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DETERMINATION OF SEROTONIN AND BUFOTENIN AS THEIR DANSYL DERIVATIVES

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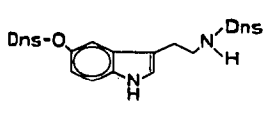
SUMMARY

A method is described which permits the determination of serotonin and bufotenin in the same tissue sample. It comprises the following steps: (a) tissue extraction with acetone–0.1 *M* hydrochloric acid (19:1); (b) reaction of the tissue extract with dansyl (Dns) chloride; (c) pre-separation of O-Dns-bufotenin from O,N-bis-Dns-serotonin and other Dns-amides on a small silica gel column (this step is dispensable if only serotonin or bufotonin is being determined); (d) TLC separation of O-Dns-bufotenin and O,N-bis-Dns-serotonin from other Dns derivatives; (e) quantitative evaluation of the separated compounds by fluorimetry for O-Dns-bufotenin and by fluorimetry or mass spectrometry for the serotonin derivative. The photometer response is linear within the range 0.1–300 nmole. With the mass spectrometric method, 2 pmole of O,N-bis-Dns-serotonin could be determined with a standard deviation of ±9%. The recovery of the amines from tissue was better than 85%.

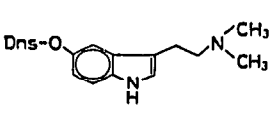
Reserpine treatment of toads caused a concomitant decrease in serotonin and bufotenin in the brain, but not in the skin of the animals. Repletion of bufotenin in the brain occurs at a higher rate than the repletion of the serotonin pool.

INTRODUCTION

The significance of serotonin (5-hydroxytryptamine) in brain physiology and pathophysiology[1,2] and the possible role of N-alkylated indoleamines in schizophrenia[3,4] prompted us to develop methods for the determination of small amounts of serotonin and bufotenin (N,N-dimethyl-5-hydroxytryptamine) that would permit the unequivocal identification of these compounds. The reaction with 5-dimethylaminonaphthalene-1-sulphonyl (dansyl) chloride (Dns-Cl) was taken as the basis of the method for the following reasons. The chemical structure of the reaction products of serotonin and bufotenin with Dns-Cl, O-Dns-bufotenin and O,N-bis-Dns-serotonin



Bis-Dns-serotonin



O-Dns-bufotenin

is well established[5]. The thin-layer chromatographic (TLC) behaviour of these Dns derivatives has been extensively studied on silica gel layers[6–10], and the serotonin derivatives (N-Dns- and O,N-bis-Dns-5-hydroxytryptamine) have been separated from some Dns-amino acids on polyamide sheets[11,12]. The method permits the determination of several compounds from the same tissue sample[8–10]. Sensitive detection and quantitation of the Dns derivatives is possible, by both fluorimetry[8–10,13,14] and mass spectrometry[15–17].

MATERIALS AND METHODS

Toads (*Bufo bufo bufo*) were purchased from K. Gutsche, Brand, G.F.R., and were kept in a terrarium and fed with meal worms.

Reserpine (Sedaraupin, 1 mg/ml, Boehringer, Ingelheim, G.F.R.) was administered into the dorsal lymph node in three portions of 3, 4 and 5 mg/kg within 1 h. Controls were injected with physiological saline solution. During tissue preparation, particular care was taken to avoid contamination of tissue with cutaneous constituents.

Male albino mice (NMRI, Ges. f. Versuchstierzucht, Hannover, G.F.R.) weighing 25–30 g were kept under standardized conditions. They had access to standard food (Altromin, Lage, G.F.R.) and water *ad libitum*.

Tissue extraction

Tissue samples were homogenized with 5 volumes of acetone–0.1 *M* hydrochloric acid (19:1) with a Potter glass homogenizer. The homogenates were centrifuged at *ca.* 800 *g* for 15 min, and the supernatants were brought to a definite volume with acetone–0.1 *M* hydrochloric acid, which was used for the re-extraction of the tissue. Aliquots of the extracts were used for the determination of bufotenin and serotonin, corresponding to 1–3 mg of tissue in the case of skin and up to 80 mg of tissue in the case of brain, intestine, etc.

Reaction of tissue extracts with Dns-Cl

The procedure, in principle, was the same as that described in detail previously[8,9], with modifications necessary for the isolation of O-Dns-bufotenin. As a tertiary amine, this compound behaves in a different manner to the usual Dns-amides.

To 0.8 ml of the extract, 0.35 ml of water and 0.1 ml of a solution of Dns-Cl in acetone (100 mg/ml) were added, and the reaction mixture was saturated with sodium carbonate. To one part of the tissue samples, known amounts of bufotenin oxalate monohydrate (EGA Chemie, Steinheim, G.F.R.) and serotonin creatinine sulphate (E. Merck, Darmstadt, G.F.R.) were added as internal standards. Standards of these amines were dansylated in the same manner. After reaction overnight at room temperature, 3 ml of acetone were added. The acetone phase was removed from the salts, which were washed again with 3 ml of acetone. The combined acetone extracts were neutralized with 0.15 ml of 66 m*M* potassium dihydrogen orthophosphate solution, then evaporated in a stream of air. The residual aqueous phase was mixed with 4 ml of methanol.

