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Identification of metabolites of 4,4′-diaminodiphenylmethane (methylene dianiline) using liquid chromatographic and mass spectrometric techniques

M. Kajbaf, O. Sepai and J. H. Lamb

MRC Toxicology Unit, Carshalton, Surrey SM5 4EF (UK)

S. Naylor

Department of Biochemistry and Molecular Biology and Department of Pharmacology, Court Level Guggenheim, Mayo Clinic, Rochester, MN 55905 (USA)

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ABSTRACT

The *in vitro* metabolism of 4,4′-diaminodiphenylmethane (methylene dianiline, MDA) was investigated using rabbit liver microsomes. Minimal clean-up of the microsomal incubations was carried out using zinc sulphate precipitation followed by solid-phase extraction on Sep-Pak C_{18} cartridges. Three metabolites were detected in hepatic microsomal incubations, namely the azodiphenylmethane (azo) azoxydiphenylmethane (azoxy) and 4-nitroso-4′-aminodiphenylmethane (nitroso) compounds. The azo and azoxy metabolites were produced enzymatically whereas the nitroso compound may have been formed via a non-enzymatic process. Reversed-phase high-performance liquid chromatography–plasma spray mass spectrometry was used to initially detect these metabolites. Fast atom bombardment mass spectrometry and fast atom bombardment tandem mass spectrometry were utilized to further structurally characterise these compounds. Comparison of mass spectral data obtained from synthesised standards with data obtained on the putative metabolites substantiated the characterisation of these compounds.

INTRODUCTION

Aromatic amines may present a health hazard to people, and in particular to workers manufacturing dye stuffs and rubber products. A prominent metabolic pathway observed for arylamines is N-oxidation which is proposed as the reaction which produces the toxic, carcinogenic or mutagenic metabolites of such compounds [1–4]. Furthermore, N-hydroxylation results in the activation of a number of arylamine and amide compounds to produce bladder carcinogens [5–8]. N-Hydroxylated compounds have been shown to conjugate with glucuronic acid but subsequent hydrolysis in the bladder results in release of the free hydroxylamine, a proven causative agent of bladder cancer in humans and in dogs [9].

The aromatic diamine, 4,4′-diaminodiphenylmethane (methylene dianiline, MDA) is widely used in industry, such as in the manufacture of isocyanates, polyisocyanates and polyurethane. The latter compound is used in the construction

Correspondence to: O. Sepai, MRC Toxicology Unit, Carshalton, Surrey SM5 4EF, UK.

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of numerous medical devices, which, during gamma-ray or autoclave sterilization, degrades to produce MDA [10]. Furthermore, the production of elastomers and epoxy resins incorporates MDA as a hardener [11]. MDA has been shown to be hepatotoxic in rats [12,13], dogs [14] and man [15–18] as well as carcinogenic in rats and mice [19,20]. MDA is mutagenic in the Ames test, but only in the presence of an S9 metabolising system [21].

MDA and N-acetylated MDA have been detected in urine samples of workers exposed to MDA [22]. The corresponding haemoglobin adducts have been detected in rats, using gas chromatography–mass spectrometry (GC–MS) [23]. Whilst there have been a number of studies [24–26] to determine exposure of workers in the workplace using conventional biomonitoring methods, no *in vitro* studies have been reported on the metabolism of MDA in either human or animal models.

In this report we present a preliminary study of the metabolism of MDA using hepatic microsomes from rabbit as the metabolic system. Two analytical methods were employed to detect metabolites in the incubation mixture: (a) high-performance liquid chromatography–plasma spray mass spectrometry (HPLC–PSP-MS; (b) off-line HPLC, fast atom bombardment MS (FAB-MS) and FAB tandem MS (FAB–MS–MS).

EXPERIMENTAL

Materials

MDA and the FAB matrix, glycerol, were obtained from Aldrich (Gillingham, UK). MDA was recrystallised from aqueous methanol, before use. [^{14}C]MDA (uniformly ring-labelled) with a specific activity of 56 mCi/mmol (Amersham International, Amersham, UK) was a gift from the Health and Safety Executive (UK).

Glucose-6-phosphate dehydrogenase (G-6-PD), glucose-6-phosphate disodium salt (G-6-P), nicotinamide-adenine dinucleotide phosphate ($NADP^+$), 3-chloroperoxybenzoic acid, potassium persulphate and magnesium chloride (AR grade) were obtained from Sigma (Poole, UK). Zinc sulphate was purchased from FSA Lab Supplies (Loughborough, UK).

Acetonitrile (HPLC grade), *p*-toluene sulphonic acid (PTSA) and sulphonic acid were obtained from BDH Chemicals (Dorset, UK).

Preparation of 4-nitro-4'-aminodiphenylmethane (nitro-MDA)

Oxidation of MDA was achieved using Caro's acid (25 g of potassium persulphate and 25 ml of concentrated sulphonic acid). The solution was adjusted to between pH 3 and 4 using 2 *M* potassium hydroxide. MDA (0.02 mol) in ethanol (20 ml) was added to the Caro's acid solution and vigorously stirred for 2 h at room temperature (20°C). The mixture was then filtered, and the filtrate left overnight at room temperature in order to complete the reaction.

The filtrate was washed with several aliquots of ethanol, and the combined ethanol washes were evaporated to dryness. The brown solid obtained was recrystallised using aqueous methanol. A single spot (R_F 0.86) was observed on thin-layer chromatography (TLC) silica gel 60 F_{254} plastic backed pre-coated plates run with toluene–methanol (9:1, v/v) visualised under UV light at 254 nm. This spot was eluted and characterised using FAB-MS.

Preparation of metabolites

The procedures outlined by Yost and Gutmann [27], Westra [28] and Chen *et al.* [29] were modified and used to produce the nitroso derivative of MDA.

MDA (17 μmol) and 3-chloroperoxybenzoic acid (35 μmol) were dissolved in cold, dry chloroform (0.9 ml) and allowed to stand at room temperature (20°C) for 90 min. The reaction was monitored using TLC (conditions as above), and three spots were observed (R_F 0.50, 0.60 and 0.76).

