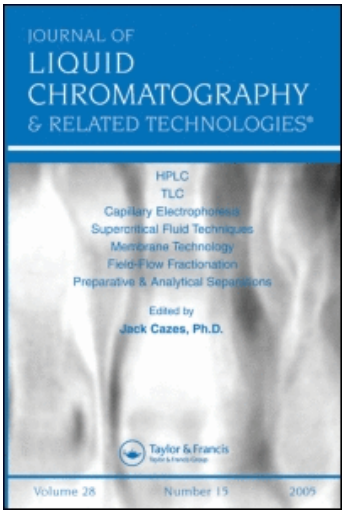

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ULTRASENSITIVE DIFFERENTIAL MEASUREMENT OF CORTISOL AND CORTISONE IN BIOLOGICAL SAMPLES USING FLUORESCENT ESTER DERIVATIVES IN NORMAL PHASE HPLC

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ABSTRACT

We report a method for differentially measuring the steroid hormones cortisol (F) and cortisone (E) in biological samples containing small quantities of either compound (50 pg F, 70 pg E). The method involves the formation of fluorescent ester derivatives of the anylates by reaction at the 21-hydroxy position with 9-anthroyl nitrile. Reversed-phase chromatography is used as a pre-derivatization sample cleanup, and solid-phase extraction on microcolumns of beta-cyclodextrin is used as a cleanup step before final separation and quantitation of the esters by normal phase chromatography with fluorescence detection. The synthetic steroid prednisolone is used as an internal standard. The derivatization consistently proceeds to a reproducible endpoint, and linearity, sensitivity, and specificity of the analysis are excellent.

INTRODUCTION

Cortisol (11,17,21-trihydroxy-4-pregnene-3,20-dione; F) and cortisone (17,21-dihydroxy-4-pregnene-3,11,20-trione; E) are interconverted by tissue- specific enzyme systems in many organisms. In some cases, better insight into the functional

status of the organism may be gained by measuring both hormones than by measuring either one alone. Such a case arises in the study of human salivary corticosteroids, since the presence of 11-hydroxysteroid dehydrogenase in the parotid gland leads to substantial conversion of F to E as F passes to the saliva from the circulation (1). The extent of conversion may vary from individual to individual or from one time to another, and thus measuring both the primary corticosteroid F and its salivary metabolite E may provide a better index of corticosteroid status than measuring only one of these compounds.

The resolving power of high performance liquid chromatographic methods makes them well suited for simultaneous differential measurement of two closely related compounds, such as E and F. However in some applications there is a need for great assay sensitivity, because of low anylate concentrations and limited sample volume. This need for sensitivity has inhibited the use of chromatographic methods and has led instead to reliance upon various immunoassay methods. We report here a method for differential determination of E and F that maintains chromatographic specificity while providing a detection limit similar to radioimmunoassay. The sensitivity enhancement is provided by measuring the fluorescence of anthroyl ester derivatives formed at the 21- hydroxy moiety common to both anylates (2).

This analytical method was applied to specimens produced by a novel physiological sampling method that uses a miniature orally-installed device (the Oral Diffusion Sink or ODS device) to accumulate the specific anylates of interest from saliva over a period of hours (3). The ODS device used to accumulate the corticosteroids contains antibodies that sequester the corticosteroids as they diffuse across a cellulose acetate membrane into the device. From an analytical point of view, the resulting sample matrix is quite consistent from one sample to another, and contains the anylates in enriched concentrations, relative to their concentrations in saliva. However, the absolute amounts of the anylates are small. The chromatographic method to be described is designed for determining the total E and F content of a single ODS device.

MATERIALS AND METHODS

Standards

Cortisol, cortisone, and prednisolone (11 beta,17 alpha,21-trihydroxy-1,4-pregnadiene-3,20-dione; PN) were purchased from Sigma Chemical Co. (St.Louis, MO), and were used without further purification. Methanolic stock solutions of each compound at 100 ug/ml were prepared, and were stored in a -20 C freezer. Dilutions of these stock solutions in methanol were made at each analytical run, and treated identically as the sample extracts described below. That is, standards were routinely carried through the sample cleanup and all subsequent steps.

