Determination of the Major Component of Some U.S.P. Adrenocortical Steroids

By FRIEDA M. KUNZE and JOEL S. DAVIS

The determination of the total steroid content and the isolation and determination of the major steroid component in pharmaceuticals by continuous ascending chromatography and a blue tetrazolium colorimetric procedure are described. Isolation of the major steroid is achieved in 1.5 to 2 hours on 8 × 8 in. paper. The method has been applied to products containing prednisolone, hydrocortisone, prednisone, and the acetates of prednisolone, hydrocortisone, and cortisone. Accuracy within plus or minus 2 per cent was achieved.

SYNTHETIC adrenal corticoids are prepared from closely related intermediates. Since the finished product may contain a number of such intermediates in varying amounts, a method to determine the ratio of the major component to the total steroid content is desirable. Polarography, gas chromatography, and gradient elution techniques were considered; however, a paper chromatographic technique was selected for its specificity and the simplicity of the equipment required.

The method presented here is not intended for the detection of every possible intermediate or side reaction product obtained by all of the various methods of production, but it does measure with an accuracy of ±2% both the total amount of steroids present and also the major steroid fraction. Prednisolone, hydrocortisone, prednisone, and the acetates of prednisolone, hydrocortisone, and cortisone in tablets have been analyzed successfully.

The procedure is divided into three steps: (*a*) extraction of the total steroid fraction of the tablets, (*b*) paper chromatographic isolation of the major component and its quantitative removal from the paper, and (*c*) colorimetric measurement of both the total steroid content and the paper fractionated component by a blue tetrazolium procedure previously described (1).

EXPERIMENTAL

Materials

(Reagent grade materials are used except as noted.) Materials used include alcohol U.S.P.; methylcyclohexane, b.p. 100–101°; formamide (redistilled reagent grade solvent); blue tetrazolium solution[1]—dissolve 50 mg. of the salt in 10 ml. of alcohol; and dilute tetramethylammonium hydroxide—dilute 1 ml. of tetramethylammonium hydroxide T.S. to 20 ml. with alcohol.

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[1] Dajac, B. T., The Borden Co., Philadelphia, Pa., has been found satisfactory and was used for these studies.

Apparatus

Chromatography Chamber.—Use a rectangular tank, about 22.5 cm. in height and 22.5 × 8.75 cm. in its other dimensions, equipped with a support for holding a 4 × 20 cm. chromatographic tray about 4 cm. from the tank bottom. Fit the tank with a lid having a slot approximately 22 cm. long and 0.6 mm. wide. Slot width is important. The lid is available from the Arthur H. Thomas Co., Philadelphia, Pa., or it can be made with glass strips separated by stainless steel shims. The strips are taped together with 0.75-in. plastic tape and may be used repeatedly without retaping.

Source of Ultraviolet Light and Viewing Apparatus.—Use a light source having an intensity of approximately 1.4 amp. at 2537 Å. units, suspended no less than 9 in. above the paper. [A Chromato-Vue model 3-C Black Light (Eastern Corp., Bayside, N. Y.) apparatus is convenient.]

Standard Preparations

Using an accurately weighed sample of U.S.P. reference standard of the steroid under test, prepare a chloroform solution containing approximately 1 mg./ml. Solution of the standard may be facilitated by the prior addition of 0.2 ml. of alcohol to the volumetric flask for each 10 ml. of chloroform to be used.

(*Caution:* These compounds are unstable in some lots of chloroform and may break down to a number of fractions in 1 to 7 days.)

Test Preparations

Bulk Steroids.—Transfer about 10 mg. of steroid, accurately weighed, to a 10-ml. volumetric flask containing 0.2 ml. of alcohol. After it is dissolved, add chloroform to volume and mix.

Steroids in Tablets.—Weigh and finely powder not less than 10 tablets. Weigh accurately a portion of powder equivalent to about 10 mg. of the active ingredient and transfer to a 125-ml. separator with the aid of 15 ml. of 8% sodium bicarbonate solution. Extract the steroid using four 25-ml. portions of chloroform, filtering each portion through chloroform-washed glass wool into a 150-ml. beaker.

Evaporate the chloroform to a volume of about 2 to 3 ml. on a steam bath with a gentle current of air. Avoid evaporating to dryness. Transfer the solution quantitatively to a 10-ml. volumetric flask with several small washings of chloroform and make to volume.

