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INVESTIGATION OF THE SALIVARY 18-HYDROXYCORTICOSTERONE:ALDOSTERONE RATIO IN MAN USING A DIRECT ASSAY

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Summary—A method for the direct determination of 18-hydroxycorticosterone (18OHB) in human saliva has been developed and validated. Saliva was collected at 30 min and 1 h intervals between 0600 and 2200 h from healthy men and women for the determination of 18OHB (SHB), aldosterone (SA) and glucocorticoids (SGC = cortisol + cortisone). SHB was highly correlated with SA ($r = 0.75$; $P < 0.001$) but even more highly with SGC ($r = 0.89$; $P > 0.001$). Multiple regression analysis confirmed that SGC was a more important determinant of SHB than was SA. Though the concentrations of 18OHB and aldosterone were highly correlated there was considerable variation in the 18OHB:aldosterone ratio during the period of saliva collection. This ratio tended to be highest in the morning and lowest in the evening and was weakly correlated with SGC level ($r = 0.62$; $P < 0.01$).

The 18OHB:aldosterone ratio in saliva approximates to, and is highly correlated with, that in plasma. We suggest that the fluctuations in SHB:SA ratio correspond to the relative rates of secretion of 18OHB and aldosterone and that this ratio is modulated either by ACTH or by cortisol. Whether this indicates that 18OHB is a by-product of glucocorticoid as well as aldosterone metabolism, or whether this implies a separate physiological role for the steroid remains to be clarified.

INTRODUCTION

It is widely believed, though not rigorously proven, that 18OHB is the immediate precursor of aldosterone. 18OHB is secreted at about twice the rate of aldosterone [1] and factors that lead to an increase in the rate of secretion of aldosterone such as angiotensin-II [2], ACTH [3, 4], negative sodium balance [4], metoclopramide [5] and postural change [6] also lead to simultaneous changes in the plasma concentration of 18OHB. It is not known however whether 18OHB possesses any physiological function [7], other than negligible mineralocorticoid activity [8], independent of its possible role as a precursor of aldosterone.

One approach to investigating whether 18OHB has an independent physiological role would be to determine whether its rate of secretion ever differs significantly from that of aldosterone. Such a divergence would be evidence of either a separate control mechanism or at least some modulation of the rate of conversion of 18OHB to aldosterone.

Since there are no *a priori* clues to a possible physiological role for 18OHB in man, it seemed appropriate to investigate spontaneous changes in the rates of secretion of 18OHB and aldosterone under basal conditions. To facilitate such studies we have used changes in the concentrations in saliva as evidence of corresponding changes in the rates of their secretion of the two steroids. We have previously shown that the concentrations of aldosterone [9–11] and 18OHB [12] in saliva are highly correlated with those in plasma. While we have previously described a direct assay for aldosterone in saliva [9] the determination of 18OHB has hitherto required a thin-layer chromatographic (TLC) step prior to radioimmunoassay. This was adequate for demonstrating the general validity of measuring 18OHB in saliva but it was not suitable for the present study on account of limited sample capacity. We have now investigated an alternative 18OHB antibody and this has permitted the development of a direct assay for 18OHB in saliva.

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In this paper we describe the validation of this direct assay for 18OHB in human saliva and its application to the study of the spontaneous changes in the 18OHB:aldosterone ratio throughout the day in healthy subjects.

MATERIALS AND METHODS

Two different, independently produced, antibodies to 18OHB have been used in this work, the preparation of which has been reported previously, antibody G [4] and antibody Y [12]. The latter report also describes the preparation of 18OHB-3-(O-carboxymethyl)oximino-2-[^{125}I]iodohistamine which is used as the ligand and the technique for the TLC purification of 18OHB which has been used in the validation of the direct assay. The antibodies were stored at dilutions of 1:1000 (G) and 1:250 (Y), in 0.1% bovine albumin in assay buffer containing 0.1% sodium azide. The ligand was stored in ethanol containing 0.1% triethylamine (ethanol-TEA) at −20°C.

Assay buffer was 0.067 molar phosphate at pH 7.4. Dextran-coated charcoal (DCC) was prepared by suspending activated charcoal (Sigma no. C-5260) in dextran–gelatin solution (1 g/100 ml). The latter was prepared by dissolving 1 g each of dextran C, swine skin gelatin and sodium azide in 1 l of assay buffer.

