MODIFICATIONS OF PAPER AND THIN LAYER CHROMATOGRAPHIC METHODS TO INCREASE SENSITIVITY FOR DETECTING N-METHYLATED INDOLEAMINES IN URINE

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N-Dimethylated indoleamines have become more and more important in studying biochemical aspects of schizophrenia. Not only do they evoke psychotomimetic effects on human beings[1–4], but the formation of these compounds in mammalian tissues has been demonstrated[5,6]. Investigators from different laboratories[7–11] have repeatedly confirmed the observations that in schizophrenic patients given a monoamine oxidase inhibitor, the amino acids methionine and to a lesser extent tryptophan produced worsening of the mental symptoms. As BRUNE AND HIMWICH[8] first suggested, tryptophan may provide primary indoleamines such as tryptamine and serotonin as precursors of N-dimethylated indoleamines with methionine acting as a methyl donator. Furthermore such a formation of N-dimethylated indoleamines may mediate the aggravation of the schizophrenic symptoms. Some authors[12–14], using paper chromatographic methods, have reported the presence of a bufotenin-like substance in the urine of schizophrenic patients, but many other investigators[15–22] failed to confirm its presence. The methods so far used by various investigators are not free of criticism. The lack of sensitive and reproducible methods for detecting very small amounts of N-dimethylated indoleamines may also account for the contradictory reports. After many attempts the present author has developed more sensitive and reproducible modifications of paper and thin layer chromatographic methods for the detection of indoleamines including N-methylated ones in the human urine.

MATERIALS

Twenty-four-hour urine collections were made from four male chronic schizophrenic patients with active symptoms. Psychoactive drugs were withheld from the patients from 4–6 weeks prior to the initiation of the urine collection and throughout the experimental period. The patients were put on a diet which excluded all preformed catechol and indoleamines, and at times they were given 30 mg per day of tranylcypromine (Parnate), a monoamine oxidase (MAO) inhibitor. Urine was kept in a refrigerator at 4.5° during the daily collection and the entire volume for 24 h was stored in a freezer at —20° after the pH was adjusted to 2 with 6*N* HCl.

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METHODS

Since the amount of indoleamines, especially N-methylated ones, excreted in the urine is very small, large volumes of urine specimens (containing 100–150–200 mg or sometimes more than 200 mg of creatinine) were applied to chromatograms. Such large volumes of urine usually contain a large quantity of solids which interfere with the chromatographic separation and identification of the amines. Therefore a high degree of concentration and purification of the amine fraction is necessary before the sample is subjected to chromatographic methods.

Preliminary concentration of urine

Frozen urine was thawed at 20° and one fourth of 24 h urine volume (260–1,825 ml) was concentrated to 50–100 ml with a rotary evaporator under reduced pressure at 36°.

Purification of indoleamines

The method of KAKIMOTO AND ARMSTRONG[23] was modified for group separation and purification of indoleamines. The concentrated urine was filtered and the filtrate adjusted to pH 4.5 by adding concentrated ammonium hydroxide in a dropwise manner. To this was added 5 g wet weight, or suspended in 10 ml distilled water, Dowex 50 W X2 resin, H^+ form, 100–200 mesh, which was previously conditioned according to KAKIMOTO AND ARMSTRONG[23]. The mixture was stirred for 45 min and then poured into a glass column (2.0 × 30 cm) and the urine was separated from the resin. The resin was washed in turn with 30 ml of distilled water, 30 ml of 0.1 *N* sodium acetate, 30 ml of distilled water and 30 ml of 50 % ethanol. The initial urine effluent and the first wash effluent with distilled water were collected and reserved for the detection of conjugated amines. The wash with sodium acetate eluted the neutral and most of the aliphatic basic substances while the aromatic bases were retained on the resin. Sixty ml of 0.1 *N* sodium acetate followed by 50 ml of distilled water were used for washing instead of 30 ml each, if removal of indole amino acids such as tryptophan or 5-hydroxytryptophan was necessary. Elution of the indoleamine fraction was made with 30 ml of 1 *N* NH_4OH in 65 % ethanol and the eluate was *rapidly* concentrated to a volume less than 0.5 ml under reduced pressure at 36° in a rotary evaporator. To this was added 3 ml of absolute ethanol and mixed well, and the mixture was concentrated again to 1 ml and then transferred to a centrifuge tube. The evaporator bulb was washed twice with 1 ml of absolute ethanol and each wash was added to the first ethanol solution in the centrifuge tube. The concentration of the eluate should be done within 30 minutes. After centrifugation the transparent supernatant was transferred into a conical-bottomed tube and the eluate was concentrated to dryness *in vacuo* over sulfuric acid. Thus a highly purified free amine fraction was obtained as a dried residue which contained indoleamines, indole amino acids, phenolic amines and a small amount of urine pigments.

