Ann. Ist. Super. Sanità, vol. 29, n. 4 (1993), pp. 607-611

Saliva as a medium for chronobiological studies: its particular potential in steroid endocrinology

Douglas W. WILSON, Richard F. WALKER and Keith GRIFFITHS

Tenovus Institute for Cancer Research, University of Wales College of Medicine, Cardiff, United Kingdom

Summary. - This paper indicates the potential of saliva as a medium for chronobiological studies. It has principally focussed on the work of the Tenovus Institute for Cancer Research, at the University of Wales College of Medicine over the past decade or more, particularly for steroid hormones, so as to give an authoritative viewpoint based on practical experience. The article discusses issues ranging from technical to clinical aspects of hormone assay in saliva, showing unadvantages and limitations of use. Increasingly, many other investigators are developing and applying assays for salivary steroids and it is timely that such assays become tools in the hands of the chronobiologist. The clinical-biochemical importance of saliva resides also in the fact that in its context it is possible to assay enzymes, toxic substances and drugs.

Key words: chronobiology, chronoendocrinology, circadian rhythms, hormone rhythms, salivary rhythms.

Riassunto (*La saliva come mezzo di studi cronobiologici: sue particolari potenzialità in endocrinologia steirodea*). - L'articolo prospetta la potenzialità della saliva come mezzo per studi di ordine cronobiologico. In particolare, viene messo a fuoco il lavoro svolto al Tenovus Institute nell'ultima decade ed oltre, specie per gli ormoni steroidei, al fine di fornire un autorevole punto di vista basato sulla esperienza. L'articolo spazia dagli aspetti tecnici a quelli clinici del dosaggio ormonale nella saliva. Constata che un numero crescente di ricercatori sta sviluppando ed applicando il dosaggio salivare degli steroidi e prevede che sia giunto il tempo che questi tipi di dosaggi divengano uno strumento nelle mani dei cronobiologi. L'importanza clinico-biochimica della saliva risiede anche nel fatto che nel suo contesto è possibile trovare tracce di enzimi, eventuali sostanze tossiche e farmaci.

Parole chiave: cronobiologia, cronoendocrinologia, ritmi circadiani, ritmi ormonici, ritmi salivari.

Introduction

It was the pioneering studies of Shannon *et al.* [1-3] which first focussed attention on the value of parotid fluid as a medium in which to measure steroid hormone concentrations. However, several factors influenced the slow rate of progress in this field of research, not least of which was the technical difficulty of reliably measuring the low concentrations of steroid found in saliva. The development of sensitive and more specific immunometric techniques [4] in the 1970s provided the required impetus to assess whether salivary steroid hormones have a practical value in clinical endocrinology and related disciplines.

During the 1980s, the Tenovus Institute, with its analytical expertise, developed reliable immunoassays for a wide variety of steroids in saliva [5, 6], and was able to show that mixed, unstimulated saliva was a useful substitute for parotid fluid, and that concentrations of neutral steroids in saliva were also largely independent of flow rate. Furthermore, steroid concentrations were found to be of the order of 5% of the plasma concentration and so resembled the non-protein bound concentration found in plasma, presumed to be the biologically active concentration available to tissues. Thus frequent sampling without paramedic assistance, the ease of collection "in the field", storage stability and easy shipment of saliva samples are additional features which make the use of saliva an attractive biological medium in chronobiological research programmes.

It is now clear from a number of clinical studies that salivary steroid assays can be useful in a chronobiological setting to study rhythms ranging from ultradian to circatrigintan and beyond [7-9]. Relevant background information and some examples of various applications are now presented to assist the chronobiologist who may wish now to take advantage of this exciting research.

Institute assays for salivary steroids

Reliable and well-validated methodologies have now been published for cortisol [10], 17 α-hydroxy-progesterone [11], progesterone [12], testosterone [13, 14], androstenedione [15], oestriol [16], and subsequent reports have confirmed our original observations.

