Simultaneous Determination of Corticosterone, Hydrocortisone, and Dexamethasone in Dog Plasma Using High Performance Liquid Chromatography

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Abstract □ A sensitive, specific and reproducible high-performance liquid chromatographic procedure, using the normal phase and radial compression system, is described for the simultaneous determination of corticosterone, hydrocortisone, and dexamethasone in plasma, with prednisolone as the internal standard. Samples were extracted with methylene chloride and chromatographed on a microparticulate silica gel column using a radial compression system with UV detection at 254 nm. The assay has been applied in pharmacokinetic studies, and a typical plasma concentration–time profile for the three corticosteroids (all with 2 ng/ml sensitivity) is presented for dogs receiving dexamethasone.

Keyphrases □ Corticosterone—high-performance liquid chromatographic determination in dog plasma □ Hydrocortisone—high-performance liquid chromatographic determination in dog plasma □ Dexamethasone—high-performance liquid chromatographic determination in dog plasma □ High-performance liquid chromatography—determination of hydrocortisone, corticosterone, and dexamethasone in dog plasma □ Pharmacokinetics—high-performance liquid chromatographic determination of corticosteroids

Many methods for the determination of corticosteroids in plasma have been described, but many of them lack specificity or are too time-consuming for routine use.

Fluorometric techniques (1, 2) measure different steroids that fluoresce with varying intensity, but overestimate the true amounts of hydrocortisone and corticosterone. Analysis using GC (3) is complicated, because steroids with a C_{17} hydroxyacetone side chain undergo thermal degradation at the temperature employed. The assay of corticosteroids by GC would also seem to require the prior formation of a stable derivative. In competitive protein binding methods, the unknown and radiolabeled hydrocortisone compete for a hydrocortisone-binding protein such as transcortin (4–6). However, endogeneous substances with significant cross reactivity confound the assay performance. Radioimmunoassay is a competitive protein binding technique using an antibody to hydrocortisone as the binding protein. If the antibody is specific and binds hydrocortisone with high affinity, a sensitive assay can be made, although separation of hydrocortisone and corticosterone is crucial to obtaining a good specificity (7, 8). All these procedures are time-consuming and difficult to automate for use in a routine pharmacokinetic study.

The recent emergence of commercial high-pressure liquid chromatographic (HPLC) equipment (coupled with a sensitive UV detector), having the advantages of simple preliminary treatment of the sample, has attracted the attention of workers studying the analysis of corticosteroids in plasma.

Separation of corticosteroids by HPLC in humans has been documented by several authors (9, 10). However, in the dog, levels of endogeneous corticoids are lower than in humans, and techniques already described are not sensitive enough to be applied in this species.

The purpose of the present study was to report the use of a new radial compression system to determine simultaneously the plasma levels of endogenous and synthetic corticoids with a sensitivity level of ~2 ng/ml.

EXPERIMENTAL

Apparatus—The apparatus used in the extraction procedure was constant shaker[1] and a refrigerated centrifuge[2]. A constant volume high pressure liquid chromatograph[3] was equipped with a single wavelength 254 nm detector[4]. The column (10 × 0.8-cm i.d.) was packed with a 10-μm nominal normal phase[5] and was included in a radial compression system[6].

Reagents—Hydrocortisone, corticosterone, dexamethasone, and prednisolone reference standards[7] were used as received. Methylene chloride[8] and acetic acid[8] (analytical grade solvents) were used without further purification.

Mobile Phase—The mobile phase was prepared by mixing exact volumes of methylene chloride, methanol, and glacial acetic acid. The solution was strained and the gas removed. The ratio of methylene chloride, methanol, and acetic acid was 96:4:0.4 (v/v/v).

Operating Conditions—The UV detector was fixed at 254 nm, with a sensitivity of 0.005 aufs. A constant flow rate of 1.5 ml/min was maintained. The radial compression pressure was 1500 psi, and the inlet pressure was 200 psi. Chart speed was 0.5 cm/min.

