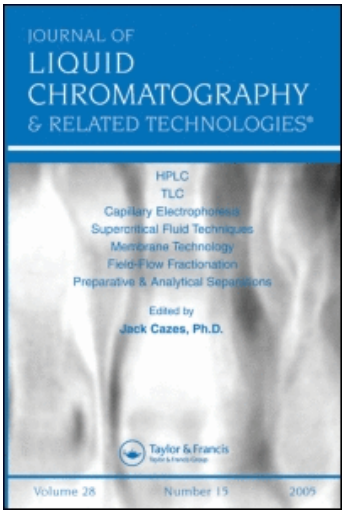
This article was downloaded by:
On: *25 January 2011*
Access details: *Access Details: Free Access*
Publisher *Taylor & Francis*
Informa Ltd Registered in England and Wales Registered Number: 1072954 Registered office: Mortimer House, 37-41 Mortimer Street, London W1T 3JH, UK



Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Differential Measurement of Cortisol and Cortisone in Human Saliva by HPLC with Uv Detection

Stephen E. Wade[a]; Albert D. Haegele[a]
[a] Hammersmith Laboratories Inc., Colorado

To cite this Article Wade, Stephen E. and Haegele, Albert D.(1991) 'Differential Measurement of Cortisol and Cortisone in Human Saliva by HPLC with Uv Detection', Journal of Liquid Chromatography & Related Technologies, 14: 9, 1813 — 1827

To link to this Article: DOI: 10.1080/01483919108049655

URL: http://dx.doi.org/10.1080/01483919108049655

PLEASE SCROLL DOWN FOR ARTICLE

Full terms and conditions of use: http://www.informaworld.com/terms-and-conditions-of-access.pdf

This article may be used for research, teaching and private study purposes. Any substantial or systematic reproduction, re-distribution, re-selling, loan or sub-licensing, systematic supply or distribution in any form to anyone is expressly forbidden.

The publisher does not give any warranty express or implied or make any representation that the contents will be complete or accurate or up to date. The accuracy of any instructions, formulae and drug doses should be independently verified with primary sources. The publisher shall not be liable for any loss, actions, claims, proceedings, demand or costs or damages whatsoever or howsoever caused arising directly or indirectly in connection with or arising out of the use of this material.

JOURNAL OF LIQUID CHROMATOGRAPHY, 14(9), 1813-1827 (1991)

DIFFERENTIAL MEASUREMENT OF CORTISOL AND CORTISONE IN HUMAN SALIVA BY HPLC WITH UV DETECTION

STEPHEN E. WADE AND ALBERT D. HAEGELE
Hammersmith Laboratories, Inc.
P.O. Box 774324
West Acres Industrial Park
Steamboat Springs, Colorado 80477

Downloaded At: 10:06 25 January 2011

ABSTRACT

Both cortisol and its dehydro metabolite cortisone are present in normal human saliva. A method for differential measurement of both compounds in 1 ml samples of saliva by HPLC/UV is described. The method uses an extraction column having a cyclodextrin bonded phase to retain the compounds of interest while allowing elution of interfering compounds. A steroid-bearing fraction is eluted from the cyclodextrin column, dried, reconstituted in a weak mobile phase, and injected on a reversed phase HPLC/UV system provided with an injector-mounted reversed phase extraction column. Samples containing corticosteroid concentrations as low as 0.5 ng/ml can be effectively analyzed by this method.

INTRODUCTION

The measurement of corticosteroid hormones in saliva presents several advantages over measurement of the same compounds in blood plasma or serum. Measurement of salivary corticosteroids allows: 1) simple and easy sample collection, 2) a noninvasive and stress- free experience for the subject, and 3) specific

Copyright © 1991 by Marcel Dekker, Inc.

representation of the "free" or non- corticosteroid binding globulin-bound fraction of the hormone [1]. This combination of practical and scientific advantages has prompted many investigators to develop immunoassay methods of sufficient sensitivity to routinely measure the principal glucocorticoid of plasma, cortisol (11,17,21-trihydroxy-4-pregnene-3,20-dione; F), in small samples of saliva. Consequently, the relationships between salivary cortisol and plasma total and free cortisol have been well studied (1-8). In general, saliva cortisol is found to be closely related to plasma free cortisol, which in turn is almost entirely determined by the concentrations of total cortisol and corticosteroid binding globulin (9).