Measurement of 18OHB in saliva

A stock solution of 18OHB in ethanol-TEA (1 mg/ml) was diluted 1:100 and then 1:50 in ethanol-TEA to give standard B2 (200 ng/ml). This was diluted 1:200 (100 μl–20 ml) in assay buffer to give the highest working standard (1 ng/ml = 2762 pmol/l) which was serially diluted in assay buffer down to the lowest standard (21.6 pmol/l). These working standards were stored at 4°C and were stable for many weeks.

The antibodies were diluted in 0.1% bovine albumin in assay buffer to give working dilutions of 1:100,000 (G) or 1:8000 (Y). To these solutions was added ligand to give approximately 25,000 cpm/ml. Saliva samples (0.2 ml) and standards (0.2 ml) were pipetted into 10.5 × 63.5 mm polystyrene (LP3) tubes. Antibody–ligand solution (0.2 ml) was added and the tubes were covered and incubated at 4°C overnight. After standing in an ice bath for at least 30 min, 0.5 ml of well-stirred, ice-cold DCC was added to the tubes in the sequence 1-*n*,*n*-1 ("mirror image"). The tubes were centrifuged at 4°C (1500 *g* for 10 min), the supernatant decanted, and after draining for a few minutes the charcoal pellet was counted in a Packard "Multi-Prias" automatic gamma counting system. This was equipped with on-line computing facility which calculated the concentrations of 18OHB in the samples. TLC eluates were assayed in exactly the same fashion.

Recovery experiments

Standard B2 (200 ng/ml) was diluted 1:100 with methanol containing 0.1% triethylamine (methanol-TEA) to give a concentration of 2000 pg/ml. This was serially diluted (1 + 1) with methanol-TEA to give standards down to 16 pg/ml. Addition of 18OHB to saliva was made by evaporating 0.1 ml of the appropriate solution to dryness in an LP3 tube and adding 0.2 ml saliva.

Measurement of plasma 18OHB concentration

This was performed exactly as described previously [13] except that antibody G was used for the radioimmunoassay of the 18OHB in TLC eluates.

Other determinations

The measurement of plasma and salivary aldosterone [9] and glucocorticoids [13] was as previously described.

Physiological investigations

9 healthy subjects (3 female, 6 male) not receiving any medication and aged 24–60 yr collected saliva samples at 30 min intervals from 0600 to 1000 h and at hourly intervals from 1000 to 2200 h. The first sample was collected while the subject was still recumbent after which the subjects got up and went about their normal daily activity. No attempt was made to standardize diet, meal times or daily activity. On a separate day both blood and saliva were collected at 0730 and 1700 h from 6 of the 9 subjects. The plasma was separated within an hour and frozen at −20°C until analysis.

Statistical analysis

Multiple regression analysis and correlations were performed using the statistical analysis programme SAS (SAS Institute, Carey, N.C.). ANOVA and Bonferroni *t*-tests were performed on means to characterize significant differences. $P < 0.05$ was considered significant.

RESULTS

Validation of the direct assay

Specificity. 18OHB was measured in 52 saliva samples using both antibodies in the direct assay and for the estimation of 18OHB in TLC eluates. It was found that in the direct assay use of antibody Y resulted in values that were, on average, 67% higher than when antibody G was used. However, when the TLC eluates were assayed the two antibodies gave-identical results. A comparison of the direct and TLC assays using antibody G for both showed that the direct assay gave values which were 14% higher (paired t; $P < 0.02$) though the two sets of values were very highly correlated. The same relationship may be discerned when only those samples on which both values are less than 100 pmol/l are considered.

When thin-layer chromatograms of saliva extracts were cut into 1 cm lengths and eluted with assay buffer all the immunoassayable activity detectable with antibody G ran parallel with authentic 18OHB but when antibody Y was used small quantities of immunoassayable activity could be detected running both ahead of and behind 18OHB, though they were insufficient to account for the discrepancy between the direct assay values obtained with the two antibodies.

Precision and sensitivity. The % free ligand for the zero standard was typically 35.0 + 1.0 (SD) (CV = 3.0%) while for the next lowest standard (22 pmol/l) the value was about 38%. This is three standard deviations higher than the zero standard and implies that an 18OHB concentration of 20 pmol/l should be distinguishable from zero with greater than 95% confidence. However, this is to ignore the possible effects of other, unknown substances in saliva ("matrix" effects). The CV of the % free ligand calculated from duplicate determinations of 17 saliva samples computed to contain less than 100 pmol/l was 1.8% while the CV of the 18OHB concentration was 10.1%. These values are similar to those obtained for standards prepared in assay buffer and confirm that measurement of 18OHB concentration down to 20 pmol/l is valid, at least in statistical terms. In practice, concentrations of 18OHB in saliva less than 50 pmol/l are uncommon and less than 30 pmol/l extremely so.