Preparation of Standard Solutions—Each steroid was dissolved in methanol at a concentration of 1 mg/ml. The operating standard mixture was prepared by a 1:100 dilution in the elution solvent. Prednisolone was used as an internal standard at a concentration range of 1 mg/ml in methanol; a working solution was prepared by a 1:10 dilution in the same solvent.

Extraction Procedure—One milliliter of plasma sample, 1 ml of 0.1 N NaOH, and 10 ml of methylene chloride were added to a 30-ml tube[9]. The tubes were shaken for 10 min and centrifuged at 8400×g for 10 min at 4°. The methylene chloride layer was aspirated and evaporated at 40° under a nitrogen gas stream to prevent oxidation. The residue was dissolved in 100 μl of elution solvent and then injected into the column.

Calibration and Reproducibility—Pooled plasma of the dog was spiked with corticosterone and hydrocortisone (10–100 ng/ml) and with dexamethasone (10–1000 ng/ml). A constant amount of 100 ng of prednisolone as the internal standard was added to each sample (10 μl of a solution containing 10 μg/ml). Pooled plasma was extracted and was performed as outlined previously.

Calibration curves were constructed by calculating the ratio of the peak height of each compound to that of the internal standard, plotting the ratio against the amount of compound added to the sample. Peak heights of hydrocortisone and corticosterone were corrected for blank plasma response representing an endogenous serum component. The corrected peak height was used as the response.

Least-squares linear regression analysis was used to determine the slope, y intercept, and correlation coefficients.

The response of the HPLC system was linear in the concentration range

[1] Evapomix, Buchler Int. Fort Lee, N. J.
[2] J. 21, Beckman.
[3] Model M 45, Waters Associates, Milford, MA 01757.
[4] Model M 440, Waters Associates, Milford, MA 01757.
[5] Radialpack B, Waters Associates, Milford, MA 01757.
[6] Module RCM 100, Waters Associates, Milford, MA 01757.
[7] Sigma Chemical Co., St. Louis, MO 63178.
[8] Merck, Darmstadt, D 6100, West Germany.
[9] Corex.

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Table I—Retention Times and Capacity Factor of Selected Glucocorticoids

Compound	Capacity Factor (K′)	Retention Time, min
Corticosterone	1.3	4.0
Dexamethasone	2	5.0
Hydrocortisone	2.5	6.0
Prednisolone	3.3	7.5

of 10–100 ng/ml for corticosterone and hydrocortisone and 10–100 ng for dexamethasone.

The analytical recovery of the compounds was measured by comparing the chromatographic peak heights from the analysis of biological samples, spiked with 100 ng of each compound and the peak height resulting from a direct injection of methanol standards.

Recovery of all compounds from plasma was 80–85% when ~85–90% of the methylene chloride layer was available for evaporation.

Assay precision was determined with successive sampling of pooled dog plasma. Ten successive samplings gave coefficients of variation <5% for all compounds: 3.39 for corticosterone, 4.90 for hydrocortisone, and 1.92 for dexamethasone.

Calculations—The concentration of corticosterone, hydrocortisone, and dexamethasone in the sample was determined from the following expression:

$$C_p : \frac{R}{a} + b \qquad \text{(Eq. 1)}$$

where C_p is the concentration of the substance in plasma (ng/ml), R is the peak height ratio (drug:internal standard), a is the slope of the calibration curve, and b is the y intercept.

Retention Time and Selectivity (Table I)—Normal-phase liquid chromatography using radial compression showed a high selectivity in the separation of corticosteroids. A good separation of the four compounds was obtained in 8 min.