Free cortisol does not, however, pass entirely unchanged from the plasma to the saliva. An enzyme activity (11-hydroxysteroid dehydrogenase, or 11-OHSD) localized within the salivary glands converts a substantial fraction of cortisol to cortisone (17,21-dihydroxy-4-pregnene-3,11,20-trione; E) in passing (10,11). No such enzyme activity exists in saliva itself. Interest in differential measurement of salivary E and F arises from concern about variations in the activity of this enzyme. If 11-OHSD activity varies, the relationship between plasma free F and saliva F will vary. However, the relationship between plasma (E + F) and saliva (E + F) will not depend upon 11-OHSD activity (10), and the ratio of E to F in saliva may conceivably be of some research interest as an index of systemic 11-OHSD activity. This paper describes a method for simultaneous, differential determination of E and F in human saliva. Saliva samples are frozen, thawed, and prepared by solid phase extraction using a cyclodextrin bonded phase. The compounds of interest and a recovery standard are then separated by reversed- phase HPLC, and measured by UV absorbance.

Downloaded At: 10:06 25 January 2011

MATERIALS AND METHODS

Steroid Standards

Cortisol, cortisone, and 11- dehydrocorticosterone (21-hydroxy-4-pregnene-3,11,20-trione, DHB) were obtained from Sigma

Chemical Co (St. Louis, MO) and used without further purification. Methanolic stock solutions of each compound at 100 ug/ml were stored in a -20 C freezer.

Tritiated F (Amersham, Arlington Heights, IL) was used to trace the recovery of F through some extraction procedures.

Solvents

HPLC grade water was produced by UV treatment (Photronix Model 816) of the product from a a deionizer system (Photronix RGW 5). Methanol, acetonitrile, and all other chemicals were purchased from Curtin Matheson Scientific (Denver, CO).

Downloaded At: 10:06 25 January 2011

Solid Phase Extraction Columns:

Prep columns having a 1 ml working volume, and a 100 mg extractant load consisting of beta-cyclodextrin bonded to a silica stationary phase, were manufactured by Astec (Whippany, NJ). These prep columns were extensively washed with hot methanol and water (HPLC grade) before first use. The columns were recycled through as many as four uses without observable loss of performance. Between uses, each column was washed with 2 ml methanol and 1 ml water, and stored in a dry condition.

Samples

Healthy volunteers collected samples of saliva by spitting into 15 ml polypropylene centrifuge tubes over the course of 2-5 minutes. Sample volumes ranged from 2 to 6 ml. Samples were frozen shortly after collection and held at -20 C until analysis (but at least 16 h).

Sample Processing

Saliva samples were thawed and centrifuged 10 min at 1000 g to pellet volumnious debris. Aliquots of of the supernatant (1.00 ml) were transferred to disposable microcentrifuge tubes, and 50

ul of 0.20 ng/ul DHB (recovery tracer) was added to each aliquot. Perchloric acid (50 ul, 9.4 N) was added to precipitate proteins. The tubes were then centrifuged 12 min at high speed (ca 10,000 g) in a Beckman Microfuge 12. Supernatants were transferred to fresh glass tubes and KOH (460 ul, 1 N) followed after vortexing by NaH_2PO_4 (250 ul, 1 M) was added to neutralize excess perchlorate and to buffer the sample. This treatment yielded a further precipitate that was allowed to settle before proceeding to extraction column treatment.

Each supernatant was applied to the top of an extraction column. The sample was aspirated through the extraction column by use of a vacuum manifold (Rainin Inst. Co, Woburn, MA). The column was then washed by aspirating 2 ml of 200 mM NaCl, followed by 2 ml 5% methanol. Retained steroids were then eluted with 1 ml methanol. The eluate was collected through a luer-fitted filter (Millex-HV, 0.45 uM, Millipore) into a 75 X 100 mm glass disposable tube. The eluate was reduced to dryness under a stream of nitrogen after addition of 100 ul of 0.1 ug/ml ascorbic acid in methanol. Samples in this condition were either chromatographed immediately as described below, or frozen at -20 C for up to 1 week before chromatography.

Downloaded At: 10:06 25 January 2011

We investigated variations in the sample processing procedure, including use of more or less extensive washes of the sample on the prep column, and use of gamma-cyclodextrin as opposed to beta-cyclodextrin bonded solid phase extractants.

