tailed inspection of the data reveals some interesting points. For example, the ranking of preparations based on mean plasma levels during the first 25 hr. (single dose) remains the same throughout the entire experiment (except for the 25–49-hr. period). Also, if the residual plasma levels are considered (values 25 hr. after the first dose, and 24 hr. after the second, fourth, and seventh doses), the following statistically significant differences emerge: at 25 hr., preparation $5 > 6$ ($p = 0.1$, least significant difference), at 97 hr., preparation $5 > 6$ ($p = 0.1$, Duncan's multiple-range statistic), and $5 > 2$ ($p = 0.05$, Duncan's multiple-range statistic). Residual plasma levels may be important in griseofulvin therapy since a minimum level of 1 mcg./ml. appears to be needed for an effective cure (1).

When the dissolution values (Table III) are correlated with the mean plasma levels (Table V), a good correlation is seen (Fig. 2) between the logarithm of the 30-min. dissolution values and mean plasma levels after 25 hr. (effect of a single dose), 49–173 hr. (the plateau region), and 0–173 hr. (total experimental period). The correlation coefficients for the 3 time periods are 0.999 ($p = 0.015$), 0.992 ($p = 0.077$), and 0.995 ($p = 0.056$). The slope of the 0–25-hr. regression line compares favorably with the slope of the single-dose treatment (0.60 in Fig. 2 *versus* 0.63 in Fig. 1). The slopes for the later time intervals (Fig. 2) are lower than the 0–25-hr. slope because differences between the mean plasma levels of the preparations are smaller for the 49–173-hr. period.

Since dissolution rates correlate well with griseofulvin absorption after a single dose, as well as after repeated drug administration, they should serve as an effective tool for selecting therapeutically useful griseofulvin preparations.

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Keyphrases

Griseofulvin absorption—human
Absorption-dissolution rate correlation—griseofulvin
Plasma levels, griseofulvin—single, multiple doses
Fluorometry—plasma griseofulvin analysis

Further Applications of Diazotized 4-Amino-6-chloro-*m*-benzenedisulfonamide

Colorimetric Determination of Pyridoxine Hydrochloride, 17β-Hydroxy-7aα-17α-dimethyl-B-homo-A-norestrane-3,6-dione and Morphine Sulfate

By TIBOR URBÁNYI and SUSAN BUDAVARI

The diazotization product of 4-amino-6-chloro-*m*-benzenedisulfonamide has been found to be a useful reagent for the colorimetric determination of a vitamin, steroid, and alkaloid. A general method, with slight modification, can be applied to those compounds which contain a phenolic or similar-to-phenolic hydroxyl group. Pyridoxine hydrochloride, 17β-hydroxy-7aα-17α-dimethyl-B-homo-A-norestrane-3,6-dione, and morphine sulfate were used as coupling components in this study. The optimum reaction conditions for coupling and color formation have been determined and the applicability of the method in the presence of commonly used ingredients is demonstrated.

A REPORT ON the colorimetric determination of estrogenic hormones by coupling with diazotized 4-amino-6-chloro-*m*-benzenedisulfonamide reagent has already been published (1). The purpose of the present investigation was to extend the application of that method to the quantitative determination of a phenolic alkaloid,

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a nonphenolic steroid, and similar-to-phenolic vitamin; morphine sulfate, 17β-hydroxy-7aα-17α-dimethyl - B - homo - A - norestrane - 3,6 - dione (steroid); and pyridoxine hydrochloride, respectively. Although the structures of these three compounds differ considerably, each has a similar functional group upon which the proposed method of determination is based.