Samples

Samples were obtained from normal human subjects by means of "Oral Diffusion Sink" (ODS) devices. The particular properties of

this device and particulars of its use will be described elsewhere. The ODS device accumulates steroids from saliva over a period of hours through their association with a binding preparation (antiserum) contained within it, and represents a fluid sample volume of about 20 microlitres.

Sample extraction

The ODS device, once appropriately exposed to saliva, was placed in a 1.5 ml microcentrifuge tube (Helena Plastics) for extraction with 1 ml 90% methanol, after addition of 1 ng prednisolone (PN) as a recovery standard, and 2.5 ug of ascorbic acid as an antioxidant. The ODS device was removed and discarded after overnight extraction. The methanolic sample extract was then dried under a stream of nitrogen at 37 deg C.

Sample cleanup

The dried residue was redissolved in 5% MeOH, and centrifuged to pellet any solids. The supernatant was injected on an automated reversed-phase HPLC system fitted with a fraction collector. The automated reversed-phase HPLC system was one optimized for measurement of corticosteroids in biological samples. The analytical performance of this system is described in detail elsewhere (4); in the present context it functioned in a preparative role only. The salient feature of the system for preparative use was its ability to produce an eluant fraction of relatively small volume, in which the compounds of interest were resolved from most potentially interfering compounds, with minimum sample wastage. The efficient use of sample derived largely from the use of a trace enrichment/sample cleanup column installed across the ports of the injection valve, as described below.

The system used a WISP autosampler (Waters) to introduce samples (100 out of 110 ul total sample volume) 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 an Upchurch guard column (2.0 x 20 mm) filled with a pellicular C18 packing, and thence to a waste receptacle. Steroids delivered in 5% methanol were strongly retained in a small band at the head of the guard column, while more polar compounds eluted to waste. The elapsed time after introduction of the sample by the WISP was monitored by a Baseline 810 chromatography data system operating on an Epson Equity II+ computer. After an interval sufficient to deliver the steroids to the guard column and to elute polar interferences, a contact closure generated by the data system caused the Valco valve to switch the Eldex pump to waste, and to direct mobile phase (water:methanol:acetonitrile 50:33.3:16.7), pumped at 0.3 ml/min by a Micromeritics 750 pump, over the guard column and directly to the analytical column (Keystone 2 x 250 mm, C8 packing). Under these conditions, the compounds of interest were eluted from the guard column within 30 seconds. At this time a signal from the data system caused the Valco valve to remove the guard column from the mobile phase stream. Subsequent signals to a solvent selector (Autochrom) mounted on the inlet of the Eldex pump caused the guard column to be washed with methanol, thereby removing late-eluting interferences, and with 5% methanol, thereby preparing it to receive the next sample from the autosampler. Meanwhile the compounds of interest were resolved from interferences on the analytical column, and column effluent was monitored by a Shimadzu SPD-2A variable wavelength UV detector set at 240 nM. A single 0.5 ml fraction containing F, E, and PN was collected into a glass 13 x 100 mm tube for each sample, by means of a timed contact closure sent to an ISCO fraction collector by the data system. This steroid fraction was again dried under nitrogen after addition of 25 ul phosphate buffer in methanol. The entire fraction was then transferred to a disposable reaction tube (1.5 ml polypropylene microcentrifuge tube, Helena Plastics) in a small volume of methanol, and the methanol was removed under nitrogen.

Alternatively, sample cleanup using reversed-phase (Analytichem C18) and silica-bonded cyclodextrin (Astec Cyclobond

I) chemistries in preparatory column format was investigated. The redissolved residue from the ODS extraction was applied to the top of a prep column and drawn into the packing by vacuum applied through a manifold. The packing was then washed with multiple- ml volumes of phosphate buffered aqueous methanol, and finally the steriod fraction was eluted with 1 ml methanol. This steroid fraction was dried under nitrogen after addition of 25 ul phosphate buffer, and transferred to a reaction tube as above.