Recovery experiments/accuracy. When 18OHB was added to saliva (25 pg to 0.2 ml) the recovery was 110 + 11%. A total of 6 saliva pools were prepared by the addition of 18OHB standards (0.1 ml) over the range 1.6–100 pg to 0.2 ml saliva. In all 6 cases the measured concentration of 18OHB was very highly correlated with the quantity of 18OHB added ($r > 0.99$), with the intercept identical to the original concentration of 18OHB. However, in 5 out of 6 saliva pools the slope of the regression line was greater than 1.00. That is, there is a tendency for the recovery of added 18OHB to be overestimated.

Parallelism with standards. When varying volumes of saliva (made up to 0.2 ml with buffer) were assayed there was a linear relationship ($r > 0.98$) between the volume of saliva and the mass of 18OHB (Fig. 1).

Physiological studies

Diurnal fluctuation in saliva corticosteroid concentrations. The saliva concentrations of all three corticosteroids (SGC, SA and SHB) showed considerable diurnal fluctuation (Fig. 2). The relationships between the concentrations of these three corticosteroids are summarized in Fig. 3. SA and SHB were significantly correlated ($r = 0.75$; $P < 0.01$), however there was considerable variation in the SHB:SA ratio (Fig. 4). This ratio tended to be highest in the morning and declined through the day. In no subject was there a peak in the SA or SHB that was not associated with a peak in the other. SHB was more highly correlated with SGC ($r = 0.89$) than with SA ($r = 0.75$, Fig. 3). Multiple regression analysis confirmed that SGC was the most significant determinant of SHB concentration (data not shown).

Corticosteroid saliva–plasma relationships. The concentrations of aldosterone, 18OHB and glucocorticoids in saliva were highly correlated

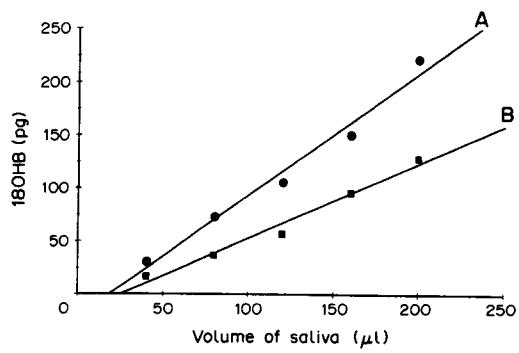

Fig. 1. Relationship between volume of saliva assayed and the quantity of 18OHB using two pools of saliva A and B. Volumes of saliva less than 0.20 ml were made up to that volume with assay buffer. Regression analysis—A: $y = 1.076x - 9.8$; $r = 0.991$; B: $y = 0.67x - 11.7$; $r = 0.989$.

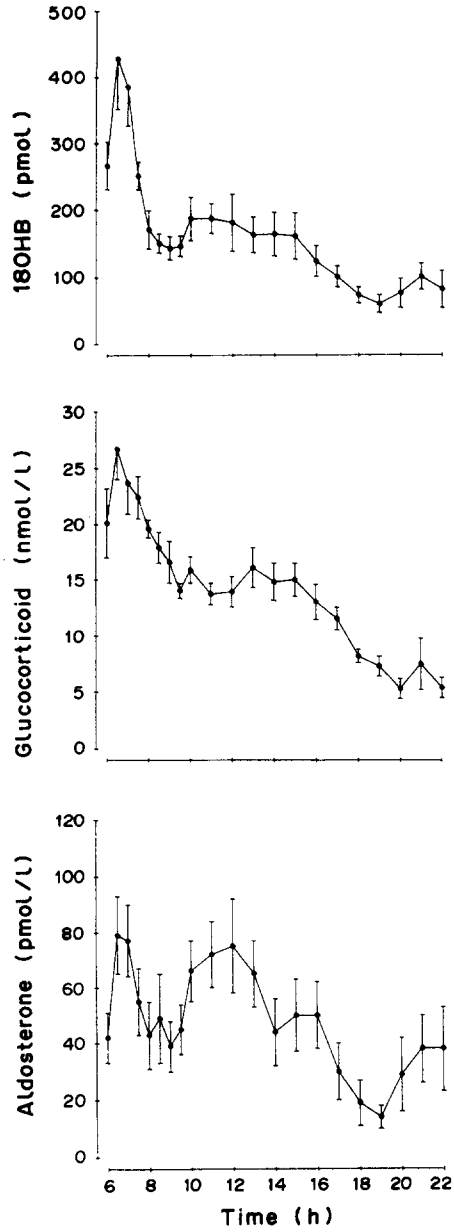

Fig. 2. Fluctuations in the diurnal concentrations in saliva of 18OHB, aldosterone and glucocorticoids ($n = 9$).

