A, however, show a difference of 2.3%, the methoxyl signal giving the higher answer. This is probably because this sample contained both estrone-3-methyl ether and estradiol-3-methyl ether as impurities in amounts which appeared, from TLC, to be significantly greater than the amount of 17α-ethinylestradiol present. Therefore, the result would be expected to be biased by an increase in the methoxyl signal.

The NMR procedure as described offers an attractive alternative assay for mestranol bulk drug. Simultaneous independent answers, from two different and isolated functional groups in the same molecule, act as a built-in check for the procedure.

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Fluorometric Determination of Reserpine and Related Compounds by Reaction with Vanadium Pentoxide

TIBOR URBÁNYI and HENRY STOBER*

Abstract ☐ A rapid and fairly specific fluorometric procedure has been developed for the routine quantitative determination of reserpine and its derivatives alone and in tablet formulations. The method is based on the formation of fluorescence induced by the oxidation of reserpine with a reagent containing vanadium pentoxide in phosphoric acid. The oxidation product exhibits a greenish-yellow fluorescence, with the maximum around 500 mμ in an acidic alcoholic solution. The dependence of the intensity of fluorescence upon the nature of the solvent, reagent concentration, and other parameters is discussed. The fluorogen developed follows Beer's law over a very wide range, from 0.004 to 2 mcg./ml. of sample solution. The advantages and disadvantages of the proposed method are discussed, and the applicability in different formulations is demonstrated.

Keyphrases ☐ Reserpine and derivatives in tablets—determination ☐ Vanadium pentoxide–reserpine reaction—fluorescence ☐ Phosphoric acid effect—reserpine–vanadium pentoxide fluorogen ☐ Fluorometry—analysis

Since the isolation of reserpine[1] from the various Rauwolfia roots was completed, its double therapeutic effect as an antihypertensive and a tranquilizer has been recognized. When the usefulness of the reserpine as an effective human medicine was realized, Szalkowski and Mader (1) developed a quantitative method for its determination. This method underwent many modifications (2, 3) which, however, did not alter the methodology of the original procedure significantly.

In recent years, photometric methods (4) have been introduced for the quantitative determination of reserpine. These methods are based on reactions with suitable reagents resulting in the formation of chromophores or fluorogens, which can be measured by colorimetric or fluorometric techniques. Colorimetric measurements (5) are applied mostly for pharmaceutical formulations where the sensitivity of the determination is not critical. Fluorescence determinations are used for reserpine in feeds and biological materials (6), where extremely sensitive and selective methods are required. A direct UV method (7) is frequently used for the determination of reserpine and has the advantage of speed of assay where the concentration of reserpine is high enough for UV absorption. Column chromatographic methods (8) are highly specific and fairly sensitive, but they are time consuming. TLC methods (9) are often used for the separation of the active alkaloids, but these methods are primarily qualitative rather than quantitative.

The development of a specific method for the determination of reserpine is difficult because of its structural similarity to other active alkaloids isolated from the Rauwolfia root. The greenish-yellow color produced

[1] Serpasil, Ciba Pharmaceutical Co.

Table I—Effect of Acids Saturated with Vanadium Pentoxide on Reserpine-Induced Fluorescence[a]

Acid	Reagent Added, ml.	Fluorescence, %
Phosphoric	0.5	85
Hydrochloric	0.5	1
Sulfuric	0.5	45
Perchloric	0.5	4
Acetic	0.5	1

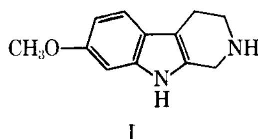
[a] The total volume was 10.5 ml.; concentration 10 mcg.

Table II—Variation of Vanadium Pentoxide Reagent on the Fluorescence of Reserpine in Ethanolic Solution[a]

Reagent, ml.	Fluorescence, %
0.25	83
0.50	82
0.75	76
1.00	72

[a] The total volume of solution was 11.0 ml.

using the general nitrite procedure for reserpine is specific for alkaloids having a 2,3,4,9-tetrahydro-7-methoxy-1*H*-pyrido[3,4-*b*]indole group (10) with the following structural formula (I):



It was observed that when reserpine was exposed to intense light, air, or oxidizing agents, a greenish-yellow colored compound was produced (11). This compound is identical with the reaction product obtained from the nitrite procedure and was identified by several authors (11, 12) as 3-dehydroreserpine, a first-step oxidation product of reserpine. Among many oxidative reagents, the official method in Pharmacopeia Belgique IV (13) is the sulfovanadic acid, which produces a greenish-yellow color with reserpine in acetone solution.

