compartments. The apparent equilibrium between the compartments was achieved within 3 hr. The apparent volume of the central compartment (V_c) was 1 liter/kg. The distribution half-life of the shallow and deep compartments (Compartments 2 and 3) were 6.14 and 32.5 min, respectively. In the postdistribution phase, the plasma levels declined with a terminal half-life of 5.45 hr (range 4.0–7.4). The plasma clearance (Cl_p) and the apparent volume of distribution ($V_{d,\beta}$) averaged 1.93 liters/hr/kg and 15 liters/kg, respectively. This unusually high apparent volume of distribution and the significant drop in plasma levels from 3–4 μg/ml at 6 min after injection to $<$200 ng/ml within 3 hr indicated extensive tissue uptake of bretylium in rats. Similar binding characteristics of bretylium were demonstrated in humans (8).

The intercompartmental distribution rate constants (k_{12} and k_{21}) between the central compartment and Compartment 2 in the same rat were close to each other and ranged from 0.8 to 5.1 hr^{-1} between rats. However, the transfer rate constant (k_{31}) from the third compartment to the central compartment was considerably smaller (mean 0.288 hr^{-1}) than either k_{13} (the intercompartmental rate constant in the opposite direction, 1.97 hr^{-1}) or the elimination rate constant (k_{10}, 1.89 hr^{-1}). This finding suggests that tissue binding in Compartment 3 (the deep compartment) is rate limiting in the elimination of bretylium from the body.

The excretion rate constant (k_{ex}) and the renal clearance (Cl_r) of bretylium in rats were 1.24 hr^{-1} and 1.27 liters/hr/kg, respectively. This renal clearance corresponds to 21 ml/min/kg and represents 66% of the total body clearance; it is about three times greater than the glomerular filtration rate of 6.64–8 ml/min/kg reported for normal rats (9, 10). It also indicates that active secretion is the major mechanism of urinary excretion of bretylium in rats. A similar mechanism of active secretion of bretylium in urine also was demonstrated for humans (4).

The major portion of the nonrenal excretion of bretylium probably occurs through the bile, as indicated by the recovery of ~30% of the dose in feces in 48 hr. If excretion in the bile is rapid immediately after dosing, there is a possibility of enterohepatic cycling. The extent of biliary excretion of bretylium has not been investigated in humans. However, on the basis of urinary excretion data, a much smaller quantity is expected to be eliminated *via* the biliary route.

In summary, these results indicate that bretylium is extensively distributed in rats, bound to the tissues, and excreted mainly unchanged in the urine, mostly by active secretion, similar to the findings in humans. Biliary excretion is the second major route of elimination of bretylium in rats. The data also show striking similarity in the pharmacokinetics of distribution and elimination of bretylium in humans and rats. This similarity might allow the use of the animal model for further *in vivo* investigations, including efficacy and drug–drug interaction studies in the rat.

REFERENCES

(1) G. Sanna and R. Arcidiacono, *Am. J. Cardiol.*, **32,** 982 (1973).

(2) D. W. Romhilt, S. S. Bloomfield, and R. J. Lipicky, *Circulation*, **45,** 800 (1972).

(3) R. Kuntzman, I. Tsai, R. Chang, and A. H. Conney, *Clin. Pharmacol. Ther.*, **11,** 829 (1970).

(4) J. Adir, P. K. Narang, J. Josselson, and J. H. Sadler, *N. Engl. J. Med.*, **300,** 1390 (1979).

(5) P. G. Harms and S. R. Ojeda, *J. Appl. Phys.*, **36,** 391 (1974).

(6) M. Gibaldi and D. Perrier, in "Pharmacokinetics, vol. 1, Drugs and the Pharmaceutical Sciences," J. Swarbrick, Ed., Dekker, New York, N.Y., 1975.

(7) A. J. Barr, J. H. Goodnight, and J. P. Sall, "SAS User's Guide," SAS Institute, Raleigh, N.C., 1979.

