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GAS CHROMATOGRAPHIC–MASS SPECTROSCOPIC CHARACTERISATION OF THE PSYCHOTOMIMETIC INDOLEALKYLAMINES AND THEIR IN VIVO METABOLITES

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SUMMARY

The use of liquid chromatography with on-line fluorescence detection has formed the basis for the separation, characterisation and quantitation of a number of metabolites of the psychotomimetic indolealkylamines N,N-dimethyltryptamine and 5-methoxy-N,N-dimethyltryptamine formed both in vitro and in vivo. Verification of the identity of metabolites has previously been facilitated by the combined use of a number of analytical techniques including multidimensional liquid chromatography and stop-flow spectroscopic analysis. We now describe the combination of liquid chromatography with gas chromatography–mass spectrometry for the unequivocal verification of a number of structurally characteristic metabolites of the psychotomimetic indolealkylamines.

INTRODUCTION

The psychotomimetic indolealkylamines N,N-dimethyltryptamine (DMT), 5-methoxy-N,N-dimethyltryptamine and 5-hydroxy-N,N-dimethyltryptamine have been detected in human body fluids, and numerous attempts have been made to relate their concentrations to the presence of psychotic illnesses in man [1–5]. Although psychotomimetic indolealkylamines are extensively and very rapidly metabolised by mammalian tissues prior to excretion [6–9] little is known about their metabolic pathways, and clinical studies continue to rely on an analysis of the parent compounds alone. There is a clear need to identify structurally characteristic and quantitatively significant metabolites of the psychotomimetic indolealkylamines and to evaluate their potential utility as indicators of the presence of these compounds in man.

Limitations of the early techniques of analysis, for example, thin-layer and paper chromatography [10,11] or counter-current distribution [9,12], have ne-

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cessitated the development of more versatile techniques for the characterisation and quantitative analysis of the psychotomimetic indolealkylamine and their metabolites. Gas chromatographic (GC) procedures have been reported [2,13–15], but the need to obtain unique and suitably volatile derivatives of the indolealkylamines and their metabolites has limited the versatility of such techniques. GC techniques have recently been successfully combined with mass spectrometry (MS) for the analysis of a number of minor metabolites of DMT formed in vivo and in vitro in the presence of tissue extracts [16,17]. However, the analysis of other major metabolites, for example, indoleacetic acid (IAA) and N,N-dimethyltryptamine-N-oxide (DMT-NO), required the use of alternative radioisotopic–thin-layer chromatographic procedures [16].

Sitaram and co-workers [3,18,19] have recently investigated the use of liquid chromatographic (LC) procedures for the separation, characterisation and analysis of the hallucinogenic indolealkylamines and a number of their potential metabolites. The applications of such techniques for the study of the metabolism of the indolealkylamine both in vitro and in vivo have recently been described [19–21]. To date the identification of metabolites has been based on retention time on multidimensional LC and on stop-flow spectroscopic analysis. Additional verification of identity has been obtained using enzyme inhibitors and studies of subcellular localisation and co-factor requirements for the synthesis of individual metabolites in vitro [21].

We now describe the combination of LC techniques with GC–MS procedures for the unequivocal verification of the identity of metabolites of the psychotomimetic indolealkylamines.

EXPERIMENTAL

Materials

Iproniazid phosphate, 5-hydroxy-N,N-dimethyltryptamine (5OHDMT), N-methyltryptamine (NMT), DMT, IAA, indoleacetaldehyde (IAC), 5-methoxyindoleacetic acid (5MeOIAA), 5-methoxy-N,N-dimethyltryptamine (5MeODMT) and diethylhexylphosphoric acid (DEHPA) were purchased from Sigma (St. Louis, MO, U.S.A.).

5-Hydroxy-N-methyltryptamine oxalate (NMS) came from Aldrich (Milwaukee, WI, U.S.A.) and trimethylchlorosilane (TMCS), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), N-trimethylsilyldiethylamine (TMSDEA) and heptafluorobutyrylimadazole (HFBI) were purchased from Pierce (Rockford, IL, U.S.A.). Pyridine, obtained from Ajax (Sydney, Australia), was dried over potassium hydroxide pellets prior to use.

