min ($n = 5$). B values were higher than in *experiment* 1 but a clearcut increase within the series was observed in the last animal only (Table 3).

DISCUSSION

Although antibody-preparations of high specificity have been produced, the minute amount of aldosterone in biological samples necessitates the separation of aldosterone from other corticoids before the binding assay. Separation of aldosterone in human peripheral plasma has been generally carried out by means of paper chromatography[2, 12–14] while Mayes *et al.*[1] has applied a column chromatography prior to paper chromatography in order to obtain the minimum blank. Ito *et al.*[14] reported also on the successful use of Sephadex LH20 column chromatography.

The present method was designed for the *simultaneous* determination of aldosterone and corticosterone in rat adrenal venous blood. When the steroids were separated by the Zaffaroni-type formamide–benzene paper chromatographical system[10], traces of formamide interfered with the corticosterone assay (not examined for aldosterone). If aldosterone and B are separated by a conventional Bush type system[9], the exact location of the relatively non-polar B requires scanning of the paper. Chromatogram scanning is time-consuming, and tritium scanners are not generally available. No scanning was necessary for the location on the thin layer chromatograms we employed.

Thin layer chromatography is considered[15, 16 etc.] to yield higher blank values in displacement analysis of steroids than paper chromatography. Nonetheless, the known advantages of t.l.c. encouraged us to apply this method for the separation of corticosteroids. Using *freshly* distilled solvents for extraction, a

Table 3. Experiment 2: aldosterone and corticosterone secretion rate in normal, surgically stressed rats. Adrenal venous blood collected by the method of Vogt[5] (single measurements)

Time of anaesthesia	9·18	10·44	11·30	12·16	13·24
Aldosterone (ng/100 g/min)	0·80	0·52	0·84	0·72	1·40
B (ng/100 g/min)	349	378	317	374	610

1:200,000 dilution of the anti-aldosterone serum and calibration standards between 10 and 150 pg (plus zero-tubes), overall assay blank values of about 20 pg were repeatedly obtained. Prewashing of the t.l.c.-plates with methanol, application of silicic acid column chromatography[1] subsequent to t.l.c. or the replacement of t.l.c. by E_2B paper chromatography[8] failed to further diminish the blank. Since t.l.c. is simpler and less time-consuming, this system was finally applied in the routine procedure.

The following requirements had to be met in order to attain an accuracy approximating 100%: (1) fresh and efficient purification of ^{3}H-aldosterone, (2) purification of the organic solvents as indicated under *Methods*, (3) the elimination of methanol at steps before evaporation and (4) use of siliconized glass tubes for evaporation. Under such conditions using a 1:200,000 dilution of antiserum, perfectly reproducible calibration curves (0–150 pg) were obtained but the results of accuracy studies in the range of 50–350 pg were not reproducible enough. For this reason the "over-sensitive" binding solution was replaced by the present one containing anti-aldosterone serum in a 1:100,000 dilution. The accuracy of the method obtained with this binding solution (98%) is within the range of 97–107% published by others[1, 2, 13, 14] for the radioimmunoassay of plasma aldosterone. The coefficient of variation of replicate determinations (an expression of precision) is 5·5–5·7% only in the report of Mayes *et al.*[1]. Our mean coefficient of variation (13%) is comparable with the results obtained by Bayard *et al.*[2] 10·5–14%), Underwood and Williams[13] (7·7–14·3%) and Ito *et al.*[14] (4·7–21%). These values of Bayard *et al.*[2] refer to a working range practically the same as ours while the other authors obtained their results in a lower working range. No data for accuracy and precision were published for the above mentioned method of Banks *et al.*[12]. The blank obtained using a 1:100,000 dilution is higher than that obtained by some other authors[1, 12, 14] and is comparable to that obtained by Bayard *et al.*[2]. This value is, however, still sufficiently low for estimating aldosterone content in adrenal venous blood.

Beta particles emitted from tritium may be absorbed by the precipitated protein in the scintillation solution. In such cases automatic external standardization may be a misleading means for the determination of the counting efficiency since it reveals a shift only in energy distribution. For this reason we removed albumin from the supernatant containing unbound aldosterone before its transfer into the scintillation vial. The precision of the aldosterone assay has been increased by this manoeuvre.

Secretion rate examinations

Aldosterone secretion rate of the surgically stressed rat was estimated in a few cases in order to compare the values obtained by radioimmunoassay with values obtained by means of double isotope dilution techniques (Table 4). Comparable mean values were obtained by the two methods used for the collection of adrenal venous blood[4, 5]. Our values (single measurements in Tables 2 and 3) are slightly lower than the majority of these data. This may be accounted for by the short (2 min) blood collection period by which the stimulation of aldosterone secretion may have been avoided. Of course, other factors such as sodium and potassium intake, anaesthesia etc. may influence the "normal" secretion rate as well.

In contrast to aldosterone, B secretion rates were considerably different in the

Table 4. Aldosterone and corticosterone secretion rate in the rat

Author	Sex*	Body weight (g)	Food Na^+ mEq/Kg	Food K^+ mEq/Kg	Anaesthesia	Flow rate μl/min	Sample	Aldosterone ng/min	Aldosterone ng/100 g/min	Corticosterone ng/min	Corticosterone ng/100 g/min
Eilers and Peterson[13]	f	175–200	—	—	ether	90	blood	2·70±0·23	—	1000±110	—
Cade and Perenich[14]	m	—	293	—	ether	210±30	blood	—	3·3±0·6	—	630±80
Palmore and Mulrow[15]	m	150–200	103	114	—	—	plasma	4·0±0·5	—	1036±90	—
Dufau and Kliman[16]	m	350–450	8·5(?)	—	pentobarbital	16–30	blood	2·2±0·54	—	220±36	—
Kinson and Singer[17]	m	168–240	67	45	ether–pentob.	80–270	blood	—	1·57±0·37	—	518±23
Abbott *et al.*[18]	m	400–528	220	250	pentobarbital	—	plasma	2·82±0·19	—	583±94	—
Melby *et al.*[19]†	f	180–200	–	—	pentobarbital	—	plasma	0·84±0·09	—	440±30	—
present paper:											
Expt. 1	m	205–230	165	150	pentobarbital	57–237	blood	2·10±0·68	1·00±0·34	211–821	100–357
Expt. 2	m	180–250	165	150	pentobarbital	105–420	blood	1·78±0·23	0·86±0·13	680–1099	349–610

*f=female, m=male.

†Aldosterone estimated by means of radioimmunoassay. The details of technique are not yet available.

two experimental series. Applying the rapid method of adrenal venous blood collection described by us [4], a low value for B output was obtained in the first animal. The subsequent values gradually increased (Table 2) reflecting the diurnal increase in ACTH secretion and/or the stress caused by the repeated disturbance of the animals. In *experiment* 2 adrenal venous blood was collected by means of cannulating the left renal vein ligated at either end [5]. The first four animals exhibited uniform but high B secretion rates as compared to the data obtained in *experiment* 1 and only the fifth animal, examined in the early afternoon, displayed an increase in B secretion rate (Table 3). (All these values were, however, within the "normal" range of the literature.) If a preliminary conclusion may be drawn from so few examinations, these data suggest the possibility that the collection of adrenal venous blood by Vogt's method [5] represents a more severe surgical stress. Thus, the responsiveness of the zone fasciculata to moderate stimuli may deserve further examinations.

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