In the USP and NF, the official method is the nitrite procedure in the presence of hydrochloric acid. The chromophores produced by these different reagents are alike and are measured colorimetrically at the absorption maximum at about 390 mμ. The greenish-yellow color recommended by the official procedure permits the determination of reserpine by fluorescence measurements, since the fluorescent product increases the sensitivity for the determination of reserpine.

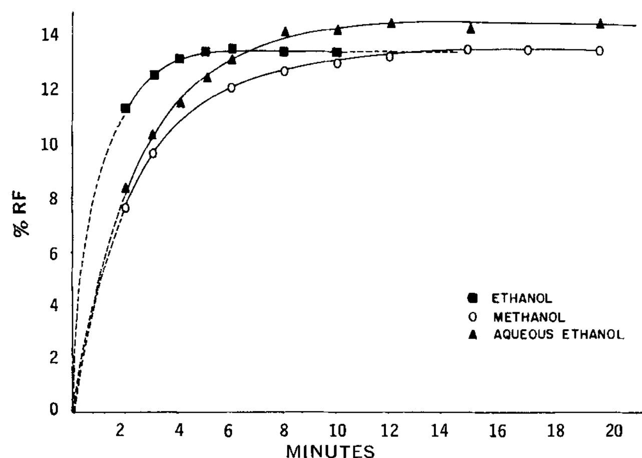


Figure 1—*Rate of formation of fluorogen with vanadium pentoxide in different solvents.*

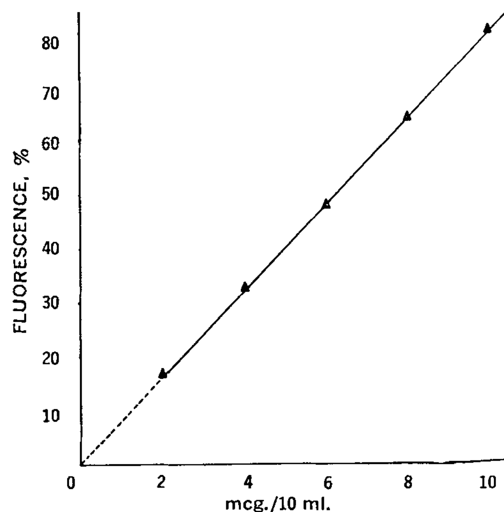


Figure 2—*Plot of fluorescence of alcoholic solutions against concentrations.*

The goal of this study was to develop a fluorescence method which would be as sensitive or better than that of the nitrite procedure and, with its simplicity, would improve the precision of the official method. The proposed method is based on the formation of a greenish-yellow fluorogen with vanadium pentoxide in phosphoric acid in alcohol or with an aqueous ethanolic solution of reserpine. On the basis of these observations, the method described can be adopted for the reserpine determination of the single-tablet assay, where extremely high sensitivities are desired. The optimal reaction conditions and the characteristics of the fluorescent species are presented.

EXPERIMENTAL

Reagents and Solutions—Concentrated phosphoric acid was saturated with reagent grade vanadium pentoxide by mechanical

Table III—Influence on Fluorescence by the Amount of Phosphoric Acid Added to Alcoholic Solution of Reserpine[a]

Phosphoric Acid Added, ml.	Reagent, ml.	Fluorescence, %
1.5	0.5	77
0.5	0.5	75
	0.5	64

[a] The total volume of solution was 12.0 ml.

Table IV—Effect of Water Content on Fluorescence in Ethanolic Solution of Reserpine[a]

Water in Ethanol, %	Reagent, ml.	Fluorescence, %
10	0.5	83
25	0.5	83
50	0.5	75

[a] The total volume of solution was 10.5 ml.

shaking for approximately 1 or 2 hr. The solution was filtered through a medium-porosity sintered-glass funnel. The saturated solution contained about 0.8 mg. of vanadium pentoxide/ml. This reagent was quite stable and could be stored for a long period without any changes in composition; alcohol USP was used as solvent.

Apparatus—An Aminco SPF-125 spectrophotofluorometer, equipped with variable slit controls and a mercury lamp for the light source, and an Aminco-Bowman recording spectrophotofluorometer with xenon lamp for the light source were used for the measurements.

