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Salivary Cortisol and Dehydroepiandrosterone Sulphate Levels in Postmenopausal Women with Primary Breast Cancer*

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Abstract—*Cortisol and dehydroepiandrosterone sulphate (DHAS) were measured in saliva from postmenopausal women with primary breast cancer and a control group of comparable age. Specimens were collected at 2-hr intervals during wake-span for two consecutive days, and circadian rhythmicity was demonstrated for each of the hormones in both populations. The marginally elevated levels of cortisol and lower levels of DHAS associated with the cancer group, and the large inter-subject variation, make it unlikely that these hormone measurements would be of value in identifying women at risk of developing breast cancer.*

INTRODUCTION

CLASSICAL epidemiological studies in the field of breast cancer clearly imply that endocrine status must be considered a determinant of risk. Early age at menarche is associated with increased risk, whereas early first-term pregnancy is protective [1]. Family history must also be considered a risk factor [2], but equally of interest, however, developing from the earlier 'discriminant function' studies of Hayward, Bulbrook and their colleagues [3, 4], has been the indication that low excretion of 'urinary androgens', in particular aetiocholanolone (< 1 mg/24 hr), also constituted a risk factor [5]. A prospective study of women in Guernsey [6] suggested that the precancer subject could be identified from controls on the basis of urinary levels of 17-hydroxycorticosteroid and aetiocholanolone up to a decade before diagnosis [7, 8], and the concept of abnormal adrenal steroid metabolism in the breast cancer subject has been supported by the studies of Deshpande *et al.* [9].

Furthermore, low plasma concentrations of DHA sulphate, DHA and androst-5-ene-3β,17β-diol have been found in women from Guernsey with a high probability of developing breast cancer [10], and these results correlated well with urinary aetiocholanolone excretion. Although Wang originally reported [11] normal plasma levels of DHA sulphate in patients with breast cancer, he was subsequently able to confirm the report from this laboratory [12] that the concentrations of DHA sulphate in plasma from women with advanced disease were subnormal. Rose *et al.* [13] and Zumoff *et al.* [14] have since reported that certain breast cancer patients have low levels of this adrenal androgen. The latter study was particularly effective, the authors recognising the unrepresentative nature and difficulties of single-sample analysis and the marked circadian fluctuation of plasma DHA sulphate, reported on a '24-hr mean plasma level' derived from the analysis of a pooled sample from aliquots of 72 specimens collected every 20 min throughout a day. Unfortunately patient numbers in the study were small.

Recent studies from this institute [15] have drawn attention to the value of salivary steroid analyses for the assessment of endocrine status and to their potential in the investigation of breast cancer aetiology. Steroid changes in saliva correlate well with those in plasma, but of particular interest is the close approximation between concentrations of the free 'biologically active' levels of steroid in plasma and the salivary hormone concentration. Such an approach provides a new opportunity to develop further endocrine studies into breast cancer using

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multiple-sampling procedures which are stress-free. Data from single-sample analysis, even collected at a fixed time during the day, can be misleading if rhythmic changes are present [16].

In this study cortisol and DHA sulphate have been measured in saliva collected throughout two days from postmenopausal women with primary breast cancer and from normal controls. The possible identification of a salivary 'discriminant function' could offer the means of recognising the individual high-risk subject, the extensive rhythm-sampling maximising the probability of separating the two groups.

MATERIALS AND METHODS

Subjects

The subjects were 50 of the volunteer postmenopausal women who had been referred by G.P.s to the Nottingham City Hospital as part of a national breast cancer screening programme. After clinical diagnosis of breast cancer had been made, each patient returned to their domiciliary environment and completed the salivary sampling laid down in the study protocol. Histological confirmation of primary breast cancer was subsequently made from a biopsy specimen prior to surgery, which was 4–6 days after the initial clinical diagnosis. Control subjects were 32 volunteer women who had been visiting a well-woman breast screening clinic for the sole purpose of attending a lecture. These controls had no familial history of breast cancer and no personal history of breast disease or endocrine cancer. Although the controls were not exactly age-matched with the patients, they did, however, span the period from 45 to 66 years of age, covering the major span of the cancer group, which was aged 49–78 years.

