Colorimetric Assay of 4,6-Unsaturated-3-keto Steroids Using Sodium Picrate Reagent

By E. P. SCHULZ and M. A. DIAZ

The observation in these laboratories that 4,6-unsaturated-3-keto steroids react with alkaline picrate to form chromogenic anionic complexes has been specifically utilized to determine 6-chloro-17α-hydroxy-pregna-4,6-diene-3,20-dione-17α-acetate in pharmaceutical dosage forms. Despite the over-all lack of specificity of the reagent, this disadvantage has been offset by the sensitivity, reliability, and simplicity of the presented analytical method. As little as 40 mcg. of steroid can be quantitatively determined at room temperature color development.

THE COMPLEXATION of the reactive α,β-unsaturated lactone group of cardiotonic glycosides (1) with alkaline picric acid to form chromogenic anions, suggested the possible use of this reagent for the assay of α,β-unsaturated-3-keto steroids.

In treating various α,β-unsaturated-3-keto steroids with alkaline picric acid in methanol, it was observed that chromogens of weak intensity were produced. Greater sensitivity was noted for α,β-unsaturated-3-keto steroids with conjugation extended to ring B by the presence of an additional double bond at the 6 position. It was also observed that the introduction of chlorine to the unsaturated 6 position of the latter type steroid greatly enhanced the intensity of the steroid-picric acid chromogen. Consequently, the efforts of this investigation were directed toward the utilization of alkaline picrate as a general means of analyzing 4,6-unsaturated-3-keto steroids and as a specific means of assaying 6-chloro-17α-hydroxy-pregna-4,6-diene-3,20-dione-17α-acetate [to be referred to in the subsequent text as chlormadinone (2)].

EXPERIMENTAL

Apparatus.—Beckman model DU spectrophotometer with 1-cm. cells, and culture tubes (screw capped type), 20 × 150 mm. were used.

Reagents.—The following reagents were used: picric acid, 0.666% in methanol (w/v), prepared by dissolving 666 mg. of picric acid (Baker's analyzed grade containing 10% water) in 100 ml. of methanol; sodium hydroxide, 8.0% in distilled water, prepared by diluting 8.0 Gm. of reagent grade material to 100 ml. with distilled water; and a steroid reference standard, prepared by dissolving 20.0 mg. steroid in 50.0 ml. methanol and diluting 10/50 with methanol so that the final concentration is equivalent to 80 mcg. steroid per 1.0 ml.

Assay Procedure for Formulated Chlormadinone.—Tablets containing 2.0 mg. of chlormadinone and 80 mcg. of 17α-ethynylestradiol-3-methylether were assayed by the following procedure.

The equivalent of two ground tablets (containing as excipients cornstarch, lactose, and magnesium stearate) were stirred for 15 minutes with 50.0 ml. of chloroform in a 125-ml. glass-stoppered flask. Exactly 10.0 ml. of the filtered extract was evaporated to dryness and the cooled residue dissolved in 10.0 ml. of methanol. A 1.0-ml. portion of the methanol phase was pipeted into a culture tube and wrapped in aluminum foil to protect the reactants from incident light. To a second culture tube, also wrapped in aluminum foil, was added 1.0 ml. of chlormadinone reference standard in methanol (80 mcg. per 1.0 ml.). This was followed by the addition of 3.0 ml. of a freshly prepared 1:1 mixture of the 0.666% picric acid and 8.0% sodium hydroxide solutions. (The combined reagent was cooled to ambient temperature before addition to the steroid solutions in the culture tubes.) A third tube (reagent blank) containing 1.0 ml. of methanol and 3.0 ml. of picric acid alkali reagent was concurrently prepared.

After 120-minute color development at room temperature, the contents of the stoppered culture tubes were each transferred to three separate 1-cm. glass cells. Absorbance values at 490 ± 2 mμ were immediately made, using as a reference the prepared reagent blank.

Calculations.—$A_p/A_s \times W \times 0.025$ = mg. chlormadinone/tablet, where A_p and A_s are the respective 1-cm. absorbance values of the analytical sample and the steroid standard as read against the reagent blank at 490 mμ and W is the mcg. weight of reference standard (*ca.* 80 mcg.) per 1.0 ml. of methanol.