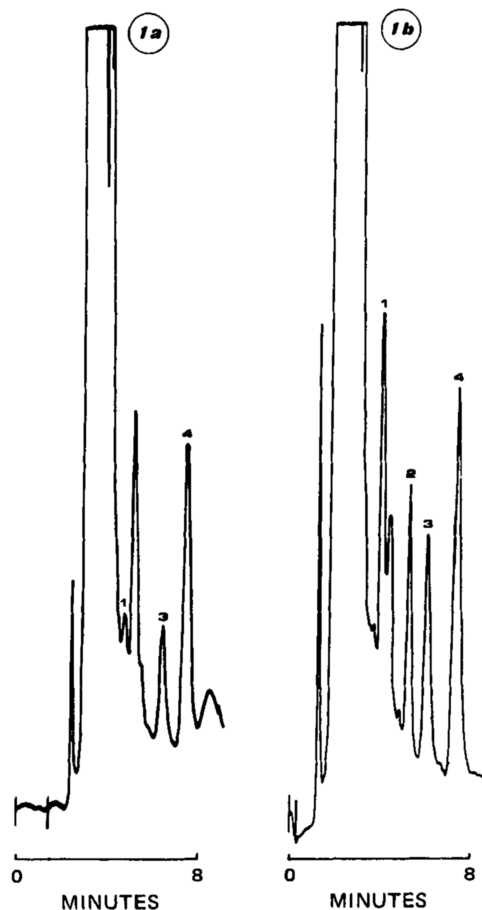

Figure 1—*Chromatogram of extracted blank dog plasma containing only internal standard (1*a*); chromatogram of extracted dog serum spiked with hydrocortisone, corticosterone, and dexamethasone (1*b*). 1, Corticosterone; 2, dexamethasone; 3, hydrocortisone; 4, prednisolone.*

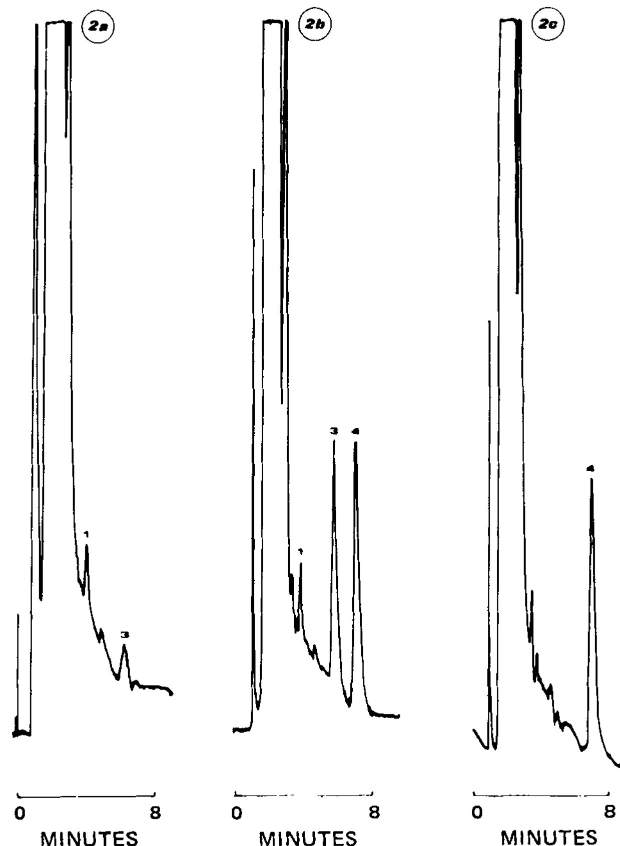

Figure 2—*Chromatogram of a plasma sample taken from a dog before the administration of corticotropin (2*a*); chromatogram of plasma 2 hr after the dose of corticotropin (2*b*); and chromatogram of extracted plasma after the injection of dexamethasone (2*c*). 1, Corticosterone; 3, hydrocortisone; 4 prednisolone.*

Drug Disposition Study—To test the ability of this method to detect endogenous corticosteroids, corticotropin[10] was administered intramusculary (20 IU) into two dogs (11–12 kg; 4–5 years of age). To prove that exogenous corticoid could be detected, dexamethasone[11] was injected intravenously (1 mg/kg). Blood was withdrawn *via* the jugular vein at 15 and 30 min and 1, 2, 4, 8, and 24 hr after the treatment and placed in tubes containing 10 U of heparin. The plasma was separated immediately and stored at −20° until analysis.

