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CAPILLARY COLUMN GAS–LIQUID CHROMATOGRAPHY SELECTED ION MONITORING ASSAY FOR [^{13}C,^{15}N]N-METHYLTRYPTAMINE IN HUMAN URINE: FAILURE TO DETECT CONVERSION OF [^{13}C,^{15}N]TRYPTAMINE IN SCHIZOPHRENIA PATIENTS

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SUMMARY

A capillary column gas–liquid chromatography selected ion monitoring-based method was developed for the measurement of [^{13}C,^{15}N]N-methyltryptamine (NMT) in human urine. The method was employed to establish the extent of conversion of [^{13}C,^{15}N]tryptamine to the correspondingly labeled NMT in schizophrenic patients in an attempt to demonstrate whether methylation of tryptamine plays a role in schizophrenia. Mass spectrometric detection in the assay procedure is via chemical ionization (isobutane) with monitoring of the MH^+ ions of the trimethylsilyl derivatives of [^{13}C,^{15}N]NMT and the internal standard, [$^{2}H_3$,^{13}C,^{15}N]NMT. The assay possesses a sensitivity limit (using 200 ml of urine) of *ca.* 0.1 ng/ml, corresponding to substrate conversion of *ca.* 0.00005% with a 75 mg dose (i.v.) of labeled tryptamine. Evidence for methylation was found with only one of seven patients studied; the extent of substrate conversion for the one individual was only 0.0001%. These results do not support the indoleamine–methylation hypothesis of schizophrenia.

INTRODUCTION

Abnormal methylation has long been considered to play a possible role in the etiology of schizophrenia[1–5]. The enzyme indoleamine-N-methyltransferase, present in human lung[6], is known to convert tryptamine to N-methyltryptamine (NMT), and also NMT to the known psychotomimetic agent N,N-dimethyltryptamine (DMT) under in vitro conditions[6,7]. *In vivo* conversion of NMT to DMT was demonstrated in the rabbit[8]. Whether DMT, which elicits a transient psychotic-like state when

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administered to humans, is found uniquely in schizophrenics is a question addressed by several groups of workers[9–12]. We developed and utilized gas–liquid chromatography (GLC)–mass spectrometric assays for DMT[13–15], but were not able to demonstrate unequivocally that DMT is present uniquely in plasma or urine of schizophrenic individuals. As an alternative approach for demonstrating whether methylation of tryptamine plays a role in schizophrenia, we have investigated whether patients are capable of converting exogenous tryptamine to NMT. The results of this study, which employed stable isotope labeling, capillary column GLC and mass spectrometric techniques, are presented in this paper.

EXPERIMENTAL

Labeled compounds

[^{13}C,^{15}N]Tryptamine, [^{13}C,^{15}N]NMT and [$^{2}H_3$,^{13}C,^{15}N]NMT were prepared by Gatto and Mertel[16].

GLC–mass spectrometry

A Finnigan 3200-6110 GC–MS–COM instrument was used. Operation was in the chemical ionization (CI) mode utilizing selected ion monitoring. The chromatographic conditions were as follows: glass capillary column 36 m × 0.33 mm coated with SE-30; oven temperature 180°C; injection port temperature 240°C; carrier gas (helium) flow-rate *ca.* 2 ml/min; retention time of the di-trimethylsilyl (TMSi) derivative of NMT 3.5 min. The mass spectrometer was operated using the following conditions; isobutane reagent gas (0.6 torr); ionizing potential 140 eV; amplifier gain 10^{-9} amp/volt; emission current 0.8 mA; electron multiplier 1500 V. Measurements were performed by selected ion monitoring, focusing the spectrometer on the ions *m/z* 320 and *m/z* 321 for [^{13}C,^{15}N]NMT, and *m/z* 324 for the internal standard.

Extraction procedure and derivative formation

Internal standard (200 ng) was added to 200 ml of urine. Extraction with methylene chloride (3 × 20 ml) was then carried out after pH adjustment (pH 9) with conc. ammonium hydroxide (centrifugation required to break the emulsion formed during the extraction). The combined methylene chloride phases were filtered (Whatman No. 2 paper), the filtrate extracted with 0.5 *N* hydrochloric acid (1 ml), and the organic phase removed by aspiration. The aqueous phase was then washed with methylene chloride (2 × 1 ml), made alkaline with 0.6 ml 2.5 *N* sodium hydroxide and extracted with methylene chloride (2 × 4 ml). The combined organic phases were then evaporated to dryness and the isolate transferred with methanol to a micro derivatization vessel (Pierce Micro Reacti-Vial or equivalent). Derivatization was carried out by treating the isolate with 10 μl of bis-trimethylsilyltrifluoroacetamide (BTSFA) and pyridine (2:1, v/v) at 70°C for 0.5 h. Three μl were injected into the GC–MS instrument for measurement.