(8) J. L. Anderson, E. Patterson, J. G. Wagner, J. R. Stewart, H. L. Behm, and B. R. Lucchesi, "Abstract of Papers presented at the 29th Annual Session of American College of Cardiology," Houston, Tex., Mar. 1980.

(9) W. E. Yarger, H. S. Aynedjian, and N. Bank, *J. Clin. Invest.*, **51,** 625 (1972).

(10) E. M. Renkin and J. P. Gilmore, in "Handbook of Physiology, Section 8: Renal Physiology," J. Orloff, R. W. Berliner, and S. R. Geiger, Eds., American Physiological Society, Washington, D.C., 1973, pp. 185–248.

High-Performance Liquid Chromatographic Measurement of Cloprednol in Human Plasma

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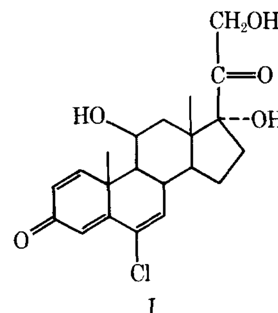
Abstract □ A rapid and specific high-performance liquid chromatographic method for the quantitative determination of cloprednol in human plasma is described. Samples were extracted using methylene chloride–ether (40:60) and then purified further by solvent and pH partitioning techniques. Cloprednol was analyzed using normal-phase chromatography and UV detection at 254 nm. The final recovery after losses during the cleanup procedure for cloprednol from human plasma was 80.8%. The lowest concentration that could be measured with confidence was 8 ng/ml.

Keyphrases □ Cloprednol—high-performance liquid chromatographic analysis, human plasma □ High-performance liquid chromatography—analysis, cloprednol in human plasma □ Anti-inflammatory agents—cloprednol, high-performance liquid chromatographic analysis, human plasma



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Cloprednol (6-chloro-11β,17α,21-trihydroxypregna-1,4,6-triene-3,20-dione, I) is a fast-acting corticoid used in the treatment of collagen and allergic diseases (1).

Recently published analytical techniques for the quantitative determination of 17-hydroxycorticoids in plasma include radioimmunoassays (2–5), competitive protein binding (6–8), GLC–mass spectrometry (9, 10), and high-performance liquid chromatography (HPLC) (11–15). The specificity in radioimmunoassays and competitive protein binding methods remains questionable due to the cross-reactivity by structurally similar compounds (16, 17). The GLC–mass spectrometric technique is specific but

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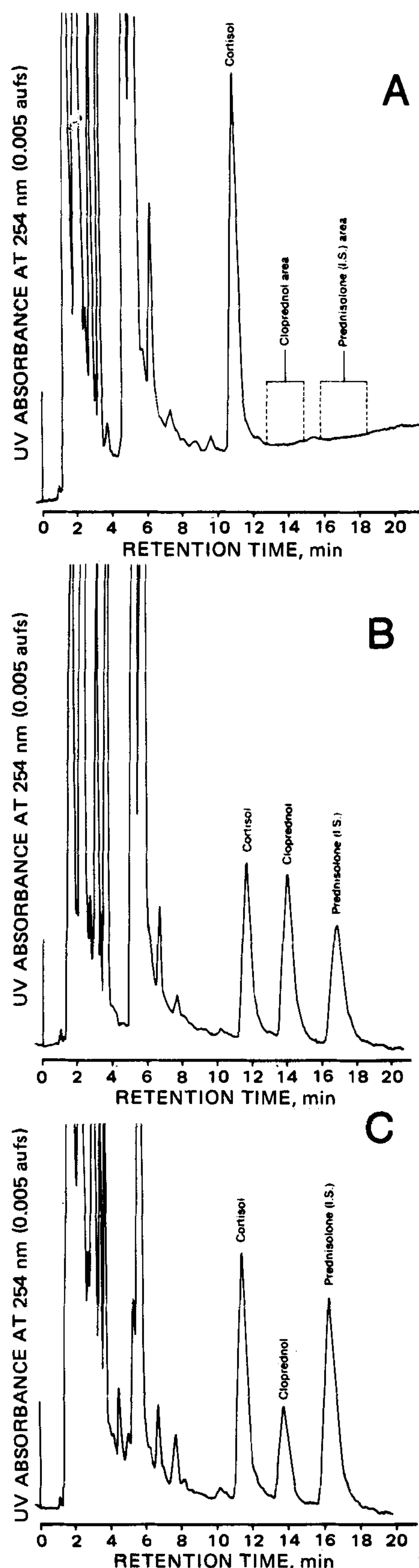

