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Research Articles

Quantitative Determination of Serotonin in *Panaeolus* Species

By J. K. WIER† and V. E. TYLER, JR.

A method was developed for the quantitative estimation of serotonin (5-hydroxytryptamine) in mushrooms, especially species of the genus *Panaeolus*. The procedure involves extraction of the dried, finely powdered carpophores with acidified alcohol, application of the extract to filter paper strips, formation of these chromatograms with *n*-propanol : 1 *N* ammonium hydroxide (5:1), development of colored serotonin spots with a modified Pauly's reagent, determination of the total color of these spots with an automatic recording electronic densitometer, and calculation of the serotonin content from a standard curve. Each step in the procedure was evaluated to determine the influence of certain variables, and the results obtained by the final method were subjected to statistical evaluation.

SEROTONIN (5-hydroxytryptamine) has received considerable attention recently due to its interesting physiological properties (1). In addition to its occurrence in certain animal tissues, the compound has been found in a number of plants (2), including carpophores of *Panaeolus* species. Several cases of cerebral mycetism reputedly have been caused by ingestion of certain species of this genus (3). Although *P. campanulatus* (Fr.) Quél. has been reported (4) to be devoid of psychotomimetic activity in the rat and in man, Tyler and Smith (5) have reported the presence of serotonin in it and in several other species of *Panaeolus*. It was of interest, therefore, to determine the concentration of serotonin in these mushrooms.

Serotonin and related indole derivatives have been quantitated by determination of their physiological effects on certain animal tissues,

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† Fellow of the American Foundation for Pharmaceutical Education, 1959–61; S. B. Penick Fellow, 1960–61. Present address: School of Pharmacy, University of North Carolina, Chapel Hill.

such as isolated intestinal strips (6), and on grass coleoptiles (7). Other methods involving absorption of ultraviolet light, fluorescence, and colorimetry (8) have also been utilized. Difficulties involved in separating serotonin from the closely related compounds with which it frequently occurs, render all existing procedures somewhat tedious to employ and more or less nonspecific. These considerations prompted us to undertake the development of a new quantitative procedure for the determination of serotonin in mushroom tissues. Consequently, a quantitative paper chromatographic assay procedure was designed and critically evaluated.

The general procedure followed in the estimation of serotonin was to extract the serotonin from dried carpophores of *Panaeolus* species by suitable means, to spot these extracts and known serotonin solutions on filter paper strips, to prepare chromatograms on these strips with suitable developing solvents and color-forming reagents, to determine the total color of the serotonin chromatographic spots with an automatic recording electronic densitometer, and to estimate the serotonin content of the mushrooms by comparison of the total color determinations of the extract spots with those produced by known quantities of serotonin.

During the course of this investigation, a preliminary communication appeared which called attention to the possibility of quantitation of serotonin and other 5-hydroxyindoles by similar paper chromatographic methods (9). This publication did not give experimental details regarding the influence of the numerous variables involved, nor did it apply the method to extracts of natural products. Preliminary experimental evidence available at the time this work came to our attention revealed that the accurate paper chromatographic determination of serotonin was by no means as simple as the publication of Carcasona, Unterharnscheidt, and Cervos-Navarro would seem to indicate. This fact was supported by continued studies.

EXPERIMENTAL

The mushrooms utilized in this investigation were specimens of the dried carpophores of *Panaeolus campanulatus* (Fr.) Quél. and *P. foenesecii* (Fr.) Kühn. which were collected in the Puget Sound region of the state of Washington. Samples of both species were authenticated by Prof. Alexander H. Smith, University Herbarium, University Museums, University of Michigan. Since fresh carpophores could not be preserved for extended periods of time, they were dried for 48 hours in a forced-air drying oven at approximately 45°. This procedure resulted in an average loss in weight (water) corresponding to 93.5% of the fresh tissue.

