Table I—Recoveries of I–III from Plasma

Compound	Dog Plasma			Human Plasma		
	n	Recovery, %	*CV*	*n*	Recovery, %	*CV*
I	7	83	7	16	67	10
II	8	59	9	16	65	8
III	8	71	12	16	59	16

range, having a standing current of 4.7×10^{-9} amp in the pulse mode with a width of 5 μsec at 44 v and a pulse interval of 220 μsec.

The partition ratios for I, II, and III between ether and physiological saline proved to be ~6, 0.5, and 0.35, respectively. The recovery from spiked samples for each substance is given in Table I.

After considerable usage, a column that otherwise appeared to be performing satisfactorily gave rise to an artifact upon injection of I. This artifact had the same retention time as II (Fig. 3). For this reason, the I standard was chromatographed at the beginning, middle, and end of the day without the two isosorbide mononitrates. The column was discarded as soon as the artifact peak was detected; however, additional useful column life was obtained by removing the top 5 cm of packing from the inlet end of the column and replacing with new conditioned packing. Under continuous use, a column lasted for approximately 1 month.

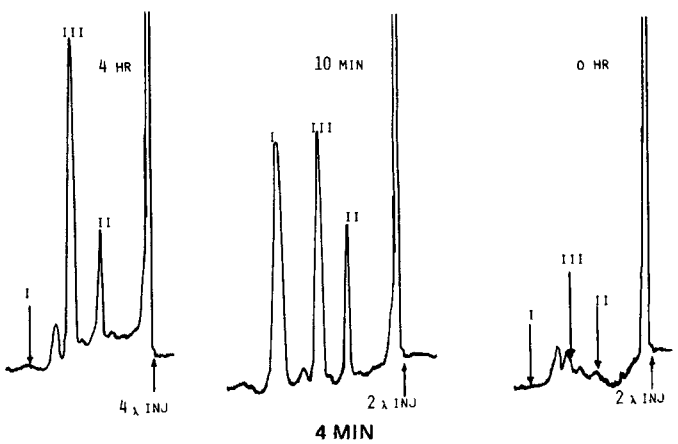

Figure 6—*Chromatograms illustrating the determinations of I–III in human plasma after sublingual administration of 10 mg of I.*

The average plasma I–III levels in four dogs are shown in Fig. 4. The results are in qualitative agreement with the data obtained in a ^{14}C-labeled study (4). There were very low and erratic blood levels of I, consistently higher levels of II, and much higher and prevalent levels of III. Very rapid absorption of I took place, since I levels were detected at 5 min postadministration.

The average plasma levels of I–III in two human volunteers are depicted in Fig. 5. Significant I levels were detected at 10, 20, and 30 min and up to 1 hr postadministration. The two metabolites reached a maximum at 30 min, and the III levels were approximately twice the II levels. Typical chromatograms are presented in Fig. 6.

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Selective Photometric Determination of Betamethasone Benzoate and Other 21-Hydroxycorticosteroids

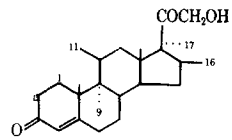
LESTER CHAFETZ ˣ and DIMITRI C. TSILIFONIS

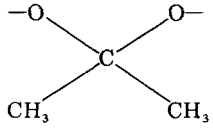
Abstract □ Condensation of the glyoxal obtained by cupric acetate oxidation of 21-hydroxycorticosteroids with acetous phenylhydrazine reagent affords a near UV chromophore. All of the tested corticosteroids, including triamcinolone acetonide, which gives low color yields in the Porter–Silber reaction and its Lewbart–Mattox modification, gave similar absorption maxima (362–370 nm) and molar absorptivities (ϵ = 17,000–20,500). Since corticosteroid 21-esters and oxidation products do not undergo the reaction, the assay method based on it is stability indicating for betamethasone benzoate and the other test compounds. Procedures are described for the assay of two topical betamethasone benzoate preparations and hydrocortisone and prednisolone tablets; recovery and precision data are given.

