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Use of particle-loaded membranes to extract steroids for high-performance liquid chromatographic analyses Improved analyte stability and detection

Gary L. Lensmeyer*, Carol Onsager, Ian H. Carlson, Donald A. Wiebe

Clinical Toxicology/Special Chemistry Laboratories and Departments of Pathology and Laboratory Medicine, University of Wisconsin Hospital and Clinics, Madison, WI 53792, USA

Abstract

Cortisol, cortisone, corticosterone, prednisone and prednisolone are extracted from serum using the novel particle-loaded octyl (C_8)-bonded silica in PTFE membrane. Extracts are directly injected, without further concentration, onto a narrow (2.0 mm) *or* conventional (4.6 mm) bore octyldecyl (C_{18}) HPLC column. Method performance data demonstrate linearity from 0.4 μg/dl (low limit of detection) up to at least 60 μg/dl. Extraction recoveries exceeded 85% and precision (between-run) R.S.D.s averaged $<5\%$. Interferences were minimal and selectivity was improved over conventional immunochemical steroid assays. When compared to large particle sorbents packed in columns or to traditional liquid–liquid extractions, the membrane extracted steroids in less time, used less reagent, and had smaller elution volumes, thereby obviating steroid instability/adsorption problems associated with traditional concentrating techniques required to improve analytical sensitivity.

1. Introduction

Commercial immunochemical assays offer a technically simple and expedient way to measure steroids in blood. However, analytically these assays are often inaccurate due to interferences from sample matrix and cross-reactivity with chemically similar steroids [1,2]. With cortisol assays, commercial antisera are non-specific and unable to differentiate between cortisol, some of its metabolites and some therapeutically administered steroids. Inaccuracy problems associated with immmunochemical assays for steroids are not always obvious or appreciated by physicans or other users of these test systems. Previously we reported descrepancies for cortisol results in a patient sample comparison study [2]; others describe similar results [3]. Sera collected from patients in a major medical center were tested both by immunoassay and HPLC with solid-phase packed-column extraction. Regression data (with $n = 134$, and $y =$ immunoassay, $x =$ HPLC) demonstrated $y = 0.851x + 7.21$, $r = 0.956$, $Sy/x = 3.8$ μg/dl. The two methods did not compare well. For several serum specimens, the immunoassay reported cortisol concentrations when none was detected by HPLC. The presence of prednisolone in serum always grossly elevated the "apparent cortisol" value. The interference problem is not limited to cross-reactivites from administered steroid drugs. Patients with a disrupted hypothalamic-pituitary-

* Corresponding author.

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adrenal axis often generate abnormal steroids that cross-react with the antisera [4] and give falsely high cortisol values. Ironically, immunoassays seem to perform best on serum specimens from healthy individuals [5,6].

Fortunately, improved analytical selectivity is available with the somewhat more complex chromatographic procedures [2–4,7,8] for steroids. Most often though, time-consuming extractions are necessary with these assays. Simplification of extraction processes is one way by which chromatography would be a cost-effective, practical, accurate and more clinically relevant alternative to immunoassays.

Advances in solid-phase extraction (SPE) technology have recently extended the benefits of this sorbent technique over traditional liquid–liquid extractions. Second generation SPE materials in the form of thin membranes or disks loaded with small particle sorbents have been introduced [9] and demonstrated advantages over large particle sorbents loosely packed in columns. Application reports of assays for environmental pollutants [9] and drugs [10–12] attest to the benefits of the membrane extraction.

Here we demonstrate the effectiveness of the thin PTFE (SPE) membrane —loaded with small-particle C_8-bonded silica— in the extraction and companion HPLC analysis of the endogenous steroids cortisol, cortisone, corticosterone and the therapeutically administered steroids prednisone, and prednisolone in patients' serum specimens. To measure overall performance, we evaluated linearity, recovery, precision and lowest limit of quantitation. Method optimization charts for each step of the process are presented. A comparative study with conventional large-particle C_8 sorbent packed in columns demonstrates the advantages of the membrane format for extraction of steroids.