Derivatization

Fluorescent derivatives of 21- hydroxysteroids (E, F, and PN) in the dried steroid fraction were formed by reaction with 9-anthroylnitrile (9-AN, WAKO Chemical Co., Dallas, TX). The reaction proceeded at room temperature in a 20 ul reaction volume after sequential addition of 9-AN (final concentration 1 ug/ul) and triethylamine (final concentration 15% w/v) dissolved in acetonitrile. Individual tubes were stoppered during the reaction, and the entire procedure was conducted under a nitrogen atmosphere in a glove bag. After 20 min reaction time, each reaction was quenched by addition of 5 ul water. Six minutes later, 50 ul of acetic acid (0.6 M, in acetonitrile) was added to neutralize the residual triethylamine. Solvents were then removed by evaporation under a stream of nitrogen at 37 deg C.

Various alternative concentrations of reagents, reaction volumes, and reaction temperatures and durations were investigated, to establish the optimum conditions for derivatization.

Postderivatization Cleanup

Anthroyl esters of the 21- hydroxysteroids were separated from reaction byproducts through chromatography on microcolumns of silica-bonded beta-cyclodextrin (Cyclobond I, Astec, Whippany, NJ). Each dried reaction product was dissolved in 90 ul 60% methanol/0.4 N monobasic sodium phosphate and applied to a

Cyclobond I microcolumn by pipette. The column was then washed with 1 ml water, 8 ml 7.5 mM phosphate buffer (pH 7.0) in 25% MeOH, and again 1 ml water. Finally the steroid esters were eluted in 1 ml 100% MeOH, and the solvent was evaporated under nitrogen.

Alternative use of reversed- phase microcolumn chromatography as a postderivatization cleanup was investigated.

Normal Phase Chromatography

Separation and measurement of the steroid esters was done by automated normal- phase HPLC, after dissolving the samples in the HPLC mobile phase. The automated normal phase chromatograph shared the autosampler, injection valve, and data system with the reversed- phase system described above. The mobile phase (33% ethyl acetate in hexane, half- saturated with water) was stored in a helium-sparged, stirred sample reservoir (Kontes), and was delivered at 0.5 ml/min by a Shimadzu LC6A pump. Samples emerging from the autosampler were first retained on a short silica column (2.1 x 30 mm silica cartridge, Brownlee), installed between the WISP and the Valco valve. The steroid esters were briefly retained on this colunm while the solvent front was eluted through the Valco valve to waste. A timed contact closure then operated the Valco valve so that a single fraction containing the steroid esters was passed onto the analytical column (Keystone Hypersil, 2 X 150 mm). This action prevented overloading of the fluorescence detector by strongly- fluorescing components of the solvent front. Since the fluorescence of the mobile phase reaching the detector was quantitatively small, mobile phase recycling proved to be practical and was routinely used. A teflon line returned the detector effluent to the mobile phase reservoir, which was sealed and developed a slight positive pressure at ambient temperature.

Quantitation was by measurement of the anthroyl ester fluorescence at 430-470 nM due to excitation at 305-395 nM (Gilson

121 Fluorometer), recorded on a flatbed recorder (Kipp & Zonen BD41) and also on the computerized data system. Sample cortisol and cortisone content were calculated by relating the peak heights (maximum fluorescence intensity) corresponding to those anylates to that of the known 1 ng PN recovery standard.

Evaluation of Sensitivity, Precision and Linearity

Precision was evaluated by analysis of multiple replicate samples representing extracts of unused ODS devices with known added amounts of E and F. Unused ODS devices were extracted in methanol as described above, and E and F corresponding to very low physiological concentrations (200 pg E, 50 pg F) or midrange physiological concentrations (800 pg E, 200 pg F) were added along with the recovery tracer. The "spiked" samples were then carried through the entire procedure. Ten replicates were processed at each dose level.

Linearity was evaluated by addition of E and F to sample material representing a pool of unused, extracted ODS devices. Additions ranged from 62.5 to 8000 pg per sample. Each dose was assayed in duplicate.

RESULTS

General

Under the stated conditions, the anthroyl esters of F, PN, and E eluted at 5.48, 7.21, and 10.2 minutes respectively. Peaks were symmetrical and completely resolved. Analysis of unused ODS devices showed a clean "method blank", i.e. the chromatographic baseline was undisturbed in the regions of interest.