5-Methoxy-N-methyltryptamine (5MeONMT) and DMT-NO were kindly donated by Dr. S.A. Barker, University of Alabama. 5-Methoxy-N,N-dimethyltryptamine-N-oxide (5MeODMT-NO) was synthesized by the method of Fish et al. [20].

All solvents used were of analytical grade or LC standard. Water was glass-distilled prior to use.

Animals

Adult male Sprague–Dawley rats in the weight range 250–350 g were subjected to a 12-h day and night light cycle. Food and water were available to all animals ad libitum.

Instrumentation

Separation and quantitative analysis of the indolealkylamines and their metabolites were performed using a Perkin-Elmer Series 3B liquid chromatograph. All samples were injected on the column using a Rheodyne 7105 injector, fitted with a 175-μl loop. The spectroscopic detectors used included a Perkin-Elmer 650-10S fluorescence spectrophotometer and a Perkin-Elmer 3000 fluorescence spectrophotometer.

MS analysis was performed using a JEOL JMS-DX 300 double-focussing magnetic sector mass spectrometer equipped with a JEOL JMA-3100 mass data analysis system.

Liquid chromatography

Separation of the indolealkylamines and their metabolites was routinely achieved using a strong cation-exchange column (Whatman Partisil 10 SCX, 25 cm$\times$4.6 mm I.D.), protected by a 3 cm$\times$2.8 mm guard column packed with Whatman Co-Pell ODS. The mobile phase was methanol–0.083 *M* acetic acid/aqueous ammonia buffer pH 4.4 (30:70, v/v) and the flow-rate was 1.5 ml/min.

Recovery of the indolealkylamines and their metabolites from urine

The indolealkylamines and their metabolites were isolated from tissues and urine of animals treated intraperitoneally with either DMT or 5MeODMT (10 mg/kg) [23]. Iproniazid pretreatment (100 mg/kg 3 h prior to indolealkylamine administration) was used to increase the concentration of structurally characteristic metabolites appearing in the urine. Detailed descriptions of the techniques used for the recovery of the metabolites have previously been reported [21,23,24].

Isolation of N-oxides of DMT and 5MeODMT from urine

Individual rats (pretreated with iproniazid phosphate) were treated with the indolealkylamine DMT or 5MeODMT. Animals were housed in metabolic cages and urine specimens collected for approxiately 3 h. To 1 ml of the urine were added 500 μl of 0.5 m*M* sodium phosphate buffer pH 7.0, 6.0 ml of water and 100 μl of 1 *M* sodium hydroxide. The samples were then extracted with 4.0 ml of 0.05 *M* DEHPA in chloroform and back-extracted with 1.75 ml of 0.4 *M* hydrochloric acid. Two 500-μl samples of the aqueous phase (sample 1 and sample 2) were removed. Following the addition of 200 μl of 0.5 *M* sodium borate buffer pH 10.0 sample 1 was adjusted to pH 10.0 with 1 *M* sodium hydroxide and extracted with 4.0 ml of dichloromethane. Zinc powder (30 mg) was added to sample 2 and the solution stirred at room temperature for 10 min. Following centrifugation, the supernatant was removed, adjusted to pH 10.0 and extracted with dichloromethane as described above.

The dichloromethane extracts from each sample were removed, dried with sodium sulphate, and the solvent was evaporated under reduced pressure using a stream of nitrogen gas. The samples were derivatised using HFBI and analysed by GC–MS.

GC–MS analysis of the psychotomimetic indolealkylamines and their metabolites

Compounds for GC–MS characterisation were isolated from the eluent fractions from the SCX column which contained the selected individual fluorescent compounds. These fractions were lyophilised using a high-vacuum drying unit (Dynavac Australia, Model FD2), and the residues were reconstituted in 100 μl of methanol.