2. Experimental

2.1. Chemicals and reagents

Pure prednisone, cortisone, prednisolone, corticosterone, fludrocortisone and methylprednisolone were purchased from Sigma (St. Louis, MO, USA). Cortisol was from the the National Bureau of Standards (Washington, DC, USA). Hydrochloric acid and all solvents (HPLC grade), including acetonitrile, methanol, ethanol and tetrahydrofuran (THF) were from J.T. Baker, Phillipsburg, NJ, USA. Sodium borate (reagent grade) was from Fisher Scientific, Fair Lawn, NJ, USA. Distilled deionized water was prepared with the Milli-Q water-purification system (Millipore, Bedford, MA, USA). Other solutions include hydrochloric acid, 0.8 *M*, methanol–water (18:82, v/v), and saturated sodium borate solution.

2.2. SPE membrane

Empore extraction disk cartridges (4 mm diameter, 500 μm thick secured in 1-ml polypropylene columns) containing octyl (C_8)-bonded silica (8 μm average particle size) enmeshed in PTFE fibrils were supplied by 3M Co., St. Paul, MN, USA (Fig.1).

2.3. Instrument and mobile phase

The HPLC system consisted of a Model 126 solvent-delivery unit and Model 167 detector

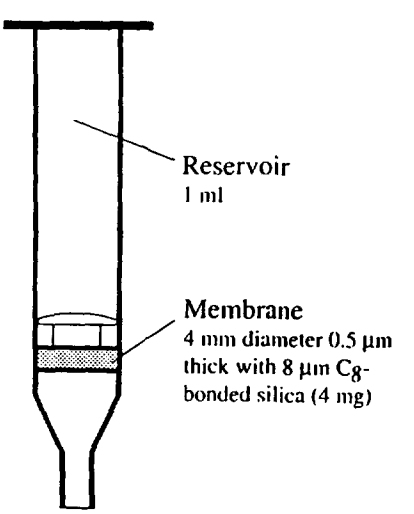

Fig. 1. Diagram of the solid-phase extraction disk cartridge.

module set at 254 nm with attenuation at 0.015 AU full scale (all from Beckman Instruments, Berkeley, CA, USA). A Model HP1050 auto-injector and Model HP3395 integrator were from Hewlett-Packard, Rolling Meadows, IL, USA. An Omniscribe strip-chart recorder (Houston Instruments) was used also. A dry-packed mobile phase silica saturating column (150 × 4.6 mm) containing 37–53-μm particle diameter silica gel (Whatman, Clifton, NJ, USA) was connected between the pump and the injector. Use of a saturator column is extremely important. Otherwise, chromatographic separation will deteriorate and the performance of the analytical column will be significantly decreased. A guard column (20 × 2 mm; Upchurch Scientific, Oak Harbor, WA, USA) was packed with 30-μm diameter particles of Permaphase ETH (DuPont Instruments, Wilmington, DE, USA) and connected to precede the Ultrasphere C_{18} (250 × 2.0 mm *or* 250 × 4.6 mm) analytical column (Beckman Instruments, Fullerton, CA, USA). All columns (silica saturating, guard and analytical) are located in an oven (Eldex column heater, San Carlos, CA, USA) maintained at 55°C. The mobile phase was water–THF (80:20, v/v). Flow-rates were 0.18 ml/min *or* 0.8 ml/min for the 2.0 mm or 4.6 mm internal diameter columns, respectively.

2.4. Standard solutions

Individual stock standards of cortisol (20 mg/100 ml), cortisone (10 mg/100 ml), corticosterone (10 mg/100 ml) and prednisolone (20 mg/100 ml) were prepared in methanol. Prednisone standard (10 mg/100 ml) was prepared in ethanol. A stock mixture solution was prepared by combining aliquots of the individual stock solutions and diluting the mixture to 500 ml with methanol. Final concentrations of this secondary stock mixture are 40 μg/dl each for cortisol, prednisone, prednisolone and 20 μg/dl each for cortisone and corticosterone. To obtain working concentrations encompassing the calibration curve (6.0, 12.0, 24.0, 36.0, 48.0 and 60.0 μg/dl for cortisol, prednisone and prednisolone; 2.25, 4.5, 9.0, 13.5, 18.0 and 22.5 μg/dl for cortisone and corticosterone) portions of the mixed stock are placed in polypropylene tubes, the solvent removed using vacuum (Speed Vac concentrator, Savant Instruments, Farmingdale NY, USA), and the steroids reconstituted with steroid-free serum. The steroid-free serum was prepared in our laboratory by sorbent adsorption of endogenous steroids with minimal disruption of native serum matrix.