The reaction mixture was evaporated, under vacuum, and the residue redissolved in 300 ml of toluene. The crude mixture was purified on a silica gel column (35–70 mesh, 10 cm × 0.7 cm I.D.). Dry toluene was used as eluent, with a flow-rate of 1 ml/min, and 5-ml fractions were

collected. Each fraction was analysed by TLC (conditions as above) and three bands were observed. Using FAB-MS these were identified as the azodiphenylmethane (azo), azoxydiphenylmethane (azoxy) and 4-nitroso-4′-aminodiphenylmethane (nitroso) products, eluting in that order (refer to Table I).

Hydroxylamine compounds are noted for their instability and tendency to degrade to, for example, the azo and azoxy compounds. Attempts to reduce nitro-MDA, using zinc dust and ammonium chloride, to yield the hydroxylamine, proved unsuccessful, and only the azo and azoxy products were detected.

HPLC separation of metabolites

Mixtures of the synthetic metabolites and crude microsomal mixtures were separated by HPLC. The HPLC system consisted of a Gilson 714 system controller software, two Model 305 pumps, a Model 811B dynamic mixer, a Model 805 monometric module and a Model 621 data master which were connected to a 1000S diode-array detector (Applied Biosystems).

All separations were carried out on a 10 μm particle size, reversed-phase μBondapak C_{18} column (25 cm × 0.46 cm I.D.) (Bio-Rad, Richmond, CA, USA).

A flow-rate of 1 ml/min was maintained during a gradient elution with water (solvent A) and acetonitrile as the organic mobile phase (solvent B). The gradient was as follows: t = 0 min, 85% A; t = 3 min, 85% A; t = 7 min, 30% A; t = 9 min, 20% A; t = 15 min, 20% A; t = 16 min, 85% A. A 100-μl injection loop was used in all experiments.

Preparation of hepatic microsomes

Male New Zealand white rabbits fed standard diet and water *ad libitum* were used as the liver source. Hepatic microsomes were prepared by the modified calcium precipitation method of Lam *et al.* [30], based on the method of Schenkman and Cinti [31], using calcium ions to precipitate a microsomal pellet at $29 \cdot 10^3$ *g*. The final pellet was suspended in a volume of phosphate buffer (0.2 *M*, pH 7.4) equivalent to twice the original liver weight.

In vitro incubation procedure

MDA (5 μmol per flask) dissolved in 20 μl of dimethyl sulphoxide (DMSO) was incubated in Erlenmeyer flasks. Incubations were carried out aerobically for 30 min at 37°C [32], using hepatic microsomes from 0.5 g of liver and a standard cofactor solution consisting of the sodium salt of NADP (2 μmol), G-6-P (10 μmol), magnesium chloride (20 μmol) and G-6-PD (1 U) in a total reaction volume of 2 ml of phosphate buffer (0.2 *M*, pH 7.4). Control flasks containing phosphate buffer without MDA and heat-denatured microsomal preparations containing MDA were also incubated.

The reaction was terminated by placing the flasks on ice and adding 100 mg of solid zinc sulphate to precipitate the protein. The contents of each flask were transferred to screw-capped glass tubes and centrifuged at $1 \cdot 10^3$ *g* 15 min. The supernatant was loaded onto a C_{18} reversed-phase Sep-Pak cartridge (Waters Assoc., Milford, MA, USA), prewashed with water and methanol, and then eluted with 5 ml of redistilled methanol. The methanol eluate was centrifuged at $1 \cdot 10^3$ *g* for 15 min in order to remove the remaining zinc sulphate [33]. The collected supernatant was evaporated to dryness in a Speedvac concentrator (Savant SVC200H).

HPLC–PSP-MS

All experiments were performed on the VG 70-SEQ (sector instrument) and Gilson HPLC systems as described above. A VG Analytical Plasma spray module was connected between the HPLC and MS systems. Samples were loaded with a 100-μl flow injector and introduced at a flow-rate of 1 ml/min, with an isocratic solvent system of water–acetonitrile (6:4, v/v).

Plasma spray ionisation was achieved by initiating a glow discharge between the probe tip and the source block. The nozzle temperature was maintained at *ca.* 220°C (4–5 A, discharge position 1) to obtain optimum sensitivity and stability.

Tandem mass spectrometry

All mass spectra were obtained on a VG 70-

SEQ instrument of EBQ_1Q_2 configuration, where E is an electrostatic analyser, B is the magnet, Q_1 is a radio frequency-only quadrupole collision cell and Q_2 is a mass filter quadrupole. EB and Q_2 correspond to mass spectrometer 1 (MS_1) and 2 (MS_2), respectively. All synthetic standards and microsomal incubation mixtures were ionised by positive-ion FAB-MS. Various matrices were tried, and 0.2 *M* PTSA in glycerol afforded the most abundant signal-to-noise ratio for MDA and all synthetic standards. Xenon atoms from a Model B11N saddle-field fast-atom gun (Ion Tech, Teddington, UK) were used at 8.5 keV. The secondary ions produced by the fast xenon atoms were accelerated out of the source region at 8 keV.

Product ion spectra. Protonated molecular ions ($[MH]^+$ also known as precursor ions) detected at a resolution of *ca.* 1000 were selected using EB (MS_1) and subjected to collision-activated dissociation (CAD) in Q_1. The collision energy used was *ca.* 20 eV, with no gas added to the collision cell (Q_1 ion gauge at $< 10^{-7}$ mbar). The resulting fragment or product ions were mass-analysed in Q_2 and product ion spectra acquired by scanning Q_2 over the mass range m/z 40–450 with ten scans being obtained in the multi-channel analysis (MCA) mode.

Precursor ion spectra. Q_2 (equivalent to MS_2) was set to transmit only the product ions of interest. The magnet was scanned to sequentially focus ions at Q_1 to induce CAD. Only precursor ions producing the specific product ions being monitored were thus detected. The collision regime used was as for the acquisition of the product ion spectra described previously.

Sample preparation for mass spectrometry

Both the synthetic standards and the microsomal mixtures (after Sep-Pak C_{18} cartridge chromatography) were dissolved in methanol (2 μl) and mixed with 1 μl of the matrix, 0.2 *M* PTSA in glycerol, on the stainless-steel probe tip and subsequently inserted into the mass spectrometer.