RESULTS AND DISCUSSION

Analyses of a typical production lot of tablets containing 2.0 mg. of chlormadinone and 80 mcg. of estrogen per tablet, resulted in assay values of 1.87 and 1.89 mg. chlormadinone per tablet. Placebo tablets gave no response to the analytical reagent, nor did 80 mcg. of the estrogen produce color. Placebo tablets, to which were added 2.0-mg. levels of chlormadinone reference standard, assayed 1.98 and 1.94 mg. chlormadinone per tablet.

Reaction Conditions.—Optimum conditions were determined only for the 6-chloro-4,6-unsaturated-3-keto steroids. Absorbance values for the other type of steroids (Table I) were determined using the conditions arrived at for the above type steroid and do not necessarily represent the maximum obtainable intensities.

Sensitivity.—The data presented in Table I indicated the following order of group sensitivity: (*a*) 6-halogenated-4,6-unsaturated-3-keto > 4,6-unsaturated-3-keto > 4-unsaturated-3-keto and (*b*) within the first group, the substitution of fluorine for chlorine at the unsaturated 6 position led to decreased sensitivity.

Specificity.—In addition to the three types of steroid found to respond to the described reagent, the data for miscellaneous steroids (80 mcg.) after 120-minute color development (*versus* reagent blank) were as follows:

(*a*) 6α,9α-Difluoro-1,4-unsaturated-3-keto steroids gave no response.

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TABLE I.—ABSORBANCE VALUES[a] FOR 80 mcg. OF VARIOUS STEROIDS

	Absorbance	Maximum, mμ
6-Chloro-17α-hydroxy-pregna-4,6-diene-3,20-dione	0.625	490
17α-Acetate of above steroid	0.564	490
6-Fluoro-17α,21-dihydroxy-16α-methyl-pregna-4,6-diene-3,11,20-trione-21-acetate	0.265	497
Pregna-4,6-diene-3,20-dione	0.356	492
17α-Hydroxy-pregna-4,6-diene-3,20-dione-17-acetate	0.311	492
6α-Methyl-pregn-4-ene-3,20-dione	0.147	497
6β-Hydroxy-pregn-4-ene-3,20-dione	0.298	493
Pregn-4-ene-3,20-dione	0.193	493

[a] Values are based on a 1-cm. cell path, as read against a reagent blank.

(*b*) 6-Chloro-1,4-unsaturated-3-keto steroids produced an absorbance of 0.030 at 492 mμ.

(*c*) 1,6-Unsaturated-3-keto steroids led to values of 0.163 at 492 mμ.

(*d*) 3-Hydroxy-4,6-unsaturated steroids gave an absorbance value of 0.052 at 487 mμ.

(*e*) 3,5-Unsaturated steroids (with no 3-keto group) exhibited values of 0.261 at 491 mμ.

(*f*) U.S.P. digitoxin gave a value of 0.140 at 476 mμ.

Solvents.—The use of either absolute or 96% ethanol in lieu of methanol to prepare the reagents and steroids standards led to a 66% decrease in intensity values.

Effect of Light.—Color development without adequate protection of the reactants from incident light led to 20% lower intensities as well as irreproducibility of color.

Color Stability.—A plot of absorbance *versus* time indicated that developed colors exhibit the least variation during the 80–140-minute interval after the addition of reagent. Each 10-minute increment during this 80–140-minute period showed an average absorbance increment of 1.8%.

Color Linearity.—Developed colors were linear within a concentration range of 40–120 mcg. of chlormadinone.

Color Development with Other Polynitro Phenols.—2,4,6-Trinitro-resorcinol; 2,4,6-trinitro-anisole; 4,6-dinitro-2-amino-phenol; and *p*-nitrophenol failed to produce chromogens under the described conditions.