2.5. Internal standards (I.S.) solution

Stock solutions of fludrocortisone (20 mg/100 ml) and methylprednisolone (30 mg/100 ml) were prepared in methanol. Portions (0.5 ml) of each stock were combined and the mixture diluted to 100 ml with water to get final concentrations of fludrocortisone 100 μg/100 ml and methylprednisolone 150 μg/100 ml. Equal parts of this mixture and of hydrochloric acid (0.8 *M*) are combined to prepare a working I.S. solution. Working solution is prepared daily. The second I.S., methyprednisolone, is used in the event of an interference with fludrocortisone, the primary I.S.

2.6. Extraction procedure

Combine 300 μl serum (or standard calibrators) and 150 μl of the working I.S. solution [0.4 *M* HCl containing fludrocortisone (50 μg/100 ml) and methylprednisolone (75 μg/100 ml)] in a polypropylene microfuge test tube (1.7-ml size). Incubate sample at room temperature for 10 min to release steroids bound to proteins. Add 800 μl saturated sodium borate solution, mix. If necessary, centrifuge (12 400 *g*) the sample mixture for 3 min to settle particulate that could plug the extraction membrane. Pour sample into the reservoir of a primed Empore C_8 extraction disk unit. To prime the membrane, place about 0.5 ml methanol in the reservoir, and force through three drops of liquid using a syringe with adapter; discard the remaining liquid. Next fill the reservoir with water and force three drops through; pour off the remaining water. The sample mixture is forced through the membrane by either (a) centrifuging (swinging

bucket) the unit at 100–120 *g* for 5 min, (b) manually applying pressure with a syringe or (c) pulling with a vacuum. We prefer centrifugation; constant flow-rates can be maintained and many samples can be processed simultaneously. After sample passes, wash membrane to remove proteins and interference. Manually force through 200 μl of water followed by 500 μl methanol–water (18:82, v/v). To elute the retained steroids, suspend unit in a 5-ml polypropylene test tube. Add 50 μl acetonitrile to reservoir and gently push liquid through; follow with 150 μl water; mix eluates. Inject 20 μl onto the narrow-bore C_{18} HPLC column (*or* 50–100 μl onto a 4.6-mm HPLC column). Relative retention time and relative peak height techniques are used for identification and quantitation, respectively. Fludrocortisone is the primary I.S. used in these calculations, except when an interference occurs; then methylprednisolone is used.

3. Results

Typical chromatograms from extracted standard calibrator, steroid-free serum, and patients' serum specimens are presented in Fig. 2. Method performance data (precision, recovery and linearity) are listed in Table 1. Extraction recovery was determined by comparing analytical reponse of steroid extracted from serum to response of unextracted steroid in acetonitrile–water (25:75, v/v). To be assured of accurate results, the assay is always calibrated with steroid standards in serum extracted in same manner as patient specimens. Linearity was determined by extracting standards in the concentration range listed in the *Standard solutions* section. The lowest limit of quantitation —described as two times baseline noise— was 0.4 μg/dl for all steroids. Studies demonstrated freedom from interference of other steroids. Table 2 lists those