For the purification of conjugated amines, the above mentioned collected effluent was hydrolyzed for 30 min on a steam bath after the pH was adjusted to 1 with concentrated HCl. After cooling, the hydrolyzed solution was filtered, adjusted to pH 4.5 and exactly the same procedures were followed as those for free amines. Both free and conjugated amine fractions thus prepared were ready to be applied to

paper and thin layer chromatograms without any further purification, and were stored *in vacuo* unless used immediately.

Paper chromatography

The purified sample of urine was dissolved in 100–200 μl of 95 % ethanol and was spotted on Whatman No. 1 filter paper, the amount equivalent to a urine volume containing 100–200 mg creatinine according to the concentration of the urinary pigment involved in the sample. Two-dimensional ascending paper chromatography was carried out using a *n*-butanol–acetic acid–water (12:3:5) solvent system for the first dimension and 20 % aqueous potassium chloride for the second. Indole compounds were visualized with *p*-dimethylaminobenzaldehyde reagent according to ARMSTRONG *et al.*[24] or with *p*-dimenthylaminocinnamaldehyde (DMCA) reagent according to HARLEY-MASON AND ARCHER[25]. The latter reagent was more sensitive for the detection of N-methylated indoleamines although the positive reaction was less specific for indole compounds. With these reagents, most phenolic amines did not develop any color.

Thin layer chromatography

The purified residue of the urine sample was dissolved in 95 % ethanol and the amount equivalent to a urine volume containing 100–200 mg creatinine was applied to a 250 μ thickness of Silica Gel G (Merck) layer on an 8 × 8 in. glass plate. Two-dimensional chromatography was carried out using isopropanol–aqueous ammonia–water (8:1:1) solvent for the first dimension and *n*-butanol–acetic acid–water (12:3:5) solvent for the second. DMCA reagent was used for the visualization of indole spots. Sometimes heat (110° for 2 min) was applied to the chromatogram after spraying with the DMCA reagent in order to facilitate the reaction and to intensify the color. Evaluation of the chromatographic pattern was made within 10 min after spraying because the faintly colored spots caused by the small quantities of N-methylated indoleamines faded very fast.

RESULTS

Paper chromatography

The R_F values and colors of the indoleamines and related compounds on the paper chromatogram are listed in Table I. Although the R_F values of these compounds can vary to some extent due to the differences in the salt concentration in the sample, one can easily identify every spot in question since the color and relative position of the individual spots on the chromatogram remained consistent. 5 μg of authentic indoleamines added to 1000 ml of urine could be detected by the present methods except for 4- and 6-hydroxyindoleamines which required more than 20 μg per 1000 ml for detection because these amines decomposed readily during the purification procedures. Methylation of amino groups of indoleamines increased the R_F values with both solvent systems: the rates of movement of tertiary amines were higher than those of the secondary amines which in turn were more rapid than those of their mother primary amines (Table I).

Free and conjugated amine fractions prepared from the urine sample were examined separately. In the free amine fraction, tryptamine, serotonin and tryptophan

TABLE I

THE R_F VALUES AND COLORS OF INDOLEAMINES AND RELATED COMPOUNDS ON PAPER CHROMATOGRAMS

Compounds	R_F		*Color*	
	BuOH–HAc–H_2O[a]	*KCl*[b]	*Ehrlich*[c]	*DMCA*[d]
Tryptophan[e]	0.50	0.62	red-purple	purple
Tryptamine[e]	0.70	0.54	red-purple	purple
N-Methyltryptamine	0.73	0.58	red-purple	purple
N-Dimethyltryptamine	0.75	0.64	red-purple	purple
N-Diethyltryptamine	0.83	0.66	red-purple	purple
α-Ethyltryptamine	0.84	0.58	red-purple	purple
Psilocin	0.75	0.52	green-blue	green
5-Hydroxytryptophan	0.36	0.42	blue-purple	blue
Serotonin[e]	0.49	0.37	blue-purple	blue
N-Methylserotonin	0.50	0.42	blue-purple	blue
Bufotenin[f]	0.60	0.51	blue-purple	blue
5-Methoxytryptamine	0.64	0.34	blue-purple	blue
5-Methoxy-N-dimethyltryptamine	0.70	0.47	blue-purple	blue
Melatonin	0.87	0.39	blue	blue-green
6-Hydroxytryptamine	0.46	0.35	green	blue-green
Kynuramine	0.66	0.69	red	red-purple
Unidentified[g]	0.68	0.78	red-purple	purple
Unidentified[g]	0.60	0.71	red-purple	purple