In instances when only serotonin was determined, the reaction mixtures were extracted with toluene in the normal manner[8,9] after the excess of reagent had been

made to react either with proline (10 mg in 50 μl of water) or with a few drops of sodium hydroxide solution. Aliquots of the toluene extract or the entire toluene phase can be immediately submitted to TLC.

Pre-separation of O-Dns-bufotenin from other Dns-amides

Glass tubes (6 × 200 mm) with a cotton-wool plug in the tipped end were filled with coarse silica gel (0.2–0.5 mm; E. Merck). The methanolic solutions (see above) were run through these columns and then the columns were eluted with a further 3.5 ml of methanol. The eluates contain Dns-amides, including O,N-bis-Dns-serotonin, excess of Dns-Cl and Dns-OH. O-Dns-Bufotenin, Dns-amino acids and some unidentified compounds were eluted from the silica gel columns with methanol–concentrated ammonia solution (9:1). The first 3 ml of the eluate were discarded; the next 3.5 ml contained, among other compounds, O-Dns-bufotenin.

Preparation of several two-dimensional thin-layer chromatograms on a single 20 × 20 cm plate

Supports (20 × 20 cm) prepared from porous polyethylene with two or more contact zones of 2 × 200 mm (see ref. 10 and Figs. 1A and 1B) were used. Depending on the intended chromatographic separation, the plate area of 20 × 20 cm is divided into two, three, four or more areas by scraping out *ca.* 2-mm wide lanes of the layer at appropriate distances. The positions of these lanes depend on the polyethylene support used. For instance, the subdivision of the 20 × 20-cm plate area into two 10 × 20-cm areas or into four 10 × 10-cm areas is used in combination with a support with two contact zones on opposite edges (Fig. 1A). This allows a chromatographic path length of 8 cm if the sample is applied at a distance of 2 cm from the plate edge. Other subdivisions of the plate area are also possible, and it is evident that the application of this support permits the use of the entire path length of the plate in a usual one-dimensional separation if the adsorbent is removed from one edge of

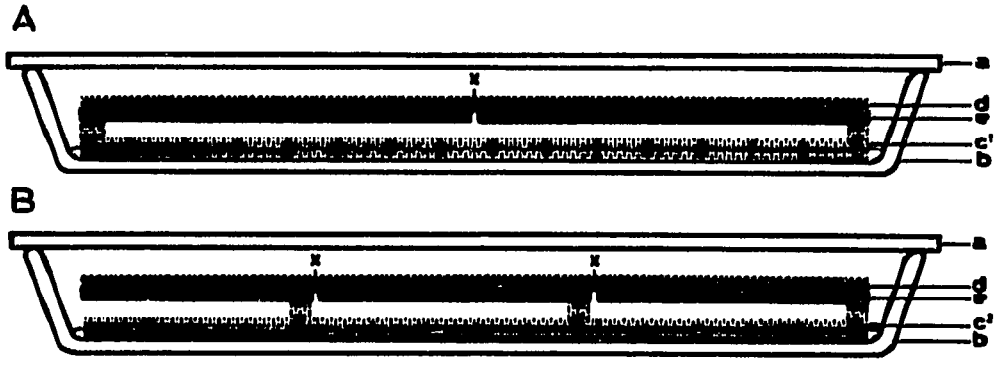


Fig. 1. Schematic diagrams to illustrate the development of thin-layer plates in a flat tank (horizontal chromatography) using porous polyethylene supports for the preparation of several two-dimensional separations on the same plate. (A) Polyethylene support with two contact zones on opposite edges; (B) polyethylene support with three contact zones for the transport of the solvent to the thin layer. a = Tank lid; b = flat tank; c = porous polyethylene support; d = glass plate; e = thin layer.

the thin-layer plate. If a support with three contact zones is used (Fig. 1B), the plate can be subdivided, for instance, into three 6.5 × 20-cm zones. Two examples of subdivisions of the plate area in combination with the use of the above types of polyethylene supports are shown in Figs. 2 and 4. In these figures, the position of the origin of the applied samples can also be seen. The plates are positioned layer-downwards on the supports (Figs. 1A and 1B), which are placed in horizontal tanks containing the solvent. Separation is complete when the solvent front reaches the scraped-out lanes.

