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## Note

# Analysis of prednisolone acetate and related corticoids in swine plasma by reversed-phase high-performance liquid chromatography

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Prednisolone acetate (PlA) is a steroidal anti-inflammatory agent commonly administered either intramuscularly or intrasinovially as an injectable suspension. As part of a continuing effort our laboratory investigated methodology for measuring PlA and its hydrolysis product prednisolone (Pl) in the presence of prednisone (P), a common oral medication which is metabolically converted to Pl, and endogenous cortisone (C) and hydrocortisone (HC) [1–3]. Earlier efforts in our laboratory involved the comparison of solid-phase extraction (SPE) and liquid–liquid extraction (LLE) and the development of a normal-phase high-performance liquid chromatographic (HPLC) assay [1].

Frey et al. [4] used a normal-phase HPLC method to examine a wide spectrum of corticoids including C, HC, P and Pl in plasma. Rose and Jusko [5] examined HC, P, and Pl in plasma, urine and saliva employing a normal-phase HPLC technique. Agabeyoglu et al. [6], Garg et al. [7] and Smith [8] described reversed-

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phase HPLC procedures for quantification of methylprednisolone (MePl) esters and the determination of their hydrolysis rates. Williams and Biehl [9] used two different reversed-phase HPLC systems to examine a wide range of topical steroidal compounds including C, HC, P and Pl, however, not at sensitivities required for plasma levels. Tretz et al. [10] examined P and others in plasma using a normal-phase HPLC system, but indicated that higher-energy UV detection increases detection sensitivities. HPLC–mass spectrometric systems such as that reported by Ost et al. [11] provided very sensitive detection and positive identification, but at high cost.

Most of the preceding work employed LLE. However, SPE of corticosteroids has been investigated by several groups [1,12,13] and while not quite as sensitive, it does provide a rapid and convenient way of processing and analyzing greater numbers of biological samples.

This study outlines a sensitive reversed-phase HPLC method employing LLE isolation to examine simultaneously P, Pl, C, HC and PlA in plasma. The viability of the assay was shown in a miniswine administered a PlA injectable suspension intraperitoneally.

## EXPERIMENTAL

### *Reagents and supplies*

C, HC, P, Pl, PlA, dexamethasone (Dex) and MePl were obtained from the U.S. Pharmacopeia (Rockville, MD, U.S.A.). Tetrahydrofuran, ethyl acetate, methanol and methyl *tert.*-butyl ether were either HPLC or spectroscopic grade from American Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and were used without further purification. Reagent-grade water was generated with a Millipore Milli-RO reverse osmosis/Milli-Q water system from Millipore (Bedford, MA, U.S.A.) Sodium fluoride and acetic acid were Baker AR grade from Baker (Phillipsburg, NJ, U.S.A.). Nitrogen gas (certified) used for sample evaporation was obtained from Roberts Oxygen (Rockville, MD, U.S.A.).

Samples were filtered through either ACRO LC3S 0.45 $\mu$m (Gelman Sciences, Ann Arbor, MI, U.S.A.) or Millex-$HV_4$ 0.45 $\mu$m (Millipore) before injection.

### *Equipment*

The chromatograpic system consisted of a Model 6000A pump, Model 710B automatic injector (both by Waters Assoc., Milford, MA, U.S.A.), Model 773 variable-wavelength detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.) and a Model 730 data module (Waters Assoc.). The HPLC columns consisted of either a 25 cm$\times$0.46 cm I.D., 6-$\mu$m spherical particle $C_{18}$ (Zorbax, DuPont Instruments, Wilmington, DE, U.S.A.) or a 22 cm$\times$0.46 cm I.D., 5-$\mu$m spherical particle $C_{18}$ (BrownLee Labs., Santa Clara, CA, U.S.A.) equipped with a 3 cm$\times$0.46 cm I.D., 5-$\mu$m spherical particle $C_{18}$ (BrownLee Labs.) guard column. Sample evaporation was carried out on a N-EVAP manufactured by Organomation Assoc. (Shrewsbury, MA, U.S.A.).

Sample filtration was performed with syringe-mounted disposable 0.45-$\mu$m PTFE filters supplied by either Gelman Sciences or Millipore.

*Chromatographic conditions*

The mobile phase consisted of tetrahydrofuran–water (25:75, v/v) which was filtered (0.45 μm, PTFE membrane) and degassed under vacuum with stirring. The detector was set at 240–242 nm and 0.004 a.u.f.s. The components were eluted isocratically at 1 ml/min (system pressure 207–221 bar) and ambient temperature (25°C).

