cating that the availability of cloprednol from all of the oral dosage forms tested was complete.

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ACKNOWLEDGMENTS

The authors thank B. Amos and J. Smithers of Syntex Research for their assistance in assaying the cloprednol plasma samples.

Quantitative Determination of Prednisone and Prednisolone in Human Plasma Using GLC and Chemical-Ionization Mass Spectrometry

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Received July 5, 1977, from the *Department of Drug Metabolism, Syntex Research, Palo Alto, CA 94304.* Accepted for publication October 28, 1977.

Abstract □ A method for the quantitative determination of prednisolone and prednisone in human plasma utilizing GLC and chemical-ionization mass spectrometry is described. Corticosteroids are extracted from plasma into ether, and the extract is purified either by passing through a magnesium silicate column or by solvent partitioning. Interference from endogenous hydrocortisone is removed by selective derivatization with Girard Reagent T. Following derivatization, prednisolone can be quantitatively separated from the water-soluble hydrocortisone derivative by simple solvent partitioning. The extracted prednisone and prednisolone are converted to their corresponding methoxyimino trimethylsilyl derivatives, and subjected to GLC–mass spectrometry. Prednisone and prednisolone plasma profiles following a 15-mg oral dose of prednisone in a human volunteer are presented. The method can measure prednisone and prednisolone in plasma at the nanogram per milliliter level.

Keyphrases □ Prednisone—GLC–mass spectrometric analysis in human plasma □ Prednisolone—GLC–mass spectrometric analysis in human plasma □ GLC–mass spectrometry—analyses, prednisone and prednisolone in human plasma □ Glucocorticoids—prednisone and prednisolone, GLC–mass spectrometric analyses in human plasma

The synthetic corticosteroid prednisone and its major metabolite prednisolone are used clinically as anti-inflammatory agents. Previously reported analytical procedures include GLC with flame-ionization detection (1), competitive protein binding assay (2), and radioimmunoassay (3, 4). The GLC method lacks sensitivity and cannot be used to follow plasma levels after the administration of a therapeutic dose.

The radioimmunoassay and competitive protein binding methods provide the required sensitivity for measurement. Specificity is apparently achieved by diluting plasma samples, thereby diluting the interference of cortisone in the prednisone assay and of hydrocortisone in the prednisolone assay. The difficulties and merits of these dilution methods recently were reviewed (5). An alternative method based on paper chromatography followed by radioimmunoassay also was described (6). Paper chromatograms require 24 hr for elution, and R_f values of the compounds of interest are obtained by comparison with R_f values of radioactive standards run on separate paper strips. Although this method has improved specificity, it is cumbersome and lengthy.

Recently, the radioimmunoassay method was used in the analysis of bioavailability and pharmacokinetic samples (7). In these studies, dexamethasone was administered to human volunteers to suppress the secretion of endogenous hydrocortisone and thus increase the specificity of the assay.

This report describes a specific and sensitive analytical method for the determination of prednisone and prednisolone in human plasma. The method is based on GLC and chemical-ionization mass spectrometry with selected ion monitoring. It can be used to study the kinetics of the interconversion of prednisone and prednisolone in humans and to acquire pharmacokinetic and bioavailability data without suppression of endogenous steroid production.

The applicability of the method is shown by the measurement of plasma prednisone and prednisolone levels in a human volunteer after oral administration of 15 mg of prednisone[1].

EXPERIMENTAL

Reagents—Methoxyamine hydrochloride[2], pyridine[2], and trimethylsilylimidazole[2] were used without further purification. Magnesium silicate[3] (60–100 mesh) and (carboxymethyl)trimethylammonium chloride hydrazide[4] (Girard Reagent T) were commercially available. Methoxyamine reagent (4% w/v) was prepared by dissolving methoxyamine hydrochloride in pyridine. Girard Reagent T (10% w/v) was prepared by dissolving it in methanol containing 1% acetic acid.