Figure 1—*HPLC chromatograms of human plasma assayed as described. Key: A, chromatogram from 3 ml of blank human plasma; B, chromatogram from 3 ml of human plasma spiked with 300 ng of cloprednol (100 ng/ml) and 200 ng of prednisolone (66.7 ng/ml) as the internal standard; and C, chromatogram of extracted human plasma sample (1 ml) 4 hr after an oral dose of 12.5 mg of cloprednol was diluted with blank human plasma to 3 ml and 200 ng of prednisolone (66.7 ng/ml) as an internal standard was added.*

Table I—Extraction Efficiency of [^{3}H]Cloprednol from 3.0 ml of Human Plasma

Extraction Step	Percent of Spiked [^{3}H]Cloprednol
Methylene chloride–ether extraction	88.7
Methanol–water layer	83.4
Methylene chloride–ether after washing (overall extraction)	80.4

requires extensive sample cleanup and time-consuming derivatization (9, 10).

A simple, sensitive, and specific HPLC method for the measurement of cloprednol in human plasma without derivatization is described here. By utilizing normal-phase chromatography, it was possible chromatographically to resolve structurally related endogenous corticoids like cortisol and drugs like prednisone. This method was applied to determine plasma concentrations in humans after the oral administration of 12.5 mg of cloprednol.

EXPERIMENTAL

Reagents and Materials—Cloprednol[1], [^{3}H]cloprednol[1], prednisolone[2], and a complete oxidizer cocktail[3] were used as received. Distilled-in-glass grade[4] methanol, hexane, and methylene chloride and absolute ethanol[5] were used. All other chemicals and reagents were reagent grade and were used as received.

Apparatus—Analyses were performed on a high-performance liquid chromatograph[6] at ambient temperature. The chromatograph was equipped with a fixed-wavelength (254 nm) spectrophotometer[7]. A commercial 4.6-mm × 25-cm prepacked normal-phase column[8] with a precolumn[9] (2 mm × 7.1 cm) was used. Samples were introduced onto the column through a septumless injector[10] with a 100-μl syringe.

A liquid scintillation spectrometer[11] was used for [^{3}H]cloprednol counting.

Chromatographic Conditions—The mobile phase was methylene chloride–ethanol–acetic acid (96:4:0.01) with the flow rate adjusted to 2.0 ml/min (1300 psi) at 0.005 aufs. The solvent mixture was degassed by stirring under vacuum before use. Prednisolone was the internal standard.

Extraction—Sample aliquots, 0.1–3 ml, were diluted with blank plasma to 3 ml. A 200-μl spiking solution of prednisolone, used as the internal standard [equivalent to 1 ng/μl of methanol–water (1:9)], was added. Additional water was added to bring the total volume to 3.5 ml. The mixture was extracted with 10 ml of 40% methylene chloride in ether by shaking on a reciprocal shaker for 10 min. After centrifugation, the organic layer was transferred to another tube and evaporated to dryness under nitrogen at 37°.

To the dry residue was added 2 ml of methanol containing 10% water. After the tubes were vortexed, the methanol–water layer was washed three times with hexane (3 ml). The hexane washings were discarded, and the remaining methanol–water layer was dried at 37° under nitrogen. The dry residue was reconstituted in 10 ml of 40% methylene chloride in ether, and the organic layer was washed with 2 ml of 0.1 *N* HCl.