[a] Butanol–acetic acid–water (12:3:5).
[b] 20% aqueous potassium chloride.
[c] Visualized with *p*-dimethylaminobenzaldehyde reagent.
[d] Visualized with *p*-dimethylaminocinnamaldehyde reagent.
[e] Compounds naturally occurring in human urine.
[f] Compounds found in schizophrenic urine in the presence of MAO blockade.
[g] Compounds inconsistently found in human urine.

were always revealed. In the presence of MAO blockade, tryptamine and serotonin spots became larger in size and more intense in color. Under the same conditions, bufotenin was detected in 4 out of more than 100 urine samples examined. As for the conjugated amine fraction, only tryptophan but neither tryptamine nor serotonin were found in the absence of a MAO inhibitor. In the presence of MAO blockade, however, conjugated tryptamine as well as serotonin were noted in many urine samples, and bufotenin was also obtained in 3 out of more than 100 urine samples.

Two more spots were occasionally observed in both free and conjugated fractions regardless of the administration of the MAO inhibitor. These two spots were also present in normal urine in a preliminary experiment. They were regarded as being indole compounds in view of their coloration on the chromatogram, but no further identification was made. No N-methylated indoleamines other than bufotenin were found in any urine sample by paper chromatography.

Thin layer chromatography

The R_F values and colors of the indoleamines and related compounds on the chromatogram are listed in Table II. Although the R_F values varied to some extent

TABLE II

THE R_F VALUES AND COLORS OF INDOLEAMINES AND RELATED COMPOUNDS ON THIN LAYER CHROMATOGRAMS

Compounds	R_F		*Color*[c]
	IpOH–NH₃–H₂O[a]	*BuOH–HAc–H₂O*[b]	
Tryptophan[d]	0.56	0.64	purple
Tryptamine[d]	0.80	0.75	purple
N-Methyltryptamine	0.77	0.69	purple
N-Dimethyltryptamine[e]	0.94	0.56	purple
N-Diethyltryptamine	0.98	0.60	purple
α-Ethyltryptamine	0.88	0.75	purple
Psilocin	0.86	0.55	yellow-green
5-Hydroxytryptophan[f]	0.55	0.57	blue
Serotonin[d]	0.68	0.70	blue
N-Methylserotonin[e]	0.65	0.60	blue
Bufotenin[e]	0.88	0.50	blue
5-Methoxytryptamine	0.78	0.72	blue
5-Methoxy-N-dimethyltryptamine[e]	0.92	0.54	blue
Melatonin	0.92	0.90	blue
6-Hydroxytryptamine	0.75	0.68	blue-green
Kynuramine[f]	0.82	0.68	red-purple
Unidentified[d]	0.34	0.46	purple
Unidentified[d]	0.65	0.94	purple

[a] Isopropanol–aqueous ammonia–water (8:1:1).
[b] Butanol–acetic acid–water (12:3:5).
[c] Visualized with *p*-dimethylaminocinnamaldehyde reagent.
[d] Compounds naturally occurring in human urine.
[e] Compounds found in schizophrenic urine in the presence of MAO blockade.
[f] Compounds inconsistently found in human urine.

from case to case, the identification of the chief spots was not difficult because of the consistency of the relative position and color development of the individual spots on the chromatogram. By the present methods, as little as 1 μg of authentic indoleamines added to 1000 ml of urine could be detected except for 4- and 6-hydroxyindoleamines which usually required more than 10 μg per 1000 ml for detection. Methylation of amino groups influenced the R_F values of indoleamines on thin layer chromatograms as well, although differently from those on paper chromatograms. With the alkaline solvent system used, tertiary amines showed larger and secondary amines smaller R_F values than their mother primary amines. With acidic solvent system, on the other hand, the R_F values were reduced with increasing number of substituent methyl groups; thus primary amines exhibited the largest, secondary amines smaller and tertiary amines the smallest R_F values (Table II).

The same urine samples examined by paper chromatography were studied by thin layer techniques. In free amine fractions, tryptamine, serotonin, tryptophan and two unidentified DMCA positive spots were consistently found in every urine sample regardless of the presence or absence of MAO blockade. In the absence of MAO blockade, faint spots suspected of being bufotenin, N-methylserotonin and/or N-dimethyltryptamine in free forms were also disclosed in a few samples. In the presence

of MAO inhibitor, tryptamine and serotonin spots enlarged and a spot thought to be bufotenin was detected almost consistently in each of the four patients studied. Not only were the R_F values and color of this spot identical with those of authentic bufotenin but also co-chromatography of the urine samples with the authentic compound did not produce any new spot. Free N-methylserotonin was also found in some urine samples under MAO blockade. Furthermore spots suspected of being N-dimethyltryptamine and 5-methoxy-N-dimethyltryptamine were also revealed occasionally under the same conditions, but they were located too close to each other and were too small in amount to make definite identification possible.