Instruments—Samples were analyzed on a gas chromatograph–mass spectrometer fitted with a chemical-ionization source and a data system[5]. Methane was used as the carrier (20 ml/min) and as the chemical-ionization reagent gas. The chemical-ionization source pressure was maintained at approximately 1 torr.

[1] Deltasone.
[2] Pierce Chemical Co., Rockford, Ill.
[3] Florisil, Matheson, Norwood, Ohio.
[4] Matheson, Norwood, Ohio.
[5] Finnigan model 3200 with model 6000 data system.

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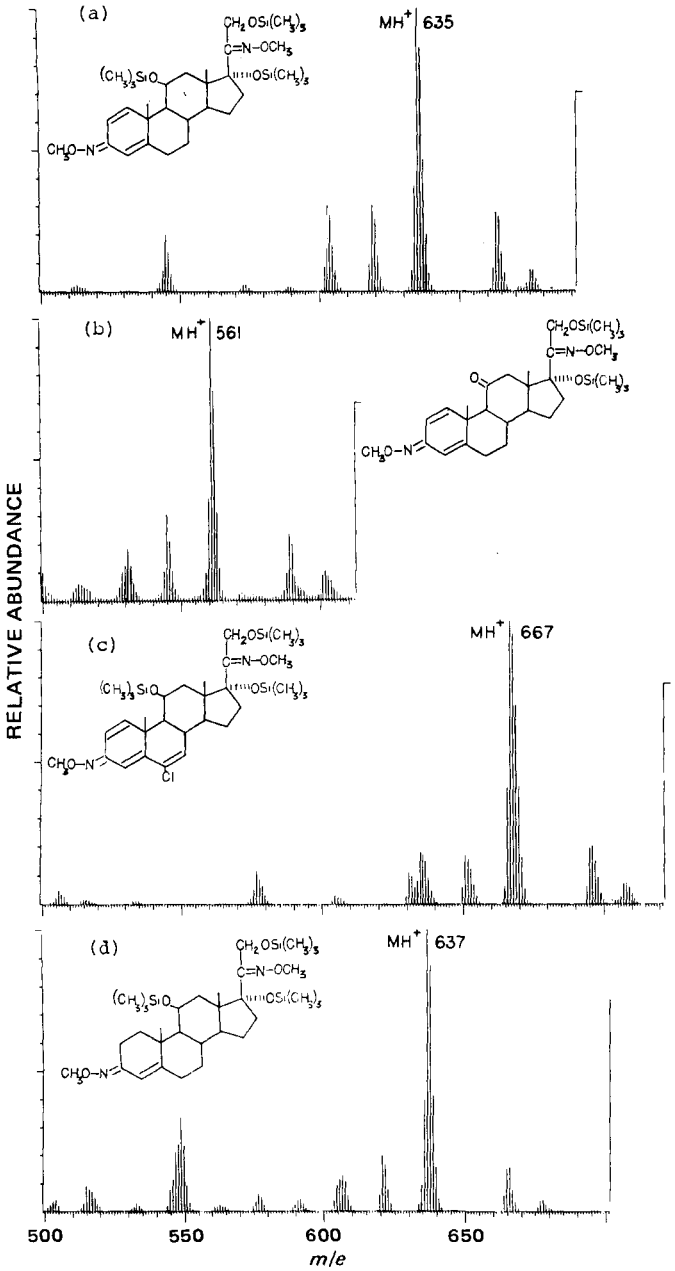

Figure 1—*Methane chemical-ionization mass spectra of methoxyimino trimethylsilyl derivatives of prednisolone (a), prednisone (b), cloprednol (c), and hydrocortisone (d).*