TABLE I.—CHROMATOGRAPHIC SOLUTIONS

Steroid	Mobile Solvent	Development Time, hr.
Prednisolone	Chloroform	2
Hydrocortisone	Chloroform	1.5
Prednisone	Chloroform:methylcyclohexane (30:7)	2
Prednisolone acetate	Chloroform:methylcyclohexane (30:15)	2
Hydrocortisone acetate	Chloroform:methylcyclohexane (30:15)	1.5
Cortisone acetate	Chloroform:methylcyclohexane (30:20)	1.5

Paper Chromatography

Prepare one paper for the test preparation and one for the corresponding reference standard. Select sheets of Whatman No. 1 filter paper approximately 20 cm. square and draw a line across each 1.25 cm. from the top and a starting line 2.5 cm. from the bottom. Mark margins on the latter at points 4 cm. from the left edge and 5 cm. from the right edge of the paper. The steroid to be determined will be streaked on the starting line between these margins. At a point 2.5 cm. from the right side, place another dot for spotting the reference standard. The reference standard used with the test preparation serves to identify the major steroid component.

Immerse the papers in the immobile solvent (formamide 40:methanol 60) for 20 seconds and remove the excess solvent by blotting lightly and uniformly between sheets of filter paper (Whatman No. 1 or the equivalent). Place the uniformly impregnated paper on a fresh piece of filter paper and allow it to dry for 10 minutes before spotting. With a micropipet, streak 0.200 ml. of the *Test Preparation* across the starting line superimposing successive portions as closely as possible. During the streaking, evaporate the solvent with a gentle current of air. Carefully blow out the pipet and wash the tip with 1 or 2 drops of chloroform. The formamide is eluted from the area by the streaking process and should be replaced with about 0.025 ml. of immobile solvent. Apply 0.010 ml. of the reference standard solution to the starting line at the designated point and again replace the eluted formamide with about 0.005 ml. of immobile solvent.

In a similar fashion, streak the second paper with 0.200 ml. of the appropriate reference standard, omitting the identity spot. The same pipet should be used for streaking the solutions on all papers.

Place the appropriate mobile solvent (Table I) in the tray of the tank about 10 minutes before beginning development. The sample and standard papers are developed in separate tanks. Insert the paper through the slot in the tank lid so that 1.25 cm. project about the lid and secure it with stainless steel pins. Set the cover with the suspended paper on the tank, insuring that the paper will dip into the mobile solvent. Seal the cover to the tank with masking tape and allow the chromatogram to develop for the length of time indicated in Table I.

Remove the chromatogram and air dry it for about 2 or 3 minutes. Place it, spotted side up, on a clean glass plate at least 9 in. from the light source equipped with a 2537 Å. unit filter. Rapidly identify the major steroid component by comparing it with the corresponding reference standard and outline the area covered by it. During this procedure, minimum exposure to ultraviolet light is imperative, but with reasonable caution, no loss is incurred. With experience, the marking can be accomplished within 10 to 15 seconds.

Carefully cut the encircled major component area from the paper. To facilitate extraction, cut it into about 10 pieces and transfer them to a 125-ml. separator. Avoid the use of Teflon-stoppered separators as the paper pulp damages the stopcock. Add 10 ml. of a 1% alcohol solution and extract with four 20-ml. portions of chloroform shaking vigorously each time for 1 minute. The paper should be pulplike when the extraction is completed. Filter the chloroform extracts through chloroform-washed glass wool into a 125-ml. glass-stoppered conical flask and evaporate the combined extracts to complete dryness on a steam bath with the aid of a gentle stream of air. Treat the developed chromatogram of the reference standard in a similar manner.

Colorimetric Procedure

Into separate glass-stoppered 50-ml. conical flasks pipet 0.200 ml. of the appropriate standard preparation and 0.200 ml. of the sample preparation (use the same pipet for measuring both samples). Evaporate the solvent to dryness on a steam bath with gentle current of air.

To each of these flasks and to the flasks containing the dried residues of the chromatographed sample and standard, add 20.0 ml. of alcohol. Stopper and swirl the four flasks to effect solution. To a fifth flask add 20.0 ml. of alcohol to serve as a reagent blank. To each flask, add 2.0 ml. of blue tetrazolium solution, mix, then add 2.0 ml. of dilute tetramethylammonium hydroxide solution to each flask and mix. Allow to stand in the dark for 90 minutes; with a suitable spectrophotometer, determine the absorbance of the solutions at the absorption peak near 525 mμ relative to the reagent blank.

DISCUSSION AND RESULTS

The U.S.P. extraction procedure was modified by use of the 8% sodium bicarbonate solution since the current official procedure permitted the extraction of excipients which interfered with the resolutions of the steroids on the chromatograms.