Preparation of Standard Solution—A solution containing 1 mcg./ml. of USP reserpine reference standard in alcohol USP was prepared.

Preparation of Assay Solution—A reserpine solution of 1 mcg./ml. in alcohol USP was prepared. To facilitate the dissolution of reserpine from the tablet mass, a sonifier bath was employed.

Procedure—Into a 25-ml. volumetric flask, pipet 10 ml. of assay solution and 0.5 ml. of reagent. Mix well and let the solution stand about 10–15 min. At the same time, pipet standard solutions into separate 25-ml. volumetric flasks containing 6, 8, and 10 mcg. of reserpine in 10 ml.; add 0.5 ml. of reagent solution to each flask, and mix well. After the standing time, set the activation slit control at 2 mm. and the emission slit control at 4 mm. at full sensitivity, and set the percent full scale setting at 100 of the instrument. Measure the fluorescence of the standard and sample solutions at 495 mμ with the activation wavelength of 365 mμ, using a 1-cm. fluorescence cell.

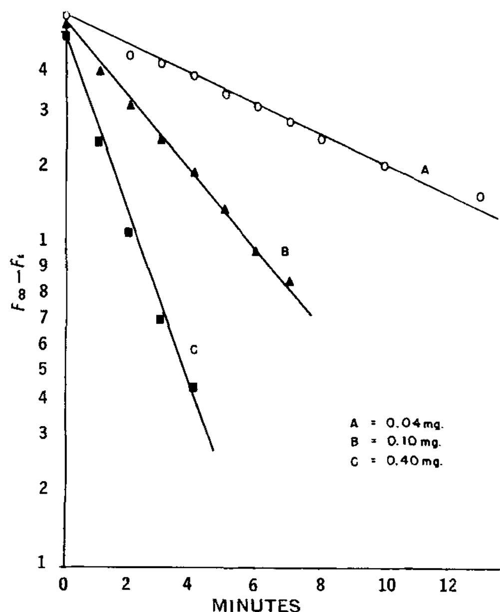


Figure 3—*Plot of the log $F_\infty - F_t$ of reserpine treated with varying amounts of vanadium pentoxide per 0.5 ml. H_3PO_4 against time. Reserpine concentration = 0.01 mg./10 ml.*

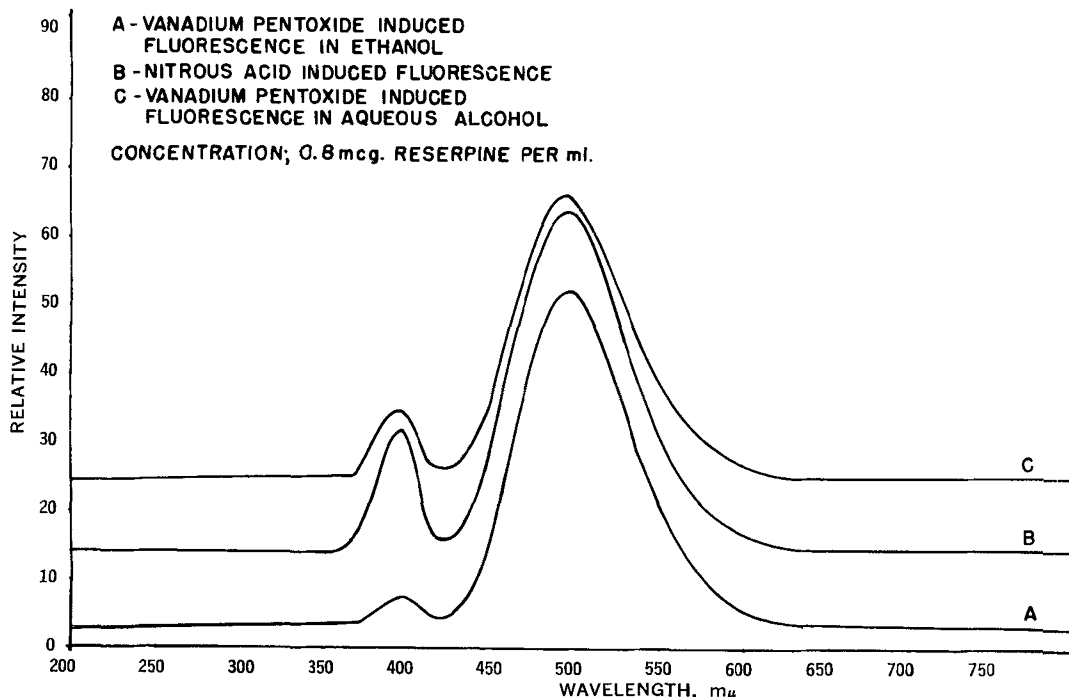


Figure 4 — *Fluorescence emission spectra of reserpine. (The base lines for Spectra B and C were displaced for visual convenience.)*