To enable GC–MS characterisation, the samples were derivatised prior to analysis using one of the following methods.

Trimethylsilyl (TMS) derivatisation [1]. Solutions of the indolealkylamine in methanol were evaporated to dryness under a stream of nitrogen. Silylation reagent mixture [pyridine–BSTFA–TMSDEA–TCMS (100:11:30:1, v/v)] was added (12 μl) and the sample heated at 85 °C for 30 min in a sealed 1-ml Reactivial. The reagent mixture was immediately analysed by GC–MS.

Derivatisation using HFBI [13]. Due to the instability of the HFBI, all reactions with this reagent were carried out in a dry box under a nitrogen atmosphere. Solutions of the indolealkylamines in methanol were evaporated to dryness under a stream of nitrogen. HFBI (80 μl) was added and the mixture heated at 80 °C for 2 h. The samples were cooled, and water (2 ml) was added. The aqueous sample was extracted with toluene (4 × 2 ml), and the organic extracts were combined. The sample was washed with distilled water (2 ml), centrifuged (1500 *g* for 5 min), and the organic layer was removed. The sample volume was reduced to less than 1 ml under reduced pressure, and then made up to 1 ml with toluene.

The following GC conditions were used for the GC–MS analysis of the derivatised samples. Column: methyl silicone bonded phase quartz capillary column (BP-1; 25 m × 0.22 mm I.D.; SGE); carrier gas: ultra-high-purity helium (0.98 bar) (Commonwealth Industrial Gases, Australia); oven temperature: 100 °C for 2 min, then increase to 300 °C at 16 °C/min; injector temperature: 280 °C.

Chemical ionization spectra of the DMT and 5MeODMT derivatives were obtained using anhydrous ammonia and isobutane, respectively, at an ion source pressure of 10^{-5} Torr and ionization energy of 200 eV. NMT and 5MeONMT derivatives were analysed using electron-impact ionization (positive-ion detection, 70 eV) at an ion source pressure of 10^{-6} Torr.

Detection of the derivatives

Following GC analysis, a reconstructed ion chromatographic (RIC) profile was obtained, and the retention time of the derivative of each authentic standard was obtained from this chromatogram. Detection of the derivatised sample was, in most cases, also achieved by the retention characteristics evident in the RIC profile. In samples of biological origin, where the individual components could not be sufficiently separated, mass chromatography was employed. This involved detection of specific ions characteristic of the individual derivatised standards. Only