A search of the literature reveals numerous assay methods for both morphine and pyridoxine. This attests to the importance of the compounds, as well as the difficulty involved in developing satisfactory analytical procedures which are quantitative and sensitive. For example, many alkaloids and vitamins can be determined by thin-layer chromatography (2, 3) with extreme sensitivity; but the results are not always quantitative. The numerous gas chromatographic methods available (4, 5) require great care in column preparation to secure reliable results. Ion-exchange methods (6, 7) generally suffer from the lack of reproducibility. Colorimetric techniques (8–10) can be quite sensitive and quantitative but they, as in the case of the USP procedure for pyridoxine in decavitamin capsules (11), are often time consuming and require rigidly controlled conditions. Among other colorimetric methods are the coupling reactions with diazonium salts. Diazotized sulfanilic acid reagent (12, 13) is often used, but this procedure fails to yield reproducible quantitative results. Aliev (14) proposes a method for pyridoxine determination which is based on the formation of a red-violet zinc complex with the stabilized diazo salt of norsulfazole. This method, as well as the others, proves less satisfactory than the proposed method for reasons of either poor reproducibility, length or complexity, or lack of sensitivity.

Alternate procedures for the analysis of the steroid cannot be cited since no previous references appear in the literature.

The need for a quantitative and sensitive method for the determination of pyridoxine and morphine as pure substances and in pharmaceutical formulations was apparent. Since pharmaceutical interest in steroids similar to the one presented here can be expected, the method for its analysis was included. The aim of this report, therefore, was to propose a practical and general colorimetric procedure for the analysis of these compounds.

EXPERIMENTAL

A Beckman model DU spectrophotometer was used to determine the absorbances, and a Cary model 11 recording spectrophotometer was used to record the spectra.

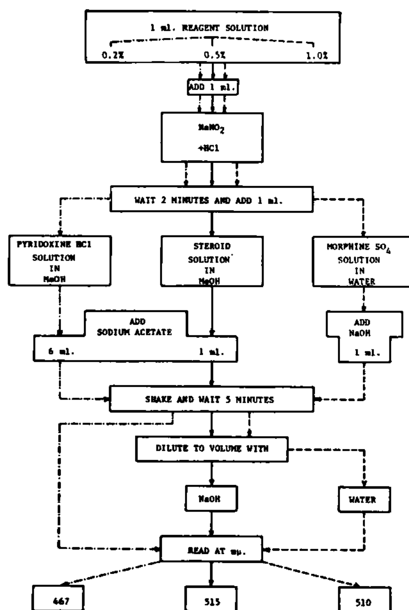


Fig. 1—Schematic diagram.

Reagents and Solutions—4-Amino-6-chloro-*m* benzenedisulfonamide (disulfonamide), 0.2, 0.5, and 1.0% methanolic solutions; sodium nitrite, 1.0% aqueous solution; hydrochloric acid, 1 *N;* sodium acetate, 1 *N* solution; sodium hydroxide, 1 *N* aqueous solution.

Preparation of Assay Solution—The compounds used in this study were dissolved in either methanol or water, whichever provided the optimum conditions for the color measurements. Solutions containing approximately 0.0025% of pyridoxine hydrochloride, 0.015% of steroid in methanol, and 0.13% morphine sulfate in water were prepared.

General Procedure for Color Development—Into separate 10-ml. volumetric flasks 1.0 ml. each of disulfonamide, sodium nitrite, and hydrochloric acid solutions were successively pipeted and mixed well. The solutions were allowed to stand 1–2 min., then 1.0 ml. of assay solution and 1.0 ml. of solvent (blank) were added. The solutions were buffered and allowed to stand approximately 5 min. at room temperature. Each flask was diluted to volume with the proper solvent, and its absorbance was determined at the given wavelength in 1-cm. cells against its respective blank.

The three compounds considered in this study belong to significantly different categories which precludes the use of a rigorous general method which can be applied to each with equal success. Since individual adaptations were made in order to obtain optimum conditions for each analysis, a schematic diagram (Fig. 1) is provided which lists the step-by-step procedure proposed.

DISCUSSION

The absorption spectra of the final coupled products of pyridoxine hydrochloride, steroid, and morphine sulfate with diazotized disulfonamide are shown in Fig. 2.

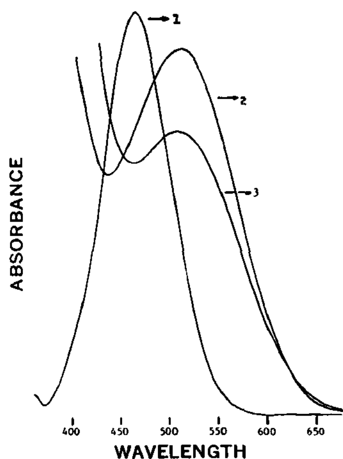


Fig. 2—Absorption spectra of coupled products. Key: 1, pyridoxine hydrochloride; 2, 17β-hydroxy-7aα-17α-dimethyl-B-homo-A-norestrane-3,6-dione; 3, morphine sulfate.