Thin-layer chromatographic separation of O-Dns-bufotenin

The fraction of the silica gel column eluate that contained O-Dns-bufotenin was evaporated nearly to dryness in a stream of air and the residue was re-dissolved in 0.1 ml of toluene. All of this solution was applied to a thin-layer plate (with a 200-μm silica gel G layer), which had been subdivided into six 6 × 10-cm areas, according to Fig. 2. On each plate, two tissue samples, two tissue samples with internal standard and two standard samples were run at the same time. In the first dimension (chromatographic path length 8 cm), the plate was developed first with methanol, in

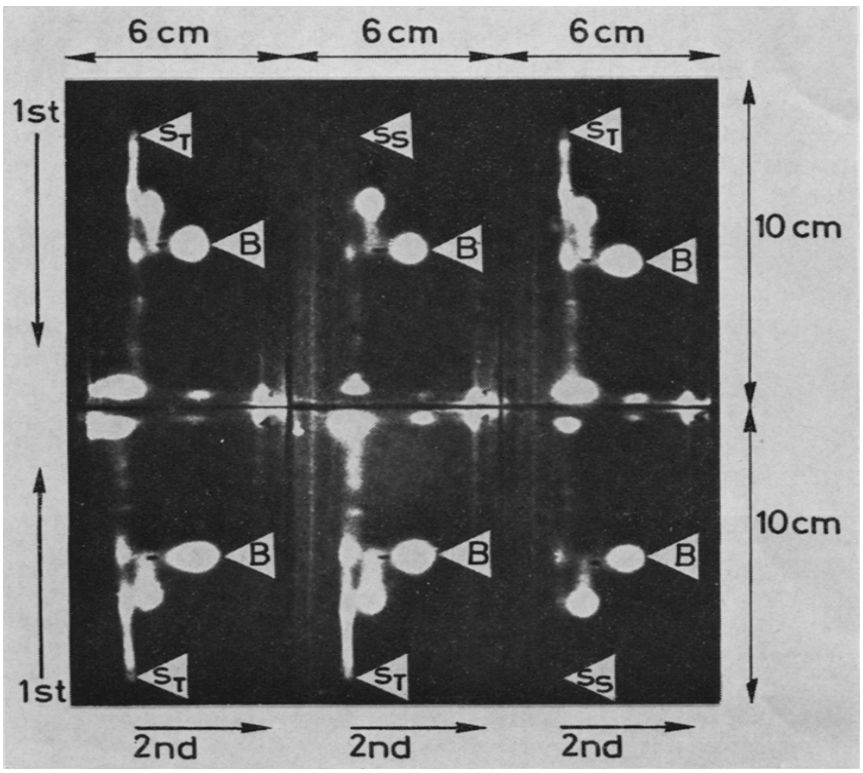


Fig. 2. Thin-layer chromatographic separation of O-Dns-bufotenin (B) from other fluorescent spots of a dansylated toad brain extract. The six two-dimensional chromatograms were prepared using the porous polyethylene supports shown in Fig. 1 (horizontal development). Solvents: 1st dimension (support with two contact zones as in Fig. 1A), (a) methanol, (b) chloroform–methanol (3:2); 2nd dimension (polyethylene support as in Fig. 1B with three contact zones), chloroform–triethylamine (5:1). S_T = Origin of tissue sample; S_S = origin of standard.

order to remove residual Dns-OH, and then with chloroform–methanol (3:2), using the support with two contact zones on opposite edges (Fig. 1A). In the second dimension (chromatographic path length 4.5 cm), chloroform–triethylamine (5:1) was used as solvent in combination with the polyethylene support shown in Fig. 1B. If the separation was not satisfactory, the plate was developed once again in the second dimension.

Quantitative determination of O-Dns-bufotenin

For increasing the fluorescence quantum yield and stabilizing the Dns derivatives, the plates were carefully sprayed with a solution of triethanolamine–2-propanol (1:4)[13]. After drying at room temperature, the plates were scanned with a commercially available spectrofluorimeter with a scanning attachment. For details of quantitative *in situ* fluorimetry see, for instance, refs. 13, 14, 18–21. Activation of fluorescence is achieved at 365 nm (mercury lamp). Either total fluorescence emission or emission at 530 nm was recorded.

Separation of O,N-bis-Dns-serotonin from other Dns derivatives

The methanol fraction of the silica gel column chromatography was evaporated nearly to dryness in a stream of air. The residue was dissolved in 2 ml of acetone and then 1 ml of water was added. The excess of Dns-Cl was then made to react by addition of 10 mg of proline dissolved in 50–100 μl of water, or was hydrolyzed by the addition of a few drops of 1 *N* sodium hydroxide solution and allowed to stand for *ca.* 30 min at room temperature. The Dns-amides were extracted from the alkaline solution with 5 ml of toluene. The toluene phase was washed with 5 ml of saturated sodium hydrogen carbonate solution and 4 ml of the toluene phase were then evaporated nearly to dryness, after which the residue was re-dissolved in 0.1 ml of toluene and applied quantitatively to a thin-layer plate (with a 200-μm silica gel G layer). The plate was prepared for two-dimensional separation (see, for instance, ref. 9) if only one sample was separated using the ascending chromatographic technique. If two samples were chromatographed on the same plate, lanes were scraped out of the layer as indicated in Fig. 4. Immediately before the samples were applied, the silica gel layer was deactivated by storage of the plate in a water vapour-saturated chromatographic tank. Development was carried out as follows: first dimension, (a) toluene, (b) cyclohexane–ethyl acetate (7:5); second dimension, chloroform–triethylamine (5:1). Finally, the plate was developed once more in the first dimension with cyclohexane–ethyl acetate (7:5). Between each run, the plate was dried at room temperature for about 3 min.