Keyphrases □ Betamethasone benzoate—UV photometric analysis, bulk drug and pharmaceutical formulations □ UV photometry—analysis, betamethasone benzoate and other 21-hydroxycorticosteroids, bulk drug and pharmaceutical formulations □ 21-Hydroxycorticosteroids, various—UV photometric analysis, bulk drug and pharmaceutical formulations □ Glucocorticoids, various—UV photometric analysis, bulk drug and pharmaceutical formulations

The Porter–Silber reaction (1) for the colorimetric determination of corticosteroids is applicable only to steroids with a 17-dihydroxyacetone side chain ($COHCOCH_2OH$) or their derivatives that are readily hydrolyzed in the strongly acid reaction medium. Its mechanism (2, 3) was elucidated as a two-stage reaction: acid-catalyzed rearrangement of the side chain to a 17-deoxy-20-one-21-al ($CHCOCHO$), followed by condensation of this glyoxal

Table I—Structures and Absorptivity Data for 21-Hydroxycorticosteroids



Steroid	Position 1–2	9	11	16	17	Absorptivity Data[a] λ_{max}, nm	α, liters/g cm	ϵ, liters/mole cm
Betamethasone	Unsaturated	α-F	β-OH	β-CH_3	α-OH	362	52.1	20,500
Betamethasone benzoate	Unsaturated	α-F	β-OH	β-CH_3	α-$OCOC_6H_5$	364	40.8	20,300
Corticosterone	Saturated	α-H	β-OH	H	H	352	56.0	19,400
Dexamethasone	Unsaturated	α-F	β-OH	α-CH_3	α-OH	362	47.5	19,700
Hydrocortisone	Saturated	α-H	β-OH	H	α-OH	364	51.9	19,000
Prednisolone	Unsaturated	α-H	β-OH	H	α-OH	366	47.0	17,000
Prednisone	Unsaturated	α-H	=O	H	α-OH	366	49.3	17,600
Triamcinolone acetonide	Unsaturated	α-F	β-OH			370	44.4	19,800

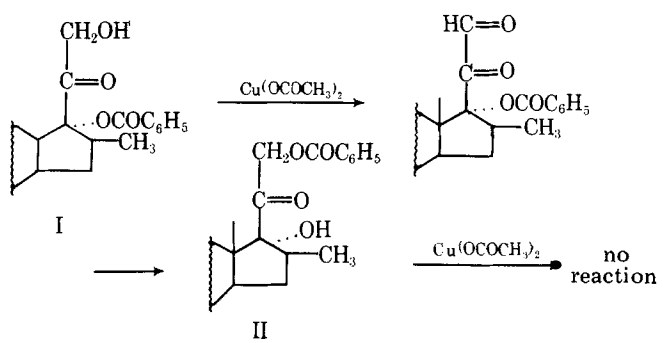
[a] Obtained using the proposed method.

with phenylhydrazine to form the protonated 21-monophenylhydrazone, the characteristic chromophore.

Lewbart and Mattox (4) extended the Porter–Silber reaction to the microdetermination of 17-deoxy-α-ketolic steroids, using oxidation with methanolic cupric acetate to produce the requisite glyoxal intermediate. This procedure was shown to be selective for betamethasone benzoate[1] (I), the 17-substituted ester, in the presence of its acyl migration product, betamethasone 21-benzoate (II) (Scheme I) (5).

The chromogenic reaction proceeded much more sluggishly with betamethasone benzoate than with other corticosteroids tested, necessitating heating at 60° for 15 min. Although the method proved useful in the assay of pharmaceutical preparations of the steroid, the exothermic reaction of the strongly acid reagent with the methanolic cupric acetate solution and the subsequent heating step were difficult to reproduce and reduced precision when many replicate determinations were performed.

Further study of reaction conditions revealed that phenylhydrazone formation could be accomplished facilely by adding an acetous solution of phenylhydrazine hydrochloride to the dried residue obtained by evaporating a mixture of cupric acetate and the steroid in methanol. Simple dilution of the reaction mixture with alcohol provides a means for the UV spectrophotometric determination of 21-hydroxycorticosteroids at about 360 nm as the (unprotonated) 21-phenylhydrazone. Details of the procedure and examples of its scope and applications are presented.