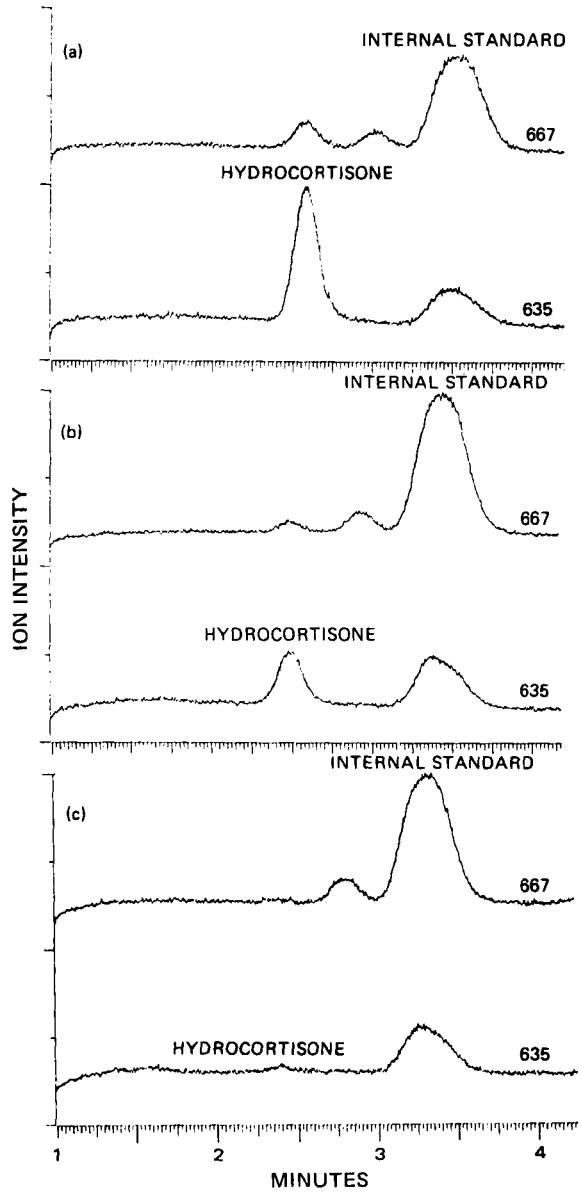

Figure 2—*Selected ion chromatograms depicting the interference of endogenous hydrocortisone at the m/e value (635) and retention time of prednisolone and its selective removal by consecutive Girard reactions. Key:* a, *before reaction;* b, *after first reaction, and* c, *after second reaction.*

GLC was performed on a 60-cm × 2-mm i.d. glass column packed with 3% SP-2250 on 100–120-mesh Supelcoport[6]. Column, injection port, and chemical-ionization source temperatures were maintained at 250, 270, and 120°, respectively. The mass spectrometer was operated in the selected ion recording mode, monitoring ions at *m/e* 561 and 667 or 635 and 667 amu.

Preparation of Magnesium Silicate Column—A 5-mm plug of silanized glass wool was placed in the neck of a disposable glass pipet (Pasteur, 14.5 cm × 7 mm). The pipet was then packed with about 300 mg of magnesium silicate (~2 cm high), and the packing was washed with 3 ml of acetone followed by 3 ml of chloroform. The column was used within 15 min of preparation.

Plasma Sample Preparation—*Method A*—To a 2-ml plasma sample in a 155 × 13-mm culture tube, fitted with a polytef-lined screw cap, were added 100 μl of the internal standard cloprednol (150 ng/100 μl of methanol), 1 ml of water, and 10 ml of ether. The tubes were shaken vigorously for at least 2 min. After centrifugation, the organic layer was transferred to a second tube and evaporated to dryness at 60° under a nitrogen stream.

The residue was transferred to a magnesium silicate column with 3 × 300-μl washes of chloroform. The column was then washed with an additional 1.5 ml of chloroform. The corticoids were eluted from the column with 10 ml of acetone, which was collected and evaporated to dryness at 60° under a nitrogen flow.

The residue was dissolved in 400 μl of Girard Reagent T and allowed to react for 45 min at room temperature. Water, 5 ml, was added, and the solution was extracted twice with 6 ml of dichloromethane–ether (3:7). Layers were separated by centrifugation after each extraction. The extracts were combined and evaporated at 60° under nitrogen. The residue obtained after the evaporation of dichloromethane–ether was taken up in 400 μl of Girard Reagent T, and derivatization was repeated. The residue obtained after the second derivatization was transferred to a 0.3-ml vial[7] with two 300-μl washes of chloroform. The chloroform was evaporated at room temperature under nitrogen after each transfer. This

[6] Supelco, Bellefonte, Pa.

