vation for I was ~3 kcal/mole less than that of cefazolin. This difference was manifested in the larger k_0 value at pH 9.4 for I. The results of this study are in agreement with expected effects of structural changes at R_1 on the pH–rate profiles of cephalosporin derivatives.

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ACKNOWLEDGMENTS

Abstracted in part from a dissertation submitted by E. S. Rattie to the Graduate School, Temple University, in partial fulfillment of the Master of Science degree requirements.

Fluorometric High-Pressure Liquid Chromatographic Determination of Hydrocortisone in Human Plasma

THOMAS J. GOEHL ˣ, GLORIA M. SUNDARESAN, and VADLAMANI K. PRASAD

Received February 28, 1979, from the *Division of Biopharmaceutics, Food and Drug Administration, Washington, DC 20204.* Accepted for publication May 8, 1979.

Abstract □ A highly sensitive and specific method for hydrocortisone determination in plasma is described. In this method, an internal standard (Δ^4-pregnen-17α,20α,21-triol-3,11-dione) was added to plasma, which was then extracted with a mixture of methylene chloride–ether (60:40). After separation and evaporation of the organic phase, derivatization was carried out with dansylhydrazine. Upon completion of the reaction, the excess dansylhydrazine was reacted with pyruvic acid and a second extraction and evaporation step was performed. The residue was taken up in 100 μl of the high-pressure liquid chromatographic mobile phase, and 5 μl was injected onto a microparticulate silica column. Elution was carried out with an ethylene dichloride–butanol–water (91:8.5:0.5) mobile phase. The effluent was monitored with a fluorescence detector (excitation 240 nm; emission 470-nm cutoff filter). A linear calibration curve was found from 5 to 150 ng/ml with the precision estimated to be ±7% (*CV*).

Keyphrases □ Hydrocortisone—analysis, high-pressure liquid chromatography, fluorometry, plasma, humans □ Glucocorticoids—hydrocortisone, high-pressure liquid chromatographic and fluorometric analysis, plasma, humans □ High-pressure liquid chromatography—analysis, hydrocortisone, human plasma, fluorometry

Hydrocortisone is a potent anti-inflammatory, immunosuppressive, and antiallergenic drug (1). Since the discovery of hydrocortisone, numerous synthetic glucocorticoids have been prepared. However, hydrocortisone remains an important agent in modern adrenocorticoid steroid therapy.

Various analytical methods have been used to assay hydrocortisone in plasma including fluorescence (2, 3), competitive protein binding (4), enzyme immunoassay (5), and radioimmunoassay (6). All of these methods have problems with specificity. More recently, high-pressure liquid chromatographic (HPLC) methods for hydrocortisone assay in plasma have appeared (7–10). Most HPLC methods exhibit the required specificity but do not provide the low nanogram per milliliter sensitivity needed for bioequivalence studies. This paper describes a highly sensitive and specific HPLC assay for plasma hydrocortisone that can be used in bioequivalence studies.

EXPERIMENTAL

Apparatus—The modular high-performance liquid chromatograph consisted of a constant-flow pump[1], a valve-type injector[2], a fluorescence detector[3] (excitation 240 nm; emission 470-nm cutoff filter), and a strip-chart recorder[4] (0.5 cm/min). A stainless steel column (4.6 mm i.d. × 250 mm) packed with fully porous, irregularly shaped 5-μm silica[5] was obtained commercially.

Chromatographic Conditions—The mobile phase was ethylene dichloride–butanol–water (91:8.5:0.5). A flow rate of 1.5 ml/min was established (2200 psig), and the column was equilibrated for 16 hr. The column was maintained at 19.5° by inserting it into a glass sleeve, which was immersed in a constant-temperature water bath[6].

Reagents and Materials—Hydrocortisone[7], Δ^4-pregnen-17α,-20α,21-triol-3,11-dione[7] (internal standard), pyruvic acid[8], and dansylhydrazine[9] were obtained from commercial sources. All were used as received except dansylhydrazine, which was recrystallized from chloroform prior to use. Solvents used were spectroanalyzed ethylene dichloride[8], HPLC-grade butanol, methylene chloride, and ether[10]. All other materials were reagent grade.