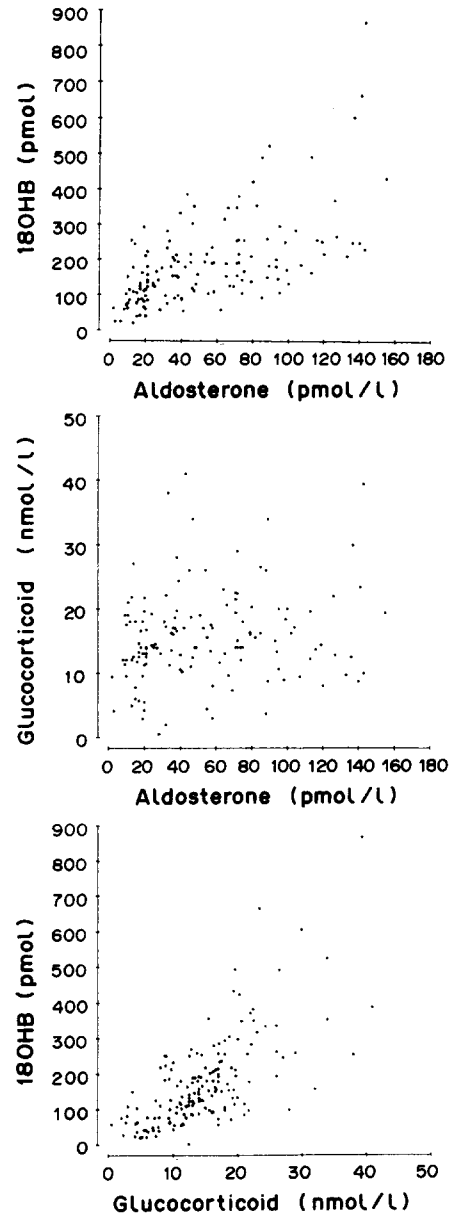

Fig. 3. Correlation of salivary 18OHB vs aldosterone ($r = 0.75$) glucocorticoid vs aldosterone ($r = 0.67$); and 18OHB vs glucocorticoid ($r = 0.89$).

with those in plasma. The values were: for aldosterone $r = 0.97$, for 18OHB $r = 0.94$ and for glucocorticoids $r = 0.75$. As shown in Table 1, the 18OHB:aldosterone ratio in saliva (4.1 ± 2.4) was not significantly different (paired t-test) from that in plasma (4.8 ± 4.3) and the two ratios are highly correlated ($r = 0.86$; $P < 0.01$).

DISCUSSION

The direct method described in this report considerably simplifies the determination of 18OHB in human saliva. Though it gives values that are statistically significantly higher than the TLC method the differences between the two methods are not significant in physiological or clinical terms. This slight loss of specificity is counterbalanced by the enhanced productivity of the direct assay and permits physiological and clinical investigations to be undertaken which would not be considered if every sample had to be chromatographed.

The two antibodies used, Y and G, were both raised in rabbits against 18OHB-3-CMO conjugated to bovine albumin. It is thus intriguing that they exhibit quite different specificity in the

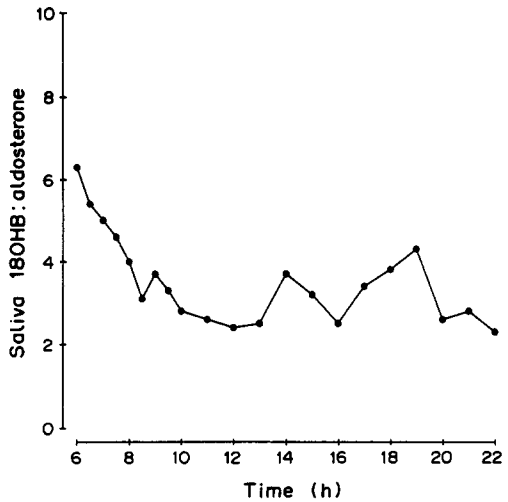

Fig. 4. Fluctuations in the diurnal variation of salivary 18OHB:aldosterone ratio ($n = 9$).

assay and this was heightened by the lack of detection of significant quantities of any substances in saliva which cross-reacted with antibody Y but not with G. This probably reflects a difference in the immune response between individual rabbits.

