A Modified Method for Estimating 17-Hydroxycorticosteroids in Plasma

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Assessment of adrenocortical activity by chemical or biological assay of corticosteroids and their metabolites in urine is of limited value: these compounds are in part excreted as water-soluble conjugates, and the existing methods for their hydrolysis are not entirely satisfactory (Marrian, 1951; Bayliss, 1952). The decrease in circulating eosinophils following administration of adrenocorticotrophic hormone or cortisone has provided only an indirect and non-quantitative index of increased amounts of circulating corticosteroids. Various methods have been used for direct estimation of adrenal steroids in blood. Vogt (1943) and Paschkis, Cantarow, Walking & Boyle (1950) used bioassay procedures involving the cold-protection and the glycogen-deposition tests, expressing the results in terms of cortisone and 11-dehydrocorticosterone (Kendall's compound A) respectively. In dogs, quantities ranging from 1 to 5 mg./100 ml. plasma were found. These methods are not suitable for clinical use. Corcoran & Page (1948) determined the quantity of formaldehydogenic material in plasma and found in human peripheral blood the equivalent of 100–400 μg. 11-deoxycorticosterone/100 ml. plasma. The exact nature of the compounds liberating formaldehyde is unknown, but Pincus (1952) suggests that some are glycols, which may or may not be steroids. Paper-partition chromatography shows that peripheral blood contains several hormones, of which 17-hydroxycorticosterone (Kendall's compound F) and corticosterone (Kendall's compound B) are present in the largest amount by weight and may prove to be clinically the most important (Hechter, 1952; Bush, 1952, 1953). Such methods are only semi-quantitative and require large volumes of plasma.

Recently, Nelson & Samuels (1952) have described a chemical method for estimating in blood and plasma steroids that give a colour reaction with phenylhydrazine in sulphuric acid. According to Porter & Silber (1950), who first described it, this colour reaction is specific for 17:21-dihydroxy-20-ketosteroids, and Nelson, Samuels, Willardson & Tyler (1951) state that by their technique the chief compound measured in blood appears to be 17-hydroxycorticosterone.

The method described here is essentially that of Nelson & Samuels (1952), but certain modifications have been effected and additional observations made to confirm the validity of the method.

METHOD

Reagents. Chloroform (B.P.) was distilled from anhydrous K_2CO_3 immediately before use. Magnesium trisilicate, Florisil, 60/100 mesh (Floridin Co., Warren, Penn., U.S.A.) was washed with ethanol and activated by heating at 600° for 4 hr. Ethanol (A.R.) was redistilled. Methanol (A.R.) was distilled from 2:4-dinitrophenylhydrazine, and then redistilled twice. The H_2SO_4 reagent was a 22·8 N-solution (62% (v/v) conc. H_2SO_4, A.R.). Phenylhydrazine hydrochloride (A.R.) was recrystallized four times from ethanol and stored in the dark *in vacuo* over anhydrous $CaCl_2$. The phenylhydrazine-H_2SO_4 reagent was a solution of 160 mg. phenylhydrazine hydrochloride in 100 ml. 22·8 N-H_2SO_4, prepared immediately before use. The 17-hydroxycorticosterone standards were solutions in methanol in concentrations ranging from 3–40 μg./ml.

Extraction. Heparinized plasma (15 ml.) is extracted in a 50 ml. centrifuge tube three times with equal volumes of $CHCl_3$. The plasma and $CHCl_3$ are emulsified with a rapid mechanical stirrer and then separated by centrifugation for 30 min. at 3500 rev./min. in a 14 cm. radius head. The lower layer is pipetted off, and the three $CHCl_3$ extracts combined in a 250 ml. flask and taken to dryness under reduced pressure at 45°. The dried extract is stored at 4° until chromatographed.

Chromatography. A closely fitting disk of Whatman no. 54 filter paper and a wad of glass wool, previously washed in methanol and oven-dried, are placed on a perforated disk at the bottom of a burette (int. diam. 11 mm.). Florisil (1·5 g.) is poured into the burette and 15 ml. $CHCl_3$ added. The Florisil is suspended by inverting the burette several times. When the adsorbent has settled a further 5 ml. $CHCl_3$ are used to wash down any powder adhering to the walls. A plug of glass wool is pressed down on top of the Florisil which is packed by applying N_2 at 5 lb./sq.in. for 5 min. The column is run at a constant rate, timed against a metronome, of 63 drops/min.