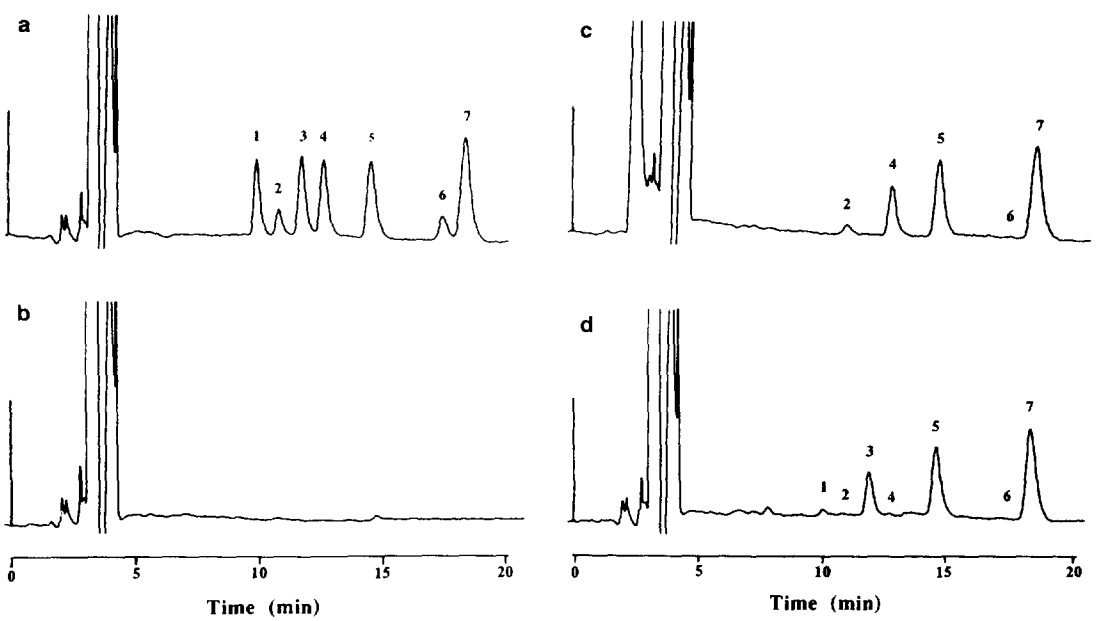

Fig. 2. Typical chromatograms of extracts from: (a) steroid-free serum supplemented with steroid standards (peaks: 1 = prednisone, 20 μg/dl; 2 = cortisone, 7.5 μg/dl; 3 = prednisolone, 20 μg/dl; 4 = cortisol, 20 μg/dl; 5 = fludrocortisone, I.S.1; 6 = corticosterone, 7.5 μg/dl; 7 = methylprednisolone, I.S.2); (b) steroid-free serum; (c) serum from healthy individual (peaks: 2 = cortisone, 2.7 μg/dl; 4 = cortisol, 13.6 μg/dl; 5 = I.S.1; 6 = corticosterone, < 0.4 μg/dl); 7 = I.S.2; and (d) serum from patient who received therapy with prednisone (peaks: 1 = prednisone, 2.3 μg/dl; 2 = cortisone, < 0.4 μg/dl; 3 = prednisolone, 12.3 μg/dl; 4 = cortisol, < 0.4 μg/dl; 5 = I.S.1; 6 = corticosterone, < 0.4 μg/dl); 7 = I.S.2. (Note: prednisone is metabolized to prednisolone.)

Table 1
Method performance data

	Precision (between-run, $n = 14$)			Recovery (%)	Linearity ($n = 6$)			
	Mean (μg/dl)	S.D. (μg/dl)	R.S.D. (%)		Slope	y-Intercept	r	Sy/x
Prednisone	5.03	0.180	3.6	87–99	0.0572	−0.0215	1.00	0.0161
	19.72	0.677	3.4	89–93				
	40.8	1.27	3.1	86–91				
Cortisone	1.98	0.056	2.8	89–92	0.0511	−0.0153	1.00	0.0206
	7.46	0.502	6.7	91–94				
	14.9	0.770	5.1	86–88				
Prednisolone	5.02	0.111	2.2	85–90	0.0565	−0.0383	1.00	0.0390
	19.75	0.603	3.1	88–93				
	40.5	1.26	3.1	86–89				
Cortisol	5.03	0.125	2.5	92–94	0.0549	−0.0551	1.00	0.0697
	19.82	0.668	3.4	86–93				
	40.3	1.41	3.5	86–89				
Corticosterone	2.08	0.168	8.0	87–93	0.0434	0.0126	1.00	0.0091
	7.53	0.344	4.6	86–91				
	14.9	0.713	4.8	87–91				

S.D. = Standard deviation; R.S.D. = relative standard deviation; r = correlation coefficient; Sy/x = S.D. of regression line at mean concentration.

compounds tested and respective retention time. Optimization data and performance characteristics are in Fiq. 3 and include the influence of: HCl concentration on recovery; acetonitrile content of wash solution on recovery; and relationship of serum sample volume size to analytical response.

