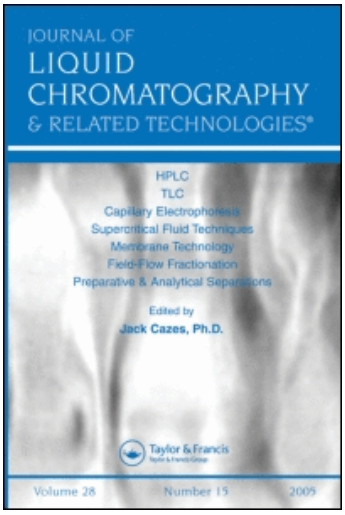

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CORTICOSTEROID ANALYSIS BY HPLC-UV FACILITATED BY USE OF AN INJECTOR-MOUNTED EXTRACTION COLUMN

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ABSTRACT

A method for integrating solid phase extraction of corticosteroids from biological samples with normal operation of reversed-phase HPLC is described. This method uses an extraction column mounted in place of the sample loop of a conventional injection valve to separate corticosteroids from some interfering compounds, and to effectively concentrate steroids from dilute samples. Both manual and fully automated chromatographs using this principle are described. A specific application to measurement of corticosterone in a rabbit serum preparation allows routine measurement of as little as 300 pg corticosterone per sample.

INTRODUCTION

Steroid hormones and related compounds can be effectively separated by reversed phase chromatography, and many can be detected by UV absorbance. Often, the hormonal steroids must be

measured in complex biological sample matrices and at low concentrations. In such cases, extraction methods have been effectively used both to separate the steroids from other, potentially interfering compounds, and to allow concentration of the steroids present in a sample into a smaller volume, for better sensitivity and control of bandwidth during chromatography. Typically, the extraction procedure is done "on the bench", and an organic fraction containing the steroids is dried by some means and then redissolved for injection into the chromatograph. This is usually the case both with the classic liquid-partition extraction (e.g. 1), and with the more recently developed solid phase extractions: when an extraction column is used, an organic eluate fraction from this unit is dried to yield a steroid-containing residue that is redissolved for chromatography (e.g. 2,3).

We have found that significant advantages, including reproducible 100% recovery of steroids through the extraction, and elimination of the drydown step common to all bench extraction procedures, can be gained by incorporating the solid phase extraction chemistry into the solvent pathway of the chromatograph. This can be done by arranging for dilute and complex samples to be introduced onto an extraction column that can later be eluted directly onto the analytical column. Practically, this is accomplished by replacing the sample loop of a conventional injection valve with an appropriately packed guard column. The conversion is easily done in a few minutes with hardware that is commercially available. It is equally applicable to manual and automated chromatographs.

We describe our experience with this approach over a period of three years, with special emphasis upon analysis of the glucocorticoid hormones cortisol and corticosterone.

MATERIALS AND METHODS

Steroid Standards

Cortisol (11 beta,17,21-trihydroxy-4-pregnene-3,20-dione, or F), corticosterone (11 beta,21-dihydroxy-3-pregnene-3,20-dione, or B), and 11-dehydrocorticosterone (21-hydroxy-4-pregnene-3,11,20-trione, OR 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.

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).

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 over 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).

This arrangement permitted considerable flexibility in the treatment of the injector column. In all cases, a cycle of treatment including injector column preparation and sample introduction occured before operation of the rotary valve, corresponding to "injection" onto the chromatograph. Solvents and samples were applied to the injector column through the valve's injection port, by means of standard Hamilton syringes with 22 ga blunt needles. Injector column preparation always included clearing the column by applying a strong mobile phase (pure methanol), and then preparing the column for maximum retention of steroids by applying a very weak mobile phase (5% methanol). Samples were then applied in aqueous solution, or in a solvent resembling the weak mobile phase. Under these conditions, the compounds of interest were very strongly retained on the injector column. In some cases, the injector column was extensively washed with high-ionic strength solvents to selectively elute non-steroidal components; in other cases, unwanted components could be eluted with 5% methanol only. When the valve was operated, the much stronger mobile phase pumped by the chromatograph eluted the compounds of interest onto the analytical column. By precise timing of the valve operation, it was possible to pass some compounds onto the analytical column while retaining others in the injector column for later disposal.

The automated system differed in using a WISP autosampler (Waters) to introduce samples 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 injector column arrangement like that described above. 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, contact closures generated by the data system were able to control injector column washing by means of an Autochrom solvent selection valve mounted on the inlet of the Eldex pump. Later contact closures operated the Valco valve to direct the flow of the Eldex pump to waste, and to direct mobile phase from the main pump (Micromeritics 750) 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.

Special Procedures

In order to more fully characterize the performance of the injector column with steroids, we conducted some studies that involved variations of the usual operating procedure. These included:

a) Absolute Recovery Study: Replicate samples of cortisol (10 ng in 50 ul 5% methanol) were injected using the injector column, or using a standard external sample loop. We compared the detector peak height response for triplicate injections by each method, taking the response observed with the sample loop to represent 100% recovery.

b) Effective Elution Volume from Injector Column: The retention time of the steroids on the injector column was too short to resolve peaks for direct detection by our UV spectrophotometer. In order to determine the retention times and

elution volumes, we resorted to fractionating the effluent from the injector column. The injector column was loaded with a sample containing both F and B, and then the rotary valve was operated to repeatedly interpose the injection column between pump and analytical column for 2 seconds at a time, with a delay of 10 minutes between each 2-second "cut". We observed the distribution of F and of B detector response among the 2-second (10 ul) fractions, to determine the volume in which each anylate was eluted.