In the conjugated amine fraction, along with consistent occurrence of tryptophan, tryptamine was consistently and serotonin was inconsistently found in the absence of MAO blockade. Bufotenin was also observed in few urine samples although the spot was very faint. In the presence of MAO blockade, conjugated tryptamine and serotonin were detected in every urine sample, and in three out of four patients bufotenin was also discovered. Neither N-dimethyltryptamine nor N-methylserotonin were found in the conjugated forms except in one patient who excreted conjugated N-dimethyltryptamine.

Further proof of identification of bufotenin was obtained using the urine collected from the patients receiving tranylcypromine, by means of the gas–liquid chromatographic techniques which were recently developed by CAPELLA AND HORNING[26]. Details of the gas–liquid chromatographic identification will be reported separately[27].

In addition to the above mentioned indoleamines, the present study disclosed kynuramine both in free and conjugated forms under tranylcypromine administration, confirming the finding of PERRY *et al.*[28] who first reported this amine as a normal constituent in human urine.

Generally speaking, thin layer chromatography is much more sensitive than paper chromatography for detecting indoleamines. In the absence of MAO blockade, conjugated tryptamine and serotonin could not be obtained by paper chromatography whereas the former was consistently and the latter was inconsistently revealed by thin layer method. When the bufotenin spot was found on paper chromatograms it could always be confirmed by thin layer chromatography using the same urine, but the reverse was not true. No N-methylated indoleamines other than bufotenin were detected by the paper method, while N-methylserotonin, N-dimethyltryptamine and 5-methoxy-N-dimethyltryptamine were found on thin layer chromatograms.

DISCUSSION

The occurrence of a bufotenin-like compound in the urine of schizophrenic patients has been reported by some authors[12–14]. Their methods, however, make the reliability of their findings doubtful. They used acetone either as a desalting agent or as a solvent in their procedures. With acetone, naturally occurring primary amines such as tryptamine and serotonin readily form artifactual substances including acetone condensation products, which frequently make the paper chromatographic identification difficult or erroneous since some of them behave very much like bufotenin on the chromatogram. In a preliminary experiment, a mixture of tryptamine and serotonin dissolved in acetone was spotted on a paper and developed with the same

solvents as used by BRUNE *et al.*[13]. Seven DMCA positive spots were obtained on the two-dimensional paper chromatogram and one of them unfortunately resembled bufotenin both in its R_F value and color development. Another criticism is concerned with the low sensitivity of the methods used by the previous authors who purified indoleamines from the urine by an extraction method, using *n*-butanol[12,14] or ether[13] at an alkaline pH, and developed the chromatogram with an ammoniacal solvent over a comparatively long period. By these methods it is almost impossible to detect the very small amounts of N-methylated indoleamines excreted in the urine. In fact, FISCHER *et al.*[12], who first reported the presence of a bufotenin-like compound in the schizophrenic urine, did not find even tryptamine which should be excreted as a normal constituent of the human urine in much higher concentrations than those of bufotenin.

Many other investigators[15–22] failed to find bufotenin in schizophrenic urine, probably because of the low sensitivity of their detection methods as summarized below:

(1) The purification of the amines was made by an extraction method[17,20,21]. Recovery of the amines by this method is lower than by the ion-exchange method, and bufotenin can be lost during extraction.

(2) Too small amounts of urine were applied to the chromatogram to reveal N-methylated indoleamines[15,17,18,20,22]. Considering the amount of bufotenin excreted in the urine, a urine sample containing more than 100 mg creatinine should be subjected to a chromatographic paper even when the urine is collected from the patient receiving a MAO inhibitor.

(3) Only paper chromatographic identification was carried out[15–19,21,22]. This method is much less sensitive than thin layer chromatography. In addition, the long development time of paper chromatography with an alkaline solvent[18,19,21,22] might result in the decomposition of the small amount of N-methylated indoleamines present.