4. Discussion

We discovered, as have other investigators, that pretreatment of serum samples before extraction with sorbents is necessary to improve analytical recovery of some steroids. Sorbent extractions are prone to give low recoveries because of competition between serum proteins and sorbent ligand for the steroid. Pretreatment frees those steroids bound to proteins [13] by either denaturating the protein or displacing the bound steroid with another compound. Heat [7] and steroid-displacing agents, such as 8-anilino-1-naphthalenesulfonic acid [13], have been used by others to pretreat serum and increase recovery. Using the membrane extraction with *no* serum pretreatment we observed recoveries of only 82, 75 and 73% for cortisol, prednisolone and corticosterone, respectively. Similar results have been reported with the packed-column sorbent format [3,7]. We discovered that HCl (0.4 M) mixed with serum in a ratio of 1:2 (v/v) effectively denatures the steroid-binding proteins and increases extraction recovery. Most important, the accuracy of the membrane extraction depends on calibration with steroid standards in serum processed in the same manner as patients' specimens.

The performance of the membrane disk and conventional SPE column format were compared. The steroid extraction was duplicated on SPE columns containing loosely packed large particle (40 μm) C_8 sorbent. Both membrane

Table 2
Interference study: relative retention times of 20 steroids chromatographed in the HPLC system

Steroid	Relative retention time[a]
Aldosterone	0.38
Triamincinolone	0.46
Metyrapone	0.49
Prednisone	0.68
Cortisone	0.74
Prednisolone	0.80
Cortisol	0.86
Fludrocortisone	1.00
Corticosterone	1.20
Methylprednisolone	1.35
11-Deoxycortisol	1.70
Dexamethasone	1.71
21-Deoxycortisone	1.82
Androsteindione	2.33
Beclomethasone	2.44
11-Deoxycorticosterone	2.83
Testosterone	3.34
17-Hydroxyprogesterone	>3.50
Progesterone	>3.50
Pregnenolone	>3.50

[a] Relative to fludrocortisone.

and column contained C_8 sorbent from the same manufacturer (Varian Sample Preparation Products, Harbor City, CA, USA). The most remarkable dissimilarity between the disk and packed column was the volume of solvent required to completely elute all retained steroids. Elution profiles (analyte recovery versus fractional volume of eluting solvent) were established to better illustrate this difference (Fig. 4). Experimentally, we passed 35-μl portions of acetonitrile, followed by 150 μl of water through a sorbent with retained steroids. An aliquot of the eluate mixture was chromatographed. The elution process was repeated until all retained steroids were completely removed from a sorbent. Recovery of steroids was calculated for each fraction. With this study, a total of 50 μl of acetonitrile was needed for complete elution off the membrane; 310 μl were required with the packed column. Overall, the membrane afforded an extract that was five to six times more concentrated than the extract from the column. The extract from the membrane disk could be injected directly into the HPLC system without sacrificing analytical sensitivity. The extract from the column needed to be evaporated/concentrated/reconstituted to achieve comparable analytical sensitivity. In our hands, the evaporation process sometimes led to lower recoveries, depending on the type of glass test tube used to hold the sample. Deactivation of the glass surface did not solve the problem.

The improved efficiency of the membrane over the packed column is due to the smaller particle size (8 versus 40 μm) and smaller sorbent mass (4 versus 100 mg) of the membrane. Both characteristics afford greater capacity per mg of sorbent and allow for smaller volume of solvent for elution resulting in a more concentrated solution of steroids and increased analytical sensitivity. Our studies demonstrate that the capacity of the 4-mm diameter C_8 membrane is more than adequate for the described steroid analysis of clinical serum specimens. However, with much larger sample volumes or when greater capacity is required, larger-diameter (7 or 10 mm) membranes should be used. When making a switch from the 4-mm diameter disk to a larger-size disk, the volume sizes of wash and eluting solutions must be reoptimized.