DISCUSSION

The most frequently reported quantitative method for the determination of reserpine and its related compounds, alone or in formulations, is still the nitrite procedure. The use of colorimetric or fluorometric determinations depends on the concentration of reserpine in the product to be analyzed, since the species assayed in both instances are identical. The colorimetric or fluorometric procedures currently used are fairly complicated (using several reagents and extractions). A simpler procedure requiring a single reagent and fewer associated steps with possibilities of automation would be desirable.

It is well known that reserpine can be fairly easily oxidized to a fluorescent product in acid solution using suitable oxidants. Several oxidizing reagents such as ceric compounds, peroxides, selenium dioxide, permanganates, periodates, and vanadium salts and its oxides were tested in these laboratories. Because the oxidation of

Table V—Comparison Data on Tablets, Using the New and Official Methods

Tablets	V_2O_5, mg./Tablet	Nitrite Assay, mg./Tablet*[a]
Reserpine (0.10)	0.101* 0.099	0.100
	0.258*	0.253
Reserpine (0.25)	0.244 0.255	0.245
Syrosingopine (1.0)	0.995 0.982* 0.994	0.982

[a] These assays were performed on chloroform extracts obtained by the official nitrite procedure. An aliquot of the chloroform extract was evaporated to dryness, the residue dissolved in ethanol, and the resultant solution assayed by the vanadium pentoxide procedure. The nonstarred results are based on analysis by vanadium pentoxide without prior extraction.

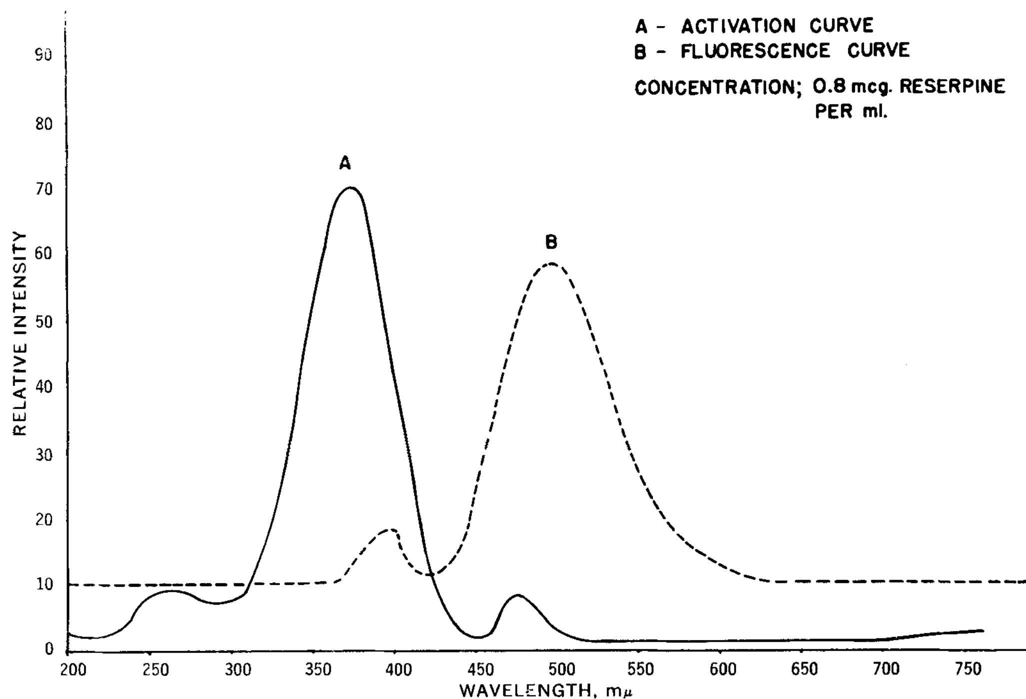


Figure 5—*3-Dehydroreserpine treated with vanadium pentoxide reagent. (The base line for Spectrum B was displaced for visual convenience.)*

reserpine occurs in acid solution, the effect of different inorganic and organic acids on the oxidants was tested. Ceric compounds dissolved in mineral acids produced fluorescence with reserpine in alcoholic solution; however, the usefulness of this reagent was impaired by a rapidly forming precipitate in the solution. The other oxidants mentioned failed to produce significant fluorescence with reserpine, regardless of the acid or solvent used for the dissolution.