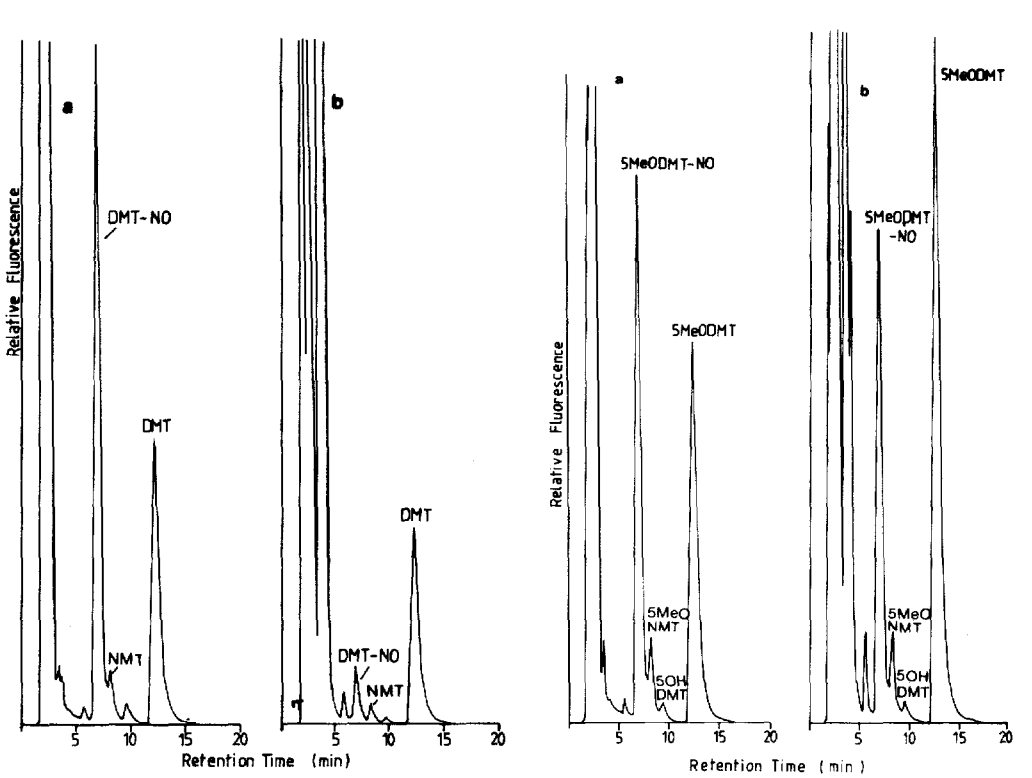

Fig. 1. Chromatogram illustrating the separation of DMT and its metabolites isolated from (a) kidney and (b) liver of animal treated with DMT (10 mg/kg intraperitoneally) 15 min before sacrifice. Tissue extracts were prepared and aliquots (100 μl) applied to the SCX column as previously described [22]. Compounds were detected by fluorescence spectroscopy (λ_{ex} 280 nm, λ_{em} 357 nm).

Fig. 2. Chromatograms illustrating the separation of 5MeODMT and its metabolites isolated from (a) kidney and (b) liver of animal treated with 5MeODMT (10 mg/kg intraperitoneally) 15 min before sacrifice. Tissue extracts were prepared as previously described [22]. Aliquots of the extracts (20 μl) were applied to the SCX column. Compounds were detected by fluorescence spectroscopy (λ_{ex} 270 nm, λ_{em} 336 nm).

ions of specific *m/e* were retrieved from the data base and used to construct a chromatogram. This restricted the interference from other endogenous compounds which chromatographed in the region of the derivatised standard.

RESULTS

Liquid chromatography

Primary chromatography of isolated metabolites was routinely performed on a strong cation-exchange column. Chromatograms illustrating the separation of DMT, 5MeODMT and their metabolites isolated from liver and kidney tissues of animals treated with DMT or 5MeODMT (10 mg/kg) are presented in Figs. 1 and 2. The metabolic profiles in urine were similar to those in kidney and liver. The use of ammonium acetate-based mobile phases coupled with lyophilisation

TABLE I

GC–MS CHARACTERIZATION OF AUTHENTIC STANDARDS OF SOME DERIVATIZED INDOLEALKYLAMINES

Compound	Retention time (min)	Major ions (relative intensity)
DMT[a,b]	9.55	386 (13), 385 (100)[c], 189 (16), 17 (19)
5MeODMT[a,b]	10.95	416 (21), 415 (100)[c], 219 (5), 162 (12)
5OHDMT[d,e]	7.77	349 (198), 348 (31)[f], 291 (22), 290 (82), 73 (100)
NMR[a,e]	10.65	566 (10)[f], 340 (24), 339 (100), 326 (41), 240 (20)
5-MeONMT[a,e]	11.99	596 (13)[f], 370 (21), 369 (100), 356 (52), 240 (25)

[a]HFB derivative.
[b]Spectrum obtained using chemical ionization.
[c]Quasi molecular ion $(M+I)^+$.
[d]TMS derivative.
[e]Spectrum obtained using electron-impact ionization.
[f]Molecular ion (M^+).

permitted the recovery of metabolites from the SCX column eluent in a form free from residual buffer salts. This form was suitable for direct derivatisation of isolated metabolites.