[1] Chromatography pump, model M6000A, Waters Associates, Milford, Mass.
[2] Sample injection valve, model U6K, Waters Associates, Milford, Mass.
[3] Model FS970, Schoeffel Instruments, Westwood, N.J.
[4] Model 9176, Varian Instruments, Palo Alto, Calif.
[5] Prepacked Hibar II column with Lichrosorb SI-60 5-μm silica, Applied Science Laboratories, State College, Pa.
[6] Model 3080, Lab-Line Instruments, Melrose Park, Ill.
[7] Sigma Chemical Co., St. Louis, Mo.
[8] Fisher Scientific Co., Pittsburgh, Pa.
[9] Regis Chemical Co., Morton Grove, Ill.
[10] Burdick & Jackson Laboratories, Muskegon, Mich.

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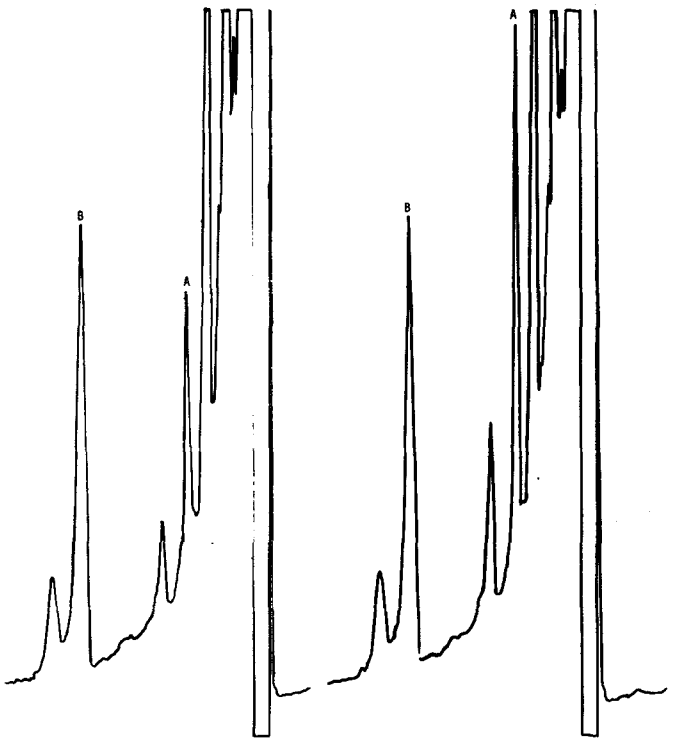


Figure 1—*Chromatogram obtained from HPLC assay of control human plasma (left) and control plasma spiked with an additional 50 ng of hydrocortisone/ml (A) (right). Both were spiked with 200 ng of internal standard/ml (B).*

Stock solutions of hydrocortisone and the internal standard were prepared individually by dissolving 10 mg of each in 100 ml of methanol and making appropriate dilutions. Plasma standards were prepared by taking small volumes of the stock hydrocortisone solutions and adding them to control plasma. The dansylhydrazine solution was prepared by dissolving 20 mg of reagent in 10 ml of absolute ethanol.

The ethanolic hydrochloric acid solution was prepared by adding 0.65 ml of concentrated hydrochloric acid to 1 liter of absolute ethanol. For the pyruvic acid solution, 40 mg of pyruvic acid was added to 10 ml of absolute ethanol. The phosphate buffer was prepared by titrating a 1.0 *M* solution of dibasic potassium phosphate to pH 8.8 with a 1.0 *M* solution of monobasic potassium phosphate.

Assay—To 1 ml of plasma in a 12-ml glass-stoppered conical centrifuge tube was added 200 ng of the internal standard (20 μl of a 10-ng/μl methanolic solution). The tubes were mixed[11], and then 6 ml of methylene chloride–ether (60:40) was added. After mixing for 30 sec, the samples were centrifuged and the organic phase was transferred to a second tube. The solvent was evaporated[12] under a gentle nitrogen stream. To the residue were added 100 μl of ethanolic hydrochloric acid and 100 μl of ethanolic dansylhydrazine.