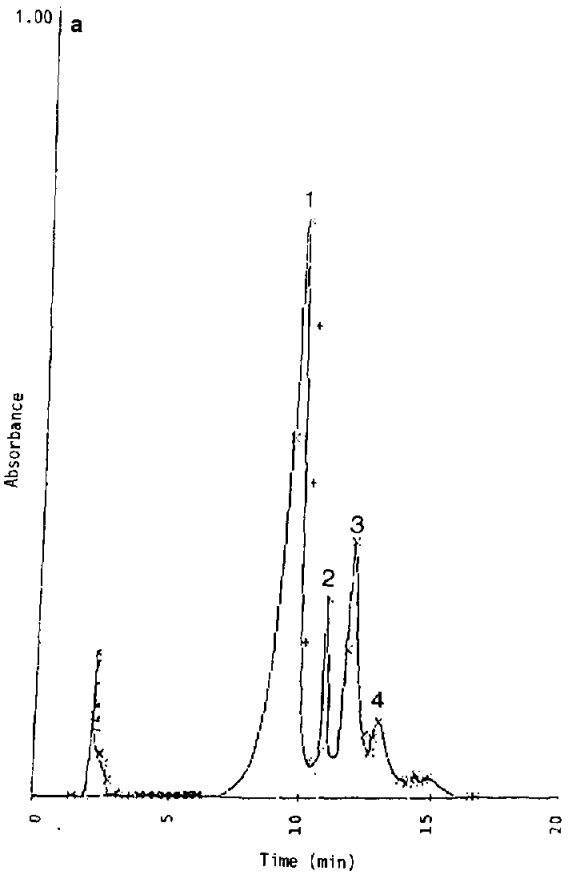

Fig. 1.

RESULTS AND DISCUSSION

HPLC–MS analyses of microsomal mixtures

Metabolites from the microsomal mixture were separated by reversed-phase HPLC using an acetonitrile–water gradient elution system with UV detection, and the HPLC chromatograms as well as the chromatogram for standards are shown in Fig. 1. The chromatograms of the microsomal incubates are denoted in Fig. 1b (test sample) and Fig. 1c (control sample). Fig. 1b has two additional absorbances not found in the control chromatogram (Fig. 1c). These were tentatively identified as the azo and azoxy compound, by comparing retention times and UV spectra with that of standards (Fig. 1a).

PSP-MS was used to directly analyse the metabolites in the incubation mixture. Optimisation of the probe temperature and flow-rate in con-

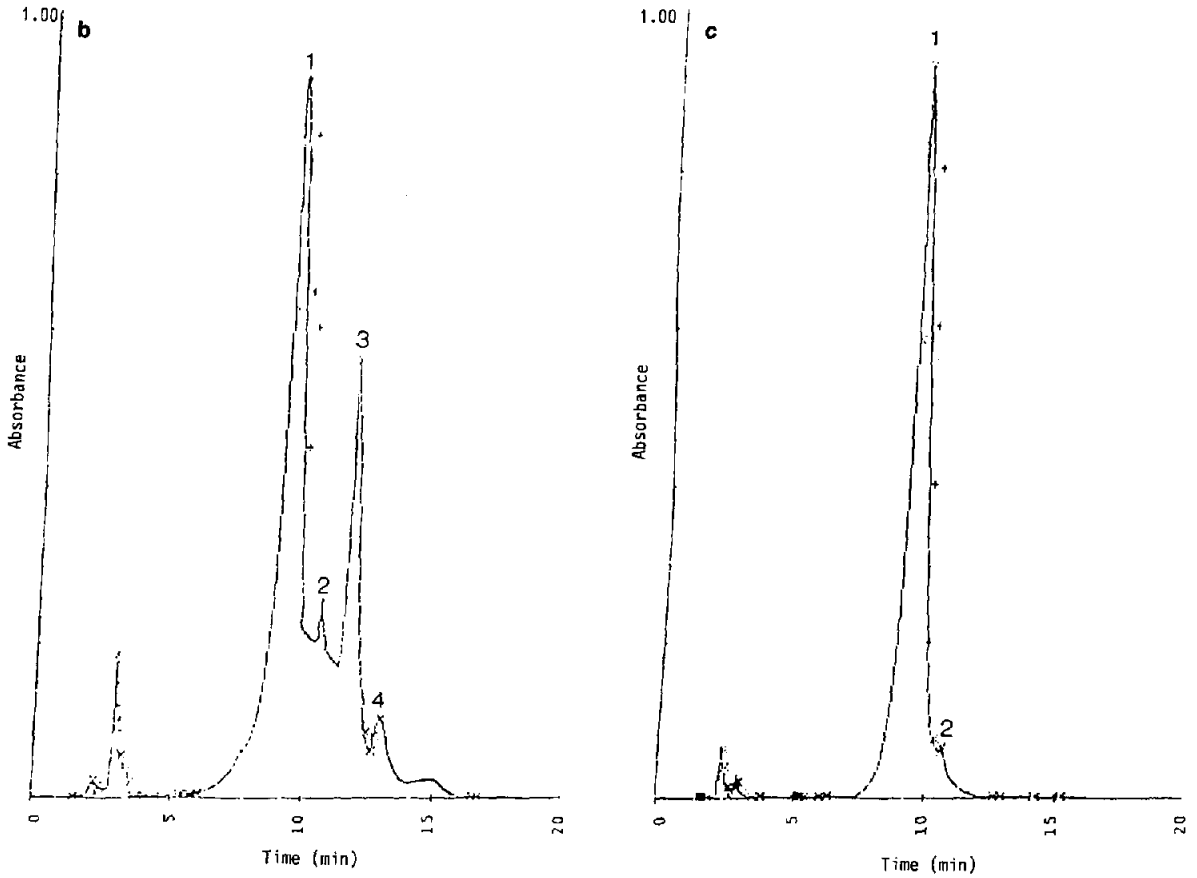

Fig. 1. HPLC of MDA and its metabolites. Peaks: 1 = MDA; 2 = nitroso; 3 = azo; 4 = azoxy. (a) Standard mixture; (b) test extracted of a microsomal incubation; (c) control extract of inactivated microsomal incubation.

junction with the ratio of eluting solvent was necessary. Maximum sensitivity and stability were produced with the probe tip at *ca.* 220°C, a flow-rate of 1 ml/min and an isocratic elution system (acetonitrile–water, 6:4, v/v).