Assays for 18-hydroxycorticosterone [17], dihydrotestosterone [18], aldosterone [19, 20], oestrone [21] and oestradiol [22] have been reported by other groups. Critical consideration should however be given to the literature, especially when attempting to compare results from one laboratory to another [23].

General, analytical and biological considerations in the use of salivary steroid assays

General considerations

Children and most adults find it easy to salivate directly into wide-necked, disposable plastic tubes of whereupon 3 ml can be collected within 10 min, but elderly subjects may experience a little more difficulty. Although some investigators have suggested the need for stimulated saliva, consensus opinion would suggest that stimuli are probably best avoided. Samples should be taken before food ingestion or otherwise the mouth needs to be rinsed-out with water to remove debris some ten min before sampling. Blood contamination arising from bleeding gums or over-zealous brushing of teeth must be avoided. Collection of samples whilst the subject is asleep is obviously difficult - though small samples can be aspirated from sleeping babies using an appropriate suction device.

Sample storage presents little difficulty. Samples, properly labelled, are usually stored in the freezing compartment in the domestic refrigerator using a plastic box with accommodating holes for as many as 40 tubes. Under cold or even temperate climatic conditions, together with a postal service of say 3 days, samples may *generally* be posted to the analytical laboratory without apparent deterioration. It is good practice, however, to expose internal quality control samples to the same conditions of storage as the samples, and to ship the consignment of samples in dry ice to the analytical laboratory. In this way, checks on the stability of the samples is inferred. In cases where the numbers of samples lead to a lengthy time-span of storage, certain samples can be assayed repeatedly to provide added assurance, and statistical considerations should be given to minimising any time-effect in the end analysis.

Analytical considerations

The concentration of steroids found in mixed saliva is approximately 5% of that circulating in plasma and resembles the non-protein bound fraction (free) available to tissues. Analytical methods must be accompanied by technical data relating to specificity, sensitivity, precision and internal quality control criteria [24]. Accuracy, and therefore bias, should be assessed using a reference method such as high resolution [25, 26], gas chromatography-mass spectrometry (GC-MS).

In the case of cortisol, for example, the correlation coefficient, gradient and y-intercept for the data from a direct assay (y), spanning the range 0-60 nMol/l, versus the GC-MS assay (x) were 0.99, 0.98, and 0.81 nMol/l respectively. It is also useful to know the intended area of application of the assay. For example, an assay for male salivary testosterone may be totally inappropriate for the female due to inadequate specificity. It is also noteworthy to distinguish steroid conjugates from the neutral steroid. The merits of direct versus indirect (solvent extraction) assays should, where applicable, be weighed-up against the advantages of high through-put, particularly where hundreds or thousands of samples may be involved in the study. Chronobiological investigations by their very nature generally require more samples, at least initially, than more conventional studies.

Biological considerations

Research at the Institute has rigorously evaluated concentrations of steroids in parotid fluid (collected using a Carlsen-Crittenden device or Curby cup) and compared these with those in mixed saliva and found good agreement [6]. For example, the correlation coefficient, gradient and y-intercept for 17α-hydroxyprogesterone in parotid fluid (y) versus mixed saliva (x), covering the range 0-20 nMol/l were 0.98, 0.96 and −0.342 nMol/l respectively; corresponding values for cortisol over the range 0-70 nMol/l were 0.99, 1.00 and 0.53 nMol/l. In citric acid stimulation tests to determine whether steroid concentrations were flow-dependent, data for the neutral steroid, in stimulated or unstimulated situations, were almost co-incident [6]. Although mixed saliva was an acceptable medium in the senses described, it was uncertain as to whether concentrations of steroids in saliva would reflect plasma levels both under so-called normal physiological conditions, or in situations where parts of the hypothalamic-pituitary-adrenal or hypothalamic-pituitary-gonadal axes were stimulated or suppressed. Matched plasma and saliva samples were collected under a variety of conditions and concentrations of steroids in saliva generally mirrored those found in circulating plasma [6]. However, certain potential pitfalls still exist and need to be recognised: mixed saliva often contains a small amount of crevicular fluid and if the hormone in question is extensively bound in plasma, then the crevicular fluid will give rise to higher values. Moreover, the salivary glands themselves contain the enzyme 115 β-dehydrogenase which can convert cortisol to cortisone. In such circumstances cortisol will probably be replenished, but cortisone will accumulate in saliva giving rise to levels much higher than the "free" found in plasma. Finally, oral bacteria in the mouth may bring about metabolic changes, but under the conditions of storage described, this has not been observed to be a problem.