Determination of O,N-bis-Dns-serotonin

The bis-Dns-serotonin spots were marked under a UV lamp. The spots were scraped out, and the silica gel was collected in the smaller section of a glass capillary tube with a construction, which was sealed with a cotton-wool plug[22]. Using this technique, quantitative elution of bis-Dns-serotonin from the adsorbent of a normal sized spot could be achieved with 30–50 μl of ethyl acetate by suction, whereby the extract was collected in the larger section of the capillary tube. After the capillary had been broken at the constriction into two pieces, the ethyl acetate extract was transferred into the glass capillary of the direct probe of the mass spectrometer. After evap-

oration of the solvent, 2 μl of the mass spectrometric standard solution (50 pmole of bis-Dns-synephrine in acetone, or another suitable amount, which was similar to that of the amount of serotonin in the probe) were added. After the acetone had been evaporated off at a temperature slightly above ambient, the sample was introduced into the ion source of the mass spectrometer.

As both O-Dns-bufotenin and O,N-bis-Dns-serotonin decompose under various conditions, especially on active surfaces, it is advisible to carry out the procedure according to a set time schedule. Evaporated samples should not be stored for longer than a few hours, but it is possible to store samples for at leas* 24 h if they are dissolved in toluene and refrigerated.

Quantitation is achieved by recording alternately the ion currents of the molecular ions of sample (O,N-bis-Dns-serotonin, *m/e* 642) and of the mass spectrometric standard (O,N-bis-Dns-synephrine, *m/e* 633), integrating the ion currents and calculating the corresponding peak area ratios[15]. For integration, we used a two-channel counter that was developed in our laboratory, but manual integration of the recorded curve areas gives the same results. A CH5 (Varian-MAT, Bremen, G.F.R.) single-focusing mass spectrometer was used at a resolution of about 1200. The electron beam energy was 70 eV and the temperature of the ion source was 250°. With our spectrometer, we can routinely measure 30–40 samples during a normal working day.

Preparation of O,N-bis-Dns-serotonin and O,N-bis-Dns-synephrine

Bis-Dns-serotonin and -synephrine were prepared as described previously[8,13] by reaction of 1 mmole of amine with 6 mmole of Dns-Cl in 60 ml of acetone plus 25 ml of water, saturated with sodium carbonate. After storage at room temperature for 6 h, the excess of Dns-Cl was hydrolyzed by addition of a few drops of 1 *N* sodium hydroxide solution. Extraction of the Dns-amides was carried out with toluene. The residue of the toluene extract was crystallized from ethyl acetate–cyclohexane. In order to separate the compounds from N-Dns-serotonin and N-Dns-synephrine, respectively, they were chromatographed on 200 × 12-mm columns of Sephadex LH-20 (Pharmacia, Uppsala Sweden) using chloroform as solvent[23]. Recrystallization from ethyl acetate–cyclohexane yielded pure compounds, which were stable for at least several months.

RESULTS AND DISCUSSION

Reaction with Dns-Cl

It has previously been shown[13] that the reaction rate of Dns-Cl with phenols is much slower than that with primary or secondary amines. An increase in pH favours the completion of the reaction with phenols.

As serotonin contains both a primary amino group and a phenolic hydroxyl group, the formation of O,N-bis-Dns-serotonin comprises two reaction steps in which the formation of N-Dns-serotonin is rapid. If the excess of Dns-Cl in the reaction mixture is not large enough (hydrolysis to Dns-OH is a significant competitive reaction) or if the mixture is not allowed to react long enough, the N-Dns derivative is observed together with the bis-Dns-derivative. This is also the case with other phenolic amines[24]. The reaction conditions lead to the quantitative formation of O,N-bis-Dns-serotonin within about 6 h, as judged from the fluorescence intensity of the chroma-

tographically separated spots and from the reaction of radioactive serotonin (5-hydroxy-[2-^{14}C]tryptamine-binoxalate, specific radioactivity 15 Ci/mole; NEN Chemicals, Dreieichenhain, G.F.R.) with Dns-Cl.

Pre-separation of O-Dns-bufotenin from O,N-bis-Dns-serotonin, Dns-OH and other Dns derivatives

During the preliminary experiments, it was observed that in the extraction of O-Dns-bufotenin with ethyl acetate from the reaction mixture, a higher recovery was achieved if tissue extract was added to a bufotenin solution than if only bufotenin was dansylated under otherwise identical conditions. Because of our previous experience with O-Dns-choline[25], it was assumed that Dns-OH forms a salt that is not extracted as easily. As in the samples without tissue a larger amount of Dns-OH is formed, owing to hydrolysis of Dns-Cl, than in the tissue samples where Dns-Cl can react with suitable functional groups; a larger amount of Dns-bufotenin is thus retained in the aqueous phase. When methanol is used as solvent, O-Dns-choline and O-Dns-bufotenin are completely separated from Dns-OH on silica gel.

Pre-separation of O-Dns-bufotenin on silica gel columns is not necessary in the case of high tissue concentrations such as frog skin. However, the pre-separation step is necessary in order to prevent overloading of the plates when the formation of large amounts of Dns-OH cannot be avoided.