5. Conclusions

The C_8 particle-loaded membrane efficiently extracts cortisol, cortisone, corticosterone, prednisone and prednisolone from serum specimens at concentrations seen clinically in patients. Most notable is the improvement in specificity when this extraction–HPLC analysis is compared to immunochemical assays prone to interferences. Method performance data demonstrates overall between-run precison R.S.D.s of 3.79% ± 1.4%, extraction recoveries that average 90% ± 3.3%, and linearity from 0.4 μg/dl (low limit of detection) to at least 60 μg/dl. When necessary, increased capacity can be achieved by choosing a larger-size diameter membrane.

In some respects both the SPE membrane and traditional large-particle packed columns per-

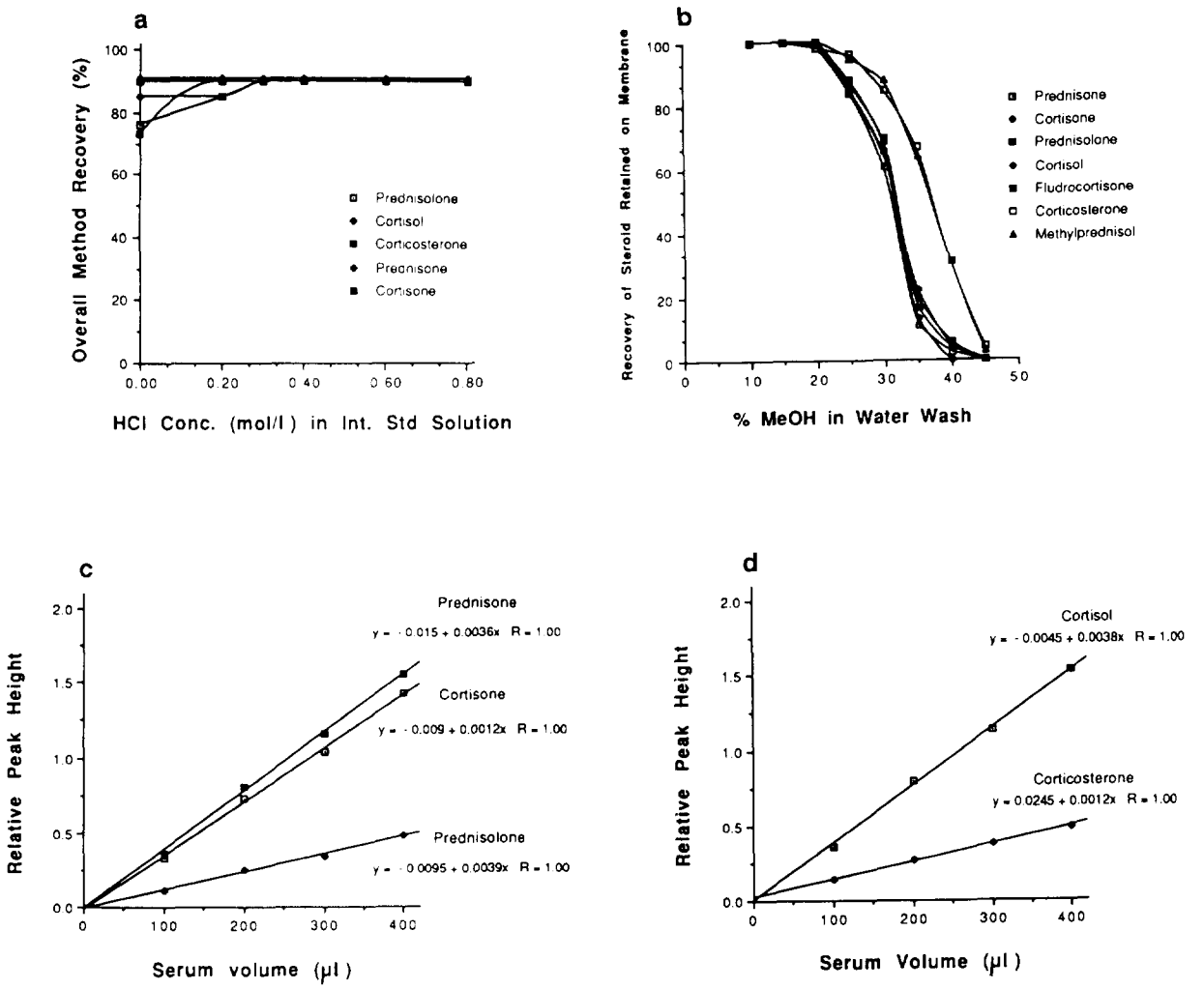

Fig. 3. Optimization charts showing influence of (a) HCl concentration in internal standard (Int. Std) solution on recovery, (b) methanol content of wash solution on recovery and (c, d) relationship of serum volume size to analytical response. Steroid concentrations tested were the same as contained in mid-range sample used in precision study.