Scheme I

EXPERIMENTAL

Materials[2]—Betamethasone benzoate and betamethasone 21-benzoate were used as received[3]. Betamethasone, dexamethasone, hydrocortisone, prednisolone, prednisone, and triamcinolone acetonide were USP or NF grades. Corticosterone was obtained commercially[4].

Cupric Acetate Solution—Dissolve 100 mg of cupric acetate monohydrate in methanol and dilute to 100.0 ml.

Phenylhydrazine Reagent—Dissolve 100 mg of phenylhydrazine hydrochloride in 1.5 ml of water, add 8.5 ml of acetic acid, and mix.

General Procedure for Test Compounds—Accurately weigh a suitable amount of 21-hydroxycorticosteroid, dissolve it in methanol, and dilute quantitatively and stepwise to obtain a solution containing about 50 μg/ml. Add 0.1 ml of cupric acetate solution to 1.0 ml of the test solution in a test tube, mix, let stand 10 min, and evaporate to dryness in a nitrogen stream at about 45°. Dissolve the residue in 0.2 ml of phenylhydrazine reagent with the aid of a vibrating mixer[5]. Let stand at room temperature for 5 min, then add 5.0 ml of alcohol by pipet, and mix. Determine the absorbance of the solution at the wavelength of maximum absorbance (Table I) against a reagent blank.

Procedure for Betamethasone Benzoate Gel or Viscous Solution, 0.025%—Accurately weigh about 2 g of the product, transfer it to a 125-ml separator with about 20 ml of water, and add 1 ml of saturated sodium acetate solution. Extract the mixture with three 25-ml portions of chloroform, shaking about 2 min with each portion. Combine the extracts in a second separator, wash with 10 ml of water, and filter the chloroform through paper into a conical flask with the aid of several small portions of chloroform. Evaporate the chloroform nearly to dryness on the steam bath with the aid of a nitrogen stream, removing the last traces of solvent without heating. Dissolve the residue in exactly 10.0 ml of methanol.

Transfer 1.0 ml of the solution under test, 1.0 ml of a standard preparation containing 50 μg/ml of betamethasone benzoate reference standard in methanol, and 1.0 ml of methanol (as the blank) to separate test tubes. Add 0.1 ml of cupric acetate solution to each, mix, and let stand 10 min. Evaporate the contents of each tube to dryness in a nitrogen stream at about 45°. Then dissolve the residue in 0.2 ml of phenylhydrazine reagent with a vibrating mixing device. Let stand at room temperature for 5 min, then add 5.0 ml of alcohol by pipet, and mix.

[1] USAN name for 9-fluoro-11β,17,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione 17-benzoate.

[2] The pharmaceutical preparations tested were formulated at Warner–Lambert Research Institute.

[3] Warner–Lambert Research Institute.

[4] Mann Research Laboratories.

[5] Vortex Genie.

Table II—Relationship of Absorbance and Concentration of Three 21-Hydroxycorticosteroids

Hydrocortisone		Prednisone		Triamcinolone Acetonide	
Concentration, μg/ml	Absorbance at 364 nm	Concentration, μg/ml	Absorbance at 365 nm	Concentration, μg/ml	Absorbance at 370 nm
10.16	0.100	10.8	0.103	9.6	0.095
20.32	0.200	32.4	0.292	28.8	0.272
30.48	0.295	54.0	0.497	48.0	0.453
40.64	0.422	—	—	—	—
50.80	0.515	—	—	—	—

Determine the absorbance of the solution from the test preparation and from the standard in 1-cm cells at the wavelength of maximum absorbance at about 364 nm, using the reagent blank in the reference cell. Calculate the quantity, in milligrams, of betamethasone benzoate in the portion of the product taken for assay by the formula $0.01C(A_U/A_S)$, where C is the exact concentration, in micrograms per milliliter, of the standard solution, and A_U and A_S are the absorbances of the solutions from the product and the standard, respectively.