[7] Reactivial.

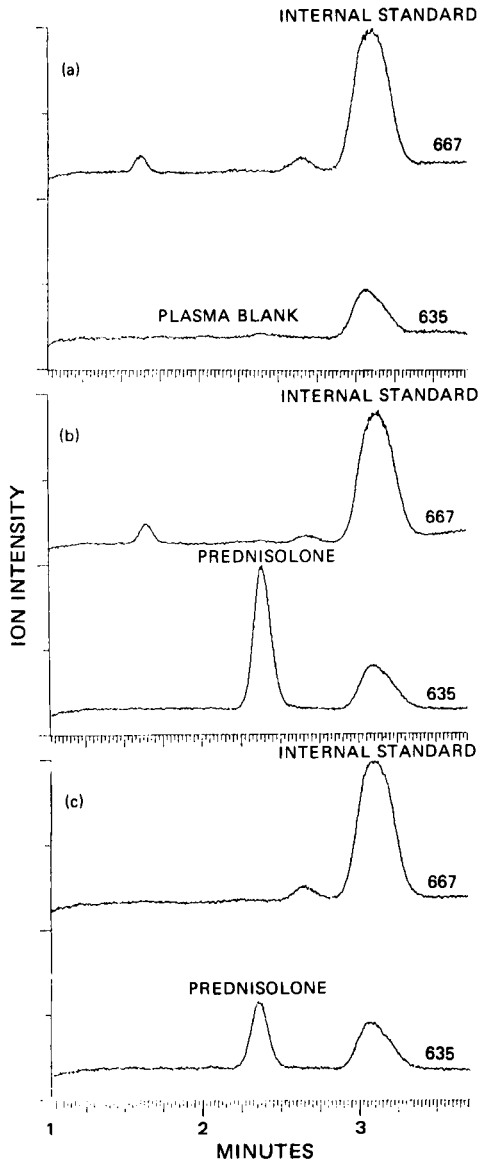

Figure 3—*Selected ion chromatograms of plasma samples obtained from a dosed subject, processed according to Method A and analyzed for prednisolone. Key:* a, *blank plasma;* b, *plasma sample after 15 min; and* c, *plasma sample after 8 hr.*

residue was then subjected to derivatization for GLC–mass spectrometry.

Method B—Corticosteroids were extracted from plasma as described for Method A. The residue obtained after ether evaporation was dissolved in methanol (2 ml) containing 20% (v/v) of 10% aqueous acetic acid. Solvent partitioning was achieved by washing the methanolic solution three times with 3 ml of hexane saturated with methanol–acetic acid. The hexane washings were discarded, and the remaining methanol–acetic acid was evaporated to dryness at 60° under nitrogen. The residue so obtained was reacted with Girard Reagent T as described for Method A, followed by derivatization for GLC–mass spectrometry.

Derivatization for GLC–Mass Spectrometry—Methoxyamine reagent, 50 μl, was added to the residue obtained by either Method A or B. The vial was capped and heated at 120° for 30 min. After cooling, 25 μl of trimethylsilylimidazole was added; the vial was then reheated at 120° for 30 min. Up to 6 μl of this mixture was injected on the GLC–mass spectrometric system for quantitation.

