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SIMULTANEOUS DETERMINATION OF PREDNISOLONE ACETATE, PREDNISOLONE, PREDNISONE, CORTISONE AND HYDROCORTISONE IN SWINE PLASMA USING SOLID-PHASE AND LIQUID—LIQUID EXTRACTION TECHNIQUES

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for the simultaneous determination of prednisolone acetate (PA), prednisolone (PO), prednisone (PN), cortisone and hydrocortisone in swine plasma is described. Extraction of the steroid mixture from swine plasma with dexamethasone as internal standard was accomplished by solid-phase extraction (SPE) or the more traditional liquid—liquid extraction (LLE) techniques. These compounds were analyzed by normal-phase HPLC with ultraviolet detection. Although a detectable sensitivity of 5 ng/ml is achieved by the SPE technique, the practical sensitivity is established as 10 ng/ml. Conversely, the practical sensitivity is 5 ng/ml for all compounds by the LLE technique. Calibration curves were found to be linear between 10 and 500 ng/ml by the SPE technique and between 5 and 100 ng/ml by the LLE technique. The average recovery of the steroids PA, PO and PN at 20 ng/ml is between 70 and 90%. PA is stable for up to 3 h in swine plasma at room temperature (22°C) but is completely converted to PO within 24 h. PA is stable in swine plasma in an ice bath for over 24 h. The usefulness of this analytical technique is demonstrated by the intraperitoneal administration of 125 mg of PA to swine and the quantitative determination of PA, PO and PN in the plasma as a function of time.

INTRODUCTION

Several high-performance liquid chromatographic (HPLC) procedures have been described for the measurement of corticosteroids in plasma. Frey et al.

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[1] have described an HPLC method for the simultaneous determination of cortisone (CN), hydrocortisone (HC), prednisone (PN) and prednisolone (PO) in plasma with dexamethasone (DX) as internal standard. Rose and Jusko [2] have reported the HPLC analysis of PN, PO and HC in biological fluids such as plasma, urine or saliva. The quantitative measurement of methylprednisolone and methylprednisolone acetate using HPLC have been described by Agabeyoglu et al. [3] and also by Garg et al. [4]. The in vitro and in vivo hydrolysis of methylprednisolone acetate to the corresponding alcohol was monitored by these workers with medroxyprogesterone acetate as internal standard. Smith [5] has described a reversed-phase HPLC procedure for the simultaneous determination of methylprednisolone, methylprednisolone hemisuccinate or hydrocortisone and hydrocortisone hemisuccinate in human serum. The internal standard for methylprednisolone and methylprednisolone hemisuccinate determination was 17-hydroxyprogesterone, whereas 11-deoxy-17-hydroxycorticosterone was used as internal standard for hydrocortisone hemisuccinate determination.

Solid-phase extraction (SPE) techniques using free or bonded silica mini-columns to isolate analytes from biological matrices were examined by Zief et al. [6]. Nine steroids, having a wide range of polarities and functional groups, were examined; PN, CN and HC were successfully recovered at 0.5-mg levels from serum, plasma or urine.

Prednisolone acetate (PA) is listed in the United States Pharmacopeia XX as a suspension for intramuscular or intrasynovial use. Until now, there have been no reports on the simultaneous determination of PA, PO, PN, CN and HC in biological fluids. The application of an SPE technique to the separation of this combination of steroids has not been reported. This paper describes the simultaneous quantitative determination of the above corticosteroids in swine plasma using SPE and liquid–liquid extraction (LLE) techniques. The steroids with DX as an internal standard were separated by normal-phase HPLC with UV detection.

EXPERIMENTAL

Reagents

Cortisone, hydrocortisone, prednisolone acetate, prednisone and prednisolone were reference standards (United States Pharmacopeia, Rockville, MD, U.S.A.). Dexamethasone (Sigma, St. Louis, MO, U.S.A.) was used as internal standard. Distilled-in-glass (HPLC- and spectroscopic-grade) methylene chloride, methanol, acetonitrile (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and spectro-grade tetrahydrofuran (Fisher Scientific, Silver Spring, MD, U.S.A.) were all used without further purification.