c) Effects of Sample Loading Volume upon Injector Column Elution Volume: As in the previous study, fractionation of the injector column effluent was used to sensitively reveal small changes in elution behavior. The injector column was loaded with 5 ng each of F and B, dissolved in 100 ul of 5% methanol. The rotary valve was then operated to deliver 2 fractions to the analytical column, separated in time by 50 seconds: the first representing the first 11 seconds (55 ul) of injector column eluate, the second representing the next 20 seconds. We observed the distribution of F between the first and second fractions, verifying that a small but detectable F peak occured in the first fraction and a much larger one in the second. The procedure was then repeated, loading the same amount of anylate in progressively larger volumes of 200, 400, and 800 ul. We compared the distribution of F into the first and second fractions as a function of total sample loading volume, expecting that any bandspreading due to increasing load volume would be revealed as increasing appearance of F in the first fraction.

d) Effects of Extensive Injector Column Washing upon Elution Volume: Samples containing 10 ng F were loaded onto the injector column and injected in two fractions as in the previous procedure. The distribution of F between the first and second fractions was observed. The procedure was then repeated with extensive washing of the injector column (1 ml 200 mM Nacl and 250 ul 5% methanol) between loading and injecting. The change in F distribution due to washing was observed, and compared to the

change produced by retarding the timing of fraction cutting by 1 second.

Evaluation of Linearity, Precision, and Sensitivity in the Analysis of Biological Samples Containing Corticosterone (B).

We applied the system described here in the analysis of samples generated using a novel sampling system, the Implantable Diffusion Sink. A prototype of this system has been described elsewhere (4). Samples generated by this system were about 200 ul in volume, and contained B in the presence of a specific anti-corticosteroid serum raised in rabbits. The protein content of a typical sample was equivalent to that of about 10 ul of rabbit serum.

Each sample or control replicate of this type was prepared for chromatography by adding DHB (2 ng) as a recovery tracer, then heating 5 min in a glass tube in a boiling water bath, then transferring to a plastic microcentrifuge tube for centrifugation of precipitated material (8 min at 10,000 g in a Beckman Microfuge 12). The supernatant was then loaded into vials for injection on the automated chromatograph.

We studied the linearity of detector response as a function of increasing amounts of authentic B (150 to 15000 pg) added to pools of dilute rabbit anti-corticosteroid serum. We evaluated the precision of replicate preparations from these pools at several concentrations, with particular reference to doses near the limit of sensitivity. In each case, the amount of B per sample was calculated from the relative detector responses to B and to the recovery standard DHB.

RESULTS

Absolute Recovery

Identical detector responses to replicate injections were observed with the injector column and with the sample loop. The absolute recovery was 100% for all the compounds investigated.

Effective Elution Volume from Injector Column

When both F and B were loaded onto the injector column in a 200 ul sample volume, and the column was eluted in 12 successive 10 ul fractions, the first 5 fractions contained no steroid. Cortisol response was observed only in fractions 6 through 9, and corticosterone only in fractions 7 through 10. Thus each steroid was eluted from the injection column and delivered to the analytical column in a volume no greater than 40 ul.

Effects of Sample Loading Volume upon Injector Column Elution Volume

The volume in which a sample was applied to the injector column (range: 100-800 ul) had no effect upon the timing of anylate elution from the injector column.

Effects of Extensive Injector Column Washing upon Elution Volume

Washing the loaded column with 1000 ul 200 mM Nacl followed by 250 ul 5% methanol speeded the elution of F from the injector column, by an amount that was still less than that due to 1 second (5 ul) of elution with the system mobile phase. The effect upon retention through the analytical column was of course undetectable.

Linearity, Precision, and Sensitivity of Measurement of B in Biological Samples

Detector response (mV) was a linear function (linear correlation coefficient r=0.999) of added B over the entire range investigated (150- 15000 pg per sample). Precision, expressed as the coefficient of variation among replicate preparations (n=6) from a single sample pool, ranged from 5% at 300 pg/sample to 3.5% at 5000 pg/sample.

DISCUSSION

These results demonstrate the effectiveness of an injector-mounted extraction column in the chromatographic analysis of corticosteroids in biological samples. The procedures described result in the quantitative extraction of corticosteroids from samples of 100-1000 ul volume, and delivery of the extracted material to the analytical column in a volume no larger than 40 ul. Appropriate manipulation of the injector column allows components of the sample that are much more polar than the anylates to be washed away before injection, and allows components that are much less polar than the anylates to be retained on the injection column for later disposal. In this way, fast chromatographic separations and high sample throughput are facilitated.

Since the injector column acts as a sample concentrator, the sensitivity of the method is best described in terms of pg per injection, rather than pg/ml. We routinely measure as little as 300 pg of corticosterone per sample with 5% CV.

We have applied this method extensively to the analysis of samples from diffusion-sink sampling devices [Wade 1984]. We have not used it extensively for analyzing more conventional serum or plasma samples. However, dialyzed rabbit immune serum is the diffusion driver used in our diffusion-sink sampling devices. Only if the dialysis treatment used to prepare our immune serum for use in the sampling apparatus removes some significant interference would we expect native serum to give less useful chromatographic results. We have examined the detection of B present in 40 ul samples of native rabbit serum, diluted to 200 ul and processed as described above. The chromatography was practically undistinguishable from that described above.

This injector column method has proved to be useful in several applications, especially when dealing with very dilute

samples. Specific applications, including analysis of the corticosteroids of human saliva (5), and a sample-preparative use (6) are described elsewhere.

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