Samples

Subjects were supplied with an instruction/record card and a compact plastic box containing an insert for holding 20 5-ml glass sample tubes. Samples of whole saliva were taken at 2-hr intervals during wake-span (07.00–23.00 hr) for 2 consecutive days with an additional sample taken at 07.00 hr on the third day. Samples were immediately placed in the plastic box and transferred to the freezing compartment of the subject's domestic refrigerator. Samples were transported to the Tenovus Institute by special courier, where they were stored at -20°C. Immediately prior to analysis samples were thawed and centrifuged at 700 *g* for 10 min to remove any particulate matter. Experiments designed to simulate the transit and storage of samples revealed no noticeable changes in either cortisol or dehydroepiandrosterone sulphate levels under these conditions.

Steroid analysis in saliva

The procedure for cortisol determination has been previously described [17]. The estimated intra- and inter-assay variations over the working range of the standard curve, expressed as % coefficient of variation (% CV), were approximately 3–8 and 10–17 respectively. An assay to determine DHA sulphate concentrations in saliva was developed using sheep antiserum raised against a DHA-3-hemisuccinate ovalbumin conjugate and a [1,2,6,7-^{3}H]-DHA radioligand. This was considered to be a valid assay for DHAS since the DHA/DHAS ratio in saliva is only about 3%. Duplicate aliquots of saliva (50 μl) were added to 100 μl of label (25,000 counts/min; sp. act. 60–90 Ci/mmol) dissolved in phosphate-buffered saline (0.01 M phosphate, 9 g/l saline, pH 7.4) and the solution incubated for 60 min. Dextran-coated charcoal (1.0 ml, 0.025 g/l dextran, 2.6 g/l charcoal) was added, tubes shaken and left at 4°C for 12 min, followed by centrifugation (700 g; 10 min) and the supernatant decanted into scintillant vials for counting. The estimated intra- and inter-assay variations (% CV) for the working range of the standard curve were 3–12 and 7–22 respectively, and the specificity of the antiserum as indicated using the method of Abraham [18] is given in Table 1. A standard curve showing the precision of the response metameter is shown in Fig. 1. The assay method was compared with a high-resolution gas chromatography–mass spectrometry procedure established in this institute and a satisfactory correlation ($r = 0.8$; $n = 12$) was observed.

Processing of RIA data and Quality Control

Procedures for the processing of RIA data have been described elsewhere [19]. Each analytical batch contained 19 samples, taken in order of collection, from one particular subject selected at random from either the cancer or the control group of women. The sequential arrangement of

Table 1. *Specificity of DHA antiserum*

Steroid	Cross-reaction (%)
Dehydroepiandrosterone (DHA)	100
DHA sulphate	92
Androsterone	<1
Aetiocholanolone	<1
17α-Hydroxyprogesterone	<1
Testosterone	<1
Androstenedione	<0.05
Androst-5-ene-3β,17β-diol	<0.005

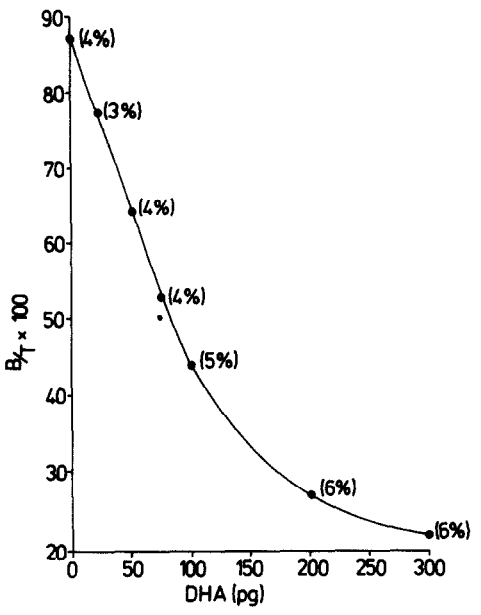

Fig. 1. Representative standard curve for DHA sulphate assay. The intra-assay precision of the response metameter at selected hormone concentrations are expressed as a % coefficient of variation. B/T represents the ratio of bound counts to total counts in the assay.

standards, subject and quality control samples was as follows: (i) duplicate standard hormone concentrations; (ii) duplicate aliquots of low, medium and high titre quality control samples; (iii) duplicate aliquots of subject samples; and (iv) a repeat arrangement of quality control samples to monitor potential intra-assay drift. Cumulative sum techniques were used to monitor changes in: (i) mean hormone concentrations; (ii) imprecision; and (iii) drift, as described previously [20]. The control parameters and target and control statistics used to monitor analytical performance are described elsewhere for use with a computer program specially developed for radioimmunoassay [21].