The column chromatographic procedure is simple and rapid. It can be easily performed with many samples at the same time if a multi-channel peristaltic pump or the more advanced sample processor of our multi-channel analytical system[26] is available.

Thin-layer chromatographic separation of O-Dns-bufotenin

Horizontal development of thin-layer plates is not new and special chambers for this technique have been constructed[27,28]. However, the simple horizontal method has never gained great attention.

The use of porous polyethylene supports for the horizontal development in flat tanks (see ref. 10 and the Materials and methods section) is advantageous for several reasons. (a) It allows two-dimensional separations of several samples on a single 20 × 20-cm plate at the same time under identical chromatographic conditions. Figs. 1A and 1B show the mode of application of two different types of supports. Figs. 2 and 4 show chromatograms that demonstrate two examples of many different possibilities for subdividing the plate area into sub-areas of different sizes and shapes. (b) The technique of pre-loading the layer with different solvents over certain areas of the plate[28] and thus influencing the chromatographic separation shows most clearly the great importance of a defined solvent vapour atmosphere. In the usual tanks for ascending chromatography, it is difficult to produce a homogeneous solvent vapour atmosphere over the whole plate area, and it is even difficult to reproduce a certain vapour gradient. In the horizontal tank technique, the solvent vapour composition is the same over the entire plate, as the distance from the solvent surface can be controlled to within a few millimetres, so that gradients in the vapour phase cannot develop. (c) Horizontal chromatography is more rapid than the ascending development.

For the separation of O-Dns-bufotenin from other fluorescent compounds of the dansylated tissue extract, the thin-layer plates are first developed with methanol

in order to remove Dns-OH (see preceding section) and separation is then achieved with chloroform–methanol (3:2). As a chromatographic path length of 8 cm is sufficient for this, the polyethylene support with two contact zones at opposite edges can be applied (Fig. 1A). If pre-separation on silica gel columns was omitted or was insufficient, streaking of fluorescent compounds is frequently observed along the length of the chromatographic path (see Fig. 6 in ref. 10). However, O-Dns-bufotenin can easily be moved out of this fluorescing zone by developing the plate in the second dimension with chloroform–triethylamine (5:1). As can be seen in Fig. 2, a chromatographic development along a few centimetres is already sufficient for this, so that, for instance, a polyethylene support of the type shown in Fig. 1B (and in Fig. 5 in ref. 10) can be applied with advantage. The suggested solvents are also applicable to the separation of O-Dns-bufotenin by ascending chromatography in the usual chromatographic tanks. Using the plate area subdivision as shown in Fig. 2, we normally apply two tissue samples, two tissue samples with internal standard and two standard bufotenin samples on each plate.

Quantitative determination of O-Dns-bufotenin

The quantitative determination of the separated O-Dns-bufotenin spot can be achieved according to the principles that were developed in our laboratory for the determination of Dns derivatives[8,9,13], *i.e.*, either by direct scanning of the thin-layer plate or by fluorimetry after extraction of the fluorescent spot. In the present work, the plates were sprayed with triethanolamine solution in order to increase the fluorescence quantum yield and stability of the Dns derivative[13]. They were then scanned at the position of the O-Dns-bufotenin spot in the direction of the second chromatographic development using the scanner previously described[29]. Any commercially available scanner with a mercury lamp and a sensitive detecting system should, however, be equally suitable. For the quantitative extraction of the O-Dns-bufotenin spot and subsequent conventional fluorimetry, methanol–concentrated ammonia solution (19:1)[8,9,13] is a suitable solvent mixture.

Recovery of bufotenin and reproducibility of its determination

Of the many Dns derivatives synthesized during the past 10 years in our laboratory (see, for instance, ref. 8), only O-Dns-bufotenin was not stable enough to serve as a standard. Therefore, the absolute recovery of bufotenin in our procedure is not known. However, we can compare the recoveries of bufotenin from tissue. As can be seen from Fig. 3, the recovery of bufotenin, which was added in different amounts to a constant amount of tissue (toad muscle), is 95%. The reproducibility of bufotenin determinations on standard solutions and on tissue samples was the same (standard deviation, $\pm 6\%$, within the range 1–30 nmole). As the fluorescence quantum yield of Dns-phenols is only about 10% of that of primary aliphatic amines[13], the sensitivity of detection for Dns-bufotenin is of the order of 0.1 nmole per spot.