After centrifugation, the acidic layer was pipetted out, and the organic layer was washed with 3 ml of 0.1 *N* NaOH. After centrifugation, the organic layer was transferred to a 15-ml conical centrifuge tube and evaporated to dryness under nitrogen at 37°. The residue was redissolved in 100 μl of methylene chloride by vigorous vortexing, and 50–75 μl was injected for quantitation.

Extraction Efficiency—To drug-free pooled plasma (3 ml) were

[1] Synthesized and supplied by Syntex Research, Palo Alto, Calif.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Oxifluor-H_2O, New England Nuclear, Boston, Mass.
[4] Burdick & Jackson Laboratories, Muskegon, Mich.
[5] Gold Shield, Terre Haute, Ind.
[6] Model 6000A, Waters Associates, Milford, Mass.
[7] Model 440, Waters Associates, Milford, Mass.
[8] LiChrosorb Si 60 5 μm, Altex, Berkeley, Calif.
[9] HC Pellisol, Whatman, Clifton, N.J.
[10] Model U6K, Waters Associates, Milford, Mass.
[11] Model 3330, Packard Instrument Co., Chicago, Ill.

Table II—Four Calibration Curves of Cloprednol Extracted from 3.0 ml of Pooled Human Plasma

Amount of Cloprednol Spiked, ng	Peak Height Ratio of Cloprednol to Internal Standard 1	2	3	4	Average	CV, %
25	0.11	0.11	0.12	0.11	0.11	4.44
50	0.24	0.21	0.25	0.24	0.24	7.37
100	0.43	0.49	0.49	0.43	0.46	7.53
200	0.98	0.99	0.95	0.89	0.95	4.72
300	1.38	1.42	1.37	1.34	1.38	2.40
Slope	0.0047	0.0048	0.0045	0.0045	0.0045	
y intercept	−0.0051	−0.0091	+0.0228	0.0013	0.0027	
Linear regression (r)	0.9983	0.9991	0.9995	0.9997	0.9997	

added a known amount of [^{3}H]cloprednol and 100 ng of cloprednol. The spiked pooled plasma sample was processed through the entire procedure. At each extraction step, an aliquot of the organic layer was taken; the amount of radioactivity extracted was determined by counting the aliquot in 10 ml of scintillation fluid[3].

RESULTS AND DISCUSSION

Cloprednol, a compound structurally similar to prednisolone, is a synthetic derivative of glycosteroids. It has an anti-inflammatory therapeutic potency twice that of prednisone and prednisolone on a milligram per kilogram basis (1).

The method described permits the analysis of underivatized cloprednol, thus making analysis simple and rapid. Cloprednol has three UV absorption peaks at 228, 256, and 299 nm with E_{max} values of 11,200, 10,400, and 10,400, respectively. In this method, cloprednol was analyzed at 254 nm because of the commercial availability of the filter for the UV detector.

Under the described chromatographic conditions, the retention times of cloprednol and prednisolone (internal standard) were 14 and 17 min, respectively. Blank plasma samples not spiked with cloprednol or internal standard were processed through the entire procedure. The chromatogram shown in Fig. 1A clearly indicates that there was no interference from endogenous substances at the retention times of cloprednol and the internal standard. The chromatogram of an extracted human plasma sample spiked with 300 ng of cloprednol and 200 ng of the internal standard is shown in Fig. 1B.

Plasma samples were extracted utilizing methylene chloride–ether (40:60). The extraction efficiency of cloprednol from 3.0 ml of human plasma was 88.7%. The extracts were purified further by solvent and pH partitioning techniques. Hexane was used to remove lipophilic substances, and acid–base washes were used to remove interference by endogenous substances.

The recovery of [^{3}H]cloprednol at each step during extraction was determined (Table I). The final recovery after losses during the cleanup procedure was 80.8%.

Linearity was established by preparing four calibration curves. The coefficients of variation (intraassay) at concentrations of 25, 50, 100, 200, and 300 ng/3.0 ml of plasma were 4.44, 7.37, 7.53, 4.72, and 2.40%, respectively (Table II). The calibration curve, as constructed by plotting the peak height ratio of cloprednol to the internal standard *versus* the amount of cloprednol spiked, was linear ($y = 0.9997$) over the range (25–300 ng/3 ml) studied.