Serotonin creatinine sulfate[1] (California Corporation for Biochemical Research) was used as a reference standard. Standard solutions of this serotonin complex were prepared in 0.1 *N* hydrochloric acid in diluted alcohol, U.S.P. XVI, and were preserved at room temperature in the dark. Under these conditions, the solutions were found to be stable for several weeks.

Extracts of mushroom carpophores were prepared by homogenizing for 20 minutes an accurately weighed quantity (approximately 1 Gm.) of a No. 200 powder of the tissues in about 7–8 ml. of 0.1 *N* hydrochloric acid in diluted alcohol, U.S.P. XVI, by means of a glass homogenizing tube fitted with a Teflon pestle. The particulate matter was separated by centrifugation. Portions of the supernatant liquid, representing the extract of a known tissue weight, were applied by means of a micropipet to 1-in. wide strips of Whatman No. 1 filter paper (designated for chromatographic use). The solutions were applied in 2–3 μl. portions, with drying between applications, to a maximum volume of 50 μl. It was found that while serotonin in the standard solutions was not affected by hot air drying, approximately 25% of the serotonin in the homogenate supernatant liquid was destroyed when the temperature of the drying air was approximately 65–70° (Table I). Consequently, forced air at room temperature was used for drying the spotted solutions.

TABLE I.—EFFECT OF HOT AIR DRYING ON PURE SEROTONIN COMPLEX AND SEROTONIN IN MUSHROOM EXTRACT

Compound on Strips	Blower Temperature	No. of Strips	Mean Serotonin Spot Size, in.²	95% Confidence Limits, in.²
Serotonin complex	cold	10	3.96	±0.116
Serotonin complex	hot	10	3.93	±0.195
Mushroom extract	cold	8	2.02	±0.079
Mushroom extract	hot	8	1.54	±0.053

Chromatograms were formed by the ascending method employing solvent systems of either the butanol phase of *n*-butanol : glacial acetic acid: water (4:1:5) or *n*-propanol : 1 *N* ammonium hydroxide (5:1). After drying for 2 hours at room temperature, the formed chromatograms were sprayed with either *p*-dimethylaminobenzaldehyde (2%) in hydrochloric acid : water (1:9), or with a diazotized sulfanilamide solution followed by an alkaline spray (Pauly's reagent) (10). In this investigation, 1 *N* ammonium hydroxide was substituted for the sodium carbonate solution usually employed in Pauly's reaction because carbonate left a residue on the paper strips which made the rate of feed of those strips through the scanning apparatus very uncertain. Chromatographic strips were sprayed while in a horizontal position to minimize the eluting effect of the spray solutions.

[1] A quantity of the compound equal to that purchased was supplied through the courtesy of Vismara Terapeutica, Casatenova brianza (Como), Italy.

The color formed by serotonin and the Pauly-ammonium hydroxide reagent was extremely stable. No appreciable loss of color could be detected after 6 hours; approximately 5% of the color intensity was lost after one week. The color formed with the *p*-dimethylaminobenzaldehyde reagent was very unstable; approximately 11% of this color was lost after only 2 hours.

Total color of the serotonin-reagent spots was determined with a Photovolt automatic recording electronic densitometer. This apparatus consisted of the following: (*a*) photometer, model 501-A with phototube C and a 485 mμ (Pauly reaction spots) narrow-band color filter; (*b*) transmission density unit, model 52C, with a 1 × 25 mm. aperture slit, a guide for the paper strips, and a synchronous motor drive assembly to advance the paper strips; (*c*) varicord, variable response recorder model 42. This apparatus moved the strips past the 1 × 25 mm. aperture which was located between the light source and the photoelectric cell. The chart paper of the recorder was moved in synchronization with the movement of the chromatographic strip past the aperture. The excursion of the pen on the recorder chart paper was proportional to the total absorption of light across the width of the paper strip. The area under the generally triangular-shaped curves traced by the recorder was a measure of the total color of the chromatographic spots scanned. This area was measured with a polar planimeter.