Chronobiological applications of salivary steroid assays: adrenal activity

Cortisol

It is now relatively easy to measure cortisol in saliva either using in-house reagents or commercial kits. There are also few controversies concerning its usefulness. At the Institute, circasemidian and circadian salivary cortisol rhythms have been studied in the newborn and older infants [27], and it is of future interest to determine if these rhythms are different in premature or sick babies. Circadian rhythms of salivary cortisol have been studied in relation to physical exercise experienced by Arctic explorers [28, 29] and concentrations have been monitored during marathon runs [30]. Salivary cortisol assays may also be used in psychiatry to study endogenous depression. Actually, it is now realised that endogenous depression probably comprises an array of illnesses with some common features, some patients however escape the counter-regulatory mechanisms that control cortisol secretion such that plasma concentrations are high. Salivary cortisol assays have already been used to "identify" DSM-111 patients, i.e. those with "Major Depression with Melancholia", as escapers from the dexamethasone test [31, 32]. Important changes in the time-structure of cortisol secretion may be important markers for the disease. In this context, salivary melatonin holds promise particularly in studies of Seasonal Affective Disorder.

Further details on the potential value of salivary cortisol assays in chronobiology in relation to mental health, "stress", exercise and sports medicine, human development and breast cancer have been reported elsewhere [8, 9].

17α-hydroxyprogesterone

The treatment of congenital adrenal hyperplasia with exogenous corticosteroid aims to impose a balance between 17α-hydroxyprogesterone, and androstendione and testosterone secretions. Inadequate treatment results in virilisation and the androgens remain high, whereas over-treatment causes suppresion of growth hormone secretion. Thus, under so-called normal circumstances, 17α-hydroxyprogesterone exhibits a circadian rhythm and it is this rhythm which, serving as an adrenal marker, must be controlled by an optimum dosage regimen of hydrocortisone to achieve adequate glucocorticoid availability and hence suppression of excess adrenal androgen production. Salivary 17α-hydroxyprogesterone profiles, constructed from samples collected by the patients themselves, at say the week-end, provide the data for the quantification of the near optimum circadian rhythm, suitably adjusted by treatment as required. Clearly, the rhythmic approach rather than the single sample of saliva is most advantageous [33].

Androstenedione

It is possible that the degree of control of treatment for congenital adrenal hyperplasia could be augmented by quantifying circadian rhythms of androstenedione in addition to 17α-hydroxyprogesterone, though the latter is generally the preferred marker. Androstenedione is also a candidate hormone for studies in human development, particularly during adrenarche, puberty and adolescence [34].

Gonadal activity

Testis: testosterone

This hormone is particularly useful in studies of male pubertal development [35]. Circadian rhythms have been characterized for each of the 5 genital or pubic hair stages. The amplitude and mesor exhibit progressive positive changes in relation to increasing genital development until these parameters are similar to those of the adult male. Time-qualified circadian rhythms, or reference ranges of salivary testosterone are being used at the Institute to investigate hyper- or hypo-gonadal activity, or delayed onset of puberty. Similar investigations may be made to study the changing activity of the hypothalamic-pituitary testicular axis in the aging male, a study of possible relevance to the development of prostate cancer. Salivary testosterone concentrations have also been used to investigate the possible suppressive effect of cortisol on testosterone secretion during strenuous exercise [28, 29], particularly in marathon runners [30]. Other examples of application of the salivary testosterone assay have been suggested elsewhere [8, 9].

It is pertinent to note that analytical difficulties exist in the reliable estimation of testosterone in female saliva [23].