Fig. 2a and b show the selected ion chromatograms of *m/z* 199, 213, 240, 393 and 409 corresponding to the $[MH]^+$ of MDA, nitroso, acetonitrile adduct of MDA, azo and azoxy, respectively, detected using PSP-MS of the microsomal mixture. These ions were similarly detected for the synthetic standards using PSP-MS, thus even with the consequent loss of good chromatography (due to isocratic elution), it was possible to confirm the tentative presence of the nitroso, azo and azoxy compounds in the microsomal incubations. The ion at *m/z* 240 corresponds to the acetonitrile adduct of MDA which was evident in all samples. The azo and azoxy peaks are absent in the control run (Fig. 3a and b).

HPLC–PSP-MS–MS was attempted to obtain structural information from the individual metabolites. However, with this method the level of metabolites was insufficient to obtain satisfactory PSP-MS–MS spectra. This sensitivity problem was also evident in work with 2-aminobiphenyl (2-ABP) metabolites where it was also not possible to obtain MS–MS spectra in this manner [34].

Based on our previous work on biological mixtures, minor metabolites can be detected with FAB-MS–MS [35–39], therefore off-line HPLC–

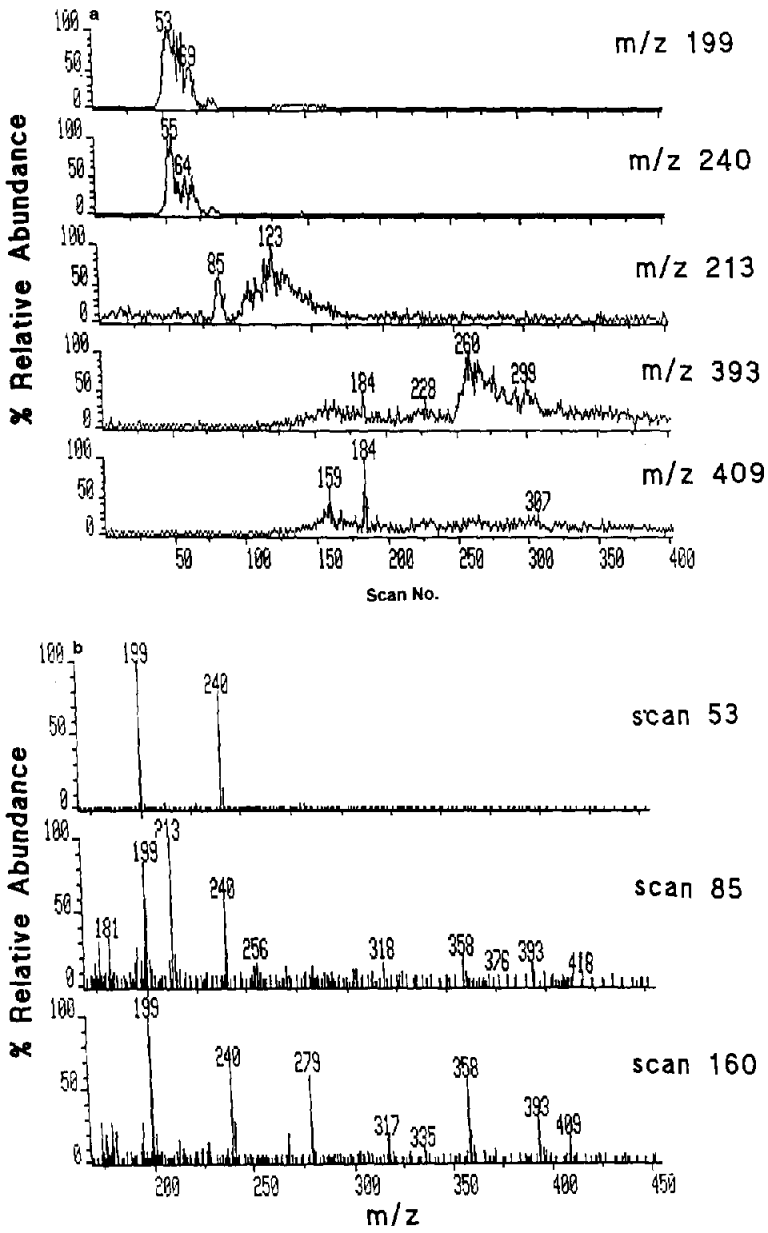

Fig. 2. (a) Plasma spray ionization selected ion chromatograms of a microsomal incubation. MDA (m/z 199) and its acetonitrile adduct (m/z 240) elute at scans 53–55. The nitroso (m/z 213) elutes at scan 85 and the azo and azoxy compounds elute at scan 160. (b) Mass spectra of scans 53, 85 and 160, showing the 199, 213, 393 and 409 corresponding to $[MH]^+$ of MDA, nitroso, azo, and azoxy metabolites.

FAB-MS–MS was used to provide definitive characterisation of the metabolites.

FAB-MS and FAB-MS–MS

Initial FAB-MS confirmed the presence of these metabolites in the incubation mixture. Ions were observed at m/z 199, 213, 393 and 409 which correspond to the protonated molecular $[MH]^+$ ions of MDA, nitroso, azo and azoxy MDA, respectively (Fig. 4). Subsequent FAB-MS–MS