Instrumentation

The instrumentation consisted of a modular HPLC system equipped with a high-pressure pump (Model 6000A), an automatic injector (Model 610B WISP), a 254-nm fixed-wavelength detector (Model 420) and connected to a data module (Model 730), all from Waters Assoc. (Milford, MA, U.S.A.). The data module was set for peak-area calculation at a chart speed of 0.762 cm/min.

The HPLC column consisted of a 25 cm × 4.6 mm I.D. stainless-steel tube packed with 5-μm silica (LiChrosorb Si-60) (Alltech/Appplied Science, Deerfield, IL, U.S.A.).

Chromatographic conditions

The mobile phase for isocratic elution was methylene chloride–water-saturated methylene chloride–tetrahydrofuran–methanol–glacial acetic acid (664.5:300:10:25:0.5). The mobile phase was degassed by sonication for 30 min prior to use. The UV detector was set at 254 nm; the sensitivity was 0.005 a.u.f.s. (absorbance units full scale). The flow-rate was held constant at 0.8 ml/min. All separations were performed at ambient temperature.

Preparation of unextracted standards

Standard steroid solutions were prepared in methanol (1 or 5 μg/ml); the appropriate amount of the standard (corresponding to 5 to 500 ng/ml of plasma) was added to a small culture tube. To this, the internal standard DX was added as a methanolic solution (corresponding to 100 ng/ml of plasma). Acetonitrile (1 ml) was added to the tube and the contents were evaporated to dryness under a stream of nitrogen at 40°C. The residue was redissolved in 150 μl of methylene chloride and vortexed for 30 s. A 100-μl volume of this solution was injected onto the HPLC column.

Solid-phase extraction procedure

SPE columns (Bond-Elut C_{18}, 500 mg Octadecyl, Analytichem, Harbor City, CA, U.S.A.) were mounted on a specially designed vacuum manifold (Vac-Elut, Analytichem) that can process as many as ten extraction columns simultaneously. Extraction columns were washed in the vacuum manifold by applying of vacuum and passing 2 ml of acetonitrile, 2 ml of 2% acetone–water and then with the vacuum off, 4 ml of water; the column should not be allowed to dry. Swine plasma (2 ml) containing the analytes was added to the columns and allowed to adsorb for 15 min before washing twice with 2 ml of water followed by two 2-ml washes of 2% acetone–water. The vacuum is turned on for 15 min. Collection tubes were then inserted and 1 ml of acetonitrile was passed through the columns under vacuum. The eluent was carefully blown to dryness under a stream of nitrogen in a water bath at 40°C. The residue was dissolved in 150 μl of methylene chloride of which 100 μl were injected onto the HPLC system.

Liquid–liquid extraction procedure

To a screw-cap tube containing 2 ml of swine plasma and the standards was added 100 ng/ml DX; the sample tube was vortexed for 30 s. A 5-ml volume of a methylene chloride–diethyl ether (50:50) mixture was added and vortexed for 15 s. The tube was centrifuged to separate layers and the organic layer was removed with a pipet. The methylene chloride–diethyl ether extraction was repeated and the combined organic extract was washed with 4 ml of 0.1 *M* sodium hydroxide and centrifuged. The organic layer was quickly separated, dried over anhydrous sodium sulfate and evaporated to dryness under a stream of nitrogen at 35°C. The residue was dissolved in 150 μl of

methylene chloride of which 100 μl were injected onto the HPLC system.

Administration of prednisolone acetate to miniature swine

A miniature swine (Hormel) weighing approximately 50 kg was placed in an inverted trough. An intraperitoneal injection of 2.5 mg/kg PA suspension (manufactured by Rubgy Labs., Rockville Centre, NY, U.S.A., FDA Inv. No. 80-200-763) was administered. The animal was placed in a sling and blood samples were drawn at 1, 2, 3, 4, 5, 6, 8, 24 and 48 h after dosage administration. The blood samples (10—15 ml) were drawn via an indwelling catheter in the vena cava into heparinized vacutainers containing 1 ml of a saturated solution of sodium fluoride (18 g sodium fluoride in 150 ml of normal saline) which prevents the hydrolysis of PA by the esterases. The vacutainers were thoroughly shaken to mix the blood intimately with the sodium fluoride and were kept in crushed ice till centrifugation at ca. 1000 *g* for 15 min at 4°C. The separated plasma was transferred to silanized screw-cap tubes and quickly frozen using a dry ice—acetone bath. The frozen samples were then stored at −20°C till analysis.

