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Note

Simplified method for the determination of plasma 18-hydroxycorticosterone

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Plasma 18-hydroxycorticosterone (18-OHB, 4-pregnene-18,21-diol-3,20-dione) determinations have been utilized to assess the terminal pathway of aldosterone biosynthesis and the production of 18-hydroxylated steroids by the adrenal zona fasciculata [1–5]. In addition, elevated plasma 18-OHB concentrations may help differentiate primary aldosteronism due to aldosterone-producing adenoma from that resulting from bilateral zona glomerulosa hyperplasia [6]. The radioimmunoassay (RIA) of 18-OHB is commonly done by RIA of 18-OHB γ-lactone, which is produced by periodic acid oxidation of 18-OHB [7]. Previous methods have utilized paper or column chromatography to purify 18-OHB γ-lactone or 18-OHB itself before RIA [2,7–10]. We are reporting a simplified method which uses bonded-phase extractions in combination with silica gel minicolumn chromatography before RIA.

EXPERIMENTAL

Bonded-phase silica derivatives (Sepralytes) were purchased from Analytichem International (Harbor City, CA, U.S.A.). These Sepralytes included methyl (C_1) and ethyl (C_2). Silica (silica Woelm R 32-63, activity grade 2) was purchased from Universal Scientific (Atlanta, GA, U.S.A.). Methanol, ethanol, ethyl acetate and isooctane were HPLC-grade solvents obtained from Fisher (Pittsburgh, PA, U.S.A.). Water was deionized and distilled. RIA-grade bovine serum albumin, Cohn Fraction II γ-globulins, boric acid and ammonium sulfate were obtained from Sigma (St. Louis, MO, U.S.A.). Solvents for high-performance

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liquid chromatography (HPLC) were filtered through a Rainin (Woburn, MA, U.S.A.) Nylon-66 0.45-μm disk filter prior to use. Syringes (3 and 12 ml) were purchased from Monoject (St. Louis, MO, U.S.A.) for use as columns and solvent reservoirs. Frits (20 μm), syringe connectors, 1.5-ml syringe-type reservoirs and a vacuum manifold (Vac-Elut) were purchased from Analytichem. A Hewlett-Packard (Palo Alto, CA, U.S.A.) HPLC system with a Rheodyne (Cotati, CA, U.S.A.) 7125 injector and a 20-μl sample loop was utilized with a Unimetrics (Anaheim, CA, U.S.A.) diol column (5-μm packing, 25 cm$\times$0.46 cm) with a Brownlee (Santa Clara, CA, U.S.A.) 10-μm diol guard column. 18-Hydroxy[1,2-^{3}H]corticosterone was purchased from Amersham (Arlington Heights, IL, U.S.A.) and purified by HPLC prior to use. A Buchler (Fort Lee, NJ, U.S.A.) vortex evaporator was used to evaporate samples. A Packard (Downers Grove, IL, U.S.A.) 460 C scintillation counter was used with a toluene cocktail containing 0.4% 2,5-diphenyloxazole, 0.008% *p*-bis(o-methylstyryl benzene) and 2% methanol.

18-OHB γ-lactone antibody

The 3-carboxymethyloxime derivative of 18-OHB γ-lactone was synthesized and conjugated to bovine serum albumin by the mixed anhydride technique [11]. Four-month-old New Zealand white rabbits were injected intradermally at multiple sites with 500 μg of immunogen and given booster injections at monthly intervals. Blood was collected one week after each booster injection. After eight weeks a suitable antibody was obtained.

Purification of 18-OHB recovery counts

18-OHB was purified by HPLC using a 5-μm diol column eluted with hexane–isopropanol (80:20, v/v) at a flow-rate of 1 ml/min. About 1 μCi of the stock solution was injected, and 1-ml aliquots were collected. The purified 18-OHB appeared in the 14–15 ml fraction, and the peak was evaporated to dryness after transfer to a glass vial and stored in toluene–ethanol (9:1, v/v) to give a final concentration of about 1200 cpm 18-OHB per 20 μl.