*Standard preparations*

Corticosteroid stock solutions were prepared at 1 mg/ml in methanol, then diluted with mobile phase to the desired concentration range (0.5–10 ng/μl). Corticosteroid acetates stock solutions were prepared in 0.5% acetic acid–methanol (v/v) prior to dilution. Acetate stock solutions appeared to have good shelf life at ambient temperatures and no hydrolysis whatever was observed when stored at 0–10°C.

*Sample work-up*

A 2-ml portion of swine plasma (treated with sodium fluoride esterase inhibitor) was placed in a 15-ml conical centrifuge tube and extracted with 2×2 ml of either methyl *tert.*-butyl ether or ethyl acetate. The extracting solvent was saturated with water and spiked at 10 ng/ml with internal standard beforehand. The plasma–organic extraction mixture was vortexed for 30 s and centrifuged at 200 *g* for 4 min to separate and clarify the layers. The 4 ml of combined organic extracts were dried over a small amount (approximately 0.5 g) of anhydrous sodium sulfate and evaporated to dryness at 35–40°C under a stream of nitrogen. The residue was reconstituted in 150 μl of mobile phase, filtered (syringe-mounted 0.45-μm PTFE filter), and 100 μl were injected on column.

*Administration and sample collection*

Miniture swine (Hormel-Hamford) weighing approximately 50 kg were placed in an inverted trough and administered, intraperitoneally, 2.5 mg/kg PlA suspension (either Meticortelone® by Schering or prednisolone acetate by Maurry Biological). Following administration the animals were placed in slings and blood samples (10–15 ml) were collected at 10, 20, 40, 60, 90 and 120 min and 3, 4, 5, 6, 7, 8 and 24 h after dosing. Samples were drawn via an indwelling catheter (vena cava) into heparinized vacutainers containing 1 ml of sodium fluoride-saturated saline (18 g per 150 ml). The fresh samples were thoroughly mixed, chilled to 0–5°C and centrifuged (5–10°C) at 510 *g* for 15 min. The plasma was transferred to silanized tubes and stored at −35°C until analyzed.

## RESULTS AND DISCUSSION

*Chromatography*

Fig. 1 illustrates a typical separation with an analysis time of approximately 40 min. The retention times are in line with those seen in normal-phase HPLC systems and adequate resolution of components was observed [4]. Other acetates such as C and HC are resolvable in this system as well. Either Dex or MePl


Fig. 1. Typical chromatograms. Conditions as outlined in the Experimental section. Approximately 10 ng of each steroid on-column. (A) DuPont Zorbax $C_{18}$ column (25 cm and matching 3 cm guard column). (B) Brownlee Spheri-5 $C_{18}$ column (22 cm and matching 3 cm guard column). Peaks: 1 = prednisone; 2 = cortisone; 3 = prednisolone; 4 = hydrocortisone; 5 = methylpredisolone; 6 = dexamethasone; 7 = prednisolone acetate; 8 = cortisone acetate; 9 = hydrocortisone acetate.

worked well as an internal standard (both elute approximately midway between the sterols and their esters). As noted in the literature [10], higher-energy UV appears to give better detection of all compounds examined, hence 240–242 nm was used instead of 254 nm which is most common in fixed-wavelength detectors. Calculations were based on peak-area ratios and the response was linear from 5 to 500 ng. As little as 1–2 ng of steroid could be detected (signal-to-noise ratio of 2) on-column, however, the practical limits were about 2.5–5 ng due to integration parameters and baseline noise.

*Recoveries*

Practical recoveries were 87 ± 13% at 20 ng/ml compared to solutions of known concentrations. Both water-saturated ethyl acetate and methyl *tert.*-butyl ether were examined as extraction solvents. Ethyl acetate dissolves more water than methyl *tert.*-butyl ether (2–3% versus 1–2%). Methyl *tert.*-butyl ether proved to