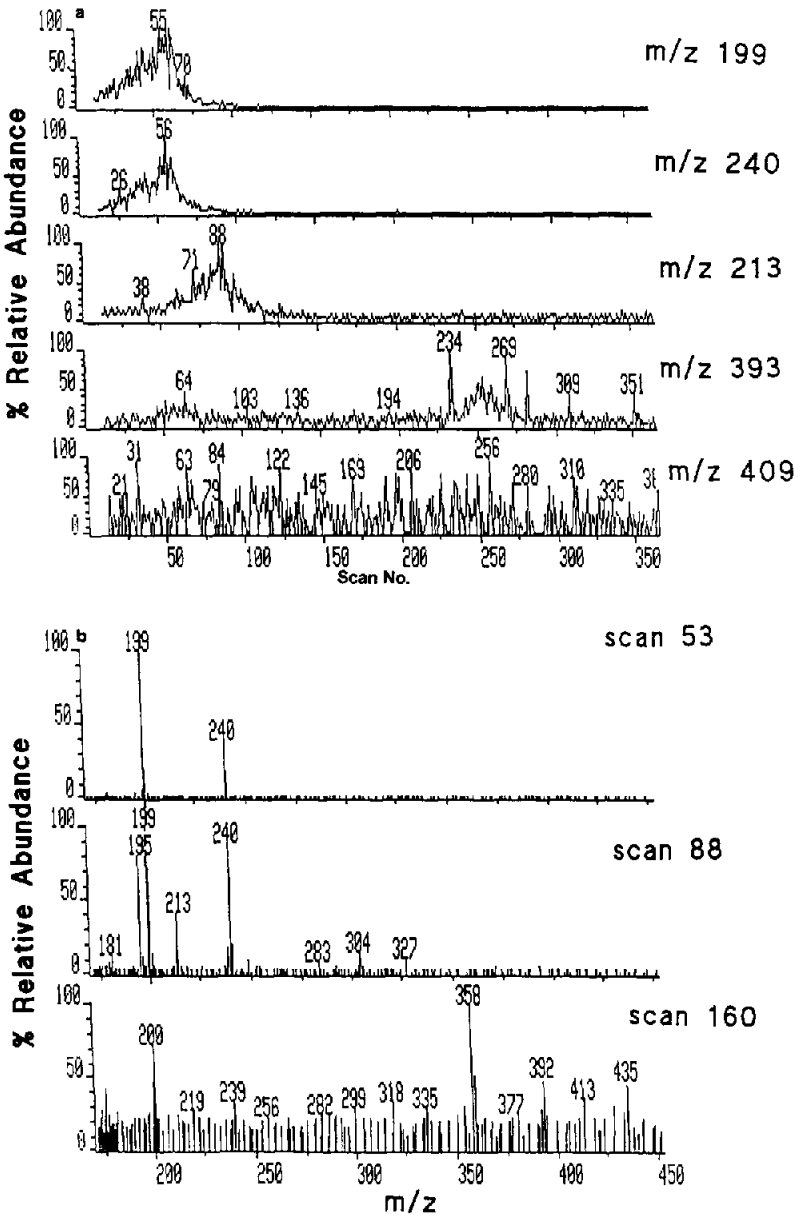

Fig. 3 (a) Plasma spray ionization selected ion chromatograms of a control inactivated microsomal incubation, showing the presence of *m/z* 199 (MDA) and *m/z* 240 (MDA–acetonitrile adduct). The nitroso (*m/z* 213) is also evident. (b) Mass spectra of scans 53, 88 and 160. The control incubation shows MDA in scan 53. The MDA–acetonitrile adduct and nitroso-MDA at scan 88 are present, however, scan 160 does not contain the azo and azoxy MH^+ ions, *i.e.* 393 and 409.

product ion spectra were obtained of the ions at *m/z* 213, 393 and 409 derived from the microsomal samples (Figs. 5a and b, 6a and b and 7a and b).

Product ion spectra of the synthetic standards

Table I shows the product ions of the relevant $[MH]^+$ ion from each of the synthetic standards. For example, the product ion spectrum for nitroso-MDA (**1**) ($MH^+ = 213$) shows product ions at

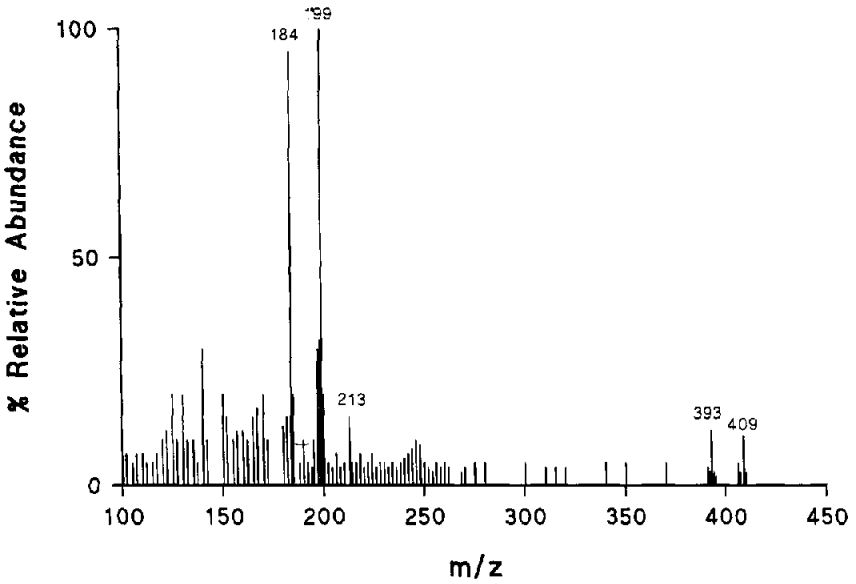

Fig. 4. Positive-ion FAB-MS (matrix 0.2 *M* PTSA in glycerol) of a microsomal incubation extract showing MDA (*m/z* 199) and its metabolites [*m/z* 213 (nitroso), *m/z* 393 (azo) and *m/z* 409 (azoxy)]; *m/z* 184 also appears in control spectra.

m/z 198 ($MH^+ - 15$) corresponding to loss of oxygen and addition of hydrogen, *m/z* 183 ($MH^+ - 30$) corresponding to loss of NO, *m/z* 120 ($MH^+ - 93$) corresponding to loss of C_6H_5-NH + H and *m/z* 106 ($MH^+ - 107$) corresponds to loss of C_6H_4 NO + H. This has been observed by other workers [40].

Product ion spectra of the synthetic nitroso, azo and azoxy standards (Table I) closely matched product ion spectra from incubation mixtures.