RESULTS AND DISCUSSION

The major requirement in establishing a specific analytical method for prednisolone and prednisone in human plasma is selective removal of the interference by the formed metabolic products and the endogenous

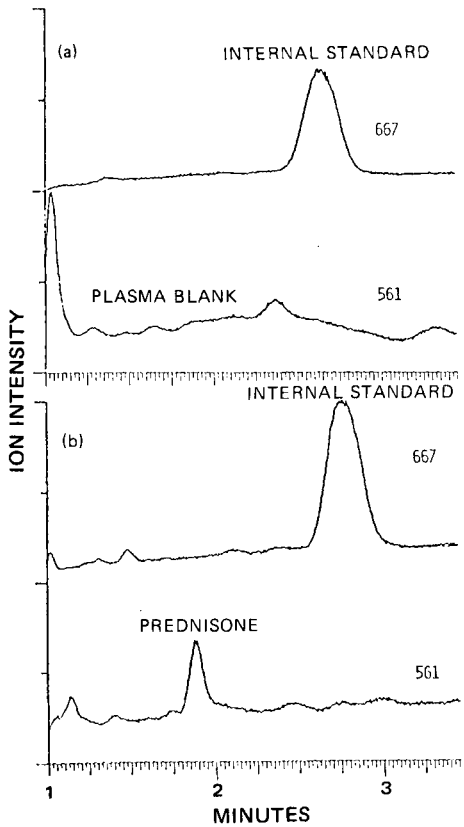

Figure 4—*Selected ion chromatograms of plasma samples obtained from a dosed subject, processed according to Method A and analyzed for prednisone. Key:* a, *blank plasma; and* b, *plasma sample after 1 hr.*

steroids. Most important of these endogenous steroids are hydrocortisone and cortisone. These compounds possess the same functional groups and differ structurally only by the absence of a Δ^1-double bond.

In a recent report on the radioimmunoassay of prednisone and prednisolone, a cross-reactivity on the order of 35% or greater was observed for the 20-hydroxy and the glucuronide metabolites formed from the two compounds (8). The effect of glucuronides on the quantitative results is important since their presence in plasma up to 4 hr after prednisolone administration has been documented (8, 9). Because of these difficulties, previous investigators resorted to chromatography before analysis (6). This alternative is lengthy and difficult and has not increased accuracy (8).

An analytical method was required for studying the pharmacokinetics and interconversion of prednisone and prednisolone in normal subjects as well as in the diseased state without the suppression of endogenous steroids. To achieve this aim, GLC and chemical-ionization mass spectrometry were utilized. Prednisone, prednisolone, and the internal standard cloprednol were derivatized to their corresponding methoxyimine trimethylsilyl derivatives prior to GLC. The chemical-ionization spectra of prednisolone, prednisone, cloprednol, and endogenous steroid hydrocortisone are shown in Fig. 1. These spectra were obtained by derivatizing pure reference compounds and subjecting them to GLC–mass spectrometry under the conditions described.

As expected, because of the presence of several trimethylsilyl groups, the molecular ion for each compound is observed as a multiplet rather than as a single ion. This multiplicity of the molecular ion imposes certain restrictions on the choice of a GLC–mass spectral internal standard. For a deuterium-labeled molecule to serve as an internal standard, it must be substituted with a large number of deuterium atoms so that its molecular ion cluster will not overlap with the molecular ion cluster of the compound under investigation. Although the synthesis of such a compound is possible, the lack of general availability makes this choice less desirable. For this reason, a structurally similar compound, cloprednol, was chosen as the internal standard. It possesses similar functional groups but elutes with a different retention time, thus making it possible to measure the compounds *via* selected ion monitoring (10).