Separation of O,N-bis-Dns-serotonin

Neuhoff and Weise[11] suggested the application of polyamide micro-sheets for the separation of O,N-bis-Dns-serotonin. However, when we applied their solvent system, we were not successful in separating O,N-bis-Dns-serotonin completely from

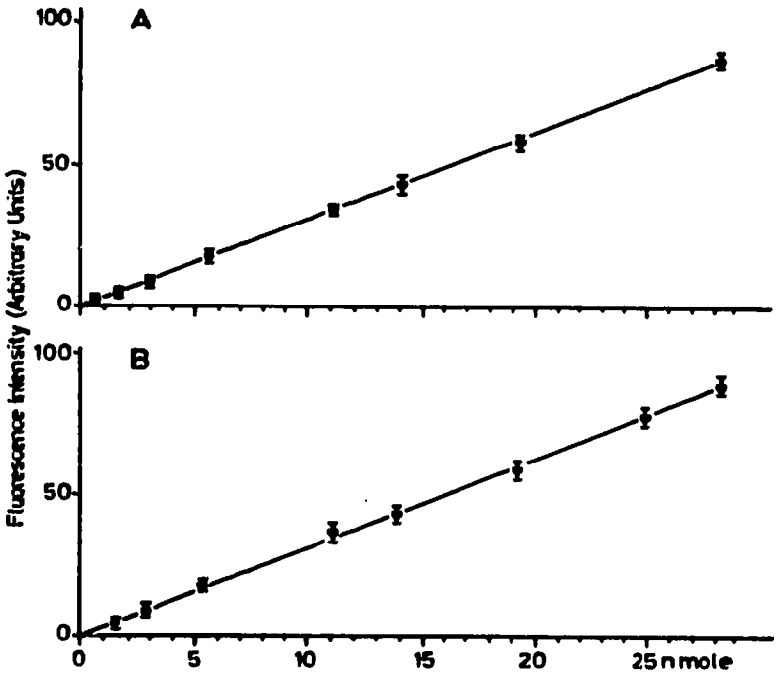


Fig. 3. Relationship between the fluorescence intensity of the O-Dns-bufotenin spot, after its chromatographic separation, and the amount of bufotenin in the reaction mixture; standard deviations indicated. (A) Samples with 50 mg of toad muscle (*Plantaris longus*); (B) samples without tissue.

all other Dns derivatives of a tissue extract. Moreover, the polyamide micro-sheets have a relatively low capacity. Amounts of Dns-NH_2 arising from tissue are normally so high that streaking occurs. Therefore, we used plates with a 200-μm layer of silica gel G and a modification of the previously described solvent system[6].

Fig. 4 shows the subdivision of the plate which permits the separation of two samples two-dimensionally together with three standards separated in only one direction. The plate is developed in a horizontal tank, using the polyethylene support shown in Fig. 1A with cyclohexane–ethyl acetate (7:5), then in the second dimension with chloroform–triethylamine (5:1). This latter separation can be performed by either ascending or horizontal development. Finally, chromatography is repeated in the first direction with cyclohexane–ethyl acetate (7:5). Before the actual chromatographic separation is performed, it is advisable to develop the plate once or twice with toluene. Toluene moves some non-polar compounds (including some side-reaction products and lipids) from the origin towards the solvent front, and thus improves the chromatographic separation.

In tissue samples that are virtually devoid of serotonin, the area of the O,N-bis-Dns-serotonin spot is "empty", even if 100 mg of tissue are extracted.

Before quantitative measurements are carried out, it is advisable to check the uniformity of the O,N-bis-Dns-serotonin spot, and its identity. For re-chromatography of the eluted spot, we recommend the use of benzene–methanol (9:1). This system not only has a powerful separation capacity for Dns-amides, but has the ad-

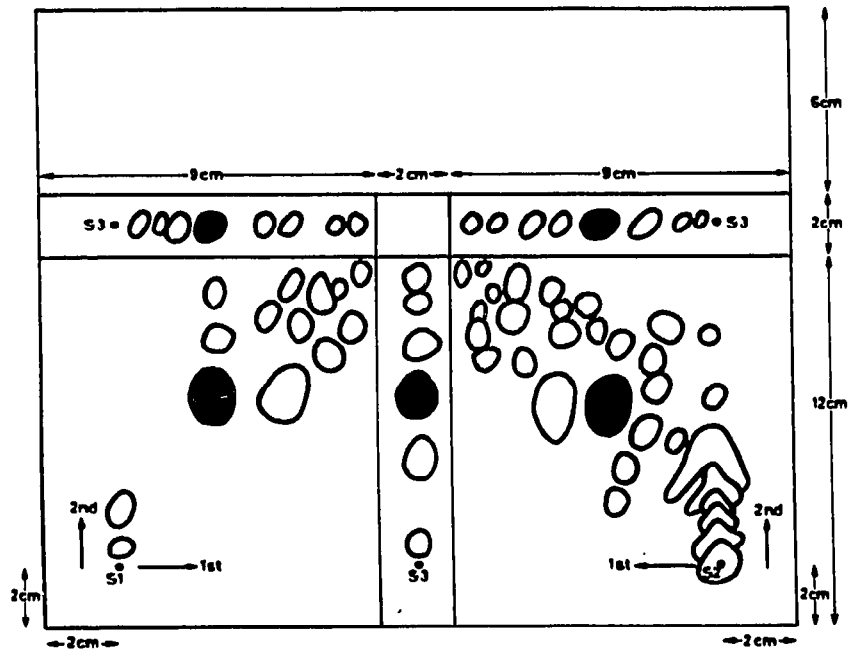


Fig. 4. Schematic diagram of the two-dimensional separation of O,N-bis-Dns-serotonin from other dansylated compounds. The shaded spot is O,N-bis-Dns-serotonin. S_1 = Origin of the standard; S_2 = origin of the tissue sample; S_3 = origins of the reference samples. Solvents: 1st dimension (horizontal development using the porous polyethylene support as in Fig. 1A), (a) toluene, (b) cyclohexane–ethyl acetate (7:5); 2nd dimension (ascending development), chloroform–triethylamine (5:1); 1st dimension, cyclohexane–ethyl acetate (7:5).