After the solution reacted for 1 hr at room temperature, 100 μl of the pyruvic acid solution was added. After 15 min at room temperature, 2 ml of phosphate buffer and 6 ml of methylene chloride–ether (60:40) were added. The samples were mixed for 30 sec and centrifuged, and the organic phase was transferred to a third test tube. After solvent evaporation, the residue was dissolved in 100 μl of the HPLC mobile phase and 5 μl was injected.

The hydrocortisone concentrations were determined from standard curves prepared by plotting peak height ratios of the dansylhydrazones of hydrocortisone and internal standard *versus* the spiked concentrations. To correct the standard curve for endogenous hydrocortisone in control plasma, the corresponding peak height ratio was subtracted from all data points used in the standard curve.

Recovery—Plasma aliquots (1 ml) were spiked with known serial hydrocortisone quantities. After the samples were treated as described, the peak heights for the hydrocortisone dansylhydrazones (corrected for endogenous hydrocortisone) were compared to the peak heights obtained from the same amount of drug that was derivatized but not extracted. The ratio of the slopes was used to estimate percent recovery.

Precision—Normal human plasma aliquots were spiked with known amounts of hydrocortisone to give several concentrations. The samples were assayed in quadruplicate as described.

RESULTS AND DISCUSSION

Before bioequivalence requirements are proposed for a drug, adequate methodology should be available. Although numerous methods exist for assaying hydrocortisone, no one method has the required specificity or sensitivity.

In initial studies with the existing HPLC methods, sensitivity seemed to be the major obstacle for bioequivalence studies. Levels of 25 ng/ml or lower were difficult to measure. One approach to increase the sensitivity of these HPLC assays would be to introduce a fluorophore into the molecule. This fluorophore would allow the use of a fluorescence detector with its inherent greater sensitivity.

Derivatization of steroid keto groups has been extensively studied (12–15). In reactions with 2,4-dinitrophenylhydrazines, Reich *et al.* (12) reported that the 3-keto group reacted faster than the 17- or 20-keto group. Van Den Heuvel and Horning (15) also found that the reaction between 1,1-dimethylhydrazine and the steroid keto groups was position dependent, with the 3-keto group reacting more easily than the 20-keto group and with no reaction for the 11-keto group.

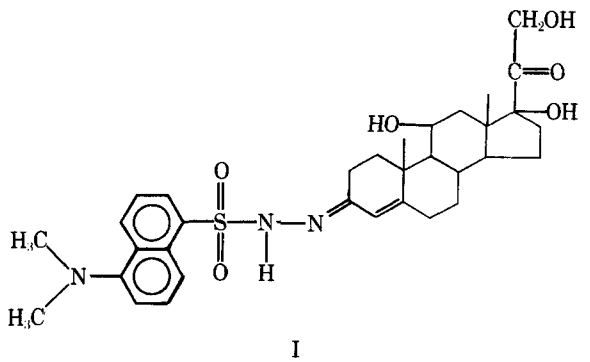
With the introduction of dansylhydrazine (11), a valuable reagent was added to the armory of derivatization reagents that could enhance chromatographic sensitivity. Dansylhydrazine was found to follow the same position dependency as the other hydrazines (13).

When the hydrocortisone dansylhydrazone (I) was prepared and subjected to HPLC analysis, two peaks were observed. These peaks were collected and analyzed by NMR. Both peaks had similar properties, pointing to the formation of *syn-* and *anti*-isomers at the 3-keto position, although no definitive conclusion could be drawn. Similar geometric isomers of the *syn-* and *anti*-types have been found for the 3-keto steroids when reacted with pentafluorobenzylhydroxylamine (14). Further evidence that the two peaks were indeed the *syn-* and *anti*-forms of the 3-keto dansylhydrazones was obtained by reacting the internal standard with dansylhydrazine.

The internal standard used in these studies is a particularly good one, since the functionality is identical, differing from hydrocortisone only in the position of the second keto group. Even though the internal standard has only one reactive keto group in the 3-position, again two peaks with similar chromatographic properties were found. These results indicate that at least with hydrocortisone and the internal standard, the 3-keto group is most reactive with dansylhydrazine and the two derivatives formed are the *syn-* and *anti*-isomers.

