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Characterization of peroxidative oxidation products of dopamine by mass spectrometry☆

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Abstract

We characterized three cytotoxic products, namely dopaminochrome (2,3-dihydro-1H-indole-5,6-dione), 2-(3,4-dihydroxyphenyl)-1-nitroethane and 2-(3,4,6-trihydroxyphenyl)-1-nitroethane. The compounds were separated from the incubation of dopamine (3,4-dihydroxyphenethylamine) with horseradish peroxidase which mimics the peroxidative activity of Prostaglandin H synthase. Incubation of 2-(3,4,6-trihydroxyphenyl)-1-nitroethane with NADPH-cytochrome c reductase led to the formation of 6-hydroxydopamine, a known neurotoxin. Several adducts were also isolated in this study. Oxidation of dopamine in the presence of N-acetylcysteine yielded a thioether conjugate namely, 5-S-(N-acetylcysteinyl)-3,4-dihydroxyphenethylamine. Reaction of the partially purified dopaminochrome with N-acetylcysteine permitted the isolation of another thioether conjugate which was tentatively identified as 7-S-(N-acetylcysteinyl)-5,6-dihydroxyindole. We also isolated the one-to-one condensation products of malonaldehyde with dopamine, norepinephrine and serotonin. The identities of these products were established by chemical synthesis and various mass spectrometric techniques.

1. Introduction

Catecholamine neurotransmitters such as dopamine may undergo auto-oxidation in the presence of molecular oxygen [1] or be oxidized by peroxidative enzymes [2] and metal ions [1–3]. The oxidized products of catecholamines such as quinones and semiquinones can serve as natural substrates for neuromelanin synthesis [4,5] and may also bind covalently to cellular protein and DNA to exert their cytotoxic effects [6].

Recent reports have demonstrated that peroxidases, such as myeloperoxidase (MPO) and prostaglandin endoperoxidase, can activate phenols and catechols to toxic metabolites [7–9]. For example, MPO/H_2O_2 mediated oxidation can hydroxylate phenol to hydroquinone and this metabolic pathway has been implicated in ben-

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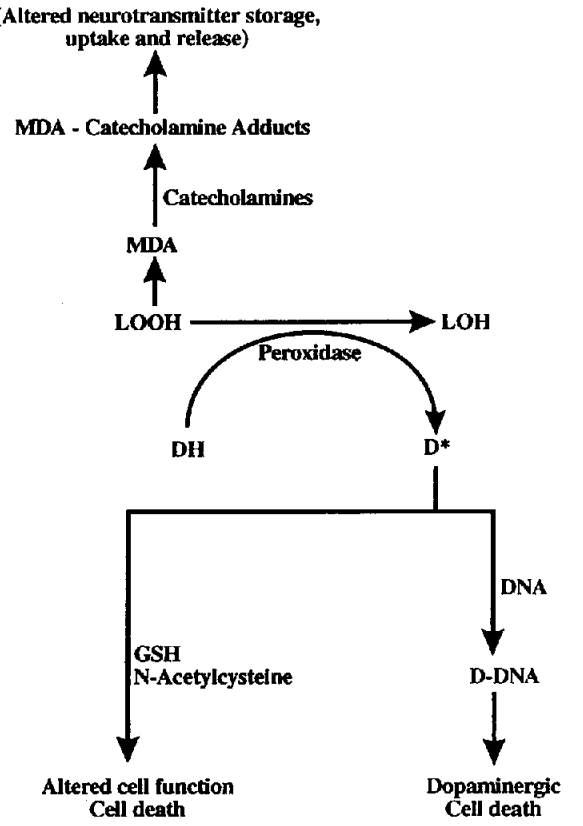
zene-induced myelotoxicity. The involvement of the peroxidase activity of prostaglandin H synthetase (PHS) in a variety of human diseases, such as nephrotoxicity, bladder cancer, uterine cancer, and lung cancer have been documented [9–13]. PHS is a bifunctional enzyme which possesses both cyclooxygenase and peroxidase activities. The cyclooxygenase activity for example, can be utilized to convert arachidonic acid (AA) to prostaglandin G_2, a lipid peroxide, which is then converted to prostaglandin H_2 by the peroxidase activity [9,10]. Since PHS activity is present in the area affected by Parkinson's disease (PD), including the caudate putamen and substantia nigra [14], the peroxidative oxidation of dopamine by PHS to toxic products is very likely to occur and may cause the neuronal degeneration in PD. The presumed co-oxidative metabolism of dopamine by peroxidases and the possible pathways that contribute to neuronal degeneration in PD are shown in Scheme 1.