Procedure for Tablets—Weigh and finely powder not less than 20 tablets. Transfer an amount of powdered tablet equivalent to about 5 mg of steroid to a separator containing 20 ml of water. Extract the mixture with three 25-ml volumes of chloroform and one 20-ml volume, filtering the extract through cotton into a 100-ml volumetric flask. Then dilute the solution to volume with chloroform and mix.

Transfer 2.0-ml portions of the solution under test and of a solution of the appropriate USP reference standard in chloroform, containing a known concentration of about 50 μg/ml, to test tubes. Evaporate the solutions to dryness with a nitrogen stream, and dissolve the residues with 1.0 ml of methanol. Follow the *General Procedure*, beginning with: "Add 0.1 ml of cupric acetate . . . ," except add 10.0 ml of alcohol.

Determine the absorbance of the solutions, *versus* a reagent blank, in 1-cm cells in a suitable spectrophotometer at the wavelength of maximum absorbance (Table I). Calculate the quantity, in milligrams, of 21-hydroxycorticosteroid in the sample taken by the formula $0.1C(A_U/A_S)$, where C is the exact concentration, in micrograms per milliliter, of the solution of the reference standard, and A_U and A_S are the absorbances of the solutions from the tablet and the standard, respectively.

RESULTS AND DISCUSSION

Scope and Selectivity—The structures, wavelength maxima, and absorptivity data of the steroids tested are presented in Table I. The molar absorptivities varied considerably less with structure than was observed previously (5).

Because the cupric acetate oxidation proceeds only with free 21-hydroxy compounds, 21-esters are not chromogenic in the reaction. This condition was verified in trials with betamethasone 21-benzoate, hydrocortisone 21-acetate, and prednisolone 21-phosphate, none of which provided a near-UV chromophore in the proposed method. The chromophore obtained in the reaction is the 21-monophenylhydrazone of the steroids.

Absence of a chromophore with the corticosteroid 21-esters tested shows that the 1,4-dien-3-one and 4-en-3-one conjugated ketone systems are not chromogenic with phenylhydrazine. Androsten-3,11,17-trione also gave no color under the reaction conditions of the proposed method, showing no interference from 11- and 17-keto functions and confirming the absence of interference from the 4-en-3-one system. Moreover, addition of 5 ml of a mixture of two volumes of 80% sulfuric acid and one volume of methanol to the reaction mixture in place of 5 ml of alcohol provided a chromophore with maximum absorbance at 425 nm for all steroids in Table I, the wavelength at which the protonated 21-monophenylhydrazone of the Lewbart–Mattox method (4) is maximum. This color, however, was unstable.

Table III—Results of Intralaboratory Collaborative Study on Betamethasone Benzoate

Betamethasone Benzoate, μg/ml	Absorbance Obtained by Chemist				
	A	B	C	D	E
4.81	0.186	0.188	0.196	0.196	0.190
9.62	0.368	0.376	0.399	0.398	0.390
14.42	0.554	0.564	0.569	0.575	0.588
19.23	0.749	0.758	0.754	0.772	0.770
24.04	0.949	0.951	0.881	0.949	0.955

Table IV—Recovery and Precision on 21-Hydroxycorticosteroid Products

Trial	Betamethasone Benzoate			Prednisolone Tablets, 5 mg
	Viscous Solution, 0.025%		Gel, 0.025%	
1	99.8	98.6	99.0	101.6
2	98.9	98.3	99.0	99.8
3	98.5	99.3	97.2	100.8
4	98.5	99.1	97.4	100.0
5	100.7	99.3	100.0	99.2
6	99.3	100.0	99.6	99.4
Average recovery	99.3	99.1	98.7	100.1
RSD, %	0.89	0.68	1.13	0.96

Görög and Szepesi (6) used a similar scheme for the photometric determination of 21-hydroxycorticosteroids: oxidation with cupric acetate in methanol, followed by condensation with *o*-phenylenediamine or 4,5-dimethyl-*o*-phenylenediamine to form quinoxalines with maxima at 318 or 331 nm, respectively, in methanol and at 331 or 351 nm, respectively, in 1 *N* hydrochloric acid. Molar absorptivities were higher in the acid medium but were about half those obtained in the procedure described here. Their method is inapplicable to betamethasone benzoate, possibly because of the steric hindrance of the 16- and 17-substituents to quinoxaline ring closure.