The specificity of the method was confirmed by chemical-ionization mass spectral studies. The HPLC-eluted peak of cloprednol from a dosed plasma sample was collected and derivatized as reported previously (18). The mass spectrum obtained from the cloprednol standard and that from the substance eluted in the chromatographic peak were identical (Figs. 2A and 2B). Based on this information, the peak was identified as cloprednol.

To show the applicability of the described method, a plasma sample obtained 4 hr after oral administration of cloprednol (12.5 mg) was processed. The chromatogram of the extracted sample is shown in Fig. 1C.

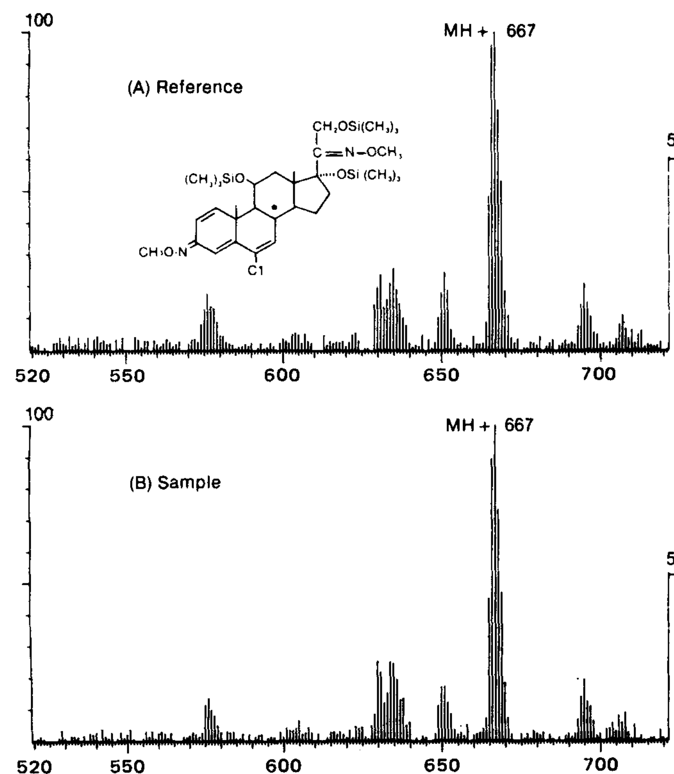

Figure 2—*Methane chemical-ionization mass spectra of methoxyimino trimethylsilyl derivative of cloprednol reference standard (A) and plasma extract (B).*

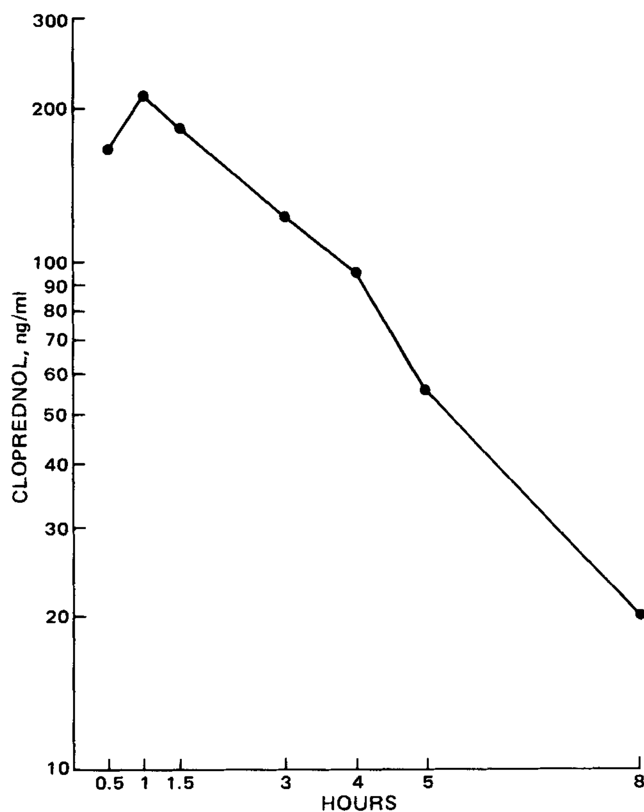

Figure 3—*Plasma cloprednol level–time profile of a human volunteer given a 12.5-mg oral dose.*