Because the density of the filter paper strips along their length was quite variable, it was necessary, for greatest precision, to apply a correction for this variation to the recorder chart base line. This was prepared by passing the blank strips through the densitometric apparatus before solutions were spotted on them and using the recording thus obtained as a base line for the curves produced from the finished chromatograms. A typical series of ten strips examined in this way, and the total spot size calculated using the variable base line, gave a mean result (95% confidence limits) of 5.35 ± 0.180 in.² in comparison to a mean of 5.20 ± 0.333 in.² when a straight base line was employed.

Standard curves were prepared for serotonin (standard solutions of serotonin creatinine sulfate) in both the *n*-butanol : glacial acetic acid : water and the *n*-propanol : 1 *N* ammonium hydroxide solvent systems, using the Pauly-ammonium hydroxide spray reagent. Table II and Fig. 1 present the data for the latter solvent system.

TABLE II.—TOTAL COLOR DETERMINATIONS OF SEROTONIN EMPLOYING *n*-PROPANOL : 1 *N* AMMONIUM HYDROXIDE CHROMATOGRAPHIC SYSTEM AND MODIFIED PAULY SPRAY

Serotonin, mcg.	No. of Strips	Mean Area, in.²	95% Confidence Limits, in.²
1	18	0.81	±0.046
2.5	18	1.98	±0.077
4	18	3.04	±0.058
5	18	3.59	±0.061
7.5	12	4.61	±0.155

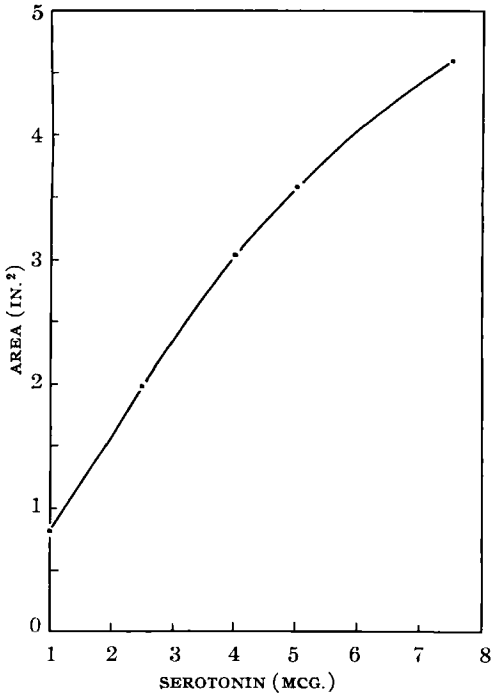

Fig. 1.—Standard curve prepared from total color determinations of serotonin spots following chromatography in *n*-propanol : 1 *N* ammonium hydroxide system and treatment with modified Pauly spray.

RESULTS AND DISCUSSION

As seen in Fig. 1, the relation of total color of chromatographic spots to the concentration of serotonin in those spots is not linear, nor does a simple logarithmic relationship exist between these two quantities. This is not an unexpected result, since the effect of the paper strip, the nature of the color-forming reaction, and the quantities of substance required in the spots for satisfactory determination of total area may all act to cause such a nonlinear relationship. The slight curvature of this line imposes no impediment to reliable assay determinations.

In order to determine the efficiency of the extraction procedure and the accuracy of the total color densitometric assay method, known amounts of serotonin were added to carpophore tissue, extracted, and quantitated by both ultraviolet light absorption and the chromatographic procedure. In addition, serotonin content of carpophore tissue was determined by a nitrosonaphthol colorimetric assay (8).