The concentrations of aldosterone, 18OHB and glucocorticoids in plasma and saliva were highly correlated. This confirms our previous findings [9–12] and indicates that measurements of the three steroids in saliva give a useful indication of their plasma concentrations. The saliva:plasma relationships are sufficiently constant such that changes in the 18OHB:aldosterone ratio in saliva correspond to that in plasma. We suggest that changes in the 18OHB:aldosterone ratio in saliva may be regarded as evidence of changes in the relative rates of secretion of the two steroids by the adrenal. This view is supported by the findings in previous studies, that the kinetics of aldosterone and 18OHB clearance from plasma are similar, though not identical [14] and in the present study, that the saliva:plasma ratios of aldosterone and 18OHB are similar and highly correlated.

Table 1. 18OHB:aldosterone ratios in saliva and plasma in 6 subjects at 0730 and 1700 h ($r = 0.86$; $P < 0.01$)

Subject		18OHB:Aldosterone Plasma	Saliva
1	a.m.	8.3	15.1
	p.m.	4.5	4.5
2	a.m.	4.3	6.0
	p.m.	1.6	1.6
3	a.m.	2.0	2.0
	p.m.	2.2	1.4
4	a.m.	2.7	3.0
	p.m.	3.0	3.0
5	a.m.	4.6	5.5
	p.m.	2.2	3.1
6	a.m.	8.8	11.1
	p.m.	5.5	1.3
Mean ± SD		4.1 ± 2.4	4.8 ± 4.3

Evidence from previous investigations suggests that 18OHB is secreted synchronously with aldosterone and probably in a fixed ratio to it. They are the only corticosteroids whose concentration in plasma is increased by angiotensin-II infusion [4]; there is a high degree of correlation between the plasma concentrations of aldosterone and 18OHB over a wide range of disorders of the pituitary–adrenal and renal–adrenal systems [15, 16] and procedures which lead to increases in plasma aldosterone level also lead to corresponding increases in 18OHB [3–6]. Under normal conditions too, 18OHB is considered to be derived predominantly from the zona glomerulosa and its secretion pattern follows that of aldosterone. It was unexpected in the present study therefore that the concentration of 18OHB in saliva was more closely correlated with that of glucocorticoids than with aldosterone, suggesting that ACTH may be more important in the regulation of 18OHB secretion than aldosterone. Although the concentrations of SHB and SA were highly correlated there was considerable variation in the 18OHB:aldosterone ratio during the period of saliva collection. This ratio tended to be highest in the morning and declined through the day (Fig. 4). This ratio was weakly correlated with SGC level ($r = 0.62$). These results suggest that the 18OHB:aldosterone ratio may be modulated by either ACTH or cortisol and that the control of secretion of 18OHB may, at least partially, be separate from that of aldosterone. Previous studies have demonstrated that exogenously administered ACTH is capable of stimulating 18OHB secretion [3] and of increasing the 18OHB:aldosterone ratio in man. The mechanism by which ACTH would have such an effect in physiological conditions is not clear however.

The finding that the SHB:SA ratio was correlated with SGC concentration ($r = 0.62$) might have been taken as an indication of differential displacement by cortisol of aldosterone and 18OHB bound to plasma proteins. The finding that the 18OHB:aldosterone ratio was similar in plasma and saliva renders this unlikely. Moreover, the similarity in the plasma:saliva ratios of aldosterone (7.2 ± 7.9) and 18OHB (6.7 ± 5.7) (calculated from samples of plasma and saliva obtained at 0730) and 1700 h and

their high degree of correlation ($r = +0.88$) suggests that similar factors determine the non-protein bound fraction of each steroid in plasma.

In conclusion, we have developed and validated a method for the direct determination of 18OHB in human saliva. We have demonstrated that SHB is highly correlated with SA but even more highly with SGC. The 18OHB:aldosterone fluctuates through the day and is correlated with SGC. These results suggest that SGC is a more important determinant of SHB than that of SA and suggest that the 18OHB:aldosterone ratio may be modulated either by ACTH or by cortisol.

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