After the $CHCl_3$ has run down to the top of the column, the plasma extract is transferred quantitatively to the burette with successive portions of 6, 4 and 2 ml. $CHCl_3$. When the extract has been adsorbed on to the column, development is carried out in sequence with 35 ml. $CHCl_3$, 25 ml. 2% (v/v) ethanol in $CHCl_3$, and 25·5 ml. 24·5% (v/v)

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ethanol in $CHCl_3$. The third eluate is collected in a boiling tube, evaporated to dryness at 45° with a stream of N_2, and stored at 4° until the colorimetric estimation is made.

In parallel with the plasma extracts, solvent alone and known amounts of 17-hydroxycorticosterone are run through columns to determine the 'column blanks' and the percentage recovery of steroid from the column.

Colorimetry. Methanol (0·6 ml.) is added from a microburette to the boiling tubes containing the dried third eluate. The walls are washed down carefully, first by rotating the tube and then by using a 0·2 ml. pipette. The methanolic solution (0·2 ml.) is added to 0·3 ml. phenylhydrazine-H_2SO_4 reagent in a small conical tube and mixed thoroughly. To correct for interfering substances which may yield a coloured solution with H_2SO_4 alone, a duplicate 0·2 ml. of the methanolic solution is added to 0·3 ml. H_2SO_4 reagent. Blanks of 0·2 ml. methanol and standards of 17-hydroxycorticosterone in 0·2 ml. methanol are run in parallel with both the phenylhydrazine-H_2SO_4 and the H_2SO_4 reagents.

The tubes are covered with glass balls and heated in a water bath at 60° for 1 hr. After cooling in tap water for 3 min., the optical density is read in cuvettes 10 mm. long by 2 mm. wide in a Beckman DU spectrophotometer at 410 mμ. (slit width, 0·06 mm.). A mask with a 1 mm. slit is placed in front of the cell carrier. Each solution is transferred to a cuvette with a separate pipette.

The standards, 'column' blanks and known amounts of 17-hydroxycorticosterone which have been run through the columns, and the unknowns in the H_2SO_4 reagent are compared with the blank of 0·2 ml. methanol in the same reagent, which in practice is set at zero intensity. Similarly, the corresponding standards, 'column blanks', column recoveries and unknowns in phenylhydrazine-H_2SO_4 reagent are compared with the blank of 0·2 ml. methanol in phenylhydrazine-H_2SO_4 reagent, which is also set at zero density.

Calculations. If A, B, etc., are the optical densities read against the appropriate methanol blank with the phenylhydrazine-H_2SO_4 reagent, and a, b, etc., those with the H_2SO_4 reagent, and the readings are: A and a for a standard containing S μg. 17-hydroxycorticosterone in 0·2 ml. methanol: B and b for the solvents run through the column ('column blank'); C and c for the unknown plasma extract which has been taken up in 0·6 ml. methanol and 0·2 ml. samples (one-third) taken for reaction with the H_2SO_4 and the phenylhydrazine-H_2SO_4 reagents; D and d for E μg. 17-hydroxycorticosterone run through the column, and one-third samples measured, then the colour given by the standards not passed through the column is $(A-a)/S$ per μg., and by the known amounts of 17-hydroxycorticosterone passed through the column $\frac{\{(D-d)-(B-b)\}}{E} \times 3$. The percentage recovery from the column is

$$x = 100 \times \frac{S}{(A-a)} \frac{\{(D-d)-(B-b)\}}{E} \times 3.$$

If M ml. of plasma have been extracted, the concentration of 17-hydroxycorticosteroids (μg./100 ml.) in the unknown is

$$3 \times \{(C-c)-(B-b)\} \frac{S}{(A-a)} \frac{100}{x} \frac{100}{M}.$$

For representative values, see Table 1, p. 525.