Chromatography

Two chromatgraphs were used with similar results in this work: one entirely manual in operation, and one entirely automated. For simplicity we will describe the manual system and its operation, and then note the distinctive features of the automated system.

Mobile phase (acetonitrile:methanol:water, 10:40:50) was delivered at 0.3 ml/min to a six-port rotary valve (SSI, College Park, PA) by a Micromeritics Model 750 pump. The rotary valve was configured for an external sample loop, but in place of the loop was mounted a 2.0 x 20 mm guard column packed with pellicular C18 stationary phase (Upchurch Scientific, Oak Harbor, WA), hereafter called the injector column. Injection into the sample port of the rotary valve caused fluid to flow over this injector column and thence to waste. Operating the rotary valve toggled the injector column between the sample loading position (injection directed over the injector column to waste, pump output directed to analytical column), and the sample delivery position (injector column interposed bewteen pump and analytical column). The analytical column (Keystone 2 x 250 mm, C8 packing) was thermostated at 50 C by a water jacket. The column effluent was monitored at 240 nM by a Micromeritics variable wavelength UV detector (Model 788). Detector output was recorded by a flatbed chart recorder (Kipp & Zonen BD41).

Downloaded At: 10:06 25 January 2011

The injector column was prepared for use by injecting 250 ul of 5% methanol. Dried sample residue from the solid-phase extraction step was redissolved in 100 ul 5% MeOH, and the entire volume was injected. This was followed by 1.0 ml of 200 mM NaCl, and 250 ul of 5% methanol. The rotary valve was then operated, placing the injector column in line for 30 seconds. The valve was then returned to the loading position, and the injector column was cleared by injection of 250 ul 100% methanol, and prepared for the next use by injection of 250 ul 5% methanol.

The automated system differed in using a WISP autosampler (Waters) to introduce samples (200 out of 230 ul total sample volume) carried in diosposable low-volume vial inserts (Sun Brokers) into a stream of 5% methanol pumped at 0.5 ml/min by a metering pump (Eldex A-60-S). This stream of 5% methanol was directed by an electrically operated Valco switching valve to a

similar injector column, and thence to a waste receptacle. The elapsed time after introduction of the sample by the WISP was monitored by a Baseline 810 chromatography data system (Waters) operating on an Epson Equity II+ computer. After an interval sufficient to deliver the steroids to the guard column, contact closures generated by the data system caused an Autochrom solvent selection valve to introduce 1 ml 200 mM NaCl to the Eldex pump, and later to operate the Valco valve to switch the Eldex pump to waste, and to direct mobile phase over the injector column and directly to the analytical column. Detector response was monitored by the data system. The operation of the automated system thus essentially duplicated the manual operations described above.

Downloaded At: 10:06 25 January 2011

Linearity and Precision Test Methods:

The performance of the chromatographs equipped with injector columns is described in detail elsewhere (12). We were concerned here to test the precision and linearity of the complete protocol, including the sample preparation on cyclodextrin extraction columns, with human saliva.

As a preliminary measure we tested the relative and absolute recoveries of E, F, and DHB through the extraction column preparation. Standards of E, F, and DHB in an aqueous buffer were injected on HPLC either directly or after solid phase extraction and reconstitution. The recovery of F was independently checked by measuring the recovery of applied tritiated F.

We definitively tested the linearity of the method by preparing an extensive series of known additions to a pooled saliva sample, and analyzing duplicate samples from the series. Saliva samples collected from two subjects between 2000 and 2300 h (a time when low corticosteroid secretion is expected) were frozen, thawed, centrifuged, and the supernatants pooled to yield 20 ml of material. Duplicate sample tubes were prepared

containing each of 7 levels of added E and F (shown here as ng E:ng F): 0:0, 2.0:0.4, 4.0:0.8, 8.0:1.6, 12.0:2.4, 20:10, and 40:20. The steroids were added as methanolic solutions containing ascorbic acid, and the methanol was removed by evaporation under nitrogen. Each tube then received 10 ng/ml of DHB in 5% MeOH as recovery standard, and 1.0 ml of the pooled evening saliva. Each saliva sample was then analyzed by the method described above, using the automated HPLC system.