RESULTS AND DISCUSSION

Although there have been HPLC procedures described for the analysis of methylprednisolone, HC and their esters from plasma, there are no techniques currently available for the simultaneous determination of PA, PO, and PN in plasma. PN administered to humans is rapidly converted to PO [7, 8]. The administration of PA to animals results in PO exclusively, however, the conversion of PO to PN is not documented. Therefore an analytical procedure which can resolve PA, PO and PN from endogenous CN, HC, the internal standard DX and other plasma components is required to determine the total bioavailability of parenteral PA suspensions.

LLE techniques as a method of enrichment of corticosteroids in plasma have been previously described [1—5]. Specifically, PO, PN and HC were extracted from plasma with a solvent mixture containing methylene chloride and diethyl ether [2, 9—13]. The use of LLE to selectively extract methylprednisolone and its acetate from plasma with methylene chloride has been reported [4]. Smith [5] acidified plasma or serum with 5% acetic acid solution, washed with hexane, and then isolated the steroids methylprednisolone and its hemisuccinate with ethyl acetate. The same procedure was used to separate HC and its hemisuccinate from plasma [5]. These multiple extractions and transfer steps require extensive use of glassware and extreme caution in handling nanogram quantities.

Because of recent advances in the chemistry of bonded-phase adsorbents, the use of SPE techniques in biological sample preparation for chromatography has become more widespread. The SPE of PN, hydrocortisone acetate and HC from urine has been reported by Zief et al. [6]. Commercially available SPE columns were used in sample preparation for the analysis of HC from plasma or serum [14]. The use of SPE columns in the sample preparation of human serum containing PN and PO has been reported by Stewart et al. [15].

The use of SPE has the potential to be faster, more efficient and more

convenient than traditional LLE techniques. We have explored the use of commercially available bonded-phase mini-columns and have compared the utility of the two extraction methods as applied to the isolation of various corticosteroids from swine plasma.

Chromatography

PA, PO, PN, CN, HC and DX, the internal standard, were resolved from the endogenous swine plasma components. A typical chromatogram of a steroid mixture consisting of 60 ng of internal standard and 30 ng each of PA, PO, PN, CN and HC on-column is shown in Fig. 1. A typical chromatogram of swine plasma containing 20 ng/ml each of PA, PO and PN and 100 ng/ml internal standard DX extracted using SPE is shown in Fig. 2a. A similar chromatogram of blank swine plasma containing 100 ng/ml DX extracted by SPE is shown in Fig. 2b.