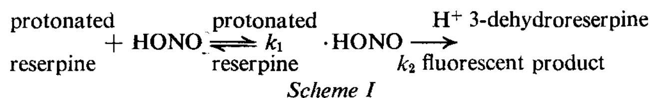
The dissolution of the pure substance or the tablet mass was carried out in alcoholic solutions. Appreciable fluorescence was observed when an alcoholic solution of the reserpine was treated with a saturated solution of vanadium pentoxide in concentrated phosphoric acid. The fluorescence yields of reserpine with vanadium pentoxide were considerably inhibited when the vanadium pentoxide was dissolved in mineral acids other than phosphoric acid. Table I illustrates the fluorescent intensity of reserpine obtained with saturated vanadium pentoxide in different acids. It is evident from these data that the maximum fluorescent intensity was achieved with phosphoric acid. The phosphoric acid reagent also proved to be more stable. From these observations it was obvious that the optimum concentration of the reagent should be investigated. The volume and the concentration of the sample solution were kept constant. The optimal fluorescent intensity was achieved using 0.25–0.5 ml. of reagent, and the data for these observations are presented in Table II. The effect of phosphoric acid upon the production of fluorescence was investigated by maintaining the reserpine and the reagent concentration constant. These data are presented in Table III and show that the variation of the phosphoric acid concentration insignificantly influenced the fluorescent intensity of the recommended assay.

During this investigation it also was observed that the fluorescence measurements were not only affected by the oxidation reagent but that the rate of oxidation was substantially affected by the solvents used in dissolution. From Fig. 1 it is apparent that the reaction proceeds most rapidly in alcohol USP but with less fluorescent intensity than in ethanolic water solutions that were tested for suitability with respect to automated procedures. This difference can be explained by the quenching effect, since the reagent imparts a slight yellow color in alcohol USP. However, this yellow color can be significantly decreased upon the addition of water. Ethanolic solutions of reserpine containing various amounts of water were analyzed by this procedure. The data presented in Table IV indicate that with 25% water, maximum fluorescence was produced. With aqueous ethanolic solution, a very faint straw-yellow color was observed; however, the fluorescence for reserpine in this solution was approximately 10–15% higher than in alcohol USP alone. Since the rate of fluorescence development is faster in alcohol USP, the slight decrease due to quenching can be overcome if one allows the fluorescence to develop in alcohol USP and then, after a suitable time, dilutes the solution to a desired volume with water.

Almost the same quenching effect as in the case of alcohol was observed when a large excess of phosphoric acid was employed for the assay. A maximum fluorescence was obtained upon addition of 0.5 ml. reagent and 0.5–1.5 ml. of phosphoric acid to 12 ml. of an ethanolic solution containing 1 mcg. reserpine/ml. These data indicate the optimum reaction conditions and the most stable fluorescence species. To determine the validity of the reaction using optimum conditions, the fluorescent intensity was plotted against the concentration of reserpine (Fig. 2). The linear plots obtained by this procedure indicate that the method is reliable. The fluorescence species appears to be stable in solution for a period of several hours. This procedure would be suitable for the determination of reserpine, alone and in tablet formulations.

Reserpine and syrosingopine[2] are fairly soluble in alcohols; tablets containing these active ingredients were extracted with alcoholic solutions. The results obtained for reserpine and syrosingopine tablets, using the new vanadium pentoxide procedure, are presented in Table V. The data were compared with the results obtained by the official nitrite procedure. The proposed new vanadium pentoxide method without an extraction procedure fails to distinguish the possible degradation products, such as reserpic acid, from the parent compound. To determine the amount of reserpic acid, the citric acid extracts remaining from the analysis of syrosingopine tablets by the nitrite procedure were subjected to the vanadium pentoxide method. Approximately 0.4% of fluorescent material based on syrosingopine was found in the citric acid extract. The differences between the extracted and nonextracted analyses indicate, therefore, that the reserpic acid concentration is negligible in comparison with the experimental error of the method. The reproducibility of the method according to the new procedure is presented in Table VI.