The fragment ion at *m/z* 106, corresponding to $[NH_2\text{-}C_6H_4\text{-}CH_2]^+$, is common in MDA and all the metabolites and was therefore selected for precursor ion scanning. A precursor ion spectrum of a mixture of synthetic azo, azoxy, nitroso and nitro-MDA was obtained. The precursor ion spectrum obtained from the microsomal incubation (Fig. 8) failed to reveal further metabolites that might have been predicted. Absence of azo and azoxy in the control mixture indicates that the azo and azoxy metabolites are formed enzymatically.

The failure to detect the hydroxylamine of MDA could be a result of this compound's tendency to condense to the azo and azoxy metabolites and break down to the amine in aerobic conditions.

Under aerobic conditions and in the presence of P-450 isoenzymes aromatic amines are oxidised to their corresponding hydroxylamines [41]. Further oxidation of hydroxylamines to the nitroso compound can occur non-enzymatically [42]. The azoxy and azo products are formed by condensation of the nitroso with hydroxylamine and amine, respectively [43,44]. It is evident that, in the presence of active microsomes, after initial enzymatic conversion of MDA to the hydroxylamine and further oxidation to the nitroso azo and azoxy occur (Fig. 9). No hydroxylamine has been detected due to the instability of this class of compound. These observations have been reported by other workers [45].

The nitroso present in the control may be produced non-enzymatically under these aerobic conditions.

CONCLUSIONS

This report demonstrates the powerful ability

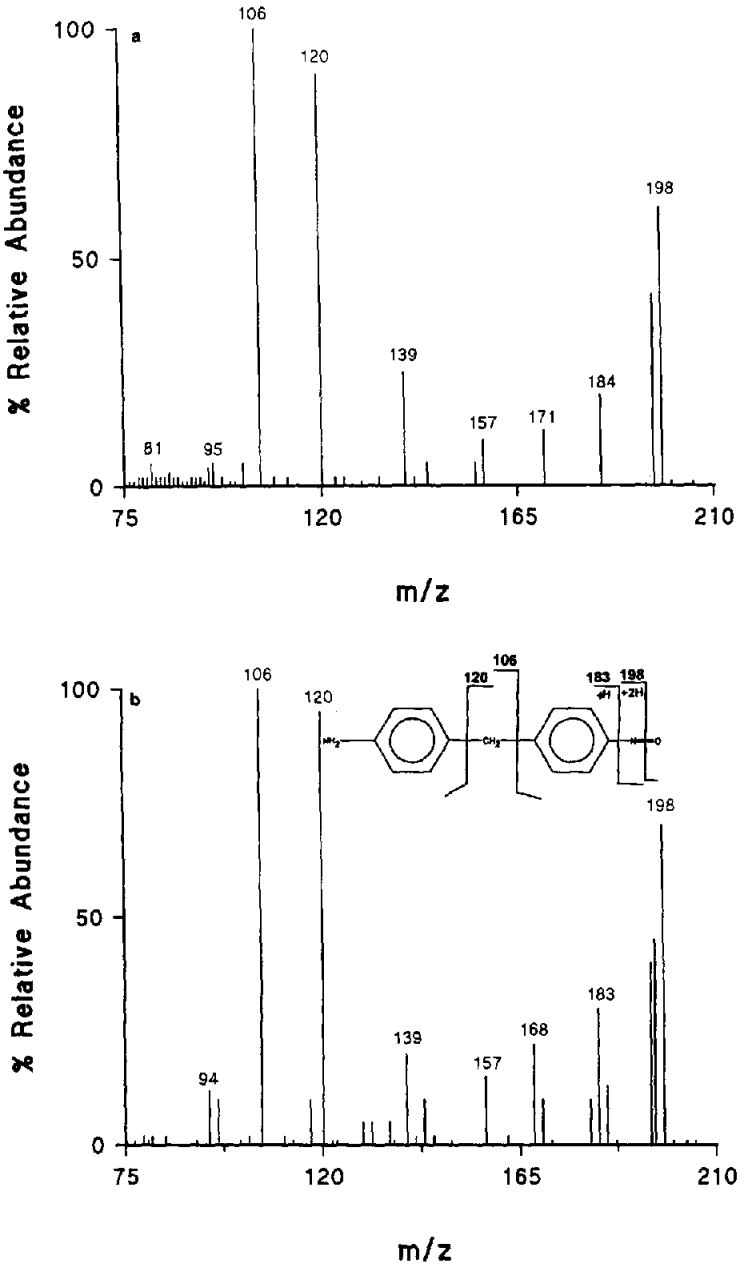

Fig. 5. FAB product ion spectra (matrix 0.2 *M* PTSA in glycerol, collision energy *ca.* 20 eV) of nitroso-MDA ($MH^+ = 213$) in (a) control incubation with inactivated microsomes and (b) test incubation with active microsomes.