RODNIGHT[15] purified the amine fraction by an ion-exchange method, but he used a relatively small amount of the urine (75–120 ml), in addition he employed a large volume of acetone for desalting the amine fraction. NISHIMURA AND GJESSING[19] examined the urine from one patient with periodic catatonia during his psychotic as well as non-psychotic phases both with and without MAO inhibitor administration. They used a large volume of urine (containing 500–1000 mg creatinine), purified the amine fraction by an ion-exchange method modified from that of KAKIMOTO AND ARMSTRONG[23], further purified bufotenin by means of column chromatography according to PERRY *et al.*[20] and identified it by two-dimensional paper chromatography. Their purification method was considered to be sensitive enough to detect 5 μg bufotenin excreted in 24 h urine, but they failed to find its urinary excretion under any conditions. Periodic catatonia examined by NISHIMURA AND GJESSING[19] probably belongs to a nosological category different from that of schizophrenia and this difference may be a cause of the discrepancy between their results and ours. PERRY *et al.*[22] using an ion-exchange method for purification, studied the urinary amines in the schizophrenic patients and failed to detect any N-methylated indoleamines on paper chromatograms. They stated that their method was sensitive enough to detect 2 μg per day of these compounds. However, if they followed the purification method of KAKIMOTO AND ARMSTRONG without modification, the very small amount of N-methylated indoleamines might decompose during the evaporating process of the eluate from the sulfonic acid resin column. The amount of the urine samples applied

to paper chromatography in their routine experiment (equivalent to 25 mg creatinine) is considered too small to reveal the tertiary indoleamines. NISHIMURA *et al.*[19] and PERRY *et al.*[22] put their patients on a plant-free diet. It is possible that urinary bufotenin and other N-methylated indoleamines are of exogenous origin, and their urinary excretion is diminished on a plant-free diet. However, PERRY reported in previous papers[28,29] that bufotenin was found in the urine of normal children with or without a MAO inhibitor, and he did not mention whether or not it disappeared on a plant-free diet[28]. In any case their final detection was made by means of paper chromatography, which, as stated above, is much less sensitive than the thin layer method and they might have found bufotenin had they also used thin layer chromatographic techniques.

SIEGEL[20] is the only previous investigator who has used thin layer techniques for detecting bufotenin. Unfortunately he purified amine fraction by an extraction method and dissolved the sample in acetone. In addition, he used an insufficient volume (100 ml) of urine, and developed the chromatograms unidimensionally.

The present methods have been devised to achieve higher purification and sensitivity as well as to minimize the decomposition of very small amounts of indoleamines. To get a highly purified amine fraction, the ion-exchange method was modified from that of KAKIMOTO AND ARMSTRONG[23]. To remove ethanol soluble contaminants, a 50 % ethanol wash of the sulfonic acid resin was added before elution. The eluate from the resin was evaporated *rapidly* to prevent a breakdown of the small amount of indoleamines. High purification made it possible to apply larger amounts of sample to the chromatograms than those used by previous workers and this helped to increase the sensitivity of the detection. Acetone was never used at any step. Paper chromatography was done without using alkaline or strongly acidic solutions as developing solvents. Two-dimensional thin layer chromatography was carried out to amplify the sensitivity and to make identification more certain. The DMCA reagent was employed for visualization since the color development of indole compounds with this reagent is known to be about ten times more sensitive than can be obtained with the *p*-dimethylaminobenzaldehyde reagent[25]. Thus by the present methods, 1 μg of authentic bufotenin added to 1000 ml of urine could be consistently detected. Very small amounts of 4- or 6-hydroxyindoleamines in urine, however, could not be found by the present methods since these amines are unstable and destroyed during the elution from Dowex resin with alkaline solution.

Only free indoleamines display biological activities. They are known to be detoxicated by two ways: conjugation and deamination by MAO. Conjugation might be a more important detoxication process for psychotomimetic N-dimethylated indoleamines than for naturally occurring amines because tertiary amines are more resistent to deamination by MAO than primary amines[30]. Thus another advantage of the present method is that the free and conjugated indoleamines are able to be examined separately.

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SUMMARY

Sensitive and reproducible modifications of paper and thin layer chromatographic methods have been developed for detecting N-methylated indoleamines in the urine. The methods consist of three main steps: (1) a preliminary concentration of urine, followed by (2) the purification of indoleamine fractions using an ion-exchange resin, and (3) identification procedures. By the present methods, free and conjugated indoleamines could be examined separately, and as little as 1 μg of indoleamines per 1000 ml urine could be consistently detected. The R_F values and the color of several indoleamines and related compounds on paper and thin layer chromatograms are presented.

The usefulness of these methods was proved in the analysis of schizophrenic urines, in which bufotenin, N-methylserotonin, N-dimethyltryptamine and 5-methoxy-N-dimethyltryptamine were revealed when the patients were receiving a monoamine oxidase inhibitor.

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