We further evaluated precision of the method at both low and high levels of both steroids, by similar methods. A pool of evening saliva was divided into 10 1.0 ml aliquots; each was assayed for native levels of E and F. A separate pool of saliva collected in the late afternoon was spiked with 20 ng/ml E and 10 ng/ml F and similarly analyzed as 10 distinct samples. The coefficient of variation for each steroid at each level was computed.

Downloaded At: 10:06 25 January 2011

RESULTS

General

We observed the following retention times in the manual system as described: E, 5.6 min; F, 6.1 min, DHB, 7.2 min. The peaks of interest were well resolved from one another and adequately resolved from the solvent front, in saliva samples from several persons. Late eluting peaks were minimized by strictly observing the 30 second timing for elution of the injection column. In the automated system, injections followed at 11.6 min intervals. Consistent results were promoted by repacking the injector column after about 100 injections. Figure 1 compares chromatograms of a) E , F, and DHB in buffer, b) a saliva sample with DHB only, and c) a saliva sample with additions of E, F, and DHB, obtained from the automated system. The retention times indicated by the data system include a delay time for operation of the autoinjector and valves; actual retention on the analytical column was similar in both systems.

Downloaded At: 10:06 25 January 2011

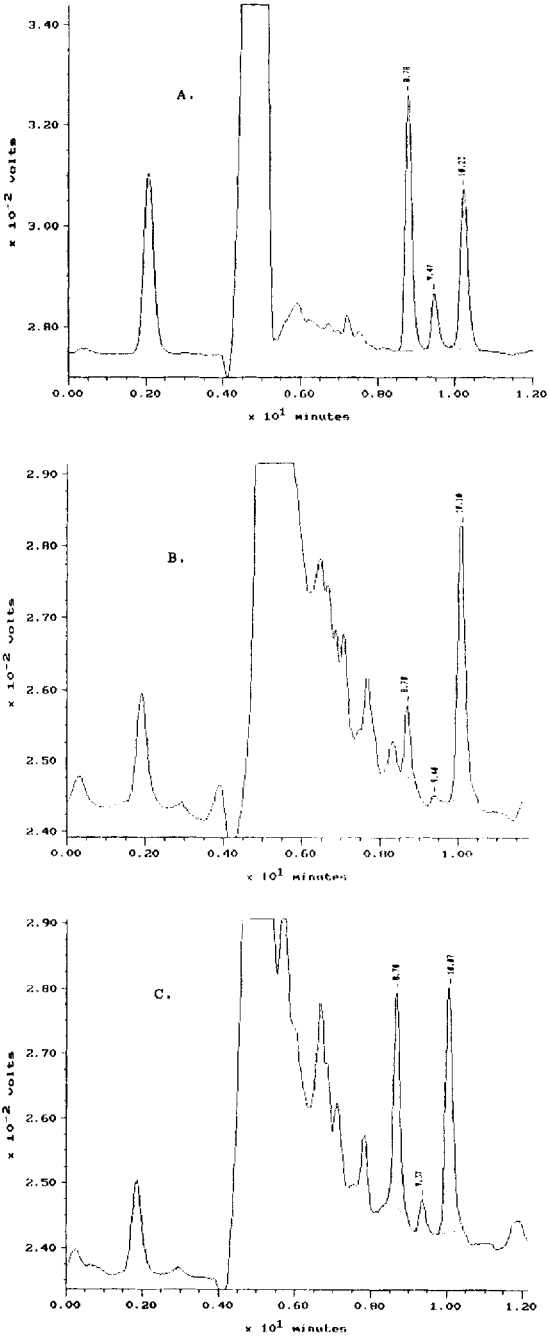


FIGURE 1. Chromatograms from the fully automated system described in the text. A: an unextracted standard containing E, F, and DHB in order of elution. B: a saliva sample with DHB (recovery tracer) added. C: the same saliva sample with 10 ng/ml added E and 2 ng/ml added F.