RESULTS AND DISCUSSION

Use of plasma. In earlier experiments whole blood after laking with water was extracted with a chloroform:ether mixture. The extract was dried, taken up in aqueous ethanol and a hexane partition carried out (cf. Nelson & Samuels, 1952). The ethanolic solution was taken to dryness and the extract treated as described above for the chromatographic and colorimetric procedures. The extracts were frequently dirty, the hexane partition was too time-consuming in a method required for clinical use, and often the agreement between duplicates was poor. The use of plasma has simplified the method considerably; hexane partition is not required and duplicates agree well.

Extraction. To determine the optimal number of extractions, duplicate samples of plasma were extracted three times with equal volumes of chloroform. The samples were then extracted a fourth time and the extracts from each pair of duplicates pooled. It was found that significantly greater amounts of steroids were not obtained by extracting more than three times. In one instance duplicate samples of plasma yielded 20·8 and 17·6 μg. 17-hydroxycorticosterone/100 ml. by triple extraction. The pair of fourth extracts pooled yielded no detectable steroid. In another experiment the amounts were 16·1 and 18·0 μg./100 ml. and 0·6 μg. in the fourth extracts.

Recovery from the column. Since each batch of Florisil may vary, it is necessary to determine the percentage recovery of 17-hydroxycorticosterone added to the column. With one batch additions in duplicate of 6·1 and 1·4 μg. gave recoveries of 95 and 96·5%, and 93 and 93% respectively. With a second batch additions of 2·7 and 6·8 μg. gave recoveries of 70 and 77% respectively. Appropriate corrections have been made for these losses. Similar recoveries were obtained with cortisone and pregnane-3α:17α:21-triol-11:20-dione (tetrahydrocortisone). Thus with a third batch of Florisil the recoveries of cortisone, 17-hydroxycorticosterone, and tetrahydrocortisone were respectively 86, 75, and 70%, the difference being related to the polarity of the compounds.

Colorimetric procedure. In the method of Nelson & Samuels (1952) the extract contained in the last eluate from the column is dissolved in 0·2 ml. methanol, the whole being treated with phenylhydrazine-sulphuric acid reagent and read against a column blank. Because substances other than 17-hydroxycorticosteroids present in the extracts give a small amount of background absorption, which decreases with increasing wavelength, Nelson & Samuels make an approximate correction by using an empirical 'absorption factor' (Allen, 1950): absorption at 410 mμ. $-\frac{1}{2}$ (absorption at 370 mμ. +

absorption at 450 mμ.). This formula assumes a rectilinear change in the optical density of the background with changing wavelength, a condition which has not been verified experimentally. In addition, the 'absorption factor' does not necessarily correct for the colour that interfering substances may produce with sulphuric acid alone. Another disadvantage of calculating the results with an 'absorption factor' is that only an unknown proportion of the reacting compounds is measured.

We have followed the technique of Porter & Silber (1950) more closely and correct for interfering colour by systematically running a control of the plasma extract with sulphuric acid. Hence the extract after chromatography is dissolved in 0·6 ml. methanol, and two duplicates of 0·2 ml. are taken, one for reaction with the phenylhydrazine-sulphuric acid reagent and the other with sulphuric acid reagent. Readings are made only at the wavelength of maximum adsorption, 410 mμ. The greater dilution of the chromatographed extract with methanol in our method has required using 15 ml. plasma instead of the 10 ml. used by Nelson & Samuels (1952), in order to obtain optical densities falling within the sensitive range of the spectrophotometer.

The values obtained with the 'absorption factor' are usually lower than those obtained with the method described above using a sulphuric acid control, although changes in the plasma concentration of 17-hydroxycorticosteroids, calculated by the two methods, run parallel. The percentage recovery of 17-hydroxycorticosterone added to plasma samples is greater using a sulphuric acid control, and falsely low recoveries may be obtained

Table 1. *Comparison of results calculated by the present method using a* H_2SO_4 *blank and by the method using an 'absorption factor' (Nelson & Samuels, 1952)*

The column blank was obtained by passing the eluants through the column. Plasma (15 ml. specimens) was obtained from subjects F.B. and E.D. *P* and *Q* were duplicates in each case. 2·7 μg. 17-hydroxycorticosterone were added to *R* from F.B. and to *R* and *S* from E.D. Letters (*A*, *a*, etc.) in table refer to the symbols used in the calculation, p. 524.)