The ultraviolet absorption technique revealed approximately 95% recovery of added serotonin; the total color densitometric procedure gave a similar value for chromatograms formed in the *n*-propanol:1 *N* ammonium hydroxide solvent system. However, this procedure when applied to chromatograms formed in the *n*-butanol:glacial acetic acid:water solvent system, showed approximately only 62% recovery of added serotonin; and the value of mushroom tissue serotonin determined from such chromatograms was approximately 62% of that indicated by chromatograms formed in the *n*-propanol:1 *N* ammonium hydroxide solvent system. Since the only difference between the two cases was in the solvent systems employed, this apparent difference in mushroom tissue serotonin

content must have been due to a difference in interaction between those two solvent systems with the substances present in the mushroom extract.

Tyler and Smith (5) have shown, by the use of two-dimensional chromatographic procedures applied to extracts of *P. campanulatus*, that the serotonin spot formed in the *n*-propanol:1 *N* ammonium hydroxide solvent system is not separable into other Pauly reagent-positive spots. Therefore, the low concentration of serotonin indicated by the chromatograms formed in the *n*-butanol:glacial acetic acid:water solvent system must have been due to a failure of a portion of the serotonin present in the extracts to migrate to the normal serotonin position on the chromatograms. As Stowe has suggested (11), such failure could be due to the existence of serotonin as more than one ionic species in the presence of this solvent system and other substances present in the extracts.

Serotonin content of mushroom tissues estimated by the total color densitometric system from chromatograms formed in the *n*-propanol:1 *N* ammonium hydroxide solvent system agreed reasonably well with that determined by the nitrosonaphthol colorimetric procedure, but consistently averaged approximately 10% higher. This would seem to indicate a lower efficiency of serotonin extraction by the procedure used for the nitrosonaphthol determination. Davis (12) has indicated that this procedure gives a low value of blood serotonin due to oxidation occurring during the extraction process.

The mean serotonin content of five samples of *P. campanulatus* determined by the total color procedure presented here was 1234 ± 39.0 mcg./Gm. (Table III). Correction for the mean water content

TABLE III.—SEROTONIN CONTENT OF DRIED CARPOPHORES OF *Panaeolus campanulatus*[a]

Sample No.	Serotonin Content, mcg./Gm.
1	1211
2	1266
3	1240
4	1193
5	1260
Mean[b]	1234 ± 39.0

[a] Extracted by homogenization in acidic diluted alcohol, U.S.P. XVI. Assayed by total color method using *n*-propanol:1 *N* ammonium hydroxide (5:1) solvent system and modified Pauly spray reagent. [b] Mean ±95% confidence limits.

gives a value of 80 mcg. of serotonin per gram of fresh mushroom tissue (0.008%). The serotonin content of *P. foenesecii*, similarly established, was 3728 ± 45.8 mcg./Gm. (dry weight) or 240 mcg./Gm. of fresh tissue (0.024%). Only one plant material, the kernel of the English walnut, *Juglans regia*, has been reported to have a higher serotonin content (170–340 mcg./Gm.–fresh weight) (13).

SUMMARY

A summary of the total color densitometric assay procedure is as follows:

1. Homogenization of a No. 200 powder of dried mushroom carpophores with 0.1 *N* hydrochloric acid in diluted alcohol, U.S.P. XVI.

2. Quantitative application of these carpophore extracts to filter paper strips with forced air drying of the spots at room temperature.

3. Formation of chromatograms by the ascending method employing an *n*-propanol:1 *N* ammonium hydroxide (5:1) solvent system.

4. Formation of colored serotonin spots with Pauly-ammonium hydroxide reagent.

5. Determination of total color of serotonin spots with an automatic recording electronic densitometer.

6. Calculation of mushroom serotonin from a standard curve prepared by chromatographing known quantities of serotonin creatinine sulfate.

The extraction procedure outlined here is rapid and simple. Although the determination of total color of the chromatographic spots is time consuming, the extreme stability of the serotonin-Pauly reagent color lends itself well to such a technique. Inspection of the 95% confidence limit values presented in Tables II and III shows that the precision of the method is quite acceptable, rendering it a useful procedure for the specific determination of serotonin in plant tissues.

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