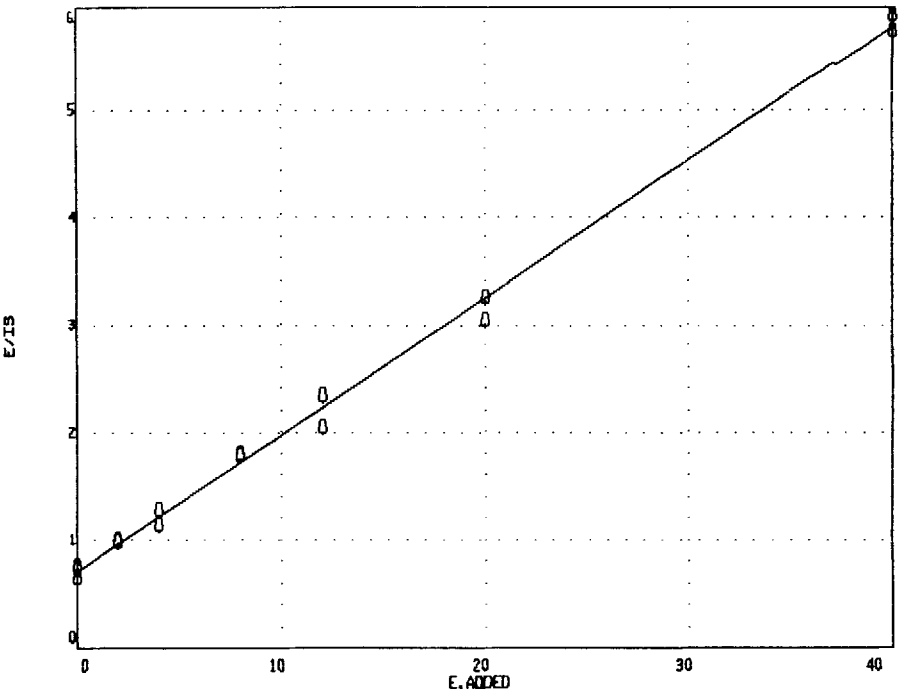


FIGURE 2. UV detector response to cortisone (E) [peak height E/ peak height internal standard; E/IS] versus nanograms of E added to 1 ml samples from an evening saliva pool. Each point represents one sample; duplicate samples were prepared at each dose level.

Downloaded At: 10:06 25 January 2011

Effects of Extraction Column Treatment upon Absolute and Relative Recoveries

The absolute recoveries (i.e. peak height following extraction divided by peak height of replicate without extraction) for E, F, and DHB in buffer were 100%, 94%, and 95%, respectively. The high rate of recovery of F through the extraction was confirmed independently by tracing the radioactivity of HPLC-purified tritiated F.

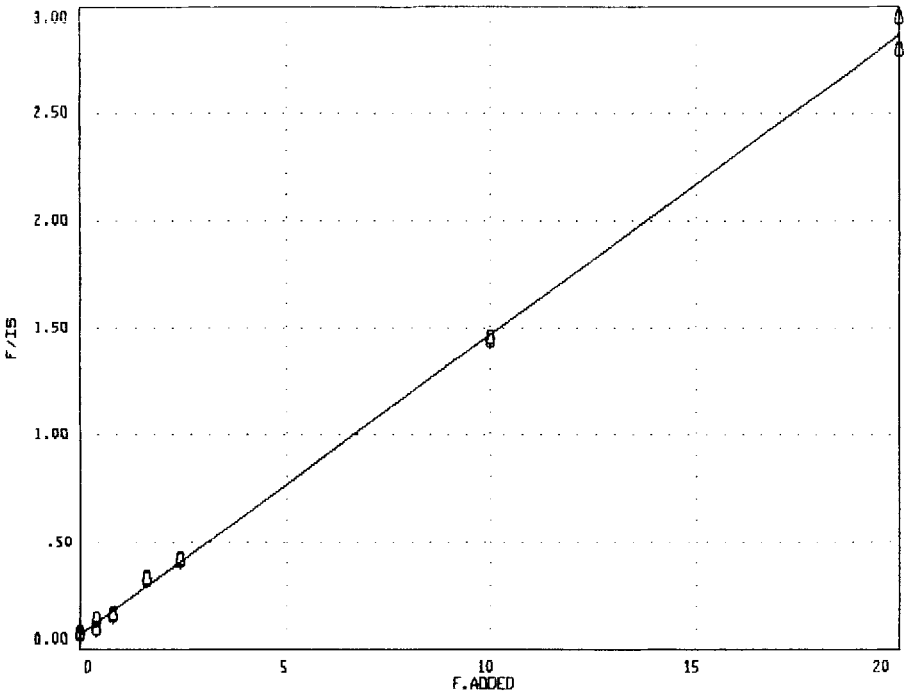


FIGURE 3. UV detector response to cortisol (F) [peak height F/ peak height internal standard; F/IS] versus nanograms of F added to 1 ml samples from an evening saliva pool. Each point represents one sample; duplicate samples were prepared at each dose level.