Attempts were made to vary the reaction conditions to change the ratio of the two peaks. No change was observed when temperature or reaction time was varied. Similar results were observed with the internal standard. The ratio was independent also of the hydrocortisone concentration. Therefore, the use of this derivatization for quantitative purposes seemed possible.

Both reversed-phase partition chromatography on a C-18 column and normal-phase adsorption chromatography on a microparticulate silica column were studied. The adsorption mode was chosen because a cleaner chromatogram was found after analysis of control plasma. The initial column stability problem was overcome by incorporating a small percentage of water in the mobile phase (16). In addition, room temperature variations affected column performance, resulting in peak shape changes



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[11] Vortex-Genie, Fisher Scientific Co., Pittsburgh, Pa.
[12] N-Evap, Organomation Associates, Shrewsbury, Mass.

Table I—HPLC Method Precision

Theoretical Concentration, ng/ml	Experimental Concentration, ng/ml, Mean[a] (Range)	*CV*, %
10	9.8 (8.1–11)	12.3
20	19 (17–21)	8.6
40	38 (38–40)	2.3
60	60 (53–65)	8.3
80	80 (78–84)	3.0

[a] $n = 4$.

that adversely affected assay precision. Instituting column temperature control resolved this issue.

After plasma was analyzed for endogenous hydrocortisone, the resulting peaks had the same retention times and peak widths as the standard. For further confirmation that hydrocortisone was present and that no interfering peaks were hidden under the dansylhydrazone peaks, the standard addition technique was used. The peak heights were additive, and the peak widths at half-height were constant.

Chromatograms obtained in the analysis of human plasma containing only endogenous hydrocortisone and 200 ng of internal standard/ml and the same plasma spiked with an additional 50 ng of hydrocortisone/ml and 200 ng of internal standard/ml are shown in Fig. 1. Two peaks were found for each of the two compounds, as discussed previously. The peaks associated with the hydrocortisone derivatization had retention times of 8.4 and 10.0 min. The peaks associated with the internal standard derivatization had retention times of 16.8 and 18.6 min. The first of these two peaks for each compound was used in calculating peak ratios.

A standard linear plasma calibration curve was obtained in the 5–150-ng/ml range. Sensitivity studies with the hydrocortisone dansylhydrazone showed that as little as 0.25 ng can be detected. The recovery of hydrocortisone added to control plasma was studied by comparing the ratio of slopes obtained for the plasma extraction standard curve and for the direct standard curve. The recovery was calculated to be 83%.

To study method precision, known drug amounts were added to control plasma. These samples were assayed in quadruplicate as described. The coefficients of variation (*CV*) for the assay of hydrocortisone over the range of 10–80 ng/ml are reported in Table I.

This method was developed for planned bioequivalence studies with hydrocortisone tablets in which dexamethasone was to be used to suppress endogenous hydrocortisone. Therefore, the possibility of dexamethasone interference in the assay had to be explored. In these studies, dexamethasone was added to plasma to give a final concentration of 200 ng/ml. This concentration was well above the dexamethasone concentration expected in the study. These samples and the same control plasma were assayed for endogenous hydrocortisone. The hydrocortisone concentration in the control plasma was determined to be the same as that in the control plasma to which dexamethasone had been added. Thus, it can be concluded that dexamethasone had no effect on the HPLC assay for hydrocortisone.

Table II—Plasma Endogenous Hydrocortisone from Three Human Volunteers

Subject	Concentration, ng/ml	*CV*[a], %
A	83.2	5.1
B	79.6	1.2
C	80.8	4.0

[a] $n = 3$.

To demonstrate the utility of this method as a routine procedure for hydrocortisone determination, plasma from normal volunteers was assayed (Table II). Results were in the accepted concentration range for endogenous hydrocortisone (17).

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ACKNOWLEDGMENTS

The authors thank M. A. Tischler and E. B. Sheinin of the Division of Drug Chemistry, Food and Drug Administration, for the NMR analyses.