RESULTS AND DISCUSSION

Concentrations of cortisol and DHA sulphate in saliva exhibited considerable between-subject variability in both cancer and control patients. Values for the arithmetic and geometric means for cortisol and DHA sulphate in saliva, together with their respective standard errors of the mean, are given in Table 2. These means and standard errors have been calculated from the average of each population computed for each time period.

Graphs of the arithmetic and geometric means, together with the standard error of the mean for each time period, are shown for cortisol and DHA sulphate in Figs 2 and 3 respectively. Chronodesms [22], tolerance levels containing 90% of the population with 90% confidence, are shown for cortisol and DHA sulphate in Figs 4 and 5, respectively.

Discriminant function and canonical variate analyses were performed at each time period on the control and cancer populations to determine whether or not a significant separation between the two groups could be achieved based solely on cortisol and DHA sulphate measurements. The analysis was carried out using the General Statistics Program, GENSTAT [23], available on the South West Universities Computer Network in the United Kingdom. Canonical variate analysis, the principles of which have been described elsewhere [19], seeks to determine those linear orthogonal combinations of variates, such as the concentrations of cortisol and DHA sulphate, which maximise the ratio of the between-group to the within-group variances. In the analysis of two groups the first canonical variate is exactly equivalent to Fisher's linear discriminant. Using raw data, little discrimination could be achieved between the two groups at each of the time periods, with the possible exception of 21.00 hr.

Cortisol levels in saliva in both groups of subjects (Fig. 2) exhibited circadian rhythmicity; levels are highest during waking hours and lowest towards 21.00 hr, with signs of elevation at 23.00 hr. It is apparent that the geometric mean, as would be expected *a priori*, gives lower values than the arithmetic mean. The results indicate that cortisol levels in patients are higher than their controls. Statistical significance, however, remains difficult to assess due to the 'error' structure between individuals.

Table 2. Mean salivary cortisol and DHA sulphate concentrations and standard errors of the mean (SEM) for normal and log-normal distributions for postmenopausal women with primary breast cancer and their controls

Steroid	Group	Arithmetic mean	SEM	Geometric mean	± SEM*
Cortisol	control	6.84	0.931	5.89	6.68–5.19
(nmol/l)	cancer	8.33	0.821	7.71	8.43–7.05
DHA sulphate	control	3.78	0.307	3.63	3.88–3.40
(nmol/l)	cancer	3.70	0.229	3.55	3.80–3.40

*SEM transposed to arithmetic scale.