RESULTS AND DISCUSSION

A chromatogram of a blank dog plasma extract and a chromatogram of plasma spiked with 40 ng of hydrocortisone, corticosterone, and dexamethasone are shown in Fig. 1. These chromatograms illustrate the response of the chromatographic system to 40 ng of corticosteroids/ml of plasma and to 100 ng of prednisolone which was used as the internal standard.

Figure 2*a* illustrates the chromatogram of a plasma sample taken from a dog before the administration of corticotropin. In addition, Fig. 2*b* illustrates the steroid concentration 2 hr after the dose of corticotropin; hydrocortisone concentrations were much greater in contrast to the small increase in corticosterone.

Figure 2*c* shows the effect of an injection of dexamethasone 12 hr postadministration. This chromatogram indicates that dexamethasone suppressed plasma corticosterone and hydrocortisone so that concentrations were no longer detectable. At this time, concentrations of dexamethasone were not detected.

Figure 3 presents a typical plasma concentration–time profile of dexamethasone after intravenous administration and the simultaneous evolution of the endogenous corticosteroids.

The simultaneous determination of corticosterone, hydrocortisone, dexamethasone, and prednisolone by this HPLC method proves to be

[10] ACTH-Choay, Choay, Paris, France.
[11] Azium, Unilabo, Levallois-Perret, France.

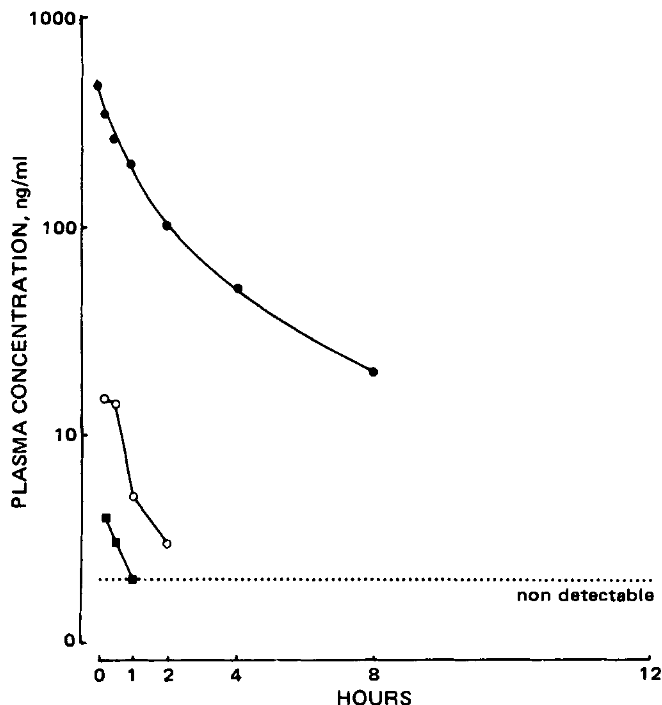

Figure 3—*Plasma concentration–time curve in the dog given a 1 mg/kg iv dose of dexamethasone. Key: (●), dexamethasone; (○), hydrocortisone; (■), corticosterone.*

efficient, precise, sensitive, and selective. It also allows the examination of the effects of dexamethasone on circulating hydrocortisone and corticosterone concentrations.

The selectivity of the extraction procedure and chromatographic system is demonstrated by the chromatogram from the blank plasma (Fig. 1*a*). Analysis of the figure indicates that there is no significant interference from endogenous compounds. It should be noted that one source of interference was encountered when the methylene chloride layer was recovered by filtration on phase-separating paper. One large interfering peak eluted at a retention time of 5 min and was labeled as an endogenous biological sample.

This chromatographic procedure differs from those previously described (11–19), as a new radial compression separation system was used. In this system a strong radial compression is applied to a soft column, thus diminishing the dead volume and the formation of preferential ways inside the column. This results in higher efficacy, with a lower pressure and increased flow rate. Moreover, it is possible to attain the same separation in 2 min with a flow rate of 6 ml/min and with only a 10% decrease in response.