Stability of Chromophore—The absorbance of the colors obtained with betamethasone benzoate, hydrocortisone, and triamcinolone acetonide was constant for at least 1 hr by scanning their spectra on a recording spectrophotometer. Spectrograms after 1 hr or more were superimposable on those determined immediately after color development.

Linearity and Precision—Solutions of hydrocortisone, prednisone, and triamcinolone acetonide, containing 10–50 μg/ml in methanol, were analyzed by the proposed method. The results (Table II) indicate that the chromophore follows Beer's law. To determine the effect of interanalyst variation, one chemist prepared a stock solution of betamethasone benzoate in methanol and five different chemists determined the absorbance of scalar concentrations at five levels by the proposed method (Table III). Each obtained rectilinear results.

Precision and Recovery on 21-Hydroxycorticosteroid Products—Experiments with placebo formulations of the gel and viscous solution and of steroid tablets showed no interference. Recovery studies were run by adding a weighed amount of the reference standard to formulation excipients or a placebo formulation and performing the assay six times to obtain the average recovery and an estimate of precision as the relative standard deviation (*RSD*), calculated by the method of Dean and Dixon (7). Two separate experiments were run with the viscous solution (Table IV). Precision data were obtained similarly with formulated products. The betamethasone benzoate gel assay had a relative standard deviation of 1.40%; for 10-mg hydrocortisone tablets, a relative standard deviation of 1.65% was found on six assays of a composite sample.

Limitations of Method—Attempts to extend the method to the assay of betamethasone benzoate creams and lotion have so far been unsuccessful owing to coextraction of materials that complex with or precipitate cupric acetate. Formulation excipient materials that form near-UV chromophores with the phenylhydrazine reagent could interfere, but none of these has been encountered in these studies.

Further Applications—The proposed procedure could easily be adapted to estimation of steroidal 21-alcohol as a synthesis precursor or hydrolysis product in corticosteroid 21-esters such as acetates, hemisuccinates, and phosphates.

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High-Pressure Liquid Chromatographic Analysis of Cimetidine, a Histamine H_2-Receptor Antagonist, in Blood and Urine

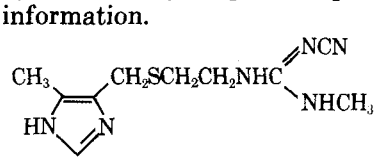
W. C. RANDOLPH [x], V. L. OSBORNE, S. S. WALKENSTEIN, and A. P. INTOCCIA

Abstract □ A method is described for extraction of cimetidine, a histamine H_2-receptor antagonist, from whole blood and urine with subsequent analysis by high-pressure liquid chromatography (HPLC). The drug is extracted from biological fluids with 1-octanol and back-extracted into dilute acid and then into a small volume of ethanol by saturation with potassium carbonate. HPLC analysis is performed on a column of 5-μm silica with a mixed mobile phase consisting primarily of acetonitrile. The method measures concentrations of cimetidine as low as 0.05 μg/ml and is reproducible. Blood levels and urinary excretion data obtained with the analytical procedure are given for a group of human subjects who received 200-mg oral doses of cimetidine.

Keyphrases □ Cimetidine—high-pressure liquid chromatographic analysis, blood and urine □ High-pressure liquid chromatography—analysis, cimetidine, blood and urine □ Histamine H_2-receptor antagonists—cimetidine, high-pressure liquid chromatographic analysis, blood and urine

The two types of pharmacological receptors in the mediation of histamine-induced responses are referred to as H_1 and H_2 (1, 2). The classical antihistaminic compounds are effective through antagonism of the action of histamine H_1-receptor sites. Several new compounds, defined as H_2-receptor antagonists, have been effective in reducing histamine-induced gastric acid secretion. Cimetidine[1], *N″*-cyano-*N*-methyl-*N′*-[2-[[(5-methyl-1*H*-imidazol-4-yl)methyl]thio]ethyl]guanidine (I), is currently undergoing trials to assess its effectiveness in the treatment of gastric and duodenal ulcer patients. The chemistry and pharmacology of this drug were discussed previously (3).