Horseradish peroxidase (HRP/H_2O_2) is a readily available enzyme which is active over a wide pH range, and can be obtained with high specific activity. The peroxidative oxidation mechanism of the enzyme has been shown to be very similar to that of PHS [9]. The utility of this model enzyme system to the study of the in vivo peroxidative oxidation of phenolic compounds has been demonstrated [8–13]. Therefore, we use this enzyme to mimic the peroxidase activity of PHS to study its peroxidation reaction with dopamine. In addition, because of the absence of molecular oxygen in this HRP/H_2O_2 system spontaneous auto-oxidation can be avoided. We characterized the products isolated from the above peroxidation. The results from this in vitro study may lead to a better understanding of the oxidation reactions catalyzed by PHS in vivo, and therefore provides insight into the mechanism underlying PD.

In this report, we also studied the reaction of malonaldehyde (MDA), the major end product of lipid peroxidation as well as a known indicator of AA release, with dopamine, serotonin, and norepinephrine. Lipid peroxides have been known to disrupt the functioning of cell membranes, thereby causing extensive cell damage. However, it is still unclear whether the lipid peroxidases are involved in dopamine metabolism and consequently cause dopaminergic neuron degeneration. The preparation and identification of the MDA–catecholamine adducts would provide standards for further investigation of the role of lipid peroxidation in neuron degeneration.



Scheme 1. LOOH = lipid hydroperoxides (e.g. prostaglandin G_2); LOH = hydroxylipids (e.g. prostaglandin H_2); DH = dopamine (as a reducing co-substrate); D* = oxidized dopamine metabolites (e.g. semiquinone or quinone).

2. Experimental

2.1. Materials

Dopamine (3,4-dihydroxyphenethylamine), horseradish peroxidase (2000 units/mg, type VI), H_2O_2 (30%), tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, double stranded DNA, sodium hydrosulfite, bovine

serum albumin (BSA), and N-cetylcysteine were purchased from Sigma (St. Louis, MO, USA). Tubulin protein was a gift from Dr. James C. Lee, St. Louis University (St. Louis, MO, USA). N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), pyridine, and Reactivials (0.5 ml) were purchased from Supelco (Bellefonte, PA, USA). Nitromethane, 2-propanol (anhydrous), potassium fluoride, sodium borohydride ($NaBH_4$), dimethylamine hydrochloride, anhydrous sodium sulfate (Na_2SO_4), 18-crown-6-ether (1,4,7,10,13,16-hexaoxacyclooctadecane), acetic anhydride, 3,4-dihydroxybenzaldehyde, and silica gel (chromatography grade, 100–200 mesh) were purchased from Aldrich (Milwaukee, WI, USA). HPLC grade methanol (MeOH), ethyl acetate, and 6-ml C_{18} Bakerbond SPE extraction columns (1000 mg adsorbent), were purchased from J.T. Baker (Phillipsburg, NJ, USA). [2,5,6-^{3}H]-Dopamine (57 mCi/mmol) was purchased from DuPont (Boston, MA, USA).

2.2. Instrumentation

UV-Vis spectra were recorded on a Beckman Acta V1 spectrophotometer (Beckman Instruments, Irvine, CA, USA). Gas chromatographic analyses were carried out with a Hewlett-Packard (Palo Alto, CA, USA) 5980 gas chromatograph equipped with a flame ionization detector and a 2 m × 2 mm I.D. glass column packed with 3% OV-17 on Chromosorb W-HP (Supelco). The column was temperature programmed from 180°C to 200°C at 10°/min. A Beckman Model 332 HPLC system equipped with Model 110A pumps, a Model 420 microprocessor, and a Model 155 variable-wavelength detector was used for analyses. All purifications were performed by a Waters (Milford, MA, USA) 300 × 10 mm I.D., 10 μm C_{18} μBondapak semipreparative column and operated isocratically.