Downloaded At: 10:06 25 January 2011

Linearity of Detector Response to E and F Added to Saliva

Figure 2 shows the detector response (peak height E divided by peak height of recovery standard, DHB) as a function of 7 levels of added E. It is clear from the figure that the relative recovery and detection of E was quite linear across the range examined (linear correlation coefficient r=0.998). The intercept of the plot indicates that the native level of E in the evening saliva pool was 5.53 ng/ml.

TABLE 1

Mean E and F Recoveries in Saliva Samples as a Function of Added E and F. Each entry represents the mean of duplicates.

Steroids Added ng/ml		Steroids Measured ng/ml		Recovery %	
E	F	E	F	E	F
0	0	5.530	0.540	100.0	100.0
2	0.4	7.804	0.899	113.7	89.6
4	0.8	9.540	1.192	100.3	81.5
8	1.6	14.076	2.363	106.8	113.9
12	2.4	17.282	3.011	97.9	102.9
20	10	24.711	10.382	95.9	98.4
40	20	45.747	20.585	100.5	100.2
		average		102.2	98.1
		s.d.		5.65	9.48

Downloaded At: 10:06 25 January 2011

TABLE 2

Mean E and F Concentration in Two Saliva Samples, and Corresponding Coefficients of Variation (CV%, n=10)

	E ng/ml	CV%	F ng/ml	CV%
Pool 1 (native levels, evening)	1.89	3.24	0.27	5.84
Pool 2 (afternoon saliva, spiked)	25.9	4.78	10.7	3.68

Figure 3 shows the corresponding analysis for F. Again, the response is completely linear (r= 0.999). The native level of F in this evening saliva pool was 0.54 ng/ml.

Table 1 summarizes the recoveries of the two steroids observed at each level of added material.

Precision of Determination at High and Low Levels of E and F

Table 2 summarizes the results of the precision study.

Effects of Departures from the Indicated Procedure

We found that extraction columns using gamma cyclodextrin, rather than beta-cyclodextrin, as the solid phase extractant were less satisfactory, in that they allowed a loss of E and DHB relative to F. With very extensive washing (i.e NaCl greater than 4 ml, in combination with 2 ml 5% methanol), this disproportionate loss could also be observed with the preferred beta-cyclodextrin columns. Substituting phosphate buffers at ph 4.4 or 7.0 for the 200 mM NaCl (pH 6.2) had no observable effect upon recoveries.

Elimination of the perchloric acid treatment of saliva led to rapid fouling and decreased reuseability of the extraction columns. We found that the extraction columns showed good performance with and allowed clean chromatography of saliva samples as large as 4 ml.

Downloaded At: 10:06 25 January 2011

DISCUSSION

The results summarized above make it clear that this HPLC/UV method with sample preparation by solid phase extraction on cyclodextrin media provides a sensitive, precise, and linear measurement of both E and F in human saliva. In view of the relatively high precision of determination for low native levels of F, we conservatively claim a sensitivity limit of 0.5 ng/ml for each steroid. This limit is based upon a saliva sample volume of 1 ml. Since samples up to 4 ml can be processed, the limit of sensitivity can no doubt be pushed considerably lower.

Other workers have combined chromatographic separation with immunoassay to attain the required combination of sensitivity and specificity in studying the corticosteroids of saliva (10,13). While immunoassay is relatively efficient for the determination of F alone, it is much less efficient when two closely related species must be determined differentially. The procedure described here provides a practical alternative to immunoassay for this application.

The combination of solid phase extraction on cyclodextrin media, followed by (in effect) a second solid phase extraction on the injector column, facilitates the analysis in two ways: 1) it provides a relatively clean sample, substantially free of UV-absorbing interferences and late peaks, to the analytical column, thus allowing relatively quick sample throughput at the final stage, and 2) the concentrating ability of the injector column allows the handling and injection of relatively large volumes of sample by the autoinjector, without attendant bandspreading on the analytical column, thus minimizing sample wastage and enhancing sensitivity. We evaluated extraction columns with both beta- and gamma-cyclodextrin functional groups, expecting that the larger internal cavity of the gamma-cyclodextrin moeity (14) might be better suited for retention of steroids. Our results do not allow any inference concerning the mechanism of retention of the steroids on these materials, but we saw no practical advantage of the gamma- over the beta-cyclodextrin material.