Preparation of Sepralyte and silica gel minicolumns

Sepralyte columns were prepared by pouring 0.13 g (0.25 ml bed volume) of C_2 Sepralyte or 0.25 g (0.5 ml bed volume) of C_1 Sepralyte into a 3-ml Monoject syringe fitted with a 20-μm frit on the bottom and top of the column bed. The columns were solvated with 5 bed volumes of methanol followed by 5 bed volumes of distilled water just prior to use.

Silica gel minicolumns were prepared by saturating 0.35 g silica gel (1 ml bed volume) with 0.35 ml of ethylene glycol–water (80:20, v/v) and packing in a 1.5-ml syringe reservoir over a 20-μm frit.

Preparation of 18-OHB γ-lactone tracer

Freshly made 1% periodic acid (100 μl) was added to approximately 3 μCi [^{3}H]18-OHB in 100 μl ethanol diluted to 1 ml with distilled water and placed in the dark for 1 h. The reaction was stopped with 100 μl of 0.2 m*M* Tris. The

resulting 18-OHB γ-lactone was extracted by passing through a solvated C_1 Sepralyte column. The column was washed with 5 bed volumes of distilled water followed by 5 bed volumes of 10% ethanol. Water was removed from the column by passing 5 bed volumes of isooctane through the column, and the sample was eluted with 5 bed volumes of acetone. The sample was evaporated to dryness under nitrogen and stored in about 1 ml of toluene–isopropanol (90:10, v/v) at −20°C.

Separation of the γ-lactones of 18-OHB, 18-hydroxydeoxycorticosterone (18-OHDOC) and aldosterone

The γ-lactones of 18-OHDOC and aldosterone were prepared as described above for 18-OHB γ-lactone. Separation of these three γ-lactones was evaluated on the same type diol HPLC column as was used for tracer purification except that a mobile phase of hexane–isopropanol–dichloromethane (77.5:7.5:15, v/v/v) at a flow-rate of 1 ml/min was used. In addition, separation provided by the silica gel minicolumn was evaluated by running each of the tracers individually initially and then by combining the three tracers before chromatography.

18-OHB RIA procedure

Approximately 1200 cpm of 18-OHB recovery counts were added to 12×75 mm glass test tubes for the samples and to three scintillation vials for total recovery count determination. Plasma samples (0.5 ml) were mixed with the recovery counts with a vortex mixer and allowed to sit for 10 min before further processing. The sample was diluted 1:1 with distilled water and passed through a solvated C_2 Sepralyte column over approximately 30 s, washed with 5 bed volumes of distilled water followed by 5 bed volumes of 10% ethanol, and then eluted with 3 bed volumes of 100% ethanol.

Oxidation to the γ-lactone. The sample was diluted with 8 ml distilled water and mixed with a vortex mixer. Freshly made 1% periodic acid (1 ml) was added to the sample, mixed, and then the sample was placed in the dark for 1 h. The reaction was stopped with 1 ml of 0.2 m*M* Tris.

Extraction of 18-OHB γ-lactone. The sample was passed through a solvated C_1 Sepralyte column fitted with a 12-ml syringe as reservoir and washed with 5 bed volumes of distilled water followed by 5 bed volumes of 10% ethanol. Water was removed from the C_1 Sepralyte column by passing 5 bed volumes of isooctane through the column, and the sample was eluted with 5 bed volumes of 100% ethanol. The sample was evaporated to dryness with a vortex evaporator.

Silica gel chromatography. Silica gel minicolumns were equilibrated with 2 bed volumes of ethyl acetate–isooctane (10:90). The samples were reconstituted with 250 μl ethyl acetate–isooctane (10:90), mixed on a vortex mixer and transferred to the column. The sample was passed through the column by use of the syringe plunger with an elution time of approximately 0.5 min. The column was washed with 6 bed volumes of mobile phase and then eluted with 2 bed volumes of ethyl acetate–isooctane (50:50).