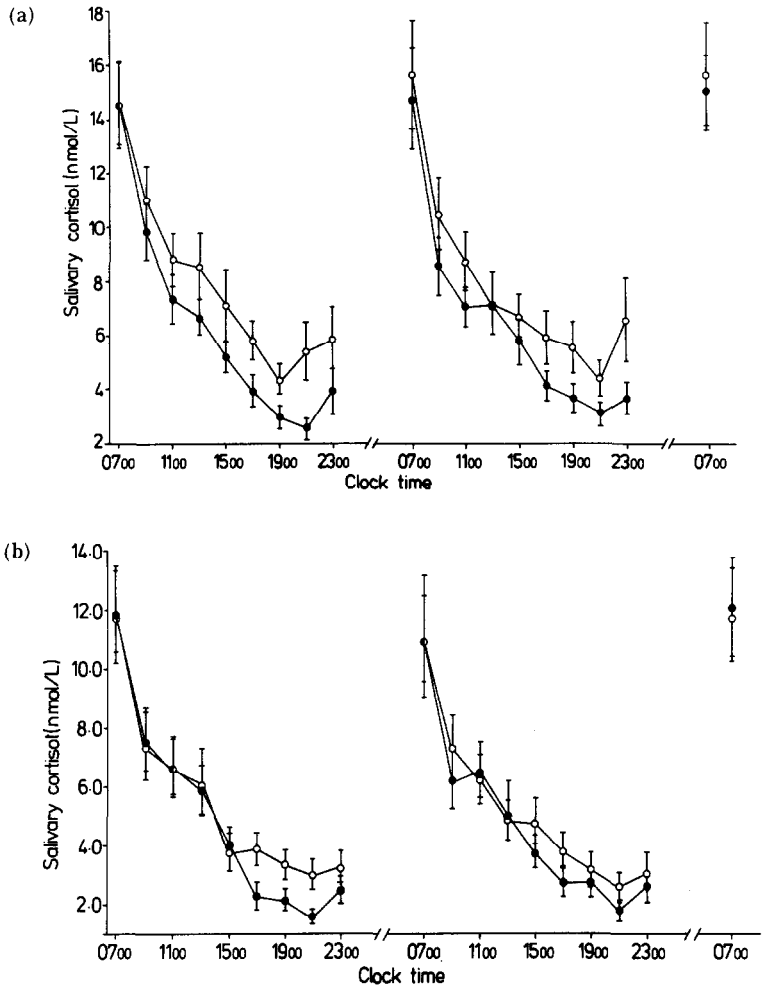

Fig. 2. Graphical representation of (a) arithmetic and (b) geometric mean salivary cortisol concentrations for each population measured at various time intervals during wake-span for 2 consecutive days. Data are for postmenopausal women with primary breast cancer (O——O) and their controls (●——●). Error bars represent standard errors of the mean.

DHA sulphate similarly exhibits circadian rhythmicity although not so pronounced, values again being highest during waking hours. When arithmetic and geometric means are compared it is interesting to note that in the latter case the results indicate that lower levels of DHA sulphate are associated with breast cancer patients, adding support to previous evidence for low androgen production in relation to breast cancer.

It is noticeable that the concentration of DHA sulphate in saliva is only 10% of that calculated for plasma-free DHA sulphate, obtained from literature values for the equilibrium association constant and plasma DHA sulphate and albumin concentrations. Despite the acceptable level of accuracy of the DHA sulphate methodology, failure to demonstrate a high correlation between matched plasma and saliva samples in other studies may indicate that the mechanism of DHA suphate secretion from the salivary glands is different from that of unconjugated steroids. Until reliable methods for determining free DHA sulphate in plasma become available these discrepancies will remain unresolved.

In conclusion, these studies have demonstrated and quantified circadian variations of cortisol and dehydroepiandrosterone sulphate in post-menopausal women with primary breast cancer and their controls. Furthermore, the advantages of salivary hormone determinations in assessing the endocrine status of individuals, particularly where large numbers of samples are required to investigate chronobiological variations, have been demonstrated. Whilst an increase in salivary cortisol and a decrease in DHAS would be consistent with previous findings, the wide variation in individual concentrations compared to the differences between groups makes it unlikely that the determination of salivary concentrations of cortisol or DHAS would be valuable in identifying a population of women at risk of developing breast cancer.