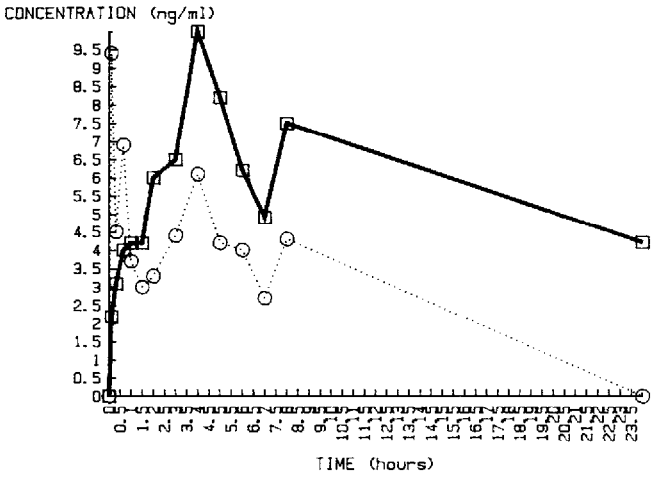


Fig. 2. Concentration versus time plot following intraperitoneal administration of 2.5 mg/kg Meticortelone (Schering) prednisolone acetate suspension to a miniswine. Solid line, prednisolone; dotted line, prednisolone acetate.

be the solvent of choice since it provided a cleaner, more easily clarified injection sample. Earlier work employed diethyl ether, methylene chloride, ethyl acetate and mixtures thereof [14,15]. Methyl *tert.*-butyl ether has a slightly lower boiling point than ethyl acetate and appears to possess desirable extraction characteristics. Use of a single solvent avoids the variation seen in mixed solvent extractions. Both ethyl acetate and methyl *tert.*-butyl ether performed well and no acidic or basic washes, which can cause hydrolysis, were required. Drying of the extracts was beneficial, but not essential since the reconstitution media were aqueous for use in the reversed-phase HPLC system. Drying did improve the clarity of injection samples which were passed through disposable syringe-mounted 0.45-$\mu$m PTFE filters prior to injection.

SPE was examined earlier in our laboratories and found to be convenient and possess good automation/mass processing potential [1]. However, it suffered from less complete recovery of selected steroids. Practical sensitivity limits using SPE was 10 ng/ml versus 5 ng/ml via LLE. Endogenous free plasma HC levels have been reported in the literature to be 2–10 ng/ml depending on time of day [16]. SPE recoveries would not allow endogenous C and cortisol measurements.

A preliminary study was carried out which involved intraperitoneal administration of two injectable PlA suspension formulations to one miniswine. Collection, work-up and analysis of samples resulted in the concentration versus time plots seen in Figs. 2 and 3. Assay sensitivity was sufficient to analyze samples containing as little as 3–4 ng in 2 ml of plasma.

Our earlier studies [1] on the stability of PlA in plasma indicated that complete ester hydrolysis occurred over a 24-h period at room temperature. Plasma treated with either sodium fluoride, an esterase inhibitor, or chilled to 0–4°C showed no loss of PlA over 24 h. To avoid uncertainty due to sample storage and handling, plasma samples were immediately treated with sodium fluoride in this

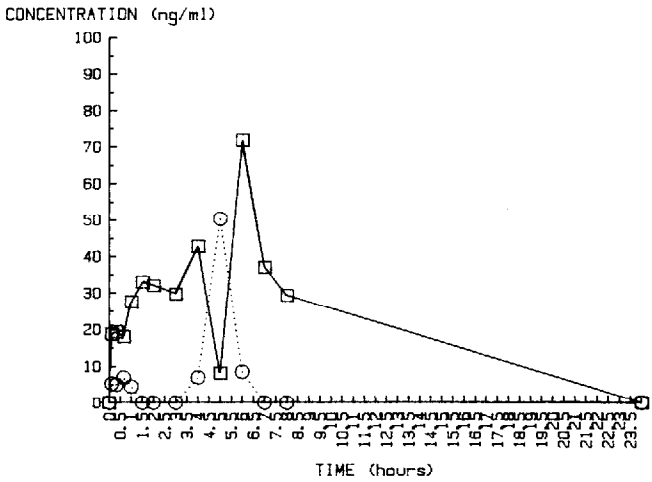


Fig. 3. Concentration versus time plot following intraperitoneal administration of 2.5 mg/kg prednisolone acetate suspension (Maurry Biological) to a miniswine. Solid line, prednisolone; dotted line, prednisolone acetate.

study. We developed this reversed-phase HPLC assay method even though many well documented normal-phase HPLC methods existed. The low volatility of aqueous injection samples used in reversed-phase HPLC are more amenable to automated injection, whereas injection samples prepared in volatile organics used in normal-phase HPLC suffer from evaporation over time and result in greater variation when autoinjection is employed.

Details of a bioequivalency study involving several PlA injectable suspension products in miniswine will be presented at a later date.

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