To measure plasma prednisolone levels, it was necessary to separate

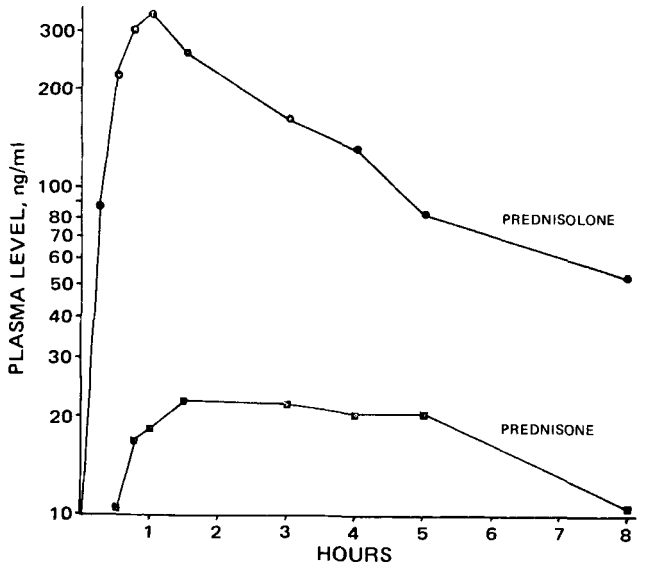

Figure 5—*Plasma prednisone and prednisolone levels achieved after the administration of 15 mg po of prednisone to a human volunteer.*

prednisolone from endogenous hydrocortisone. These two compounds differ by only 2 mass units and, after derivatization to the methoxyimine trimethylsilyl derivatives, their molecular ion clusters overlap each other. Since the basal levels of hydrocortisone change during the day, it is not possible to measure prednisolone in the presence of changing concentrations of hydrocortisone.

Attempts to resolve derivatized prednisolone from hydrocortisone chromatographically for reproducible quantitation at low nanogram levels were not promising. These two compounds could be separated *via* selective derivatization with Girard Reagent T. Derivatization of ketones with Girard Reagent T to form water-soluble products was reported previously (11, 12). The possible application of this reaction in the improvement of the radioimmunoassay for prednisolone also was discussed (13). The reported method does not provide complete separation of hydrocortisone from prednisolone, and extensive modification of the procedure was adopted. It also was necessary to repeat the Girard reaction a second time to remove detectable interference from hydrocortisone (Fig. 2).

Plasma samples to be analyzed could be processed by two alternative procedures, Methods A and B, as described under *Experimental.* Method A was better than Method B in providing less overall background in the mass spectrometer, whereas Method B was simpler and faster. Plasma cleanup procedures similar to Methods A and B previously were reported (1, 14) and were modified for the present study.

The clinical applicability of the developed method was established by the measurement of plasma prednisone and prednisolone levels in a human volunteer after the oral administration of 15 mg of prednisone. Plasma samples were processed *via* either Method A or B, and compounds of interest were measured *via* selected ion monitoring. Figure 3*a* shows the selected ion chromatogram obtained for prednisolone from a blank plasma sample processed according to Method A and then subjected to GLC–mass spectrometry and selected ion monitoring. Figures 3*b* and 3*c* show selected ion chromatograms for prednisolone from plasma samples obtained from a volunteer 15 min and 8 hr after prednisone administration, respectively. The ions selected for measurement were MH^+ 635 for prednisolone and MH^+ 667 for the internal standard.

Figures 4*a* and 4*b* show the selected ion chromatogram obtained for prednisone from a blank plasma sample and a plasma sample from a volunteer 1 hr after drug administration, respectively. Ions selected for measurement were MH^+ 561 for prednisone and MH^+ 667 for the internal standard.

Prednisone and prednisolone could be measured simultaneously in one single analysis. In the present study, however, each compound was monitored separately to gain higher sensitivity.

Plasma concentrations in unknown samples were determined with respect to the calibration curves prepared by plotting the peak height ratio *versus* the amount of compound spiked per milliliter of plasma. The calibration curves prepared for prednisolone (concentration range of 0–150 ng/ml) and prednisone (concentration range of 0–40 ng/ml) were linear. Plasma prednisone and prednisolone levels achieved in a human volunteer after the administration of 15 mg of prednisone are shown in Fig. 5. The two compounds reached peak plasma levels within 2 hr after the administration and then decayed with different apparent half-lives.

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ACKNOWLEDGMENTS

Presented at the Pharmaceutical Analysis and Control Section, APhA Academy of Pharmaceutical Sciences, Orlando meeting, November 1976.

The authors thank Dr. James Strand for arranging the clinical study, Ms. Jackie Smithers for the column technique, and Dr. Richard Runkel for support and encouragement.