This paper describes methodology for extraction and concentration of cimetidine from whole blood and urine with subsequent analysis by high-pressure liquid chromatography (HPLC). Numerous samples from various clinical studies were analyzed by this method, which proved highly satisfactory in providing necessary bioavailability information.



I

[1] Tagamet, Smith Kline & French Laboratories, Philadelphia, Pa.

EXPERIMENTAL

Instrumentation—A constant flow liquid chromatograph[2] was used in conjunction with a variable wavelength UV detector[3] set at 228 nm. The stainless steel column, 3.2 mm i.d. × 25 cm long, was obtained prepacked with 5-μm silica[4]. A rotary injection valve[5] was used for introducing samples at the column inlet.

Reagents—Certified grade 1-octanol[6] was used for extraction of cimetidine from biological fluids. The HPLC mobile phase consisted of UV grade acetonitrile[7], glass-distilled methanol[7], glass-distilled water, and concentrated ammonium hydroxide (1000:60:20:5).

Standard Solutions (Blood)—Dissolve 10 mg of cimetidine in 10 ml of methanol. Add 0.05 ml of this drug solution to 25 ml of heparinized whole human blood and mix thoroughly. From this blood stock, containing 2.0 μg of cimetidine/ml, make appropriate dilutions with heparinized whole blood to provide a concentration range of 0.1–2.0 μg/ml. Weigh 30 mg of metiamide, *N*-methyl-*N′*-[2-[[(5-methyl-1*H*-imidazol-4-yl)methyl]thio]ethyl]thiourea (internal standard), into a 100-ml volumetric flask. Dissolve and bring the contents to volume with methanol.

Standard Solutions (Urine)—Dissolve 50 mg of cimetidine in 10 ml of methanol. Pipet 1 ml of the drug solution into a 25-ml volumetric flask, and bring to volume with control human urine. Make dilutions of the urine stock (containing 200 μg of cimetidine/ml) with control urine to provide a concentration range of 10–150 μg/ml. Dissolve 30 mg of metiamide in 10 ml of methanol for use as the internal standard.

Extraction (Blood)—Heparinize and freeze whole blood samples as quickly as possible after collection. Prior to extraction, thaw the samples and pipet 5-ml aliquots of the lysed blood into 40-ml centrifuge tubes. Add 10 μl of the internal standard solution (containing 3 μg of metiamide) to each sample and adjust to pH 9 with 1 *N* NaOH. Add 10 ml of 1-octanol to each tube, place horizontally on a reciprocating shaker, and shake 15 min at low speed (about 60 oscillations/min).

After shaking, centrifuge the tubes briefly to obtain a sharp separation of layers. With a pipet, remove 9.5 ml of the octanol layer to a second 40-ml centrifuge tube, add 5 ml of 0.03 *N* HCl, and shake horizontally on a reciprocating shaker at high speed (about 120 oscillations/min). Again, centrifuge to achieve phase separation and aspirate and discard the octanol layer. Transfer 4.5 ml of the aqueous phase to a 12-ml centrifuge tube, add 0.3 ml of absolute ethanol, and vortex briefly to mix. Add sufficient granular potassium carbonate to saturate the solution (about 5.5 g), and vortex vigorously for 30 sec. Centrifuge the tubes at 1300×*g* for 5 min, thereby separating the ethanol as a layer over the aqueous phase. Remove 0.2 ml of the ethanol fraction to a small vial for HPLC analysis.

[2] Perkin-Elmer 1220.
[3] Perkin-Elmer LC-55.
[4] Altex Scientific Inc., Berkeley, Calif.
[5] Valco Instruments Co., Houston, Tex.
[6] Fisher Scientific Co.
[7] Burdick and Jackson.