2.3. Mass spectrometry

Low resolution electron ionization (EI) mass spectra were acquired on a Finnigan 3200 quadrupole gas chromatography–mass spectrometer equipped with a Teknivent (St. Louis, MO, USA) Vector 1 data system. All EI mass spectra were recorded under the following conditions: injection port temperature 200°C; jet separator temperature, 240°C; ionizing energy, 70 eV; trap current 200 μA. The gas chromatographic separations were performed in a similar way as described above. Samples were introduced into the mass spectrometer via GC or direct insertion probe. A VG ZAB-SE (VG Analytical, Cheshire, UK) double-focusing mass spectrometer was used to measure exact masses by scanning or peak matching at a resolution of 10 000–12 000 and to observe metastable peaks by scanning the accelerating voltage or by linked scanning of the electric sector and magnetic field at constant B/E. Fast atom bombardment (FAB) mass spectra were also acquired using the VG ZAB-SE mass spectrometer. Glycerol was used as the matrix, and ionization was performed by a xenon gun at 8 keV, 1 mA.

Thermospray liquid chromatography–mass spectrometry (TSP-LC–MS) experiments were performed on a Vestec (Houston, TX, USA) 201 N mass spectrometer equipped with a Teknivent Vector 1 data system. Mass spectra were recorded in the positive-ion mode using discharge ionization for the enhancement of sensitivity. The operating conditions were as follows: tip temperature, 185°C; control, 150°C; block, 300°C; and vapor temperature, 240°C. HPLC was carried out using two Shumadzu (Tokyo, Japan) LC-6A pumps, a Shumadzu LC-6A controller and a 250 × 4.6 mm I.D., 5 μm Supelcosil C_{18} column (Supelco). The following gradient was used: 5% methanol (0–5 min); linear gradient to 30% methanol (5–15 min); linear gradient to 80% methanol (15–25 min); 80% methanol (25–60 min). The flow-rate was 1.0 ml/min.

2.4. Preparation of TMS derivatives

Trimethylsilyl (TMS) derivatives were prepared in Reactiviąls using dry pyridine (50–100 μl) and BSTFA (100 μl). The reaction mixture was kept at room temperature for at least 2 h, and then diluted with acetone (100 μl). Under

these conditions the amino group was not derivatized.

2.5. Isolation of oxidation products from in vitro incubation

A mixture containing 10 ml of 0.1 *M* Tris-HCl buffer (pH 7.2), 6 m*M* dopamine, 140 m*M* H_2O_2, and 25 units of HRP was incubated at room temperature. An aliquot of the reaction mixture was removed and the UV-Vis absorption spectra were recorded after 1.0 min (Fig. 1, scan A) and 3 min (Fig. 1, scan B). After 5 min, the reaction mixture was quick-frozen (dry ice–acetone) and lyophilized. The residue was dissolved in 2 ml of water (nitrogen purged). The deep reddish solution was poured onto a C_{18} extraction column and the column was eluted with 5 ml of water. The reddish eluents were lyophilized, redissolved in 500 μl of methanol–water (30:70, v/v), and used for TSP-LC–MS analysis. The mass spectrum of the major peak (compound 1, eluting at 5 min) is given in Fig. 2. Three minor components [$t_R = 3.5$ min (15%), $t_R = 3.7$ min (10%) and $t_R = 15$ min (8%)] were also observed but not identified.

The purple material adsorbed on the column was eluted with 5 ml of 0.1 *M* sodium hydrosulfite in water. The faint yellow eluent was lyophilized and the residue was redissolved in 500 μl of acetonitrile. An aliquot (100 μl) was transferred to a Reactivial, evaporated under nitrogen, and TMS derivatives were prepared. GC–MS showed a dopamine peak ($t_R = 9.5$ min) and two other major components (compounds 2 and 3). The remainder of the solution was used for the purification of compound 3.