Presision and Sensitivity

Table 1 summarizes the results of the precision study. Clearly, precision was excellent above 200 pg per sample, and

TABLE 1. Within Assay Precision for Replicate Samples (n=10) Processed by Reversed-phase Sample Cleanup, 9-AN Derivatization and Postderivatization Cleanup, and Normal Phase HPLC with UV Detection. Abbreviations: F= cortisol, E= cortisone, CV%= coefficient of variation.

STEROID	pg/sample added	c.v.%
F	50	10.7
	200	3.86
E	200	5.90
	800	3.52

deteriorated significantly near the lower limit of expected anylate dose. The signal to noise (S/N) ratio corresponding to the 50 pg F additions was about 7/1.

Linearity

Figure 1 shows detector response (peak height E/ peak height PN) as a function of E added to blank ODS extracts. Log scales are used due to the large linear range involved. Clearly the response is linear over all parts of the range examined (linear correlation coefficient r= 0.998). Figure 2 presents the corresponding data for F.

Evaluation of Variations in Procedure

The preliminary separation of the steroid esters from the solvent front and from later- eluting components provided by the silica injector column proved to be essential to practical operation at high sample volumes. In the absence of this refinement, overloads of the fluorometer were common. In the system as described, fluorometer overloads did not occur, and injections were repeated at 17- minute intervals. Recycling the volatile mobile phase in a closed system resulted in very reproducible retention times over the course of as much as 4 weeks of low- volume operation. Typically, a single 1 l batch of mobile phase was used for as many as 50 injections, regardless of whether these injections took place over a day or over a month.

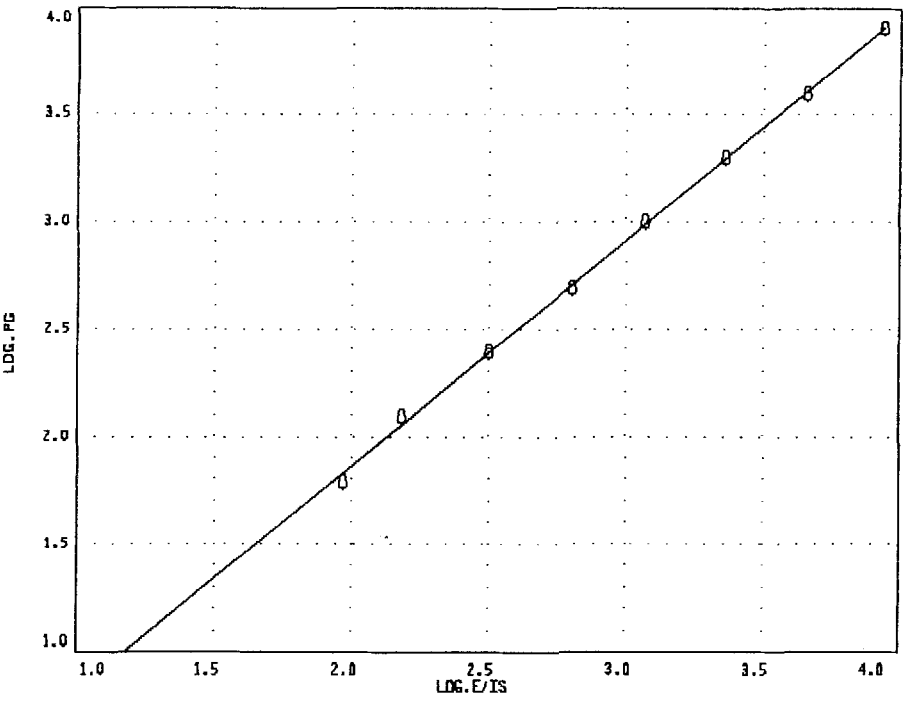

FIGURE 1. Log-log plot of fluorescence detector response to cortisone (E) ester [(peak height E/ peak height internal standard) x 1000] versus amount of E added to material representing extracted sample. Each point is the mean of duplicates.