Pyridoxine Hydrochloride—It is apparent that while both the alkaloid and steroid exhibit broad absorption maxima in the 510–515-mμ region, the vitamin absorption maximum appears considerably lower at 467 mμ. This hypsochromic displacement of the absorption maximum in the case of vitamin, along with the much greater color intensity of its coupled product, can be attributed to the fact that the pyridoxine has the most favorable coupling position which is *para* to the phenolic hydroxyl group as well as *ortho* to the ring nitrogen, and thus exhibits strong electron density. Only coupling *ortho* to the hydroxyl group is possible for the morphine and the steroid. The intensity of absorbance was significantly affected by the choice of diluents and the pH of the solution. The color intensity of the final solution was increased 5–10% when the pyridoxine to be determined was dissolved in methanol rather than water. Furthermore, the fact that the intensity was significantly greater when acetate buffer was chosen as the diluent rather than sodium hydroxide is consistent with the observation of Stamm and Zollinger (15). They reported that a 10-fold increase in the concentration of acetate ions catalyzes the coupling reaction and produces five times more *para* coupling.

The most intense and reproducible color was obtained when the final pH was between 5.3 and 6.0. Therefore, 1 *N* sodium acetate, which brought the solution to the suitable pH upon dilution, was utilized as the diluent. Although the orange color developed immediately upon dilution with sodium acetate, it was found advisable to wait about 3 min. before reading the solution to avoid interference from the bubbles which develop.

Table I—Molar Absorptivities

Compd.	ε ($\lambda_{max.}$)
Morphine sulfate	2.1×10^4
Steroid	8.7×10^4
Pyridoxine hydrochloride	35.7×10^4

The relationship between the concentration and the absorbance at 467 mμ was found to be linear over a range of 0–0.04 mg./assay. The molar absorptivity value is considerably larger than the values for morphine and steroid as seen in Table I.

Steroid—The synthetic steroid used in this study is significantly different from the phenolic estrogens considered in the previous publication (1). It is nonaromatic and has the structural formula in Scheme I.

Experimental data have indicated that the enol form of the steroid predominates in the enol–keto equilibrium, thereby making its determination as a coupling component with diazonium salts possible. Based on the above, it was evident that the pH of the solution at the coupling stage should be critically controlled. Sodium acetate was found to be the best buffering agent for this purpose. Although a stable color was obtained omitting the sodium acetate, the results were not reproducible, and a bathochromic shift was observed. An attempt was made to vary the sodium acetate buffer concentration and also to replace it with sodium bicarbonate, sodium carbonate, and sodium hydroxide solutions. Color instability was noted, and the Beer's law plots did not intersect the origin. The most reproducible values were obtained when coupling was carried out with 1 *N* sodium acetate solution at pH 5 ± 0.3, and the coupling time was 5 min. Color developed immediately upon the addition of 1 *N* sodium hydroxide and remained constant for at least 30 min. (Fig. 3). No absorption peak was obtained when the solution was diluted with sodium acetate. Beer's law was valid for this steroid at 515 mμ over a range of 0–0.25 mg./assay.

Morphine Sulfate—The rate of coupling and the color intensity of the coupled product with diazo-