2.6. HPLC purification and microsomal reduction of compound 3

Compound 3 was isolated by HPLC using methanol–water (25:75, v/v). The peak eluting at 6.5 min was collected. Product from multiple injections was pooled and the solvent was evaporated. The dry residue was dissolved in water (100 μl), and 100 μl of NADPH cytochrome c reductase (5 mg/ml) was added to start an anaerobic incubation as previously reported [15,16]. After reduction, the reaction mixture was lyophilized. The residue was dissolved in acetonitrile (500 μl) and purified by HPLC using methanol–water (15:85, v/v). The major product (compound 4, eluting at 5.5 min) was collected. Product from multiple injections was pooled, evaporated under nitrogen, and TMS derivative was prepared for GC–MS analysis. The mass spectrum is given in Fig. 5. Compound 4 was 99% pure as assessed by HPLC and thin-layer chromatography on silica gel (solvents: chloroform–methanol, 30:70, v/v, $R_F = 0.30$; and diethylamine–methyl ethyl ketone–methanol, 1:1:3, v/v, $R_F = 0.55$).

2.7. Assessment of the reactivity of the dopamine oxidation products

Binding to DNA, BSA, and tubulin protein

To a mixture consisting of 50 μM [2,5,6-^{3}H]-dopamine (0.25 μCi), 3 units of HRP, 1.0 mg/ml of DNA, BSA, or tubulin protein, in a total volume of 0.5 ml Tris-HCl buffer (pH.7.2), was added 100 μM of H_2O_2 to start the reaction. The incubation was carried out at room temperature for 5 min. The binding of the dopamine metabolites to DNA, BSA, or tubulin protein was determined by the previously reported method [17].

Reaction with N-acetylcysteine

A mixture of dopamine (1.5 m*M*), 8 units of HRP, and Tris-HCl buffer (pH 7.2) in a total volume of 6 ml was prepared. The reaction was started by the addition 600 μM of H_2O_2. The deep reddish reaction mixture was allowed to stand for 5 min.

To one third of the reaction mixture, N-acetylcysteine (500 μM) was added. Immediate decolorization resulted. The mixture was lyophilized, dissolved in 1 ml of water, and poured onto a C_{18} extraction column, which was then washed with water until the eluent was colorless (5 × 1 ml). The light reddish material adsorbed on the column was eluted with 3 ml of methanol. The products were further purified by HPLC using methanol–water (10:90, v/v). The major peak, eluted at 17 min, was collected. Product from multiple injections was pooled, evaporated

under nitrogen, and used for TSP and FAB analyses.

Another one third of the reaction mixture was quick-frozen, lyophilized, redissolved in 0.5 ml of water, and poured onto a C_{18} extraction column. The column was eluted with 3 ml of water. The fraction was lyophilized, redissolved in methanol–water (10:90, v/v, 200 μl), and 200 μM of N-acetylcysteine in water (200 μl) added. After 0.5 h the pale yellow reaction mixture was diluted with 600 μl of water, poured onto a C_{18} extraction column, and washed with water (9 ml). The light yellow material adsorbed on the column was eluted with 2 ml of MeOH and the eluent was evaporated to dryness under nitrogen. The light yellow powdery material was further purified by HPLC using methanol–water (10:90, v/v). The major peak, eluting at 11 min, was collected. Material from multiple injections was pooled, evaporated under nitrogen, and used for TSP and FAB analyses.

2.8. Preparation of MDA–catecholamine adducts

A mixture containing 6 ml of 25 mM Tris-HCl buffer (pH 7.5), 3 mg of liver microsomal protein [18], 150 μM NADPH, 3 mM adenosine diphosphate, and 50 μM $Fe(NO_3)_3$ was incubated at 37°C for 10 min. To each of the above 2 ml deproteinized samples, 500 μM of the individual catecholamines (in 500 μl ethanol) were added and the mixtures were heated in boiling water for 10 min. Each mixture was adjusted to pH 6.5 with 0.1 M NaOH, lyophilized, and the residue was extracted with MeOH (300 μl). The MDA–catecholamine adducts were purified by HPLC using methanol–water (40:60, v/v). Retention times for the adducts were: MDA–serotonin, 6.1 min; MDA–norepinephrine, 7.1 min; MDA–dopamine, 7.5 min.