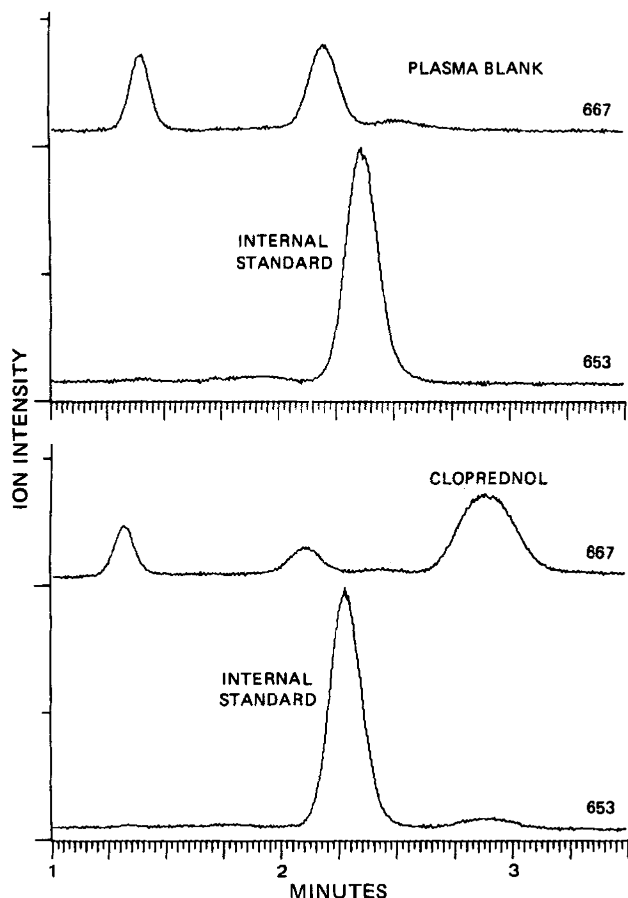

Figure 4—*Selected ion chromatograms of blank plasma (3 ml) spiked with the internal standard (top) and a plasma sample obtained 90 min after the administration of a 12.5-mg oral dose of cloprednol (bottom).*

The developed method also established a plasma level–time profile of a normal human volunteer following an oral dose of 12.5 mg of cloprednol (Fig. 3).

To validate the present HPLC method, plasma specimens were assayed by both HPLC and GLC–mass spectrometric[12] methods. To analyze the plasma samples by chemical-ionization mass spectrometry, the samples were extracted with ether and the extracts were cleaned by using miniature adsorption columns. A structural analog was used as an internal standard. The corticoids were converted to their methoxyimino trimethylsilyl derivatives to render them stable to analysis by GLC–mass spectrometry. A typical ion chromatogram obtained using GLC–mass spectrometry is shown in Fig. 4.

Numerous plasma samples from volunteers dosed with cloprednol were analyzed by both procedures. The excellent correlation between the two methods is indicated by the slope of 1.04 obtained from the scatter diagram shown in Fig. 5.