ditional advantage that Dns-NH_2 moves slower than O,N-bis-Dns-serotonin[8]. This is important when very large tissue samples are taken, where streaking of Dns-NH_2 could prevent fluorimetric evaluation.

Quantitative determination of O,N-bis-Dns-serotonin

With low tissue concentrations of serotonin, where small amounts of O,N-bis-Dns-serotonin have to be separated from large amounts of other tissue constituents and from side-reaction products of the dansylation, fluorimetric evaluation of O,N-bis-Dns-serotonin spots obtained by the chromatographic procedure (see Fig. 4) may be hazardous because even moderate tailing or overlapping of neighbouring spots may influence the quantitative result.

For the purpose of determining specific radioactivities, we suggest re-chromatography with benzene–methanol (9:1), followed by extraction with dioxan, fluorimetry of the dioxan solution and determination of the radioactivity in the same solution, after addition of PPO scintillant. Although this method is feasible in principle for the determination of serotonin in tissue as well, we prefer the mass spectrometric evaluation of the O,N-bis-Dns-serotonin spot of a two-dimensional chromatogram (Fig. 4) because of its superior sensitivity and specificity. Quantitative mass spectrometry seems at present to be the most reliable technique for this purpose. In

our method[15], a suitable standard is added to the sample and the ion currents of the molecular (or fragment) ions of sample and standard are measured alternately during evaporation of the compounds from the direct inlet probe of the mass spectrometer. Integration of the ion currents is performed with a two-channel counter or by manual integration of the recorded curves. The ratio of the integrated ion currents of the sample and the standard independent of instrumental sensitivity changes during the measurement or during successive runs. As has been demonstrated[15], O,N-bis-Dns-synephrine (O,N-bis-Dns-1-hydroxy-1-(*p*-hydroxyphenyl)-2-methylaminoethane) fulfils the requirements of an internal standard for the determination of O,N-bis-Dns-serotonin by this method. If deuterated serotonin is available, another version of the integrated ion current technique can be used[16,17]. We should point out here, because our work has been erroneously interpreted[17], that the mass spectrometric standard in our procedure is added to the sample in the capillary of the direct probe of the mass spectrometer and is not chromatographed together with the sample.

Using O,N-bis-Dns-synephrine as a mass spectrometric standard, the calibration curve was linear in the range between 1 and 40 pmole of serotonin, as can be seen in Fig. 5. With larger amounts, the curve deviates from linearity[15], the reason for which is not known[15,17]. In addition to fragmentation on the capillary surface, there may be other factors that cause the non-linearity of the calibration curve for O,N-bis-Dns-serotonin. The standard deviation in the above range was $\pm 9\%$.

The recovery of the procedure was determined by dansylation of known amounts of serotonin creatinine sulphate and comparison of the ratios of the peak areas for these

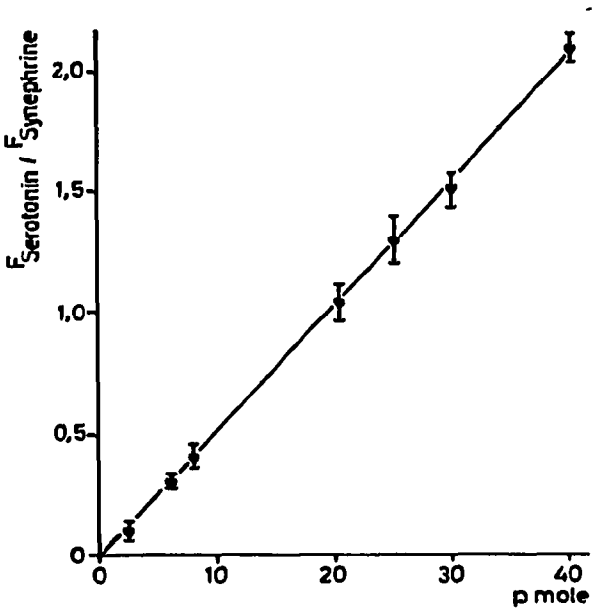


Fig. 5. Calibration graph (with standard deviations indicated) for O,N-bis-Dns-serotonin. Ratios of the integrated ion current curves of various amounts of O,N-bis-Dns-serotonin and 50 pmole of O,N-bis-Dns-synephrine.

samples with standard samples of authentic O,N-bis-Dns-serotonin. Due mainly to destructive processes on the active silica gel surface, the overall recovery of O,N-bis-Dns-serotonin is only 20%. The losses during tissue extraction are nearly negligible compared with the losses during the chromatographic procedure.