RIA. The eluate was mixed with a vortex mixer, and then 300-μl aliquots were obtained for triplicate determinations for RIA and an 800-μl aliquot for recovery

count determinations. The RIA samples were evaporated to dryness in 12×75 mm glass test tubes. RIA buffer (0.5 ml of 0.05 *M* borate with 0.2% bovine serum albumin, 0.1% γ-globulin, pH 7.8) containing approximately 3000 cpm [^{3}H]18-OHB γ-lactone and a 1:1000 dilution of the 18-OHB γ-lactone antibody was added to each tube. A standard curve was constructed in triplicate with standards, which were added in 10-μl aliquots to the 12×75 mm glass test tubes and evaporated to dryness before addition of 0.5 ml of the identical RIA buffer. The assay was incubated overnight at 4°C before bound/free separation by the addition of 0.5 ml of saturated ammonium sulfate, mixing with a vortex mixer and centrifugation at 3500 *g* for 10 min. The supernatant was transferred into 20-ml polyethylene counting vials, and 10 ml of counting cocktail were added. The samples were mixed well, equilibrated in the dark for 3 h and counted.

RESULTS

The antibody prepared for 18-OHB γ-lactone had very little cross-reactivity with γ-lactones of steroids other than 18-OHDOC γ-lactone (Table I). For this reason, the purification system was designed to separate these two γ-lactones adequately. HPLC separation of the γ-lactone standards of aldosterone, 18-OHDOC and 18-OHB was monitored by UV detection at 254 nm. The elution pattern was evaluated with individual standards initially, and then a combined sample was evaluated. The separation was further monitored by including [^{3}H]18-OHDOC or [^{3}H]18-OHB to confirm the identify of the proper UV peak when the steroids were mixed. With this HPLC system, 18-OHDOC γ-lactone and aldosterone γ-lactone eluted together in the 6-ml elution fraction and 18-OHB γ-lactone eluted in the 11–12 ml elution fractions. Thus, excellent separation was obtained.

The separation of the γ-lactones of 18-OHDOC and 18-OHB by silica gel mini-column chromatography was evaluated with the use of the tritiated tracers of these steroids. Aldosterone γ-lactone was not evaluated because its cross-reactivity with the antibody was low, plasma levels of aldosterone are low, and the HPLC study had demonstrated a similar chromatographic behavior of the γ-lactones of

TABLE I

18-HYDROXYCORTICOSTERONE γ-LACTONE ANTIBODY CROSS-REACTIVITIES

Steroid	Cross-reactivity (%)
18-OHDOC γ-lactone	100
18-OHB	0.66
Androstenedione	0.35
Testosterone	0.1
Progesterone	0.001
Corticosterone	0.007
17α-Hydroxyprogesterone	0.005
Cortisol	<0.005
Aldosterone γ-lactone	<0.005

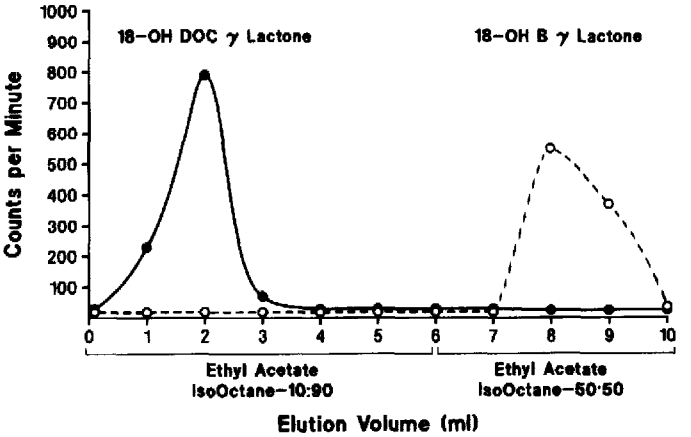

Fig. 1. Elution pattern of the γ-lactones of 18-OHDOC and 18-OHB with silica gel minicolumn chromatography. A one-step gradient elution was performed with 6 ml ethyl acetate–isooctane (10:90) followed by 4 ml ethyl acetate–isooctane (50:50).