Preliminary studies showed that the assay of plasma levels achieved after the

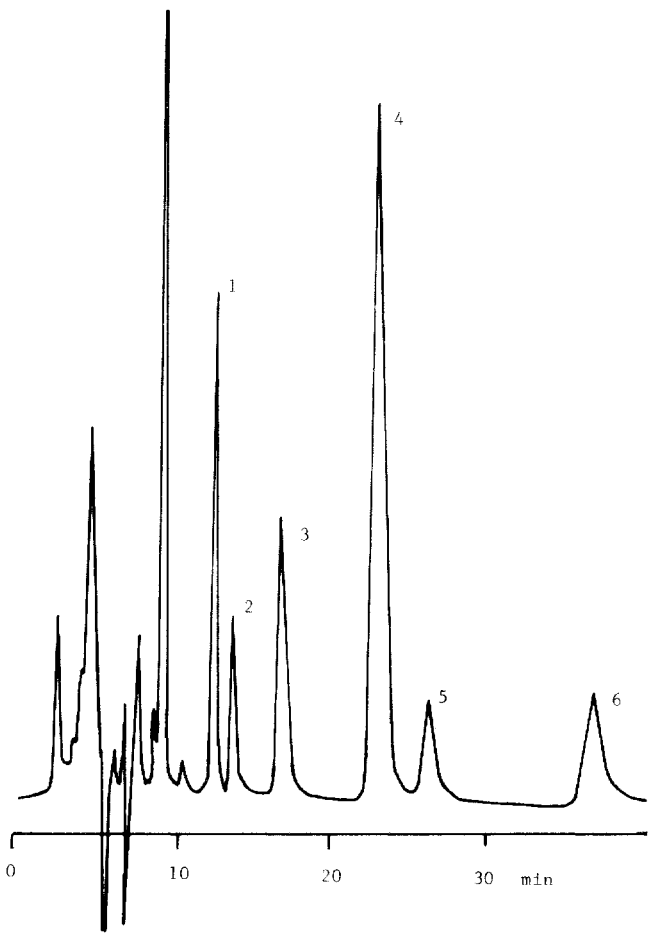

Fig. 1. Chromatogram of a standard steroid mixture. Chromatographic conditions are described in text. The 100-μl injection contained 30 ng each of prednisolone acetate (1), cortisone (2), prednisone (3), hydrocortisone (5) and prednisolone (6) and 60 ng of dexamethasone, the internal standard (4). Detection sensitivity was 0.005 a.u.f.s.