We have investigated the specificity of this system toward the hormones of interest, in the presence of other steroids that are likely to be present in saliva under normal or pathological conditions. As might be expected, the degree of specificity is extremely high. The reversed- phase sample cleanup excludes corticosterone (11,21-dihydroxypregnene-3,20-dione), which can be present in trace amounts in normal human saliva, and 17-hydroxyprogesterone, which can be elevated in the saliva of subjects with various forms of congenital adrenal hyperplasia. The selectivity of the 9-AN reagent and the normal- phase chromatographic resolution provide further specificity. Table 2

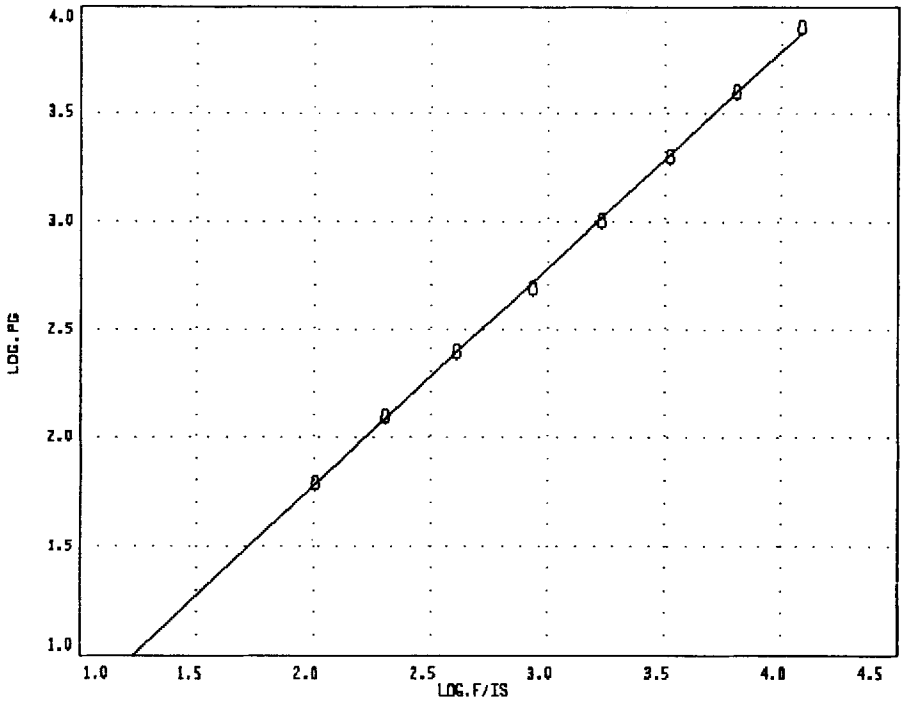

FIGURE 2. Log-log plot of fluorescence detector response to cortisol (F) ester [(peak height F/ peak height internal standard) x 1000] versus amount of F added to material representing extracted sample. Each point is the mean of duplicates.

lists compounds which have been specifically investigated and which have been found to be excluded from the reversed-phase HPLC fraction. In fact, only two compounds have been found that co-elute with the anylates through the reversed-phase step, form derivatives in the presence of 9-AN, and appear in the same neighborhood as the anylates on normal phase chromatography: prednisolone (the recovery standard), and 11-dehydrocorticosterone.

The precision of the system as described depends to a large degree upon the constancy of the "peak height ratio" among the

TABLE 2. Steroids Excluded by Reversed-Phase Chromatography.

dexamethasone corticosterone deoxycorticosterone 11-beta-hydroxyandrostenedione 11-deoxycortisol androstenedione 17-hydroxyprogesterone prednisone

anylates and the recovery standard. This, in turn, depends upon reproducible recoveries of the three compounds in the various cleanup steps and in the derivatization. We have investigated the effects of various deviations from the procedures described above upon the peak height ratio and upon overall yield (sensitivity), and note the following as particularly important:

1) Omission of ascorbic acid from the sample extracts during the first drydown step depressed yields overall, and led to variability in apparent recovery of standards. Omission of phosphate buffer from the prederivatization drydown had similar effects. Use of a vacuum oven rather than a nitrogen- stream evaporation exacerbated these losses.