Optical density readings

	Phenylhydrazine-H_2SO_4 reagent Wavelength (mμ.)			H_2SO_4 reagent Wavelength (mμ.)
	370	410	450	410
Methanol blank (set at)	0·000	0·000	0·000	0·000
Standards				
0·81 μg./0·2 ml.	0·072	0·095 (*A*)	0·042	0·001 (*a*)
2·70 μg./0·2 ml.	0·224	0·288	0·127	0·004
4·06 μg./0·2 ml.	0·367	0·463	0·208	0·012
Column blank	0·060	0·046 (*B*)	0·036	0·033 (*b*)
Recoveries from column	0·103	0·110 (*D*)	0·060	0·026 (*d*)
(using 2·7 μg. 17-hydroxycorticosterone)	0·102	0·110	0·057	0·034
Plasma F.B. *P*	0·194	0·170 (*C*)	0·120	0·105 (*c*)
Q	0·184	0·165	0·113	0·097
R	0·203	0·203	0·117	0·077
Plasma E.D. *P*	0·191	0·183	0·136	0·132
Q	0·178	0·157	0·112	0·098
R	0·223	0·215	0·147	0·113
S	0·245	0·240	0·173	0·127

Results obtained by the two methods of calculation corrected for the column blank and losses on the column

(Percentage recovery of steroid added to plasma is shown in parenthesis.)

	17-Hydroxycorticosteroids (μg./100 ml. plasma)	
	Present method	Method of Nelson & Samuels
Plasma F.B. *P*	13·9	9·0
Q	14·4	10·9
R	30·3 (93)	25·3 (85)
Plasma E.D. *P*	10·2	12·5
Q	12·3	8·4
R	23·9 (70)	18·2 (41)
S	26·8 (85)	18·8 (44)

using the 'absorption factor', if interfering substances give a high optical density at wavelengths other than 410 mμ. One example of this is shown in Table 1 where the values obtained by the two methods are compared. The recovery of 17-hydroxycorticosterone added to duplicate plasma samples from subject E.D. was 70 and 85% by the present method and only 41 and 44% using the 'absorption factor'.

Sensitivity. As found by Nelson & Samuels (1952), within the range of 0·6–4·0 μg. 17-hydroxycorticosterone, the intensity of the colour developed follows Beer's law. 1 μg. 17-hydroxycorticosterone gives an optical density of about 0·100. Using 15 ml. plasma and taking 0·2 ml. of the final 0·6 ml. methanolic solution of the extract, plasma containing 10 μg./100 ml., which is an average normal value, will give theoretically an optical density of 0·050. In practice, owing to other chromogenic material which is corrected for by the sulphuric acid reagent control, readings from 0·080 to 0·200 are obtained, depending on the amount of interfering substances present in the particular specimen of plasma.

Recovery of 17-hydroxycorticosterone added to plasma. Table 2 shows the percentage recovery when 17-hydroxycorticosterone (2·3–13·5 μg.) is added to 15 ml. samples of plasma. The recovery varies from 68 to 93%.

Table 2. *Percentage recovery of 17-hydroxycorticosterone added to 15 ml. samples of plasma*

(Steroid added in 1 ml. absolute methanol, except for the last three examples where it was added in 1 ml. 25% (v/v) methanol in saline.)

17-Hydroxycorticosterone (μg.)			Recovery of added steroid (%)
Added	Expected	Found	
2·3	3·8	3·2	75
2·3	3·8	3·3	80
4·5	6·0	4·5	67
7·4	8·9	6·9	73
8·1	8·9	7·7	85
13·5	15·0	10·7	68
2·7	4·8	4·6	93
2·7	4·4	3·6	70
2·7	4·4	4·0	85

Accuracy of the method. Over a period of 12 months duplicate samples of thirteen specimens of plasma from patients with various non-endocrine conditions have been assayed at random. The concentrations of 17-hydroxycorticosteroids ranged from 5·0 to 20·4 μg./100 ml. Differences between duplicates varied from 0·0 to 2·8 μg. and are considered as deviations from an expected zero. The sum of squares is 25·66 with a mean square of 2·14 for 12 degrees of freedom. At the 0·05 probability level, with a t value of 2·17, a difference between estimates of 3·3 μg. 17-hydroxycorticosteroids/100 ml. plasma ($t \times$ standard deviation) is significant for the range 5·0–20·4 μg./100 ml.