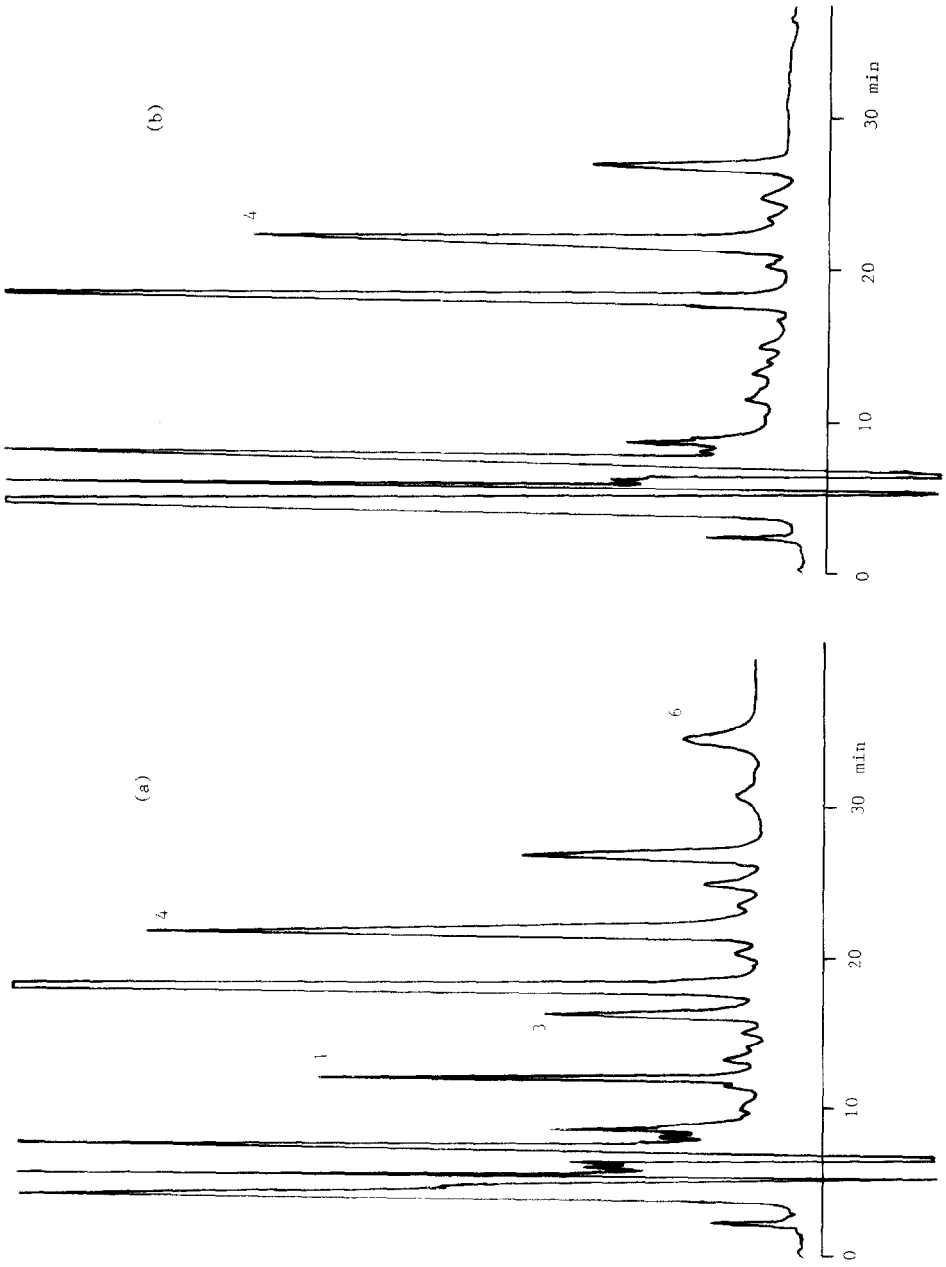

Fig. 2. Chromatograms of plasma extracts obtained by solid-phase extraction. (a) Control swine plasma (2 ml) containing 100 ng/ml dexamethasone, the internal standard (4), and 20 ng/ml each of prednisolone acetate (1), prednisone (3) and prednisolone (6); (b) control swine plasma (2 ml) containing 100 ng/ml dexamethasone (4). Detection sensitivity was 0.005 a.u.f.s.

intraperitoneal administration of PA to swine would require a sensitivity of lower than 10 ng/ml. Because this sensitivity could not be achieved by the SPE technique, an LLE procedure with methylene chloride—diethyl ether (1:1) was developed. A typical chromatogram resulting from the LLE of swine plasma spiked with 20 ng/ml each of PA, PO and PN and 100 ng/ml DX is shown in Fig. 3a. Similarly, results of blank swine plasma spiked only with 100 ng/ml internal standard DX and extracted by LLE are shown in Fig. 3b.

Recovery

All calculations were carried out on the basis of peak-area ratios between drug and the internal standard. The ratios from extracted plasma samples were compared to the peak-area ratios obtained from unextracted solutions of the steroids at known concentrations to determine the percentage recovery. The average (n = 5) recoveries at the 20 ng/ml level for PA, PO and PN were 70, 90 and 70%, respectively, using the SPE technique. Conversely, the LLE technique resulted in recoveries of 80, 90 and 90% (n = 5) for PA, PO and PN, respectively. Thus the recoveries by LLE technique are slightly higher than those obtained with the SPE procedure. The lower recoveries for PA and PN in the SPE technique can be attributed to a possible loss of these compounds by irreversible adsorption to the bonded material.

Linearity of response and sensitivity

Pooled swine plasma spiked with PA, PO and PN at concentrations of 5—500 ng/ml of each steroid were processed by the two extraction techniques. The logarithm of peak-area ratios of drug/internal standard were plotted as a function of the logarithm of the concentration [16]. Equal weights used in linear least-squares regression resulted in large deviations at the lower concentrations and weighting eliminated this problem. The average slope, y-intercept and correlation coefficients for the data obtained by the two extraction techniques are shown in Table I. It is evident that both methods are comparable. The data obtained below the 10 ng/ml concentration with the SPE technique were extremely variable; therefore, these data were not considered

TABLE I

PRECISION DATA OF THE SPE AND LLE TECHNIQUES FOR THE DETERMINATION OF PREDNISOLONE ACETATE, PREDNISONE AND PREDNISOLONE

Precision date were obtained using log peak area versus log concentration.