2.9. Chemical syntheses

Synthesis of 2-(3,4-dihydroxyphenyl)-1-nitrostyrene

3,4-Dihydroxybenzaldehyde (1.0 g, 6.7 mmol), nitromethane (500 mg, 8.2 mmol), dimethylamine hydrochloride (700 mg, 8.6 mmol) and 18-crown-6-ether (100 mg) were dissolved in 10 ml methanol. The reaction mixture was diluted with toluene (100 ml), heated to reflux, and potassium fluoride (100 mg, 1.7 mmol) was added in one portion. After 2 h, the mixture was cooled, and the toluene layer was separated, washed with water, dried with Na_2SO_4, and evaporated under vacuum to give a reddish oil (700 mg). This material was 97% pure as assessed from GC of its TMS derivative. High-resolution mass measurement on the molecular ion of the TMS derivative of 2-(3,4-dihydroxy)-1-nitrostyrene showed a composition of $C_{14}H_{23}NO_4Si_2$ (calcd.: 325.1166, found: 325.1165).

Synthesis of 2-(3,4-dihydroxyphenyl)-1-nitroethane [19]

To a stirred mixture of the above 2-(3,4-dihydroxy)-1-nitrostyrene (500 mg, 1.54 mmol), silica gel (10 g), 2-propanol (15 ml), and chloroform (125 ml), $NaBH_4$ (500 mg, 13 mmol) were added in 50-mg portions over a period of 15 min at 25°C. The reaction mixture was stirred for another hour. By that time, the deep reddish color had completely disappeared. After removal of excess $NaBH_4$ with HCl (6 M), the mixture was filtered and evaporated in vacuum. The syrupy residue was diluted with water, and extracted with ethyl acetate (25 ml). The extract was washed with water, dried with Na_2SO_4, and evaporated to 1 ml under nitrogen. The solution of 2-(3,4-dihydroxyphenyl)-1-nitroethane (98.7% pure by GC analysis of its TMS derivative) was stored under nitrogen.

3. Results and discussion

The spectral properties of the products resulting from the oxidation of dopamine with HRP/H_2O_2 are shown in Fig. 1. Upon initiation by H_2O_2, the reaction mixture developed absorptions at 450–500 nm and 300–400 nm. Absorptions in these regions have been attributed to 2-hydroxy-1,4-benzoquinone, 1,2-benzoquinone, and dopachrome (2,3-dihydro-2-carboxy-1H-indole-5,6-dione) chromophores [20–25]. The TSP mass spectrum of compound 1 shows an $[M+H]^+$ ion at m/z 150 (Fig. 2A), which

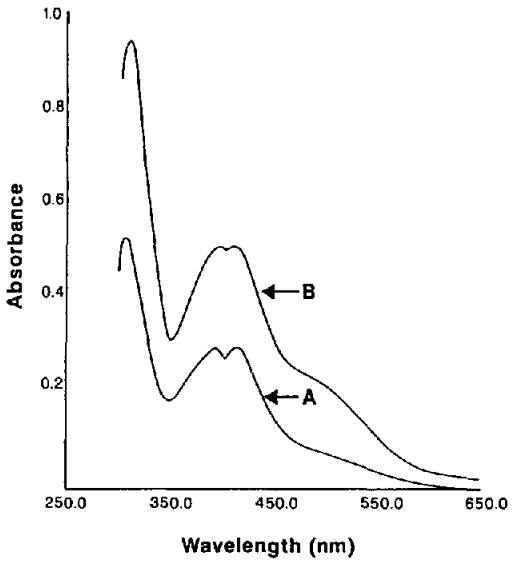


Fig. 1. The UV-Vis absorption spectra of the reaction mixture containing dopamine and HRP. The oxidation was initiated by the addition of H_2O_2. A = scan after 1.0 min, B = scan after 3.0 min.

corresponds to a dopaminochrome or its dihydroxyindole tautomer. Compound 1 also gives absorption maxima at 305 nm and 480 nm which are similar to the absorption maxima reported for iminoquinones (300–303 nm and 473–480 nm), such as adrenochrome (2,3-dihydro-3-hydroxy-1-methyl-1H-indole-5,6-dione) and dopachrome [20,21,24]. Therefore, the isolated material is most likely a dopaminochrome (2,3-dihydro-1H-indole-5,6-quinone).