Ovary: progesterone

Considerable scope still exists for indirect studies of ovarian progesterone secretion during pubertal development, maturity and the time-span of ovarian decline. Circadian variations in salivary progesterone concentration during the luteal phase are considerable [7]. Studies from this Institute have also quantified circatrigintan patterns of salivary progesterone concentrations found in the mature, premenopausal woman [36]. Semi-quantified changes in circatrigintan progesterone concentration profiles have been monitored before, during and following menarche in British girls [37]. Salivary progesterone concentrations in post-pubertal girls living in Britain and Thailand, who were matched for chronological and gynaecological age, have also been compared; the British girls exhibiting the circatrigintan pattern of the mature premenopausal woman sooner than the Thais [38]. Other proposed applications have been cited elsewhere [8, 9].

Other applications

The potential scope of saliva in chronobiological studies is substantial. Undoubtedly as the potential usefulness of human saliva continues to be explored, drug monitoring will take up a prominent position in future research. A review of the current literature demonstrates that theophylline, digoxin, anti-convulsant, anti-arrhythmic, anti-inflammatory, anti-microbial, anti-biotic and analgesic drugs can be measured in saliva. Other analytes in saliva, such as β-human chorionic gonadotrophin, carcinoembryonic antigen, insulin, growth factors, thyroxine, melatonin, phyto-oestrogens and related compounds, and many other substances await their potential exploitation, either as their molecular entity or possibly as distinct metabolic fragments.

Acknowledgements

The authors acknowledge the generous financial support of the Tenovus Organisation and wish to thank the many colleagues who have collaborated in various clinical studies.

Submitted on invitation.
Accepted on 18 February 1993.

REFERENCES

1. SHANNON, I., PRIGMORE, J., BROOKS, R. & FELLER, R. 1959. The 17-hydroxycorticosteroids of parotid fluid, serum and urine following intramuscular administration of repository corticotropin. *J. Clin. Endocrinol. Metab.* **19**: 1477-1480.

2. KATZ, F.H. & SHANNON, I.L. 1964. Identification and significance of parotid fluid corticosteroids. *Acta Endocrinol.* **46**: 393-404.

3. SHANNON, I.L., BEERING, S.C. & JENSON, R.L. 1966. Dexamethasone suppression tests employing parotid fluid. *J. Clin. Endocrinol.* **26**: 967-969.

4. CAMERON, E.H.D., HILLIER, S.G. & GRIFFITHS, K. (Eds). 1974. *Proceedings of the 5. Tenovus workshop: steroid immunoassay.* Alpha Omega Publishing Ltd., Cardiff. pp. 1-334.

5. READ, G.F., RIAD-FAHMY, D., WALKER, R.F. & GRIFFITHS, K. (Eds). 1984. *Proceedings of the 9. Tenovus workshop: immunoassay of steroids in saliva.* Alpha Omega Publishing Ltd., Cardiff. pp. 1-346.

6. READ, G.F., FAHMY, D.R., WILSON, D.W. & GRIFFITHS, K. 1983. A new approach for breast cancer research: assays for steroids in saliva. In: *Commentaries on research in breast disease.* R.D. Bulbrook & J.D. Taylor. (Eds). Alan R. Liss Inc., New York. vol. 3, pp. 61-92.

7. WILSON, D.W., READ, G.F., HUGHES, I.A., WALKER, R.F. & GRIFFITHS, K. 1984. Hormone rhythms and breast cancer chronoepidemiology: salivary progesterone concentrations in pre- and post-menarchal girls and in normal premenopausal women. *Chronobiol. Int.* **1**: 159-165.

8. WALKER, R.F., READ, G.F., WILSON, D.W., RIAD-FAHMY, D. & GRIFFITHS, K. 1990. Chronobiology in laboratory medicine: principles and clinical applications illustrated from measurements of neutral steroids in saliva. In: *Chronobiology: its role in clinical medicine, general biology and agriculture.* Alan R. Liss Inc., New York. Part A. pp. 105-117.