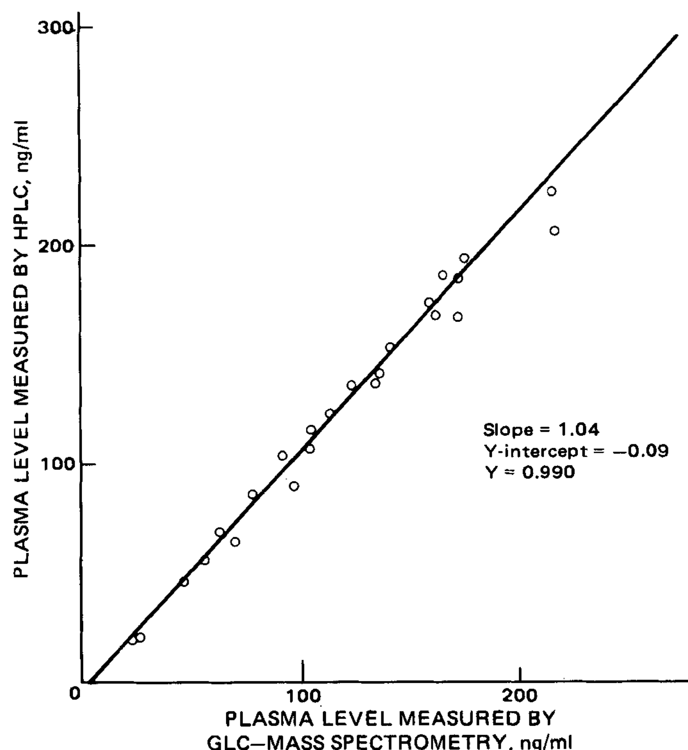

Figure 5—*Correlation between HPLC and GLC–mass spectrometric methods for plasma cloprednol analysis.*

REFERENCES

(1) E. Ortega, C. Rodriquez, L. J. Strand, S. Bessler, and E. Segre, *J. Clin. Pharm.*, **16,** 122 (1976).

(2) W. A. Colburn and R. H. Buller, *Steroids*, **21,** 833 (1973).

(3) W. A. Colburn, *J. Clin. Endocrinol. Metab.*, **41,** 868 (1975).

(4) J. Chakraborty, J. English, V. Marks, M. C. Dumasia, and D. J. Chapman, *Br. J. Clin. Pharmacol.*, **3,** 903 (1976).

(5) E. Rolleri, M. Zannino, S. Orlandini, and R. Malvano, *Clin. Chim. Acta*, **66,** 319 (1976).

(6) D. H. Sandberg, C. Z. Bacallao, and W. W. Cleveland, *Biochem. Med.*, **4,** 383 (1970).

(7) A. K. Turner, C. J. Carroll, J. L. Pinkus, D. Charles, and S. C. Chattoraj, *Clin. Chem.*, **19,** 731 (1973).

(8) G. C. Cashmore, J. D. Few, and C. H. Fowles, *Ann. Clin. Biochem.*, **13,** 371 (1976).

(9) I. Bjorkhem, R. Blomstrand, O. Lantto, A. Lof, and L. Svensson, *Clin. Chim. Acta*, **56,** 241 (1974).

(10) J. P. Thenot and E. C. Horning, *Anal. Lett.*, **5,** 21 (1972).

(11) F. K. Trefz, D. J. Byrd, and W. Kochen, *J. Chromatogr.*, **107,** 181 (1975).

(12) J. C. K. Loo, A. G. Butterfield, J. Moffatt, and N. Jordan, *ibid.*, **143,** 275 (1977).

(13) J. C. K. Loo and N. Jordan, *ibid.*, **143,** 314 (1977).

(14) G. E. Reardon, A. M. Caldarella, and E. Canalis, *Clin. Chem.*, **25,** 122 (1979).

(15) J. Q. Rose and W. J. Jusko, *J. Chromatogr.*, **162,** 273 (1979).

(16) S. W. Schalm, W. H. J. Summerskill, and V. L. W. Go, *Mayo Clin. Proc.*, **51,** 761 (1976).

(17) A. V. Tembo, M. R. Hallmark, E. Sakmar, H. G. Bachmann, D. J. Weidler, and J. G. Wagner, *J. Pharmacokinet. Biopharm.*, **5,** 257 (1977).

(18) S. B. Matin and B. Amos, *J. Pharm. Sci.*, **67,** 923 (1978).

[12] Unpublished data.