We checked our method using mouse brains, as the serotonin concentration in this tissue is well established. From determinations in four brains (four samples of tissue were analyzed from each brain), a mean value of 4.6 ± 0.2 nmole/g (wet weight) was found. This value is in good agreement with that of Udenfriend[30] (4.5 nmole/g). The values given by Pletscher[31] are higher (4.8–5.7 nmole/g).

Bufotenin and serotonin in the tissue of toad

Paying attention to the precautions which were mentioned in the Materials and methods section, we analyzed different tissues of the toad for their bufotenin and serotonin concentrations. The results are given in Table I.

TABLE I

BUFOTENIN AND SEROTONIN CONCENTRATIONS IN SOME ORGANS OF THE TOAD
Mean values ±S.D. on at least five samples.

Tissue	*Bufotenin (nmole/g, wet weight)*	*Serotonin (nmole/g, wet weight)*
Brain	21.6 ± 1.7	11.7 ± 0.5
Skin	3140 ± 210	23.5 ± 1.8
Small intestine	11.7 ± 0.7	—
Muscle	<0.5	—
Blood	<0.5	—

High concentrations of bufotenin in amphibian skin were found 40 years ago by Wieland and co-workers[32,33]. The presence of bufotenin in toad brain was established only a few years ago[34].

Our results for the concentration of serotonin in toad skin are considerably lower than those cited by Erspamer[35] (114 nmole/g). We assume that in the early experiments, interfering substances may not have been completely separated from serotonin.

The concentration of serotonin is lower than that of bufotenin not only in the skin but also in the brain of the toad. This is analogous to the lower concentration of noradrenaline compared with adrenaline in amphibian brain[36]. It is certainly premature to discuss a possible transmitter function of bufotenin in amphibian brain, but the few available data at least favour such an assumption. Further, as can be seen in Fig. 6, treatment of the toads with reserpine depletes concomitantly the brain pools of bufotenin and serotonin. However, no depletion of these amines was observed in the skin of the reserpine-treated animals. The repletion of the depleted pools seems to occur at different rates: 25 h after the administration of reserpine, the bufotenin concentration was found to be already about 50% of the control, whereas the serotonin concentration did not increase between 16 and 25 h after administration of reserpine.

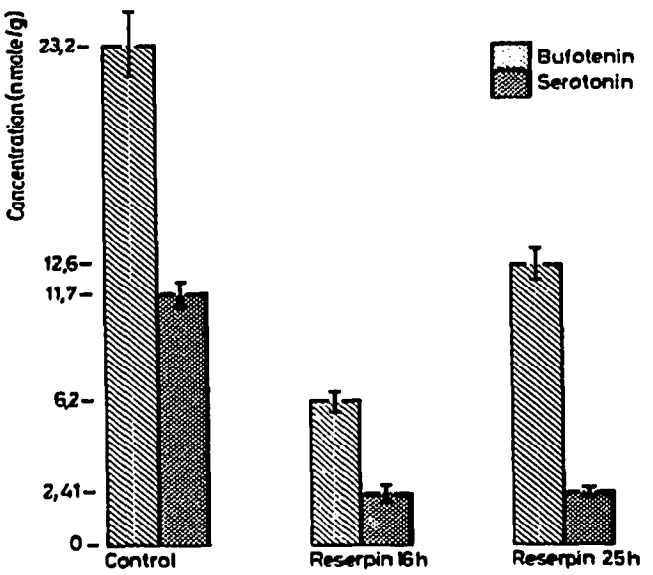


Fig. 6. Influence of pre-treatment with reserpine (12 mg/kg) on the serotonin and bufotenin content of toad brain (standard deviations are indicated in the histogram).

CONCLUSION

Several methods are available for the determination of serotonin and bufotenin. Usually, fluorescence of the reaction product with ninhydrin[30,37–39] or *o*-phthaldialdehyde[30,40] is measured, although the inherent fluorescence of indoles in acidic media has also been used until recently for the determination of serotonin[30,41]. Many different procedures have been suggested for its pre-separation from interfering compounds, but it is not easy to obtain reliable results[42].

Methods for the determination of bufotenin and other methylated indoleamines have been reviewed recently[3]. From the available information, it appears that chromatographic behaviour, distribution in different solvents and fluorescence spectra are insufficient criteria for the unequivocal identification and specific determination of these compounds. In consequence, combined GLC–MS has been applied recently, both for the determination of serotonin[43] and for the identification of alkylated indoleamines[44].

Our method is comparable to GLC–MS insofar as derivative formation precedes chromatographic separation and mass spectrometric identification and determination. The methods are also comparable in their detection sensitivity. For routine determinations, GLC–MS may have some advantages, but our TLC procedure permits the separation of extracts of larger tissue samples and is the more versatile method. It can easily be adapted to varying separation problems and, most important, nearly every step in the procedure can be controlled visually for efficiency. If fluorimetry is used for quantitation, or if only a part of the chromatographically purified sample is used for the quantitative evaluation by mass spectrometry, further experiments can be carried out with the isolated substance.

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