Calibration

Calibration mixtures were prepared, each containing 200 ng [$^{2}H_3$,^{13}C,^{15}N]NMT and [^{13}C,^{15}N]NMT levels of 0, 20 or 40 ng. Regression analyses were carried out on the peak height ratios of the ions of interest (I_{320}/I_{324} and

TABLE I

INTENSITY RATIOS I_{320}/I_{324} AND I_{321}/I_{324} OF ISOLATES FROM CONTROL URINE (200 ml) SPIKED WITH NMT

[^{13}C,^{15}N]NMT (ng) = 374 I_{320}/I_{324} − 3.5, r = 0.9952; [^{13}C,^{15}N]NMT (ng) = 339 I_{321}/I_{324} − 13.9, r = 0.9999.

Volume urine (ml)	*[^{13}C,^{15}N] NMT (ng)*	*Internal standard (ng)*	I_{320}/I_{324}	I_{321}/I_{324}
200	0	200	0.013	0.041
200	20	200	0.057	0.100
200	40	200	0.119	0.159

I_{321}/I_{324}) *vs.* the amount of [^{13}C,^{15}N]NMT present in the mixture after isolation from 200 ml urine (Table I).

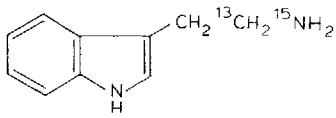
Patient group and procedure

The patient group consisted of seven DSM III-diagnosed chronic schizophrenic male patients, mean age (± S.D.) = 27 ± 2 years. All patients were actively symptomatic with multiple delusions and hallucinations at the time of study. Five of the seven patients were non-medicated (at least 3 weeks drug-free); the patient with the detectable level of [^{13}C,^{15}N]NMT in the post-dose urine was medicated with thiothixene.

All patients had urine collections obtained for the 24 h prior to the procedure; urine was then collected in two separate containers for the first 2 h post infusion and the remaining time up to 24 h post infusion. Urine was collected in bottles containing 5 ml of 10% EDTA as a preservative. Collections were kept refrigerated until completed. Urine samples were stored at −80°C until analyzed. [^{13}C,^{15}N]Tryptamine · HCl (100 mg) was infused into an antecubital vein using an infusion pump to deliver approximately 0.025 mg/kg/min up to maximum of 1 mg/min. No behavioral or cardiovascular side effects were noted in response to the infusion.

RESULTS AND DISCUSSION

Abnormal methylation of tryptamine to DMT (presumably via NMT) has been proposed as a possible cause of certain types of schizophrenia. We have therefore developed an approach for determining whether schizophrenic patients are capable of converting the endogenous indoleamine tryptamine to NMT. A key component in this study is [^{13}C,^{15}N]tryptamine (see below). If the abnormal methylation hypothesis is correct, the labeled compound would be converted to [^{13}C,^{15}N]NMT (and ultimately [^{13}C,^{15}N]DMT) at a higher than normal rate. Recognition of a substrate



conversion of 0.0001% with a 75 mg dose of labeled tryptamine was desired so that even a slight extent of methylation could be detected. This means that the assay must possess a sensitivity limit (using 200 ml of urine) of 0.1 ng/ml of [^{13}C,^{15}N]NMT. The mass spectrometric detection system, necessary to distinguish between the labeled biosynthesis product and any endogenous NMT, was operated in combination with dropping glass needle (splitless) injection capillary column GLC (which exhibits minimal adsorption losses for sample components and allows analysis of the entire sample aliquot).

Our capillary column GLC–MS assay for DMT[15] involved monitoring of the side chain fragment ion, [$CH_2=N(CH_3)_2$], m/z 58; while monitoring an ion of this low mass did run the risk of interference, the use of capillary column GLC with its superior resolving power lessened the possibility. In the current work we preferred to monitor higher mass ions from [^{13}C,^{15}N]NMT and its internal standard, and thus turned to chemical ionization (CI)-MS with isobutane reagent gas. These compounds (and their TMSi derivatives; *vide infra*) give intense pseudomolecular ion clusters suitable for monitoring and quantification purposes. The CI mass spectra of tryptamine and [^{13}C,^{15}N]tryptamine, and [^{13}C,^{15}N]NMT and [$^{2}H_3$,^{13}C,^{15}N]NMT, are