GC–MS of standards

Authentic samples of both N-methylated and N,N-dimethylated indolealkylamines were derivatised as described in Experimental. Preliminary investigation revealed that the electron-impact ionization of DMT and 5MeODMT resulted in mass spectra having molecular ions of very low relative abundance. As a consequence chemical ionization was preferred for these compounds, with ammonia being used as the reagent gas for DMT and isobutane for 5MeODMT. This resulted in spectra with quasi molecular ions ($m \neq 1$) at *m/e* 385 and 415, respectively. In both cases these were also the peaks of highest relative intensity.

The N-methylated derivatives (NMT and 5MeONMT) could be analysed using electron-impact ionization. In each case, molecular ions were observed (at *m/e* 566 and 596, respectively), and although they were not the base peaks they were of sufficient relative intensity to permit unique characterisation. The base peak in both cases was observed at M–227, possibly arising from a McLafferty type rearrangement involving the loss of a $CH_3NCOHC_3F_7$ fragment.

Electron-impact ionization was also employed for the TMS derivative of 5OHDMT resulting in the appearance of a prominent molecular ion at *m/e* 348 and a base peak at *m/e* 290. The retention characteristics of these derivatised standards as well as their mass spectral data are given in Table I.

GC–MS analysis of metabolite samples

As a result of rapid renal clearance the highest concentrations of the metabolites of DMT and 5MeODMT were detected in urine [23]. The use of MS characterisation was therefore evaluated on metabolites isolated from urine. The SCX column eluent fractions were obtained as described in Experimental. These sam-

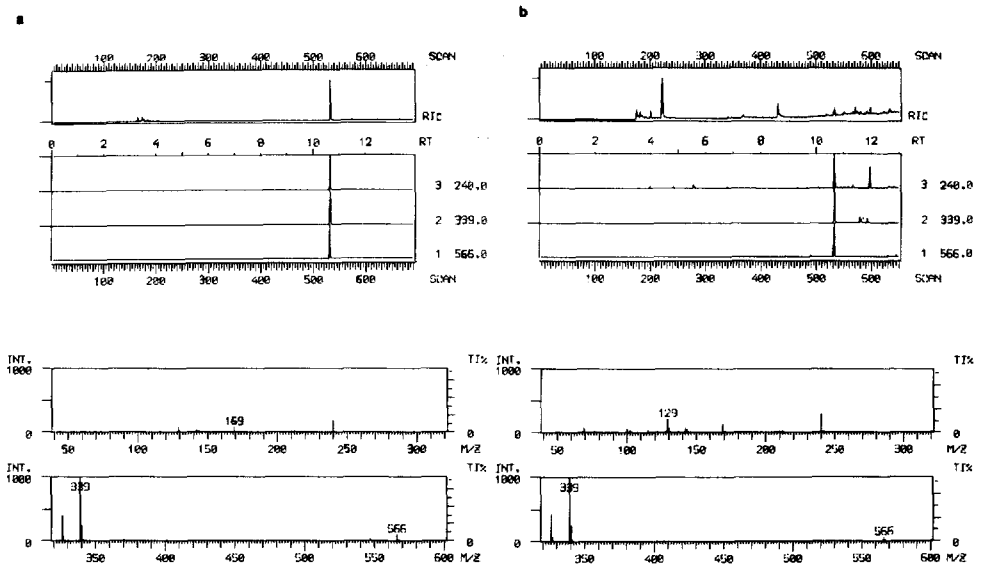

Fig. 3. RIC profiles, mass chromatograms and EI mass spectra of (a) authentic NMT and (b) NMT isolated from urine. DMT (10 mg/kg intraperitoneally) was administered to animals pretreated with iproniazid phosphate, and the urine was collected for 3 h. Urine extracts were prepared [23] and a 100-μl aliquot was applied to the SCX column. Eluent fractions containing NMT were collected and derivatised with HFBI. GC–MS of the isolate and of authentic NMT was peformed as described in Experimental.

ples were then subjected to derivatisation and GC–MS analysis under identical conditions to the respective standards. The RIC profiles of the derivatized biological extracts indicated the inevitable presence of impurities in these samples. These impurities, especially when the metabolite levels were low, sometimes prevented the simple identification of the retention characteristics of the derivatised metabolite from the RIC profile alone.