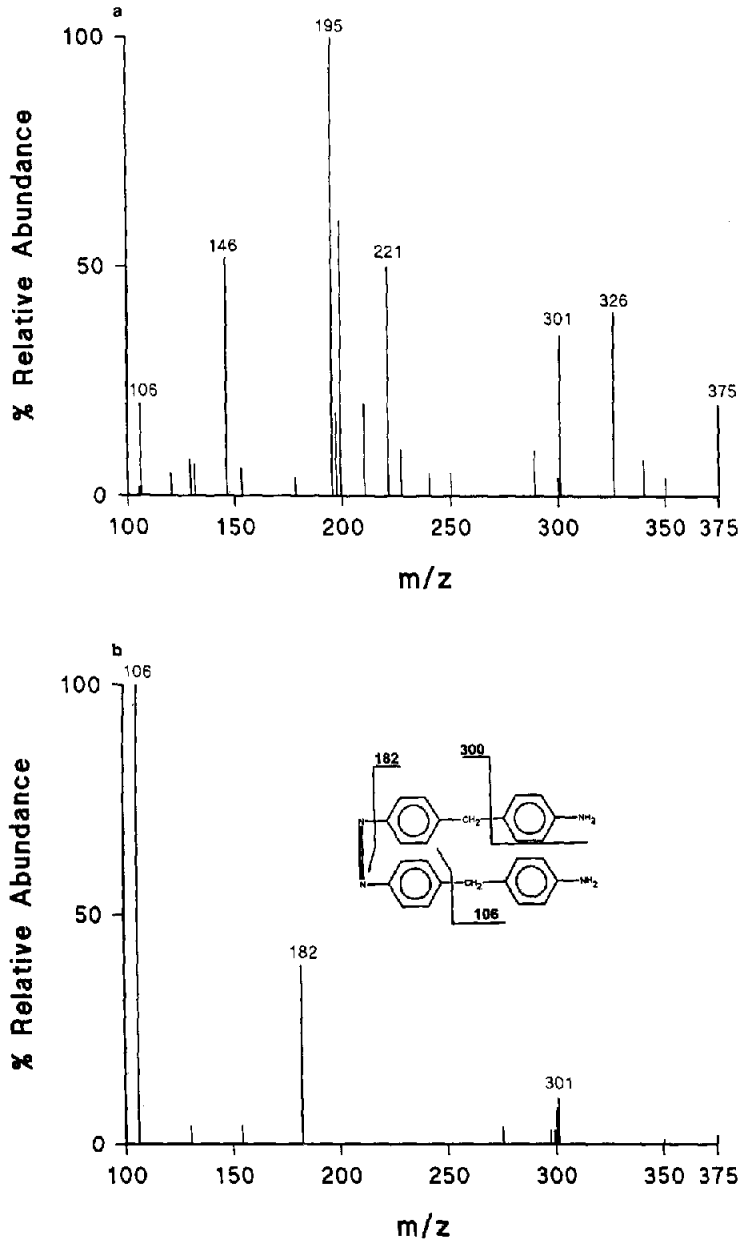

Fig. 6. FAB product ion spectra (matrix 0.2 *M* PTSA in glycerol, collision energy *ca.* 20 eV) of azo-MDA ($MH^+ = 393$) in (a) control incubation and (b) test incubation.

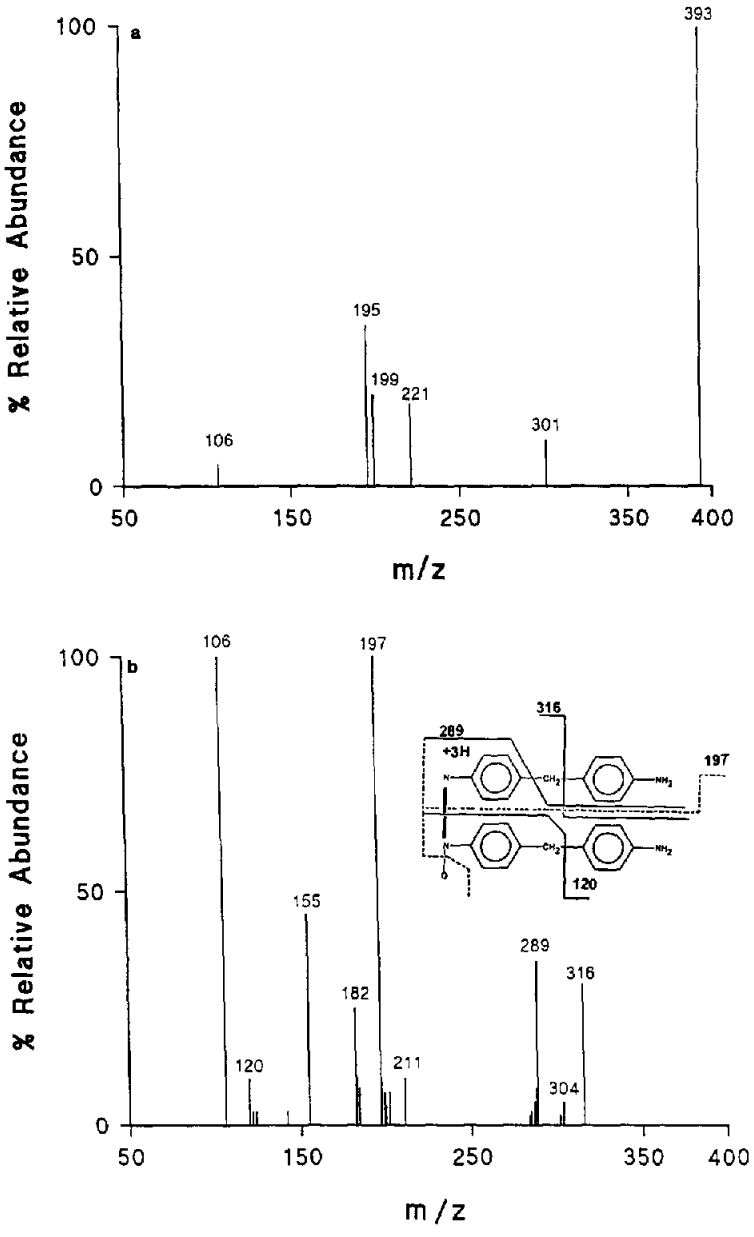

Fig. 7. FAB product ion spectra (matrix 0.2 *M* PTSA in glycerol, collision energy *ca.* 20 eV) of azoxy-MDA ($MH^+ = 409$) in (a) control incubation and (b) test incubation.

of HPLC–PSP-MS, FAB-MS and FAB-MS–MS to analyse a complex biological mixture with minimal clean-up procedures. Two metabolites of MDA have been identified from a rabbit microsomal incubate, namely azo and azoxy compounds. These two metabolites have not previously been reported and have special significance as they could result from the condensation of hydroxylamine, a known carcinogen, toxin and mutagen. Comparison of FAB-MS with HPLC–PSP-MS confirmed the presence of these metabolites. The nitroso compound was identified in