2) Use of either reversed- phase or cyclodextrin prep columns, rather than automated reversed- phase HPLC, gave unsatisfactory sample cleanup. The steroid fractions prepared by these alternate methods, although generally clean- looking upon inspection, gave a crowd of peaks upon derivatization. Others have reported satisfactory performance with dual, serial prep column cleanup of samples (reversed phase followed by normal phase) destined for 9-AN derivatization and normal phase chromatography (2). We did not investigate the use of two prep column chemistries in series.

4) Use of reversed- phase (C18) rather than cyclodextrin chemistry in the postderivatization cleanup gave unsatisfactory results upon normal- phase chromatography.

5) The absolute yield of the steroid esters could not be increased by increasing the amount of 9-AN used. However,

increasing the amount of 9-AN increased the formation of other, unidentified products that interfered with normal phase chromatography. The formation of the anthroyl esters proceeded at ambient room atmosphere and humidity, but yields were more reproducible and interferences less pronounced when the reactions were conducted under a dry nitrogen atmosphere in a glove bag.

The stated conditions provided an optimal balance between reaction yield and chromatographic interference, and result in reproducible relative yields of E, F, and PN esters. Over the course of 13 separate derivatizations during four months of operation, the coefficient of variation of the E/PN ratio in standards was 6.15%.

We specifically investigated the effects of 9-AN concentration upon peak height ratio. Increasing the amount of nitrile in each reaction tube from 20 to 40 micrograms had no effect upon the relative yields of E, F, and PN esters.

Varying the reaction duration at room temperature between 10 and 40 minutes revealed that peak height ratios stabilized between 10 and 20 minutes and were unaffected by longer reaction times.

DISCUSSION

The procedure described here is relatively laborious, chiefly because of the need for both pre-derivatization cleanup of the sample, and post- derivatization separation of the steroid esters from other products. The performance, however, is excellent in terms of sensitivity and precision. The reaction conditions are robust, in the sense that concentrations of the nitrile reagent and duration of the reaction can vary within broad limits, without any significant effect upon relative yields of the anylates and the recovery standard.

The results presented here are all derived from samples representative of a unique sampling technology, the Oral Diffusion Sink (ODS) (3). It seems likely that sample materials of other origins can be analyzed with appropriate attention to the pre-derivatization cleanup procedure.

In routine practice, samples containing as little as 50 pg F or 75 pg E could be analyzed. Each injection onto the normal phase HPLC system represented about one third of the starting material, due to injection of less than the total available volume by the autosampler. The absolute sensitivity of the normal phase HPLC/fluorescence system (i.e the smallest amount of steroid ester distinguishable from zero) corresponded to about 9 pg of steroid per injection.

The limit of sensitivity in this method is imposed not by S/N ratio at the detector, but by resolving power of the chromatography. In this connection, the abundance of the steroid esters relative to other fluorescent products in the reaction mixture is important. When a 10+ ng quantity of relatively clean steroid is available for derivatization, the relative abundance of the desired products is large, and injection of 0.1% of the reaction mixture, representing as little as 10 pg of starting material, can give a highly reproducible peak on normal phase chromatography (2). However, in the present case the nature of the sample can require the derivatization of as little as 50 pg of steroid, and a single normal-phase HPLC injection may represent 35% of the reaction mixture. Under these conditions, the peak of interest is resolved from other, much larger peaks only with difficulty. Nevertheless, we have been able to routinely resolve and measure peaks representing as little as 18 pg steroid at the detector, from samples containing as little as 50 pg total steroid.

On the whole, this represents a practical analytical sensitivity comparable to that acheived with radioimmunoassay, and equivalent to the best sensitivity reported using other HPLC methods (5,6). Other HPLC approaches to ultrasensitive measurement of steroids have used post-column reaction systems to generate chemiluminescent derivatives for detection (5,6).

In cases where sensitive differential determination of E and F in samples of limited quantity is required, this method provides an alternative to multiple immunoassay. In cases where sample quantity is less limiting, so that total amounts of steroid in the sample exceed 500 pg, a simpler chromatographic approach may be more appropriate (7).

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