This was overcome by the use of mass chromatography wherein ion chromatograms were obtained of a single, specific *m/e* which was characteristic of the compound under investigation. The utility of this technique is demonstrated in Fig. 3.

The RIC profile of the derivatised NMT standard shows the presence of one major peak; however, in the sample the presence of impurities preclude the identification of the derivatised NMT solely from the RIC profile. The mass chromatogram, however, shows clearly that only one peak is present in the sample that possesses all the ions characteristic of the standard. Following the retrieval of the full spectrum from the data base the peak could be identified as that arising from derivatised NMT. A final check was provided by a comparison of the retention characteristics of the standard and sample.

For each metabolite sample at least three of the most characteristic ions were scanned, and the mass chromatograms were recorded. In all cases only one peak was seen to display all of these ions. The spectrum of this peak was then retrieved from the data base for comparison with that of the standard. The mass spectral

TABLE II

GC–MS CHARACTERIZATION OF THE DERIVATIZED INDOLEALKYLAMINE METABOLITES ISOLATED FROM URINE

Compound	Retention time (min)	Major ions (relative intensity)
DMT[a,b]	9.52	386 (17), 385 (100)[c], 189 (13), 17 (19)
5MeODMT[a,b]	10.97	416 (18), 415 (100)[c], 219 (14), 162 (10)
5OHDMT[d,e]	7.73	349 (11), 348 (33)[f], 291 (24), 290 (90), 73 (100)
NMT[a,e]	10.67	566 (55)[f], 340 (25), 339 (100), 326 (43), 240 (29)
5MeONMT[a,e]	11.95	596 (23)[f], 370 (21), 369 (100), 356 (63), 240 (15)

[a]HFB derivative.
[b]Spectrum obtained using chemical ionization.
[c]Quasi molecular ion $(M+1)^+$.
[d]TMS derivative.
[e]Spectrum obtained using electron-impact ionization.
[f]Molecular ion (M^+).

data of the derivatised samples are given in Table II. In every case the fragmentation pattern of standard and sample is identical, with only minor deviations in relative intensity being observed. These deviations were probably due to the corrections for baseline noise which are necessary in data analysis of this type. The chromatographic properties of the standard and sample were also similar with retention times being identical to within 2 s (Tables I and II).

Analysis of the N-oxides

N-Oxides are notoriously difficult to characterise using MS, as they tend to rapidly lose the oxygen [24]. Softer methods of ionization such as fast atom bombardment (FAB) have been used successfully [25]; however, as a source of FAB was not available, alternative methodology was developed.

Identification of DMT-NO and 5MeODMT-NO was achieved by Zn/HCl reduction of the N-oxide to the corresponding parent copound, prior to derivatisation with HFBI and subsequent GC–MS analysis. To confirm the effective reduction of the N-oxide to its parent compound, samples of urine spiked with DMT-NO or 5MeODMT-NO were analysed on the SCX column before and after Zn/HCl treatment. The absence of peaks for DMT-NO or 5MeODMT-NO and the emergence of DMT and 5MeODMT peaks confirmed the effective conversion of the N-oxides to their parent compounds.

Urine specimens obtained following indolealkylamine administration normally contained not only the N-oxide metabolites but also significant levels of the parent compounds [23]. To determine the magnitude of the increase in the levels of DMT and 5MeODMT following Zn/HCl treatment, the urine was divided into two fractions during analysis, only one of which was treated with Zn/HCl. Following derivatisation, the samples were analysed using GC. As expected, increased levels of DMT and 5MeODMT were evident in the treated samples. Fig. 4 illustrates the increase in the levels of 5MeODMT following Zn/HCl treatment of a urine sample obtained from an animal injected with 5MeODMT.