The quinone products adsorbed on the extraction column after the separation of dopaminochrome were reduced to hydroquinones with aqueous sodium hydrosulfite. GC–MS analyses of the TMS derivatives showed two peaks with retention times of 5 min (compound 2) and 7 min (compound 3) respectively, in addition to the dopamine peak. The mass spectrum of the TMS derivative of compound 2 (Fig. 2B) shows a molecular ion at m/z 327 and a base peak at m/z 193. High resolution measurement of the molecular ion gives an elemental composition (Table 1) indicating the presence of two TMS groups and two additional oxygen atoms compared to dopamine. The presence of a 1,2-benzoquinone absorption at 393–395 nm in the oxidation mixture combined with the mass spectral data suggests that the amino group in dopamine has been oxidized to a nitro group. Oxidation of an amino group to a nitro group in peroxidative metabolism has been previously reported [26]. Daughter-ion scan for the molecular ion supports the fragmentations which are shown in Table 1. These data indicate that the compound is 2-(3,4-dihydroxyphenyl)-1-nitroethane. The structure was further confirmed by chemical synthesis. The mass spectrum of the TMS derivative of compound 2 is identical to that of synthetic 2-(3,4-dihydroxyphenyl)-1-nitroethane. The fragmentation pathways of this compound and related 2-phenyl-1-nitroethanes are currently being investigated.

The mass spectrum of the TMS derivative of compound 3 (Fig. 2C) shows a molecular ion at m/z 343. High-resolution mass measurement gives a composition of $C_{14}H_{25}Si_2NO_5$ (calcd.: 343.1271, found: 343.1273) which indicates the presence of one additional oxygen atom compared to compound 2. The fragment ions observed at m/z 328, m/z 313, m/z 295 and m/z 267 correspond to $[M-CH_3]^+$, $[M-NO]^+$, $[M-H_2NO_2]^+$, and $[M-(CH_3+CH_3NO_2)]^+$, respectively. The base peak at m/z 179 most probably arises from m/z 267 by the loss of $TMSCH_3$. The observation of two rather than three TMS groups may arise from the interaction of the *ortho* hydroxyl group with the nitro group to form a cyclic structure, which deters the silanization. The above information suggests that the third hydroxyl group is probably present in the aromatic ring. This conclusion is supported by the fact that the oxidation mixture has an absorption spectrum (Fig. 1B) similar to that reported for 2-(2-aminoethyl)-5-hydroxy-*p*-benzoquinone [23]. The structure of this compound was further established by the reduction of compound 3 with microsomal NADPH-cytochrome c reductase [27] which resulted in the formation of compound 4. The mass spectrum of the TMS derivative of compound 4 is given in Fig. 2D and the results of high-resolution measurement on the molecular ion and major fragments are given in Table 2. The mass spectrum