Steroid	Slope	y-Intercept	Correlation coefficient
Solid-phase extraction (prepared with plasma spiked at 10–500 ng/ml)			
Prednisolone acetate	1.003	−1.795	0.999
Prednisone	1.052	−1.942	0.999
Prednisolone	1.095	−2.130	0.999
Liquid—liquid extraction (prepared with plasma spiked at 5—100 ng/ml)			
Prednisolone acetate	0.968	−1.874	0.999
Prednisone	1.011	−1.944	0.998
Prednisolone	1.121	−2.092	0.996

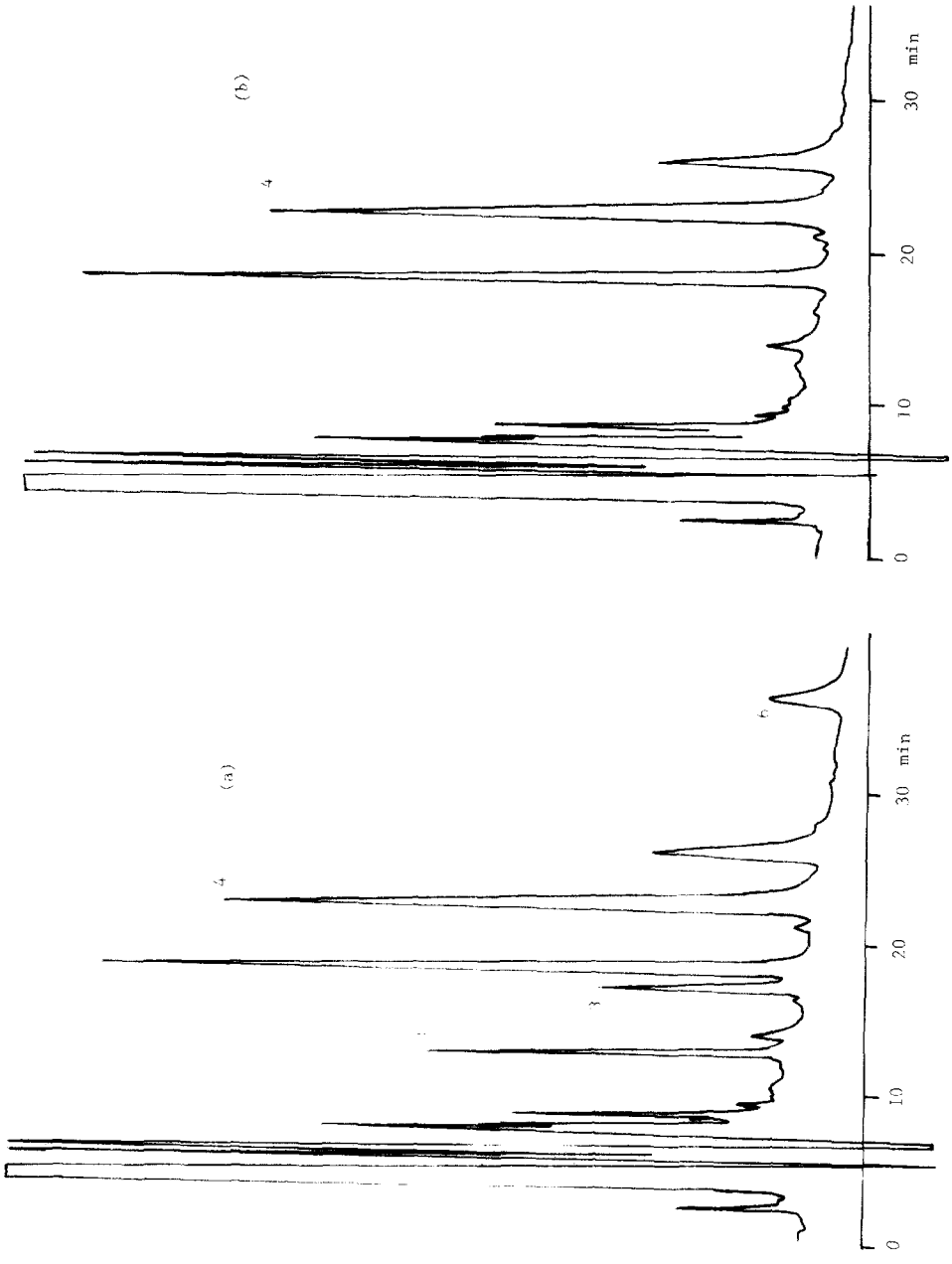

Fig. 3. Chromatograms of plasma extracts obtained by liquid–liquid extraction. (a) Control swine plasma (2 ml) containing 100 ng/ml dexamethasone, the internal standard (4), and 20 ng/ml each of prednisolone acetate (1), prednisone (3) and prednisolone (6); (b) control swine plasma (2 ml) containing 100 ng/ml dexamethasone (4). Detection sensitivity was 0.005 a.u.f.s.

in the calculation. However, good linearity and excellent correlation was demonstrated even at the 5 ng/ml concentration with the LLE procedure.

The sensitivity of this method appears to be dependent upon the method of extraction. For example, in the case of the SPE technique, even though the detection limits were between 5 and 10 ng/ml, a practical sensitivity limit was about 10 ng/ml because of the possible irreversible adsorption of the steroids on to the bonded material. In the case of the LLE technique, even though 1 ng/ml could be detected by direct injection on to the column of a standard solution, a practical sensitivity limit was established as 5 ng/ml from an extracted plasma sample.

Within-day and inter-day variability

Within-day and inter-day variability was studied by spiking 2 ml of plasma with each of PA, PO and PN at 20 and 100 ng/ml and then processing by either of the two extraction techniques. The results are summarized in Tables II and III. The SPE technique gave acceptable values for within-day variability (Table II) but the SPE inter-day variability was higher than inter-day variability for the LLE technique (Table III). The within-day coefficient of variation for the LLE techniques was between 1.8 and 6.7% for PA, PO and PN. The inter-day coefficient of variation was slightly higher (i.e. between 3.5 and 8.1%) and one value for PA was much higher.