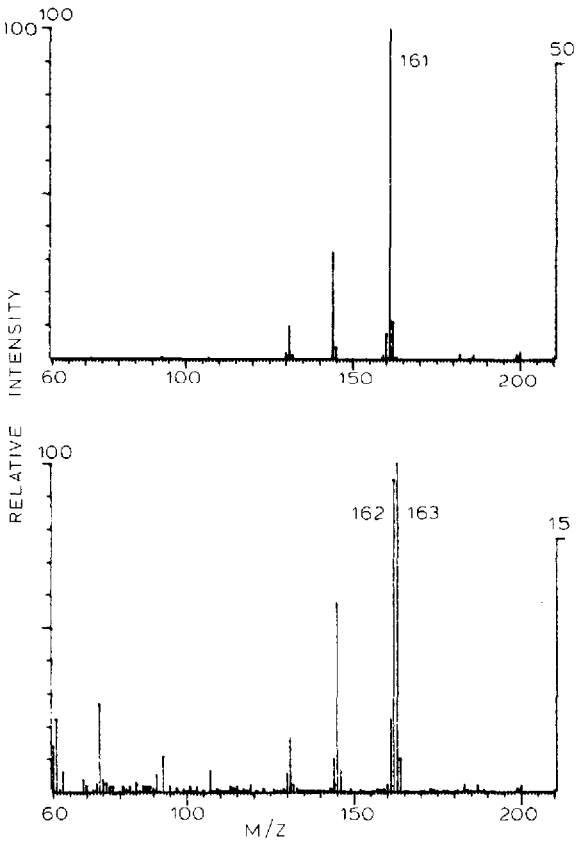


Fig. 1. Chemical ionization (isobutane) mass spectra of tryptamine (upper panel) and [^{13}C,^{15}N]tryptamine (lower panel). Conditions given in the Experimental section.

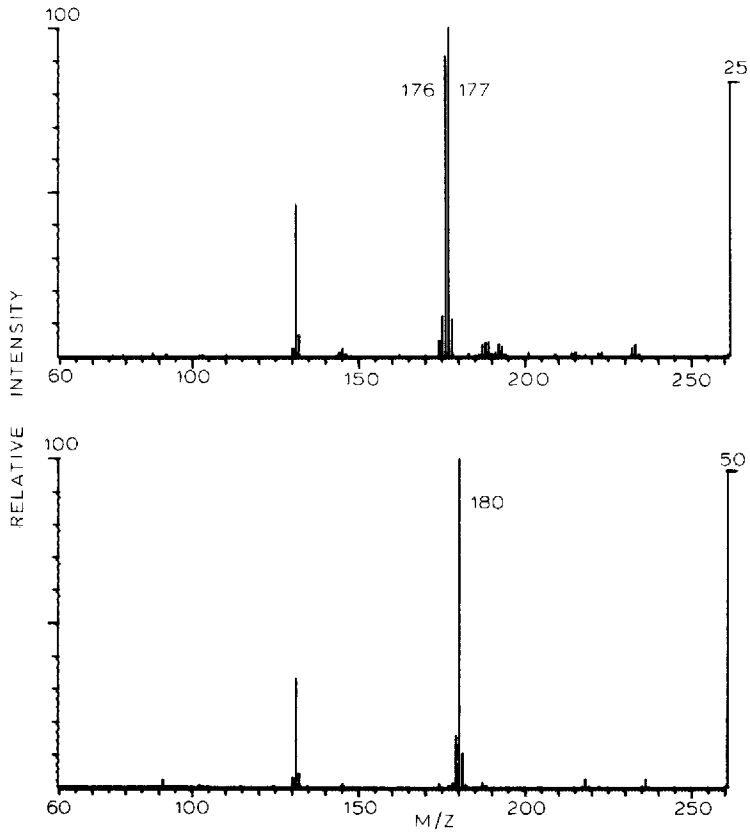


Fig. 2. Chemical ionization (isobutane) mass spectra of [^{13}C,^{15}N]NMT (upper panel) and [$^{2}H_3$,^{13}C,^{15}N]NMT internal standard (lower panel). Conditions given in the Experimental section.

presented in Figs. 1 and 2, respectively. The labeled tryptamine contains 90 atom-% excess ^{13}C and 50 atom-% excess ^{15}N, resulting in the observed doublet at *m/z* 162, 163 (compared to the single signal at *m/z* 161 for the normal tryptamine); however, the internal standard was prepared not by a route employing the labeled tryptamine as precursor, but rather a route which allowed generation of labeled NMT containing 90 atom-% excess ^{15}N (Fig. 2)[16].

Derivatization with BSTFA is employed to minimize any on-column losses and to eliminate interference noted with some urine samples when the analysis is carried out on the free amine. A mixture of the mono and di-TMSi derivatives is formed occasionally, but extended heating of the derivatization reaction mixture normally results in essentially complete conversion to the di-TMS derivative (the one of choice). Ions arising from each of these derivatives are monitored (*m/z* 248, 249 for the mono-TMSi derivative, and *m/z* 320, 321 for the di-TMSi derivative). The monitoring of two ions for each derivative gives an added measure of confidence to the result. Response for the internal standard is observed at *m/z* 252 (NMT-TMSi) and *m/z* 324 (NMT-di-TMSi).