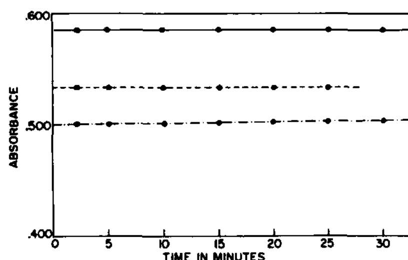
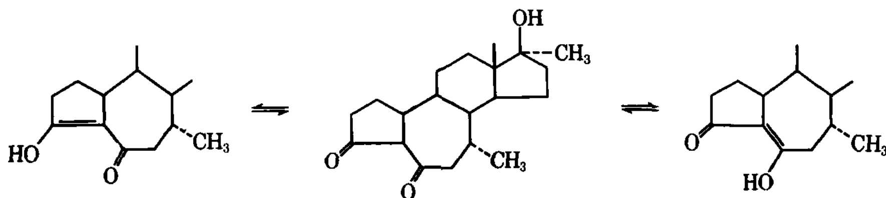
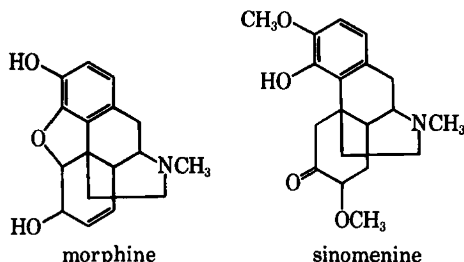


Fig. 3—Absorbance of final solution versus *time. Key:* ———, *pyridoxine hydrochloride;* ---, *steroid;* ·-·, *morphine sulfate.*



Scheme I

nium compounds are strongly affected by the structural formula of the coupling agent. Alkaloids, having *ortho* or *para* positions to the phenolic hydroxyl unsubstituted, give diazo reactions; however, the intensity of the reaction is dependent upon whether the free coupling site is at the *ortho* or *para* position. One notes that sinomenine (16), with the free *para* coupling position, gives a forty times stronger coupling reaction with diazobenzenesulfonic acid than morphine, where only *ortho* coupling can occur.



The experimental conditions in this study were carefully investigated to obtain the optimum coupling reaction with morphine sulfate. The concentration of disulfonamide solution was increased to 1% to provide sufficient reagent for the amounts of morphine sulfate added. An increased amount of sodium nitrite raised the color intensity of the coupled product, but the color stability was diminished. Morphine sulfate was dissolved in water, rather than methanol or 1 *N* hydrochloric acid, to obtain linear absorbance *versus* concentration plots. Sodium hydroxide (1 *N*) was abandoned as a solvent despite good color stability and satisfactory adherence to Beer's law because reproducible results were not obtained.

The morphine sulfate was allowed to couple in sodium hydroxide solution. Although acetate at coupling intensified the final color, acetate was not used since plots of absorbance *versus* concentration did not intersect the origin and color fading was noted. An intense red color developed immediately upon addition of sodium hydroxide at coupling, and the 5-min. waiting time ensured the stabilization of absorbance values upon dilution with water. Water was selected as the diluent because the final absorption of the coupled product was about 10–15% higher than when sodium hydroxide was employed. No absorption peak was observed when sodium acetate was used as diluent. Stable and reproducible results were obtained when the pH of the solution was 10.5 ± 0.5.

The absorbance was linear at 510 mμ for concentrations up to 1 mg. morphine (as the sulfate) per assay. As seen in Fig. 3, the plot of absorbance against time was quite constant and showed only about 2% increase in color intensity after 30 min.

Mixtures—The data in the tables confirm that the method already described may be satisfactorily applied even though the compounds studied are in the presence of similar vitamins or alkaloids and excipients commonly used in pharmaceutical preparations. In each of the synthetic mixtures analyzed, the ratio of the active ingredient to the additive was that of the average found in commercial preparations (if existing) rather than the optimum for analysis procedures.

It can be seen from the data in Table II that a 10- to 100-fold increase in excipient concentration resulted in a 100–101% recovery of active ingredient in the presence of sucrose, lactose, and starch.

One can conclude from Table III that pyridoxine hydrochloride can be accurately determined though compounded with a considerable amount of any of the other common B vitamins and without the need for prior separation. However, interference due to ascorbic acid, which is also often found in vitamin B complex formulations, should be first eliminated by a simple oxidation process (14).

As evident from Table IV, morphine may be assayed in the presence of certain related opium alkaloids since those alkaloids without a phenolic group, or with a substituted phenolic group, do not interfere.

The unique character of the steroid investigated here illustrates the extended applicability of this method. Since steroids with similar structural formulas are not found in the literature, 12 steroids with alcoholic or ketonic groups at the 3 position were chosen to check the applicability of the proposed method. Androsterone, ergosterol, dehydrocholic acid, *etc.*, did not give coupling reactions with diazotized disulfonamide. Of course, steroids with phenolic hydroxyl group interfere; however, such combined formulations would not normally be expected.