TABLE II

COMPARISON OF PLASMA 18-HYDROXYCORTICOSTERONE DETERMINATIONS UTILIZING HPLC OR SILICA GEL MINICOLUMN CHROMATOGRAPHY

Sample	HPLC		Silica gel minicolumn chromatography	
	n	18-OHB (ng/dl)	*n*	18-OHB (ng/dl)
Water	2	0 ±0	23	1.6±1.0
Plasma	4	41.6±5.9	23	37.4±3.8

aldosterone and 18-OHDOC. The initial mobile phase [ethyl acetate–isooctane (90:10)] in the two-step gradient elution chromatography eluted essentially all of the 18-OHDOC. The second mobile phase [ethyl acetate–isooctane (50:50)] eluted the 18-OHB γ-lactone in the second and third 1-ml fraction. A typical silica gel minicolumn separation is shown in Fig. 1. Separation of the γ-lactones of 18-OHB and 18-OHDOC was excellent with this method. RIA values of a water blank and a plasma pool were obtained with HPLC in comparison to silica gel minicolumn chromatography (Table II). The values obtained were close with both methods. The coefficient of variation was 10.2% for the plasma pool with the silica gel minicolumn separation. Recovery studies ($n=2$) demonstrated an average recovery of 113% with the water blank and 109% with the plasma pool.

DISCUSSION

Several assay procedures are available for 18-OHB. Several methods [2,5] have utilized conversion of 18-OHB to the γ-lactone. This provides the advantage that most steroids with an α-ketol side-chain are converted to etienic acids, which can be easily separated. In addition, 18-hydroxylated steroids are sensitive to the for-

mation of dimers in the presence of traces of acids [12,13]. Conversion to the γ-lactone prevents this problem and avoids the need to add triethylamine to all chromatographic solvents to maintain a basic pH.

We have utilized bonded-phase extraction at two steps to facilitate the assay. Initial extraction of 18-OHB with C_2 Sepralyte is achieved with about an 85–90% recovery, whereas it is slightly less with C_1 Sepralyte. Washing of the sample with water and 10% ethanol removes most plasma proteins, lipoproteins and other more polar compounds. We noted that a 10% ethanol wash was also required after conversion to the γ-lactone to remove cross-reacting materials. 18-OHB is eluted with 100% ethanol and can be diluted directly before oxidation to the γ-lactone. This is an easy and rapid procedure for extracting 18-OHB from plasma. We have found that C_2 Sepralyte is preferable to longer-chain bonded-phase packings because fewer interfering materials are retained and a smaller volume of ethanol is satisfactory for elution, thereby allowing simple dilution before oxidation to the γ-lactone.

The second bonded-phase extraction recovers 18-OHB γ-lactone. We noted that C_1 Sepralyte worked as well as C_2 Sepralyte for this purpose, and it allowed elution with slightly less ethanol, thus reducing the time for drying. We found it necessary to wash the sample with water and 10% ethanol to remove compounds which cross-reacted with the antibody in the subsequent RIA. The columns for both of these bonded-phase extractions can be regenerated for subsequent reuse by washing with 10 bed volumes of 100% methanol followed by 5 bed volumes of distilled water. We noted no change in assay results with reuse up to ten times.

Silica gel minicolumn chromatography provided satisfactory separation of the γ-lactones of 18-OHB and 18-OHDOC. Other types of chromatography including paper [7] and Sephadex LH-20 [2] chromatography have been utilized, but both of these methods are more tedious. In addition, HPLC has been utilized in the assay of plasma 18-OHB [9,10]. Though this procedure may facilitate the determination of multiple steroids from a single plasma sample, it is more tedious and expensive to perform than is our method. Thus, the method for determining 18-OHB which we are reporting offers several advantages over previous methods.

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