Concentration of Alkali.—Variations in the sodium hydroxide content while maintaining the concentration of picric acid constant indicated that the method is particularly sensitive to the concentration of alkali. A 4 to 8% decrease in alkali content led to 7% lower absorbance values. Concentrations of sodium hydroxide greater than 8% led to precipitation of the picric acid. The use of 8.0% potassium hydroxide also precipitated the picric acid.

Concentration of Picric Acid.—Varying the picric acid concentration from 0.637 to 0.693%, while maintaining the alkali content at 8.0%, produced no significant changes in the observed absorbance values.

Stability of Reagents.—The combined reagent, after 24-hour aging of the uncombined components showed a 6% decrease in absorbance values; the combined reagent after 24-hour aging at ambient temperature showed 13% lower values.

Interferences.—Chloroform soluble carboxylic acids (stearic acid, carbapol, alginic acid, etc.) and excipients tending to precipitate picric acid (tertiary amines, proteins, etc.) interfered. Interference due to chloroform-soluble carboxylic acids was resolved by re-extracting the chloroform–steroid phase with 0.1 *N* sodium hydroxide.

The second type interference was overcome by precipitation of these materials from the chloroform phase, using a 1% solution of silicotungstic acid in 6 *N* hydrochloric acid.

The described reagent may also be applied to the analyses of residues from chloroform extracts of steroids formulated in cream bases, after the removal of interferences from the organic phase by means of 0.1 *N* aqueous sodium hydroxide. A cream base (propylene glycol, stearic acid, Spans, and Tweens) gave no response to the reagent using the above extraction technique.

Reproducibility.—Using freshly prepared reagents, the day-to-day variation of chlormadinone (80 mcg.) absorbance values ranged from 0.466 to 0.564. However, triplicate determinations of the same steroid standard solution and using the same reagents indicated that the mean average deviation of absorbance values was ±2.2%. Since steroid standards were determined concurrently with the steroid isolated from the formulated sample, the analytical error also was within the above range. The above deviation was reduced to a range of ±1.1% by using larger volumes of reactants and by minimizing the lapsed time between absorbance measurements. For example, colors developed on 3.0 ml. of steroid (240 mcg. chlormadinone) with 9.0 ml. of picric acid-alkali reagent led to the latter mean average deviation.

Purity of Steroids.—In all instances, steroids were used which indicated only one spot after thin-layer chromatography on silica gel.

Mechanism of Reaction.—The available data, though not rigorously elucidating the mode of reaction, indicated the following results:

(*a*) The bis 2,4-dinitro-phenylhydrazone of chlormadinone, as prepared by the method of Cohen and Bates (3), reacted with the alkaline picric acid reagent to form a chromogen absorbing at 490 mμ. This indicated that complexation did not occur at the unavailable 3 or 20 keto positions of the steroid molecule.

(*b*) The ultraviolet spectrum of the isolated complex showed a maximum at the same wavelength (284 mμ) as that of unreacted chlormadinone. This suggested intact structure of the 4,6-unsaturated-3-keto moiety in the complexation product.

(*c*) Conversion of the sodium form of the complex anion to the hydrogen form yielded, after column chromatography on silica gel and elution with 90:10 chloroform–methanol, a pure product which analyzed C, 54.3%, H, 4.6%, and N, 6.9%. Calculated values for a 1:1 complex of 6-chloro-17α-hydroxy-pregna-1,4-diene-3,20-dione ($C_{21}H_{27}O_3Cl$, mol. wt. 362.89) and picric acid ($C_6H_3N_3O_7$, mol. wt. 229.11) were: C, 54.7%, H 5.1%, and

N, 7.0%. This demonstrated that the reaction involves a 1:1 complexation of picric acid and chlormadinone as well as alkaline hydrolysis of the steroidal 17-acetoxy entity. This is consistent with the mechanism (4) proposed for the anionic complexation of picric acid and α,β-unsaturated lactones.

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Enzymatic Methylation of Nicotinamide by *Claviceps purpurea*

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Soluble supernatant fractions from two strains of *Claviceps purpurea* were tested for the presence of enzymes capable of methylating tryptamine, 5-hydroxytryptamine, tyramine, and nicotinamide. The latter compound was the only one of the substrates tested in which methylation took place.