Stability of PA, PO and PN in swine plasma

Plasma samples treated with sodium fluoride were spiked with PA and PO at 100 ng/ml each. Samples kept at room temperature (22° C) or in an ice bath were analyzed by SPE at 0, 1, 2, 3, 5 and 24 h after they were prepared. The

TABLE II

WITHIN-DAY AND INTER-DAY VARIABILITY FOR THE MEASUREMENT OF SPIKED PLASMA SAMPLES: SOLID-PHASE EXTRACTION

Steroid	Expected concentration* (ng/ml)	Found concentration (mean ± S.D.) (ng/ml)	n	Coefficient of variation (%)
Within-day variability				
Prednisolone acetate	20.0	20.1 ± 1.1	8	5.5
	100.0	103.1 ± 6.3	9	6.1
Prednisone	20.0	18.1 ± 0.8	8	4.4
	100.0	93.4 ± 3.4	8	3.6
Prednisolone	20.0	18.8 ± 1.9	7	10.0
	100.0	103.9 ± 8.8	8	8.4
Inter-day variability				
Prednisolone acetate	20.0	20.1 ± 1.9	3	9.5
	100.0	99.9 ± 12.5	3	12.5
Prednisone	20.0	19.2 ± 1.9	3	9.9
	100.0	97.8 ± 14.3	3	14.6
Prednisolone	20.0	19.8 ± 1.9	3	9.6
	100.0	99.9 ± 14.1	3	14.1

*A 2-ml plasma sample was spiked at the indicated concentration.

TABLE III

WITHIN-DAY AND INTER-DAY VARIABILITY FOR THE MEASUREMENT OF SPIKED PLASMA SAMPLES: LIQUID–LIQUID EXTRACTION

Steroid	Expected concentration* (ng/ml)	Found concentration (mean ± S.D.) (ng/ml)	n	Coefficient of variation (%)
Within-day variability				
Prednisolone acetate	20.0	20.8 ± 1.4	5	6.7
	100.0	98.9 ± 6.2	6	6.3
Prednisone	20.0	20.3 ± 0.8	4	3.9
	100.0	101.8 ± 1.8	5	1.8
Prednisolone	20.0	20.3 ± 0.8	5	3.9
	100.0	101.6 ± 2.2	5	2.2
Inter-day variability				
Prednisolone acetate	20.0	19.7 ± 1.6	3	8.1
	100.0	80.3 ± 15.5	3	19.3
Prednisone	20.0	20.1 ± 0.7	3	3.5
	100.0	95.6 ± 3.9	3	4.1
Prednisolone	20.0	21.0 ± 0.8	3	3.8
	100.0	95.4 ± 6.1	3	6.4