9. WILSON, D.W., WALKER, R.F., READ, G.F. & GRIFFITHS, K. 1990. Chronobiology in laboratory medicine: principles and clinical applications illustrated from measurements of neutral steroids in saliva. In: *Chronobiology: its role in clinical medicine, general biology and agriculture.* Alan R. Liss Inc., New York. Part A. pp. 119-130.

10. WALKER, R.F., RIAD-FAHMY, D. & READ, G.F. 1978. Adrenal status assessed by direct radioimmunoassay of cortisol in whole saliva or parotid saliva. *Clin. Chem.* **24**: 1460-1463.

11. WALKER, R.F., READ, G.F., HUGHES, I.A. & RIAD-FAHMY, D. 1979. Radioimmunoassay of 17 (alpha)-hydroxyprogesterone in saliva, parotid fluid, and plasma of congenital adrenal hyperplasia patients. *Clin. Chem.* **25**: 542-545.

12. WALKER, R.F., READ, G.F. & RIAD-FAHMY, D. 1979. Radioimmunoassay of progesterone in saliva: application to the assessment of ovarian function. *Clin. Chem.* **25**: 2030-2033.

13. TURKES, A., TURKES, A.O., JOYCE, B.G., READ, G.F. & RIAD-FAHMY, D. 1979. A sensitive solid phase enzyme immunoassay for testosterone in plasma and saliva. *Steroids* **33**: 347-359.

14. WALKER, R.F., WILSON, D.W., READ, G.F. & RIAD-FAHMY, D. 1980. Assessment of testicular function by the radioimmunoassay of testosterone in saliva. *Int. J. Androl.* **3**: 105-120.

15. TURKES, A.O. & READ, G.F. 1984. *Proceedings of the 9. Tenovus workshop: immunoassay of steroids in saliva.* Alpha Omega Publishing Ltd., Cardiff. pp. 260-270.

16. TRURAN, P.L., READ, G.F. & WALKER, S. 1982. Radioimmunoassay for salivary estriol, with use of an ^{125}I-radioligand and a solid-phase separation technique. *Clin. Chem.* **28**: 2393-2397.

17. LEE, C.Y., FEW, J.D. & JAMES, V.H.T. 1988. The determination of 18-hydroxycorticosterone in saliva and plasma: comparison with aldosterone and glucocorticoids. *J. Steroid Biochem.* **29**: 511-517.

18. BAXENDALE, P.M., JACOBS, H.S. & JAMES, V.H.T. 1983. Plasma and salivary androstenedione and dihydrotestosterone in women with hyperandrogenism. *Clin. Endocrinol.* **18**: 447-457.

19. McVIE, R., LEVINE, L.S. & NEW, M.I. 1979. The biologic significance of the aldosterone concentration in saliva. *Pediatric Res.* **3**: 755-759.

20. FEW, J.D., CHAUDRY, S. & JAMES, V.H.T. 1984. The direct determination of aldosterone in human saliva. *J. Steroid Biochem.* **21**: 87-92.

21. LUISI, M., SILVESTRI, D., MALTINI, G., CATARSI, A.L. & FRANCHI, F. 1980. Radioimmunoassay of oestrone in male saliva. *Lancet* **2**: 542-543.

22. CHEARSKUL, I., RINCON-RODRIGUEZ, I., SUFI, S.B., DONALDSON, A. & JEFFCOATE, S.L. 1982. Simple direct assays for measuring oestradiol and progesterone in saliva. In: *Radioimmunoassay and related procedures in medicine.* IAEA, Vienna. pp. 265-274.

23. READ, G.F. 1989. Hormones in saliva. In: *Human saliva: clinical chemistry and microbiology.* J. Tenovuo (Ed.). Vol. 2. CRC Press, Boca Raton. pp. 147-176.

24. WILSON, D.W., GASKELL, S.J. & KEMP, K.W. (Eds). 1981. In: *Proceedings of the 8. Tenovus workshop: quality control in clinical endocrinology.* Alpha Omega Publishing, Cardiff. pp. 1-275.

25. GASKELL, S.J., PIKE, A.W. & GRIFFITHS, K. 1980. Analysis of testosterone and dehydroepiandrosterone in saliva by gas chromatography-mass spectrometry. *Steroids* **36**: 219-228.