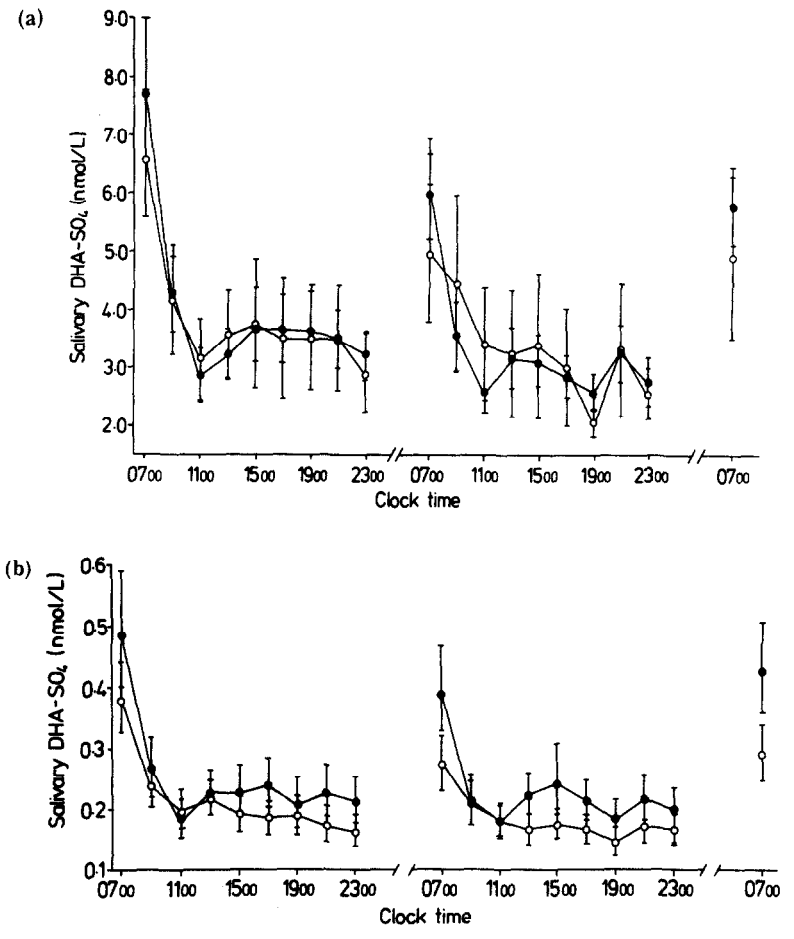

Fig. 3. Graphical representation of (a) arithmetic and (b) geometric mean salivary DHA sulphate concentrations for each population measured at various time intervals during wake-span for 2 consecutive days. Data are for postmenopausal women with primary breast cancer (○——○) and their controls (●——●). Error bars represent standard errors of the mean.

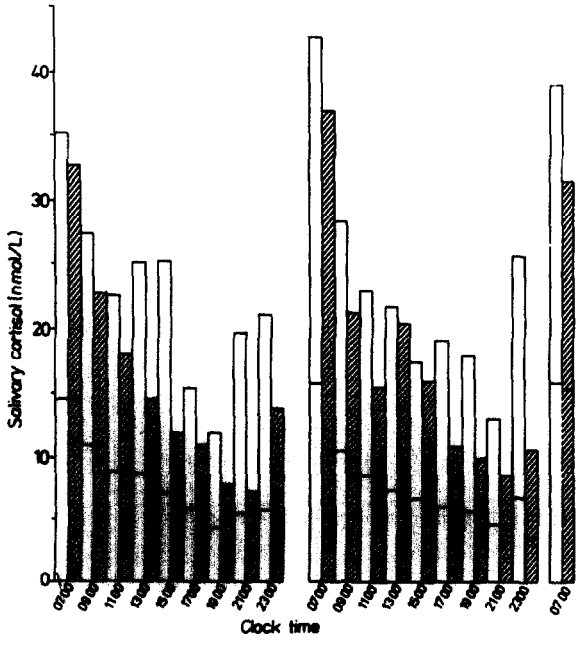

Fig. 4. Salivary cortisol chronodesms for postmenopausal women with primary breast cancer (open columns) and their controls (hatched columns). Chronodesms contain 90% of the respective populations with 90% confidence.

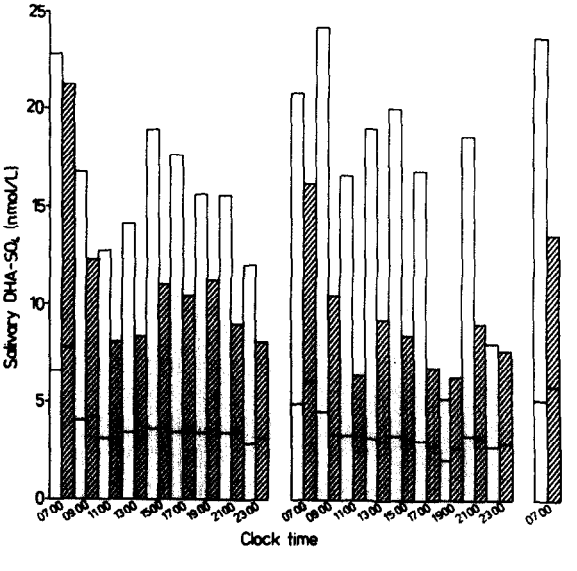

Fig. 5. Salivary DHA sulphate chronodesms for postmenopausal women with primary breast cancer (open columns) and their controls (hatched columns). Chronodesms contain 90% of the respective populations with 90% confidence.

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