*A 2-ml plasma sample was spiked at the indicated concentration

TABLE IV

STABILITY OF PREDNISOLONE ACETATE (PA) AND PREDNISOLONE (PO) IN SWINE PLASMA AT 100 ng/ml CONCENTRATIONS AT 4 AND 22°C

Time (h)	Remaining (%)			
	4°C		22°C	
	PA	PO	PA	PO
0	100	100	100	100
1	107	96	106	104
2	106	97	97	121
3	100	94	94	114
5	107	-	82	143
24	93	100	0	198

results shown in Table IV indicate that PA and PO are stable in an ice bath up to 24 h. However, at 22°C PA is quantitatively converted to the more stable PO within 24 h. PA is reasonably stable up to 3 h at 22°C in swine plasma at the concentration studied. These findings are consistent with reports on the stability of methylprednisolone acetate which was found to be stable for at least 2 h at room temperature in human plasma [14].

Swine study

Plasma concentration–time curves for PA, PO and PN in a mini-pig after intraperitoneal administration of a commercial PA suspension at 2.5 mg/kg

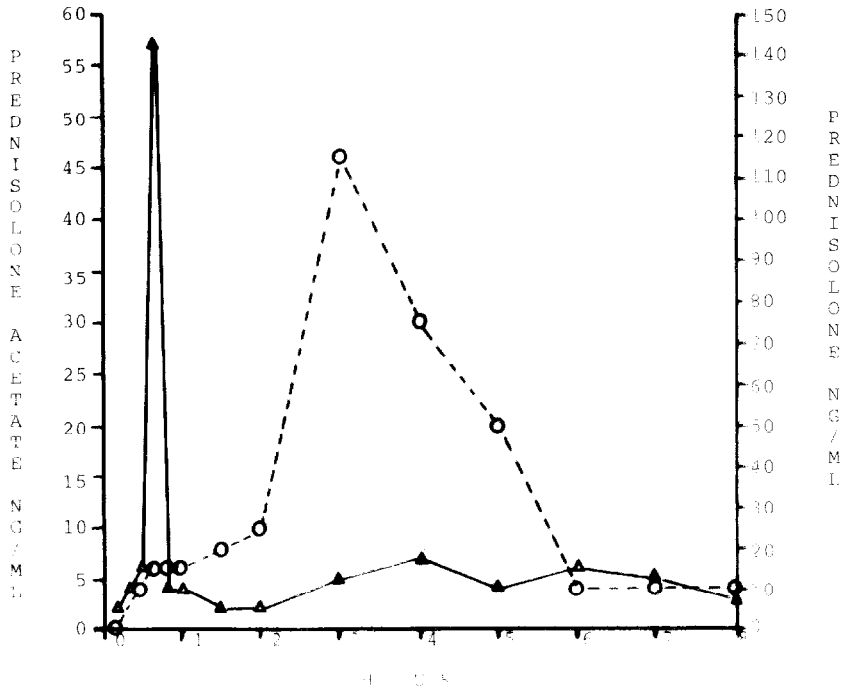

Fig. 4. Plasma concentration time curve for PA ( ) and PO ( ) in mini-pig after intraperitoneal administration of 125 mg PA suspension to a mini pig.

(total dose administered 125 mg) are shown in Fig. 4. Each data point represents an average of three determinations using the LLE technique. This method was employed because of the lower inter-day variability as compared to the SPE technique. The data show that PA peaks rapidly within 45 min after administration. The rapid decrease in PA is followed by a gradual increase in PO which peaks at about 3 h from the time of administration. Details of a bioequivalency study conducted on several commercial PA suspensions shall be presented later [17].

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