SEVERAL PSYCHOTOMIMETIC indole substances (1), such as bufotenin and psilocybin, which are found in certain fungi are *N*-methylated tryptamine derivatives. Lysergic acid, a derivative of which is the most potent psychotogen known, also contains an *N*-methyl group, and a residue of this compound occurs in the alkaloids of ergot.

It has been reported (2) that the *N*-methyl group in the ergot alkaloids is probably derived from a transmethylation reaction from methionine.

Cantoni (3) first reported that *S*-adenosylmethionine was an active methyl donor and has studied in great detail the methylation of nicotinamide. Axelrod (4) has isolated an enzyme from rabbit lung that is capable of methylating a number of naturally occurring compounds to psychotomimetic derivatives utilizing *S*-adenosylmethionine as the methyl donor.

The literature is replete with information concerning the biosynthesis of the ergot alkaloids from *C. purpurea*, but relatively little information is available concerning the activity of the enzymes found in this organism. The present investigation has been conducted in an attempt to determine if a cell-free extract of the fungus possesses an enzyme system which can methylate tryptamine, 5-hydroxytryptamine (5-HT), tyramine, and nicotinamide, since their methylated derivatives have been associated with psychotomimetic activity.

EXPERIMENTAL

Cultures.—Two strains of *C. purpurea* (Fries) Tulasne were used in this investigation. One of the strains, designated CPM, was isolated from sclerotia obtained from the Department of Plant Pathology, University of Minnesota, Minneapolis. The other, an alkaloid producing strain, designated 47A, was obtained as a slant culture from the College of Pharmacy, University of Washington, Seattle.

The fungi were cultured in 50 ml. of Abe's medium (5) in cotton-stoppered 250-ml. conical flasks grown as still cultures in the dark at room temperature. The cultures were harvested for extraction at periods of from 21 to 27 days.

Preparation of Enzyme Fraction.—The mycelial tissue of both CPM and 47A cultures was separated from the culture medium by centrifugation, washed three times with distilled water, recentrifuged, and finally pressed between sheets of filter paper.

The enzyme fraction was prepared from 6–7 Gm. (fresh weight) of mycelial tissue, which was homogenized with 15 ml. of phosphate buffer (0.1 *M* K_2HPO_4, pH 7.9) in a VirTis 45 homogenizer for 10 minutes in a chilled vessel in an ice bath. After the homogenate was obtained, it was centrifuged at 2500 r.p.m. (755 × *g*) for 10 minutes and the resultant supernatant fraction recentrifuged at 16,000 r.p.m. (31,000 × *g*) for 20 minutes in a refrigerated Servall centrifuge at 0–3°. The soluble supernatant fraction was then separated and employed as the enzyme preparation.

In addition to the above method, three other means were tested for extraction of the mycelium: (*a*) homogenizing the mycelium with buffer as previously described, then subjecting the homogenate to ultrasonic vibrations, (*b*) grinding the mycelium in buffer solution with sand, and (*c*) grinding with alumina. After several preliminary trials, it was decided to use homogenization only as the extraction method for further investigations since this method proved most successful.

Enzymatic Activity of Soluble Fractions.—Nicotinamide,[1] tryptamine hydrochloride,[1] tyramine hydrochloride,[1] and 5-hydroxytryptamine creatinine sulfate[1] were tested with the soluble supernatant fraction using *S*-adenosylmethionine[2] as a methyl donor to determine if the soluble fraction contained an enzyme(s) capable of methylating these physiologically active naturally occurring substances.

Nicotinamide—The incubation mixture consisted of 0.005 mole of *S*-adenosylmethionine (0.2 ml.), 0.01 *M* nicotinamide (0.3 ml.), and an amount of the soluble supernatant fraction equivalent to 6

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[1] Nicotinamide, tryptamine hydrochloride, tyramine hydrochloride, and 5-hydroxytryptamine creatinine sulfate were obtained from Nutritional Biochemical Corp., Cleveland, Ohio.
[2] *S*-Adenosylmethionine was obtained from Calbiochem, Los Angeles, Calif.