Responses were considered significant when the signal to noise ratio was >1.5. In such conditions, using a detector sensitivity of 0.005 aufs, levels as low as 2 ng/ml, giving 0.5-cm peaks, could be quantitated.

In conclusion, the described method offers a simple, rapid, and reliable determination of corticosteroids for use in pharmacokinetic studies.

REFERENCES

(1) F. W. Funck and L. Sicha, *Med. Exp.*, **7,** 1 (1962).
(2) B. Favre and J. P. Moatti, *Ann. Rech. Vét.*, **8,** 111 (1977).
(3) W. L. Gardiner and E. C. Corning, *Biochim. Biophys. Acta*, **115,** 524 (1965).
(4) A. W. Meikle, L. G. Lagerquist, and F. H. Tyler, *Steroids*, **23,** 193 (1973).
(5) M. Hichens and A. F. Hogans, *Clin. Chem.*, **20,** 266 (1974).
(6) M. C. Dumasia, D. I. Chapman, M. S. Moss, and C. O'Connor, *J. Biochem.*, **133,** 401 (1973).
(7) S. W. Schalm, W. H. J. Summerskill, and V. L. M. Go, *Mayo Clinic. Proc.*, **51,** 761 (1976).
(8) D. C. Anderson, B. R. Hopper, B. L. Lasley, and S. S. Yen, *Steroids*, **28,** 179 (1976).
(9) W. Wortmann, C. Schnabel, and J. C. Touchstone, *J. Chromatogr.*, **84,** 396 (1973).
(10) J. Q. Rose and W. J. Jusko, *ibid.*, **162,** 273 (1979).
(11) J. C. K. Loo, A. G. Butterfield, F. Moffat, and N. Jordan, *ibid.*, **143,** 275 (1977).
(12) J. C. K. Loo and N. Jordan, *ibid.*, **143,** 314 (1977).
(13) S. E. Tsuei and J. J. Ashley, *ibid.*, **145,** 213 (1978).
(14) M. D. Smith, *ibid.*, **164,** 129 (1979).
(15) M. D. Smith and D. J. Hoffman, *ibid.*, **168,** 163 (1979).
(16) G. Cavina, G. Moretti, R. Alimenti, and B. Gallinella, *ibid.*, **175,** 12 (1979).
(17) M. Schoneshoffer and H. J. Dulce, *ibid.*, **164,** 17 (1979).
(18) N. R. Scott and P. F. Dixon, *ibid.*, **164,** 17 (1979).
(19) M. C. Petersen, R. L. Nation, and J. L. Ashley, *ibid.*, **183,** 131 (1980).

NOTES

Rapid Enzymatic Preparation of [^{14}C]D-Leucine from [^{14}C]DL-Leucine

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Received July 6, 1981, from the *Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Kentucky, Lexington, KY 40506.* Accepted for publication September 25, 1981.

Abstract □ A rapid enzymatic method for the preparation of [1-^{14}C]D-leucine is described. [1-^{14}C]D-Leucine was obtained from [1-^{14}C]DL-leucine by oxidative deamination of the L-isomer using immobilized L-amino acid oxidase. The total preparation (including ion exchange purification) was accomplished in 40 min with an 83% yield. The methodology is applicable to the production of [1-^{11}C]D-leucine, a potential imaging agent for tumor localization.

Keyphrases □ [^{14}C]D-leucine—rapid enzymatic preparation for tumor localization □ Enzymatic preparation—D-leucine isomers, [^{11}C]D-leucine production for tumor localization

Amino acids labeled with gamma-emitting radionuclides (carbon 11 or nitrogen 13) have been investigated for tumor localization, pancreatic scanning, and the study of normal physiological processes. Several ^{13}N-labeled L-amino acids have been synthesized enzymatically and their tissue distribution and pancreatic uptake studied in animals

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