As little as 300 pg of NMT applied to the column can be detected as its TMSi

derivative. This does, in fact, permit an assay sensitivity limit for [^{13}C,^{15}N]NMT of *ca.* 0.1 ng/ml urine. Aliquots of control urine and control urine spiked with [^{13}C,^{15}N]NMT were carried through the isolation procedure and analyzed for the labeled indoleamine. Essentially no response was observed at the retention time of NMT with the unspiked urine, but with the spiked urine positive results were obtained (Fig. 3). In a related experiment, four 200 ml aliquots of control urine were each spiked with 40 ng of [^{13}C,^{15}N]NMT (0.2 ng/ml) and subjected to the assay. Mean intensity ratios of 0.13 (I_{320}/I_{324}; C.V.-% = 8.0, n = 4) and 0.18 (I_{321}/I_{324}; C.V.-% = 6.2, n = 4) were obtained, leading to concentrations of 0.22 and 0.24 ng/ml, respectively.

The assay procedure has been employed to measure [^{13}C,^{15}N]NMT concentrations in the urine of seven schizophrenic individuals given [^{13}C,^{15}N]tryptamine. Labeled NMT was found in the post-dose urine of only one person, and at levels (0.3 ng/ml, 0–2 h; 0.2 ng/ml, 3–11 h) corresponding to substrate conversion of only 0.0001% for the 11-h collection period. A previous study on the *in vivo* (rabbit; indoleamine-N-methyltransferase is found in the lung of this animal species) conversion of NMT (administered via the ear vein) to DMT suggested a maximum conversion of 0.5%. As NMT is much superior (factor of 20) to tryptamine as a

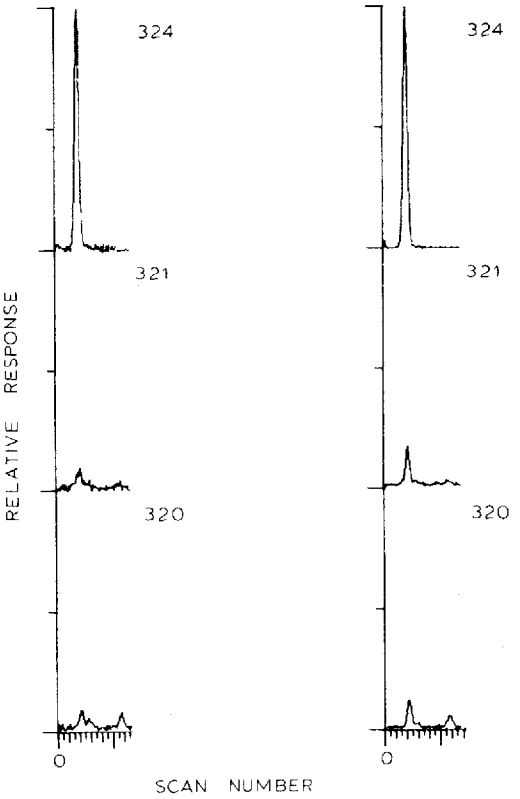


Fig. 3. Selected ion (m/z 320 and m/z 321, [^{13}C,^{15}N]NMT; m/z 324, [$^{2}H_3$,^{13}C,^{15}N]NMT) monitoring plots resulting from analysis of isolates from control urine (left panel) and control urine spiked with 0.1 ng [^{13}C,^{15}N]NMT/ml urine (right panel).

substrate for the rabbit lung enzyme[8], the poor extent of conversion of tryptamine by humans is not surprising. Study of the conversion of NMT to DMT by humans was not attempted as an approved U.S. Investigational New Drug application did not exist, whereas there were human experiences for tryptamine and it is a normal body constituent. Analysis for DMT using tryptamine as a substrate was not attempted since the levels of DMT (involving a two-step conversion) would necessarily be extremely low. No evidence was found for the presence of endogenous NMT in the urines investigated, but endogenous tryptamine was detected in the predose urines; both labeled and unlabeled tryptamine were found in the post-dose urines.

The results of the current study demonstrate that only one of seven actively symptomatic schizophrenics was capable of even a very minor extent of methylation of tryptamine to NMT. This strongly suggests that, within the limits of our analytical approach, we cannot support the hypothesis that methylation of tryptamine to DMT (requiring a second methylation step) plays a key role in schizophrenia. It is possible, of course, that urinary excretion of NMT does not reflect brain levels of the amine, or that the biosynthesized NMT was metabolized and thus not detected by the assay.

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