26. GASKELL, S.J. & FINLAY, E.M.H. 1984. Identification and quantification of steroids in saliva by gas chromatography-mass spectrometry. In: *Proceedings of the 9. Tenovus Workshop: immunoassay of steroids in saliva.* Alpha Omega Publishing Ltd., Cardiff. pp. 33-42.

27. FRANCIS, S.J., WALKER, R.F., RIAD-FAHMY, D., HUGHES, D., MURPHY, J.F. & GRAY, O.P. 1987. Assessment of adrenocortical activity in term newborn infants using salivary cortisol determinations. *J. Pediatr.* **111**: 129-133.

28. CAMPBELL, I.T., WALKER, R.F., RIAD-FAHMY, D., WILSON, D. & GRIFFITHS, K. 1982. Circadian rhythms of testosterone and cortisol in saliva: effects of activity-phase shifts and continuous daylight. *Chronobiologia* **IX**: 389-396.

29. CAMPBELL, I.T., WILSON, D.W., WALKER, R.F. & GRIFFITHS, K. 1985. The use of salivary steroids to monitor circadian rhythmicity on expeditions in the Arctic. *Chronobiol. Int.* **2**: 55-59.

30. COOK, N.J., READ, G.F., WALKER, R.F., HARRIS, B. & RIAD-FAHMY, D. 1986. Changes in adrenal and testicular activity monitored by salivary sampling in males throughout marathon runs. *Eur. J. Appl. Physiol.* **55**: 634-638.

31. HARRIS, B., COOK, N., WARNER, N., READ, G.F., WALKER, R.F., THOMAS, R. & RIAD-FAHMY, D. 1988. Anxiety in the dexamethasone suppression test monitored with saliva. *Biol. Psychiatry* **23**: 698-704.

32. HARRIS, B., WATKINS, S., COOK, N., WALKER, R.F., READ, G.F. & RIAD-FAHMY, D. 1990. Comparisons of plasma and salivary cortisol determinations for the diagnostic efficacy of the dexamethasone suppression test. *Biol. Psychiatry* **27**: 324-327.

33. HUGHES, I.A., DYAS, J., ROBINSON, J., WALKER, R.F. & FAHMY, D.R. 1985. Monitoring treatment in congenital adrenal hyperplasia: use of serial measurements of 17-OH-progesterone in plasma, capillary blood, and saliva. In: Congenital adrenal hyperplasia. M.I. New (Ed.). *Ann. N.Y. Acad. Science USA* **458**: 193-202.

34. YOUNG, M.C., WALKER, R.F., RIAD-FAHMY, D. & HUGHES, I.A. 1988. Androstenedione rhythms in saliva in congenital adrenal hyperplasia. *Arch. Dis. Child* **63**: 624-628.

35. BUTLER, G.E., WALKER, R.F., WALKER, R.V., TEAGUE, P., RIAD-FAHMY, D. & RATCLIFFE, S.G. 1989. Salivary testosterone levels and the progress of puberty in the normal boy. *Clin. Endocrinol.* **30**: 587-596.

36. WALKER, R.F., WILSON, D.W., TRURAN, P.L., READ, G.F., RICHARDS, G., WALKER, S.M. & RIAD-FAHMY, D. 1985. Characterization of profiles of salivary progesterone concentrations during the luteal phase of fertile and subfertile women. *J. Endocrinol.* **104**: 441-446.

37. READ, G.F., WILSON, D.W., HUGHES, I.A. & GRIFFITHS, K. 1984. The use of salivary progesterone assays in the assessment of ovarian function in postmenarcheal girls. *J. Endocrinol.* **102**: 265-268.

38. DANUTRA, V., TURKES, A., READ, G.F., WILSON, D.W., GRIFFITHS, V., JONES, R. & GRIFFITHS K. 1989. Progesterone concentrations in samples of saliva from adolescent girls living in Britain and Thailand, two countries where women are at widely differing risk of breast cancer. *J. Endocrinol.* **121**: 375-381.