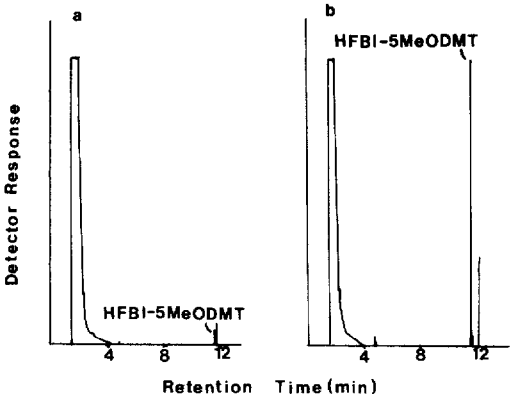

Fig. 4. Chromatogram of 5MeODMT (and 5MeODMT-NO) isolated from rat urine and derivatised with HFBI (a) without and (b) with prior Zn/HCl acid reduction. 5MeODMT (10 mg/kg intraperitoneally) was administered to animals pretreated with iproniazid phosphate. Urine was collected and 5MeODMT and 5MeODMT-NO were extracted as described in Experimental. Samples were assayed by GC with flame ionization detection on a capillary BP column.

The GC–MS analyses of the derivatives were also consistent with their identification as DMT and 5MeODMT in terms of both retention characteristics and mass spectra.

DISCUSSION

LC when combined with techniques such as fluorescence spectroscopy offers a number of major advantages over existing thin-layer, paper and gas chromatographic techniques for the separation and detection of the indolealkylamines and their metabolites. Given that the fluorescence characteristics conferred on the indolealkylamines by the indole moiety is preserved during metabolism such LC–fluorescence techniques permitted the simultaneous analysis of both their parent compounds and their metabolites. Using on-line fluorescence detection sub-nanogram quantities of the indolealkylamines and their derivatives can be detected [18].

The application of such diverse models of LC as cation-exchange, normal-phase and reversed-phase chromatography offers great flexibility for the separation and chromatographic characterisation of structurally related derivatives of these compounds [19,21]. Assigned identities may be further supported by stop-flow spectroscopic analysis. However, for the unequivocal identification of individual metabolites it is essential to obtain GC–MS confirmation of assigned identities. Since detection by fluorescence spectroscopy is non-destructive and recoveries following chromatography are essentially quantitative [18], compounds can potentially be isolated from the column eluent and subjected to such analytical characterisation.

Characterisation of the metabolites recovered from the SCX column eluent was greatly facilitated by the removal of ammonium acetate-based mobile phase by lyophilisation.

Identification of DMT and 5MeODMT in the lyophylisates of appropriate SCX column eluent fractions was achieved using GC in combination with chemical ionisation MS following derivatisation with HFBI. In each case compounds with retention characteristics and mass spectra comparable to those of the derivatised standards were present confirming the identities of the parent compounds. Similarly GC–MS was successfully used to identify the monomethylated metabolites of DMT and 5MeODMT by comparison of the electron-impact mass spectra and retention characteristics of the derivatised compounds. The effective derivatisation of authentic 5OHDMT was achieved using TMS reagents. Subsequent GC–MS analysis enabled the confirmation of the identity of the hydroxylated metabolite of 5MeODMT.

Due to the difficulty incurred during the analysis of N-oxides using MS [24] alternative techniques were developed to enable the effective characterisation of DMT-NO and 5MeODMT-NO using GC–MS. Reduction of the N-oxides to their parent compounds prior to heptafluorobutyryl derivatisation and subsequent GC–MS proved successful. Comparison of the RIC profiles and CI spectra of the derivatised samples and standard confirmed the presence of DMT and 5MeODMT following reduction of their respective N-oxides.

In addition to providing unequivocal verification of the identity of metabolites of DMT and 5MeODMT previously characterised by multidimensional LC and stop-flow spectroscopic analysis the inclusion of GC–MS has yielded an integrated analytical strategy to facilitate the identification of further metabolites of the psychotomimetic indolealkylamines.

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