The paper chromatographic method used is a modified form of the ascending continuous-flow technique described by Fischbach and Levine (2), and Mitchell (3). Using this technique, the mobile solvent can ascend the paper for an indefinite period, since the portion of the paper that emerges from the tank through the slit in the cover acts as a wick for the evaporation of the mobile solvent. The major advantage of this technique is that it makes possible the use of a short paper for the rapid resolution of closely related compounds while maintaining discrete, sharply defined zones. Conventional R_f values are meaningless here, but the systems are designed to move the major component from the line of origin to a point midway on the paper in 1.5 to 2 hours. Because of the small chamber volume

and short development time, only a short chamber equilibration period and minimum temperature control are required.

Initially, a series of control samples was carried through the paper chromatographic separation, elution, and the color development stages. These control samples were prepared mixtures of prednisolone containing hydrocortisone and various other steroids likely to be present in commercial preparations of these products. The samples contained approximately 85% prednisolone, 10% hydrocortisone, 3% epihydrocortisone, and 2% epiprednisolone. Colors were developed by one or more of the reactions using triphenyltetrazolium, phenylhydrazine, or isonicotinic acid hydrazide. The average recovery of prednisolone from 54 determinations was 98.2% with a maximum deviation of plus or minus 5%.

The colorimetric techniques presented certain problems. For the phenylhydrazine or isonicotinic acid hydrazide reactions, additional steps were necessary to remove traces of paper and formamide residue which interfered with color development. The oxygen sensitivity of triphenyltetrazolium chloride and the precise timing required by that procedure are disadvantages when a number of samples are to be assayed simultaneously. The simplicity of the blue tetrazolium technique recommended it as the method of choice.

The feasibility of allowing colors to develop overnight was investigated. While the precision of standards read at 90 minutes and again at 18 hours did not change, erratic values were obtained after 18 hours for samples exposed to ultraviolet light during the paper scanning procedure. Hamlin *et al.* (4) have pointed out the sensitivity of these steroids to ultraviolet light and the particular susceptibility of $\Delta^{1,4}$-3-keto steroids to photolytic degradation.

A study was made by comparing the absorbance of untreated standard solutions and of standards which, after streaking on impregnated papers, were exposed to short-wave light (2537 Å.) for periods of 0, 15, 30, and 60 seconds. The papers were extracted without chromatographic development. After 90 minutes of color development time with blue tetrazolium reagent, the maximum deviation of absorbance between standards and extracts of papers exposed 0, 15, and 30 seconds was less than 1.0%, while the absorbance of the extract of the paper exposed for 60 seconds was 3.0% below the unexposed standard. Checked again after 18 hours of development, the absorbance of the standards and of the papers exposed for 0 and 15 seconds was identical, while the absorbance of the papers exposed for 30 and 60 seconds was 4 and 9% lower than the standard. The above results indicate the necessity for viewing and marking the chromatograms rapidly, preferably in less than 30 seconds.

Table II.—Analysis of Simulated Tablet Formulations

U.S.P. Ref. Std.	Prior to Chromatography, % Recovery	After Chromatography, % Recovery
Hydrocortisone	99.5	101.4
	99.5	99.5
	100.6	100.0
	Av. 99.9	Av. 100.3
Prednisolone	99.3	100.5
	100.1	99.8
	99.3	98.2
	Av. 99.6	99.1
		Av. 99.9
Prednisone	99.5	101.0
	99.3	100.5
	99.3	100.1
	Av. 99.4	Av. 100.6
Hydrocortisone acetate	100.0	98.1
	99.8	99.2
	99.8	Av. 98.7
	Av. 99.9	

Table III.—Analysis of Commercial Powders

	Prior to Chromatography, % Claim	After Chromatography % Claim
Prednisolone	98.5	95.4
	98.5	96.5
	98.5	96.7
	Av. 98.5	Av. 96.2
Prednisone	99.4	96.2
	99.4	95.2
	99.4	93.6
	Av. 99.4	Av. 95.0
Prednisolone acetate	101.4	100.8
	101.4	100.4
	101.4	99.5
	Av. 101.4	Av. 100.3

A mixture comprising 19 parts of prednisolone to 1 part of hydrocortisone was subjected to extraction, paper fractionation, and blue tetrazolium color development. In 12 determinations, recovery of the major steroid fraction was 97.4 ± 1.0%, when compared directly with the standard solution. When the standard was subjected to chromatographic separation and extraction before comparison with the sample, the recovery of prednisolone averaged 99.5%.

Failure to achieve complete recovery from the papergrams is not due to pipet error, paper residue, effect of light, incomplete extraction, loss during evaporation, or reagent contaminants. Since recently distributed U.S.P. reference standards were used in the control studies, it is assumed that the loss is due to tailing on the papergrams rather than to impurity of the standards. While the dangers inherent in the use of procedural standards are well recognized, they are minimized in this case by the relatively complete recoveries found by direct comparison with the standard. Since the factors contributing to incomplete recoveries appear to be constant, the improved results obtained with procedural standards justify their use.