formed similarly, however we observed distinct advantages with the membrane. Remarkably, the steroids could be eluted off the membrane in a small volume and the eluate directly injected onto the HPLC system without additional concentration or loss of sensitivity. Extracts from the SPE column required an additional solvent evaporation/concentration/reconstitution step. Overall, when compared to the SPE column, the membrane gave smaller elution volumes, afforded five times greater analytical sensitivity, circumvented instability problems associated with conventional concentrating processes, and simplified extraction to give a faster analysis.

The improved concentrating ability of the membrane can be attributed to the robust configuration of the sorbent within an inert support. The small 8-μm diameter particle size particles tightly held within PTFE gives greater surface area and increased capacity per mg of sorbent than conventional 40-μm particle sorbents packed in columns. Most important though, this

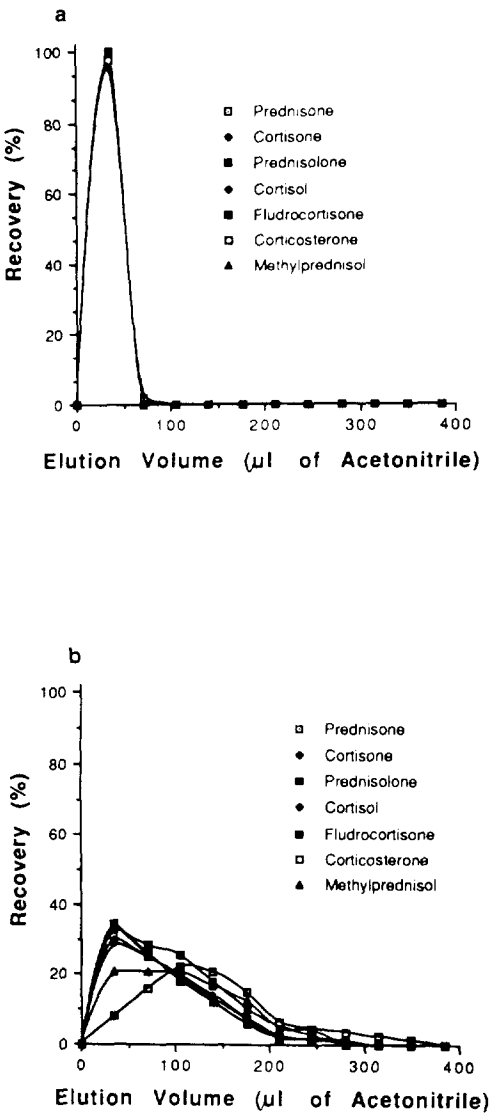

Fig. 4. Comparison of elution volume required to completely recover steroids retained on (a) SPE C_8 disk membrane (4 mm) and (b) SPE C_8 packed column (100 mg).

second generation form of solid-phase material is a new tool capable of decreasing the complexity of highly selective chromatographic procedures to offer accurate and clinically relevant steroid results.

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