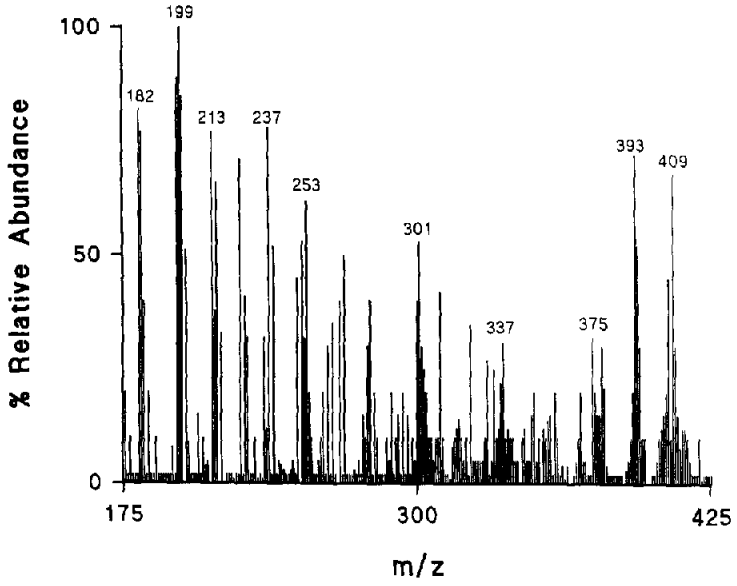

Fig. 8. FAB precursor ion spectra (matrix 0.2 *M* PTSA in glycerol, collision energy *ca.* 20 eV) of ions derived from a microsomal incubation showing precursor ions of *m/z* 106; at *m/z* 199, MDA; *m/z* 213, nitroso; *m/z* 393, azo; and *m/z* 409, azoxy.

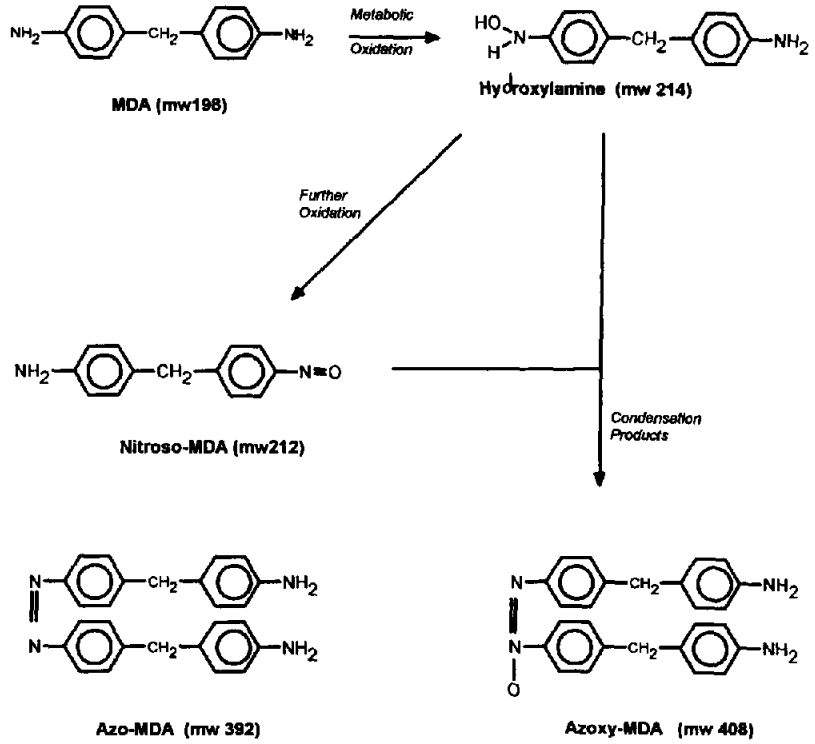

Fig. 9. Metabolites of MDA identified in test incubations. For further details see text and Table I.

TABLE I

FRAGMENT IONS OBSERVED IN THE DAUGHTER ION SPECTRA OF THE SYNTHETIC STANDARDS

Values in parentheses are precentages.

Fragment ion (*m/z*)	Possible structure of ion detected[a]
Nitroso	
198 (100)	$[N\text{-}R\text{-}CH_2\text{-}R\text{-}NH_2 + 2H]^+$
183 (10)	$[R\text{-}CH_2\text{-}R\text{-}NH_2 + H]^+$
120 (50)	$[O{=}N\text{-}R\text{-}CH_2]^+$
106 (70)	$[CH_2\text{-}R\text{-}NH_2]^+$
Azo	
300 (10)	$[CH_2\text{-}R\text{-}N{=}N\text{-}R\text{-}CH_2\text{-}R\text{-}NH_2]^+$
182 (32)	$[R\text{-}CH_2\text{-}R\text{-}NH_2]^+$
106 (100)	$[CH_2\text{-}R\text{-}NH_2]^+$
Azoxy	
316 (36)	$[CH_2\text{-}R\text{-}NO{=}N\text{-}R\text{-}CH_2\text{-}R\text{-}NH_2]^+$
289 (60)	$[R\text{-}N{=}N\text{-}R\text{-}CH_2\text{-}R\text{-}NH_2 + 3H]^+$
197 (100)	$[N\text{-}R\text{-}CH_2\text{-}R\text{-}NH_2 + H]^+$
182 (25)	$[R\text{-}CH_2\text{-}R\text{-}NH_2]^+$
120 (20)	$[CH_2\text{-}R\text{-}NO]^+$
106 (100)	$[CH_2\text{-}R\text{-}NH_2]^+$
Nitro	
212 (40)	$[R\text{-}CH_2\text{-}R\text{-}NO_2]^+$
183 (25)	$[R\text{-}CH_2\text{-}R + NH_2 + H]^+$
136 (15)	$[NO_2\text{-}R\text{-}CH_2]^+$
106 (15)	$[NH_2\text{-}R\text{-}CH_2]^+$
93 (62)	$[R\text{-}NH_2 + H]^+$
MDA	
182 (8)	$[NH_2\text{-}R\text{-}CH_2\text{-}R]^+$
106 (100)	$[NH_2\text{-}R\text{-}CH_2]^+$
93 (2)	$[R\text{-}NH_2 + H]^+$

[a] R = Phenyl.

control as a possible non-enzymatic by-product.

Direct analysis of microsomal metabolites by FAB-MS is not normally possible without a prior chromatographic purification stage. This requirement may be avoided by the use of MS–MS which, as has been demonstrated here, is capable of analysis of relatively impure extracts. To be definitive over structure identification it is, however, still appropriate to incorporate HPLC purification of the materials prior to MS analysis and to compare both chromatographic and MS data with those from synthetic standards.

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