Specificity. The specificity of the method depends on three factors: the extraction with a lipid solvent, the chromatographic purification of the extract, and the colorimetric reaction. The colour reaction is relatively specific for 17:21-dihydroxy-20-ketosteroids, although certain other compounds such a methyltestosterone, oestradiol and pregnenolone react with sulphuric acid to give a coloured compound and therefore an erroneously high sulphuric acid reagent control (Porter & Silber, 1950). By experiment it was found that pregnenolone in amounts equivalent to 25 μg./100 ml. plasma did not cause a high sulphuric acid reagent blank and that this compound was eluted from the columns before the third eluate containing 17-hydroxycorticosteroids. No interference was caused by methyltestosterone or oestradiol in amounts equivalent to 10 and 12 μg./100 ml. plasma respectively.

The colour developed may be due to any 17:21-dihydroxy-20-ketosteroid. Those which are known or thought to be present in blood include cortisone, 17-hydroxycorticosterone, 17-hydroxydeoxycorticosterone, the dihydro and tetrahydro metabolic reduction products of cortisone (pregnane-17α:21-diol-3:11:20-trione) and pregnane-3α:17α:21-triol-11:20-dione and pregnane-3α:11β:17α:21-tetrol-20-one (Burton, Zaffaroni & Keutmann, 1951*a*; Baggett, Glick & Kinsella, 1952; Dohan & Richardson, 1952; Schneider, 1952).

An attempt was made to characterize as far as possible the steroids in peripheral blood that give the phenylhydrazine-sulphuric acid reaction. Large samples of plasma were pooled and worked up as described above. A proportion of the chromatographed extract equivalent to 15 ml. plasma was assayed by the colorimetric method: the remainder was partitioned by paper chromatography using a toluene:propylene glycol system (Burton, Zaffaroni & Keutmann, 1951*b*). In an extract of 200 ml. pooled plasma 30 μg. 17-hydroxycorticosteroids (expressed as 17-hydroxycorticosterone) were found by colorimetry. By paper chromatography about 30 μg., as judged visually, of material giving a blue colour with triphenyltetrazolium chloride were found, 10 μg. of which were present in an area corresponding to the position of 17-hydroxycorticosterone. The remainder of the colour was given by a compound or compounds more polar than 17-hydroxycorticosterone and which appeared as an ill-defined streak corresponding in part to the position of tetrahydrocortisone, the only pure compound of this type available to us. In an extract of 680 ml. plasma about 50 μg. 17-hydroxycorticosteroids were found by colorimetry. Paper chromatography showed 40 μg., as judged visually, of

compounds reacting with triphenyltetrazolium chloride. About half this material occupied an area corresponding to 17-hydroxycorticosterone. The other half was more polar, some of it corresponding to tetrahydrocortisone and some of it even more polar. No material running at a similar speed to cortisone was detected in either extract.

Nelson *et al.* (1951), using a similar technique, state that most of the colour developed is due to 17-hydroxycorticosterone. Our observations suggest that only about one-third to one-half is due to this compound. The discrepancy may be due to the fact that the plasma we used was obtained from subjects with cardiac failure, hypertension or chronic bronchitis; and theirs from patients whose adrenals had been stimulated with adrenocorticotrophin and whose blood would be expected to contain a greater amount of 17-hydroxycorticosterone. It seems, therefore, that the method measures both 17-hydroxycorticosterone and certain other compounds which may be either adrenal secretory substances or their metabolic products.

Values obtained in various endocrine and non-endocrine conditions will be published elsewhere.

SUMMARY

1. A modification of the method of Nelson & Samuels (1952) for estimating 17-hydroxycorticosteroids in plasma is described. The method is sufficiently simple for clinical use.

2. About one-third to one-half of the material estimated appears to be 17-hydroxycorticosterone, the remainder being more polar compounds.

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