TABLE II—DETERMINATION OF MORPHINE, PYRIDOXINE, AND STEROID IN THE PRESENCE OF COMMON EXCIPIENTS

Additives	Morphine	Pyridoxine	Steroid
Sucrose			
Added, mg./g.	90.0	120.0	10.0
Found, mg./g.	90.4	120.0	10.0
% Recovery	100.4	100.0	100.0
Starch			
Added, mg./g.		120.0	
Found, mg./g.		121.0	
% Recovery		101.0	
Lactose			
Added, mg./g.	90.0	120.0	10.0
Found, mg./g.	90.5	120.0	10.1
% Recovery	100.6	100.0	100.7

TABLE III—DETERMINATION OF PYRIDOXINE HYDROCHLORIDE IN THE PRESENCE OF RELATED VITAMINS

Additives	% Pyridoxine Recovered[a]
Thiamine hydrochloride	100.1
Riboflavin	100.0[b]
Niacinamide	99.6
Cyanocobalamin	99.9

[a] The compound–additive ratio 1:5. [b] Determined against sample blank.

TABLE IV—DETERMINATION OF MORPHINE SULFATE IN THE PRESENCE OF RELATED ALKALOIDS

Additives	% Morphine Recovered[a]
Papaverine hydrochloride	101.5
Codeine sulfate	100.8
Dionin	100.4

[a] The compound–additive ratio 1:1.

This method with its sensitivity, rapidity, and color stability provides a significant improvement over the procedures mentioned in the literature.

SUMMARY

Pyridoxine hydrochloride, steroid, and morphine sulfate which contain either phenol c, or similar-to-phenolic hydroxyl groups, form colored azo dyes with diazotized disulfonamide.

The color intensity was significantly increased when coupling occurred *para*, rather than *ortho*, to the phenolic hydroxyl group. Certain compounds showing group similarities, as well as commonly used excipients in pharmaceutical formulations, do not interfere in this determination.

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Keyphrases

Pyridoxine HCl—analysis
Morphine—analysis
17β-Hydroxy-7aα-1,7α-dimethyl-B-homo-A-norestrane-3,6-dione—analysis
Colorimetric analysis—spectrophotometer
4-Amino-6-chloro-*m*-benzenedisulfonamide, diazotized—color reagent

In Vivo and *In Vitro* Activity of a Vasoactive Drug 9-(3,5-Dimethylpyrazole-1-carboxamido)-7-methyl-4,6,6*a*,7,8,9,10,10*a*-octahydroindolo[4,3-*fg*] Quinoline Maleate

By PAUL W. WILLARD and CLARENCE E. POWELL

An indoloquinoline compound prepared from lysergic acid base has been found to have an α-blocking action on *in vivo* and *in vitro* preparations. The compound reverses the blood pressure response to epinephrine from pressor to depressor in pithed and decerebrate cats. In renal hypertensive dogs, the compound produced a significant sustained decrease in blood pressure. On rat vas deferens preparations, the spasmogenic effect of epinephrine and norepinephrine was blocked by the compound similar to, but not in the same degree as that observed with dibenamine. The primary effect of the compound appears to be an interference with sympathetic transmission postsynaptically at the α-receptor sites resulting in peripheral dilatation.

Compounds derived from lysergic acid are known to have a variety of pharmacological properties. Among these properties are effects on blood pressure and other cardiovascular parameters (1). Recently, a chemically unique series of indoloquinoline compounds prepared from the lysergic acid base[1] produced a sustained lowering of blood pressure in unanesthetized and anesthetized animals. This study was made to determine the mechanism of actions whereby one of the members of the series lowered blood pressure.

The compound, 9-(3,5-dimethylpyrazole-1-carboxamido)-7-methyl-4,6,6*a*,7,8,9,10,10*a*-

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[1] Compounds were prepared by Dr. William L. Garbrecht of the Process Research and Development Division, Eli Lilly and Co., Indianapolis, Ind.