Approximately 10 mg. of accurately weighed U.S.P. reference standard was added to 200 mg. of a simulated tablet mixture and carried through the extraction, paper chromatography, and blue tetrazolium color development steps. The excipient mixture contained 80% starch, 15% lactose, 3% talc, 0.6% stearic acid, 0.5% magnesium stearate, 0.6% tragacanth, and 0.3% sodium alginate. Triplicate aliquots of standard solutions and tablet extracts were used for direct color development prior to chromatography, and triplicate papers of both standard solutions and tablet extracts were developed and determined by the colori-

metric procedure. From inspection of Table II, it is seen that quantitative recovery was achieved by the extraction and chromatographic techniques. The analyses reported in Table III were carried out in triplicate by the assay method described.

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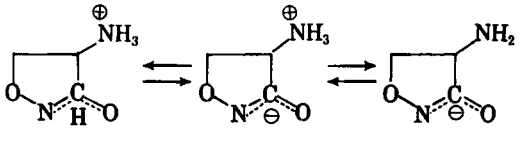
Stability of Cycloserine in Buffered Aqueous Solutions

By LOUIS MALSPEIS and DAVID GOLD

The pH rate profile of the hydrolysis of cycloserine in aqueous buffers is reported. Apparent pseudo first-order plots of the hydrolytic degradation are observed over the entire pH range. Minimal over-all velocity occurs in the vicinity of pH 12. A linear log k-pH plot is found for the protonated molecule and the dipolar ion in the pH ranges 0.4 to 2.3 and 3.0 to 7.0. The hydrolysis is catalyzed by undissociated acetic acid, and the acetic acid-catalyzed reaction appears to be first order with respect to cycloserine, regardless of whether the protonated molecule or the dipolar ion is present in the solution. Primary kinetic salt effects were not detected in hydrolyses involving the protonated, dipolar, or anionic molecules. The kinetics suggest that in the acid-catalyzed hydrolysis the substrate is the protonated cycloserine molecule. An intermolecular electrophilic-nucleophilic mechanism involving this substrate is considered.

THE STRUCTURE of the antibiotic cycloserine[1] has been shown to be D-4-amino-3-isoxazolidone (1, 2); the dipolar ion structure, as deduced from its infrared absorption spectrum and from potentiometric titration in aqueous solution, is II.



I II III

Reports of qualitative observations of the degradation of cycloserine in aqueous solution indicate that the compound is relatively stable in alkaline solution, whereas it is readily hydrolyzed in acid solution. Kuehl *et al.* (1) noted that cycloserine was facilely degraded to serine and hydroxylamine upon acid hydrolysis and that it was relatively stable to alkali. Hidy *et al.* (2) reported that mild acid hydrolysis yielded D-serine and hydroxylamine, while prolonged hydrolysis gave DL-serine and hydroxylamine. In aqueous solution at pH 6, the compound was 63% degraded in 20 hours at room temperature (3). In 15% sodium hydroxide solution at 100°, Ratouis and Behar observed that the compound was 38% degraded in 6 hours (4). Felder and Tiepolo reported that cycloserine is about 2% degraded in 24 hours at physiological conditions (5). The decomposition products were stated to be hydroxylamine, serine, and 3-aminoxyalanine in acidic solution and 2,5-bis(aminoxymethyl)-3,6-dioxopiperazine and 2,5-bismethylene-3,6-dioxopiperazine in neutral solution (5).

Analogous results were reported for 4-benzamido-3-isoxazolidone. This amide was stable to boiling 2 *N* sodium hydroxide, while acid hydrolysis produced ring opening in addition to amide cleavage (6). Ratouis and Behar (4) identified serine and serine amide as the products of the acid-catalyzed hydrolysis.

Inconsistent with these observations is the report of Nishimura and Shimohima (7), who found that the stability point of cycloserine in aqueous solution is at pH 6.0.

Structurally, cycloserine may be viewed as a cyclic *O*-alkyl hydroxamic acid. The observed stability of cycloserine to alkaline hydrolysis indicates that *N*-amide substitution with an oxygen atom results in a compound which is much less susceptible to nucleophilic attack. Simple amides are approximately equally susceptible to nucleophilic and to electrophilic attack (8); the pH rate profile of the hydrolysis of simple amides exhibits a minimal over-all velocity

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[1] Marketed as Seromycin by Eli Lilly and Co., Indianapolis, Ind.