Downloaded At: 10:06 25 January 2011

The results we have observed in several months of using this assay are in good agreement with those reported by others using immunoassay methods: E is more abundant than F in saliva under most conditions, and the concentration of both glucocorticoids together varies from roughly 1 to 30 ng/ml as a result of diurnal variation and physiological stimuli (10,13).

ACKNOWLEDGEMENT

This work was supported by grant number MH42031 from the National Institute of Mental Health to Hammersmith Laboratories.

REFERENCES

1) Vining, R. F.; McGinley, R. A. The measurement of hormones in saliva: possibilities and pitfalls. Journal of Steroid Biochemistry 27: 81, 1987.

2. Bolufer, P.; Gandia, A.; Rodriguez, A.; Antonio, P. Salivary Corticosteroids in the Study of Adrenal Function. Clinica Chimica Acta 183: 217, 1989.

3. Guechot, J.; Lepine, J. P.; Cohen, C.; Fiet, J.; Lemperiere, T.; Dreux, C. Simple Laboratory Test of Neuroendocrine Disturbance in Depression: 11 p.m. Saliva Cortisol. Neuropsychobiology 18: 1, 1987.

4. Kahn, J.-P.; Rubinow, D. R.; Davis, C. L.; Kling, M.; Post, R. M. Salivary Cortisol: A Practical Method for Evaluation of Adrenal Function. Biological Psychiatry 23: 335, 1988.

5. Laudat, M. H.; Cerdas, S.; Fournier, C.; Guiban, D.; Guilhaume, B.; Luton, J. P. Salivary Cortisol Measurement: A Practical Approach to Assess Pituitary-Adrenal Function. Journal of Clinical Endocrinology and Metabolism 66: 343, 1988.

6. Peters, J. R.; Hall, R.; Walker, R. F.; Riad-Fahmy, D. Immunoassay of Steroids in Saliva, Proc. 10th Tenovus Conf., Cardiff, Wales, 1984, p317.

7. Mccracken, J.T.; Poland, R.E. Saliva and Serum Cortisol Dynamics Following Intravenous Dexamethasone in Normal Volunteers. Life Sciences 45: 1781, 1989.

8. Walker, R. F.; Walker, S.; Riad-Fahmy, D. Immunoassay of Steroids in Saliva, Proc. 10th Tenovus Conf., Cardiff, Wales, 1984, p115.

9. Dunn, J. F.; Nisula, B. C.; Rodbard, D. Transport of Steroid Hormones: Binding of 21 Endogenous Steroids to Both testosterone-binding Globulin and Corticosteroid-Binding Globulin in Human Plasma. Journal of Clinical Endocrinology and Metabolism 53: 58, 1981.

10. Brooks, F. S.; Brooks, R. V. Immunoassay of Steroids in Saliva, Proc. 10th Tenovus Conf., Cardiff, Wales, 1984, p322.

11. Edwards, C. R. W.; Burt, D.; McIntyre, M. A.; De Kloet, E. R.; Stewart, P. M.; Brett, L.; Sutanto, W. S.; Monder, C. Localisation of 11 Beta-Hydroxysteroid Dehydrogenase-Tissue Specific Protector of the Mineralocoticoid Receptor. The Lancet Oct: 986, 1988.

12. Wade, S.E. and Haegele, A.D. Corticosteroid analyses by HPLC-UV facilitated by use of an injector-mounted extraction column. Journal of Liquid Chromatography (in press) 1991.

13. Meulenberg, P.M.M. and Hofman, J.A. Differences between concentrations of salivary cortisol and cortisone and of free

Downloaded At: 10:06 25 January 2011

cortisol and cortisone in plasma during pregnancy and postpartum. Clinical Chemistry 36:70, 1990.

14. Szejtli, J. Application of Cyclodextrins in the Chromatography. Starch 39:357, 1987.

Received: December 22, 1990
Accepted: February 27, 1991

Downloaded At: 10:06 25 January 2011