After a practical determination of reserpine by fluorescence measurements, an attempt was made to determine if the reaction products from the nitrite and vanadium pentoxide methods were identical. Haycock *et al.* (12) studied the kinetics of nitrous acid-induced fluorescence and suggested that the fluorescent product in reserpine by nitrous acid is a two-step reaction which can be explained by Scheme I:



Scheme I

[2] Singoserp, Ciba Pharmaceutical Co.

Table VI—Reproducibility of Vanadium Pentoxide Method[a]

Sample	Assay, mg./Tablet
A	0.2580
B	0.2540
C	0.2580
D	0.2500
E	0.2570
Average	0.2554
	$SD \pm 0.0017$

[a] Analysis of 0.25-mg. reserpine tablets.

A linear relationship was obtained by plotting the logarithm of unreacted reserpine expressed as $F_\infty - F_t$, where F_∞ is the final fluorescence of the solution and F_t the fluorescence at time t, *versus* the time required for fluorescence development with nitrous acid. A plot of this type is linear for several different concentrations of nitrite reagent and indicates first-order kinetics with respect to reserpine concentration. Preliminary data of this type have been obtained for the fluorescence produced by the reaction of reserpine with vanadium pentoxide reagent. A plot of the logarithm of $F_\infty - F_t$ *versus* time for different concentrations of vanadium pentoxide produces straight lines, indicating an apparent first-order reaction with respect to reserpine concentration as in the nitrite case (Fig. 3).

To establish that the fluorescent species is the same, 3-dehydroreserpine was synthesized from reserpine, and the synthesized product was compared with the fluorescent species produced by vanadium pentoxide reagent. Satisfactory combustion analysis was obtained for the synthesized product, and the TLC data indicated only a few trace impurities. The activation and fluorescence spectra of 3-dehydroreserpine in the presence and absence of vanadium pentoxide reagent were essentially identical to that of reserpine treated with vanadium pentoxide reagent (Figs. 4 and 5). Since the spectra were generated by an Aminco-Bowman spectrophotofluorometer, the activation and fluorescence maxima show slight differences in wavelengths from those obtained on SPF-125. This difference between the Aminco-Bowman and SPF-125 is most likely due to the difference in sources for the two instruments. The spectra were generated in ethanol and aqueous alcoholic solutions. The fluorescence yields of reserpine treated with vanadium pentoxide reagent were 106% based on the fluorescence obtained from an equimolar amount of 3-dehydroreserpine treated similarly. Some fluorescence quenching is observed for 3-dehydroreserpine treated with reagent compared to a solution of 3-dehydroreserpine treated with phosphoric acid only.

An attempt was made to isolate the fluorescent species of reserpine generated by vanadium pentoxide reagent by making the reaction mixture basic with sodium hydroxide and extracting with chloroform. Approximately 250 mcg. of reserpine was allowed to react with vanadium pentoxide reagent for several hours. The reaction mixture was made basic with sodium hydroxide, diluted to 100 ml. with water, and extracted with chloroform. The chloroform extract was evaporated to dryness, and the residue was taken up in ethanol. The UV spectrum of this solution did not compare well to the UV spectrum of 3-dehydroreserpine in ethanol, but it was practically identical to that obtained for 3-dehydroreserpine treated with vanadium pentoxide reagent and extracted as described. Rather than indicating a fluorescent species other than 3-dehydroreserpine, the different UV spectra obtained for 3-dehydroreserpine in the absence of vanadium pentoxide and after extraction from a basic solution containing vanadium pentoxide might possibly be explained by the formation of an extractable complex between 3-dehydroreserpine and a vanadium species.

The authors feel that the proposed method, with its simplicity and efficiency, would provide a suitable procedure for the routine analysis of reserpine and may contribute to the progress of an automated technique for reserpine.

CONCLUSION

A new fluorometric method has been developed for the determination of reserpine and related compounds in bulk and tablet formulations. The proposed method is based on the formation of a fluorogen with vanadium pentoxide. The intensity of the fluorogen was significantly increased when the reagent was saturated in phosphoric acid. This method, with its simplicity and rapidity, can be used for the quantitative determination of reserpine. The possible formation of 3-dehydroreserpine as the fluorescent product was postulated, and the optimum reaction conditions, were evaluated. Since the mechanism of the reaction is not completely evident and is strongly dependent on the reagent and the acid used for the reaction, these parameters will be the subject of a further investigation.

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