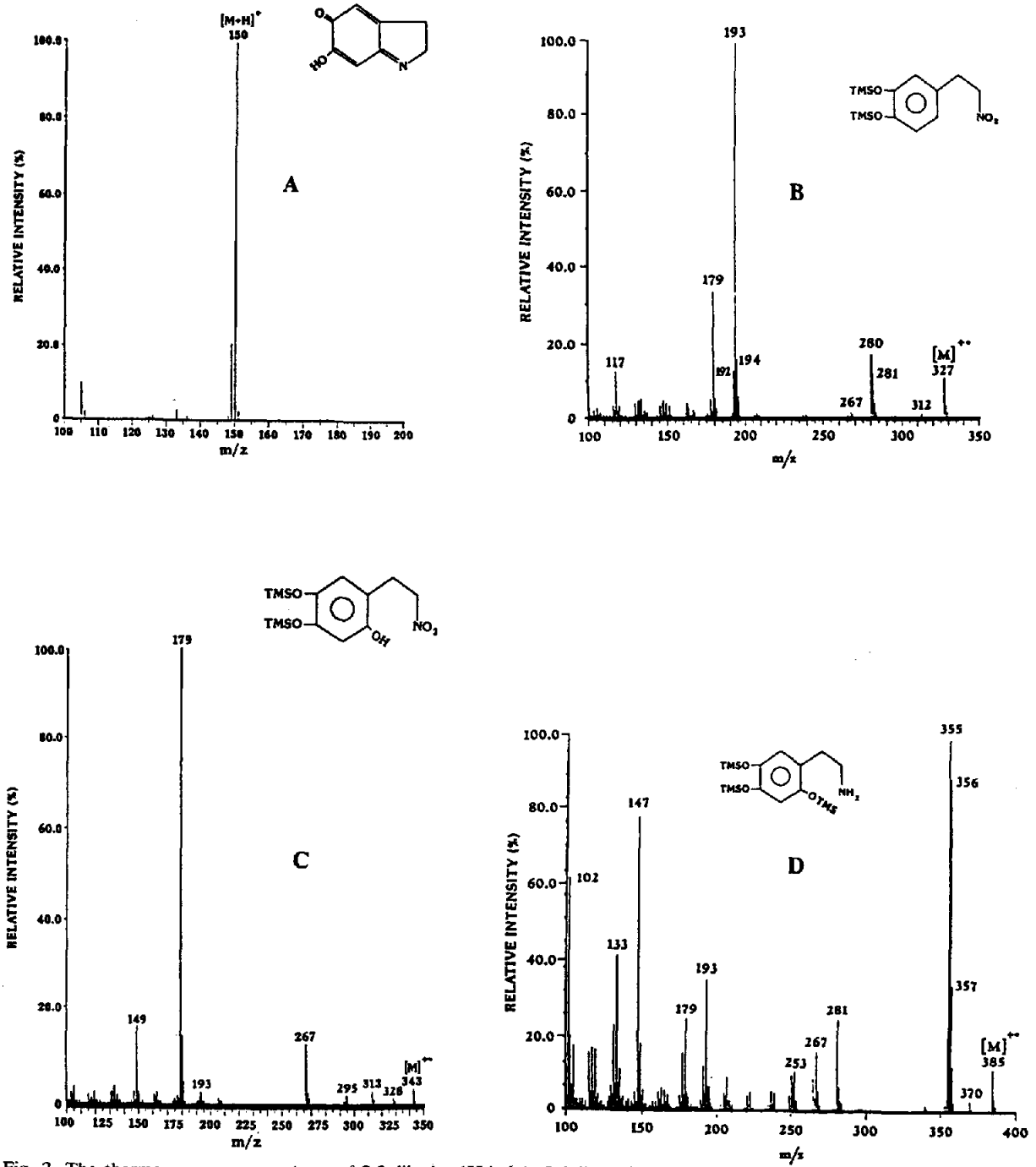


Fig. 2. The thermospray mass spectrum of 2,3-dihydro-1H-indole-5,6-dione (dopaminochrome, compound 1) (A), and the EI mass spectra of the TMS derivatives of 2-(3,4-dihydroxyphenyl)-1-nitroethane (compound 2) (B), 2-(2,4,5-trihydroxyphenyl)-1-nitroethane (compound 3) (C), and 2,4,5-trihydroxyphenethylamine (compound 4) (D).

Table 1
High-resolution mass measurements of the TMS derivative of 2-(3,4-dihydroxyphenyl)-1-nitroethane (compound 2)

Formula	Calculated	Found	Ion assignments
$C_{14}H_{25}NO_4Si_2$	327.1322	327.1320	$[M]^+$
$C_{13}H_{22}NO_4S_2$	312.1087	312.1090	$[M - CHD_3]^+$
$C_{14}H_{25}O_2Si_2$	281.1393	281.1379	$[M - NO_2]^+$
$C_{14}H_{24}O_2Si_2$	280.1315	280.1318	$[M - HNO_2]^{\cdot+}$
$C_{13}H_{23}O_2Si_2$	267.1237	267.1230	$[M - CH_2NO_2]^+$
$C_{10}H_{13}O_2Si$	193.0685	193.0647	$[M - (CH_2NO_2 + TMSH)]^+$
$C_9H_{11}O_2Si$	179.0528	179.0531	$[M - (CH_2NO_2 + TMSCH_3)]^+$

and chromatographic properties of compound 4 are identical to that of authentic 6-hydroxydopamine (6-OHDA). This confirms the structure of compound 3 as 2-(2,4,5-trihydroxyphenyl)-1-nitroethane.

When N-acetylcysteine was added to the oxidation mixture and to partially purified dopaminochrome (compound 1) separately, two N-acetylcysteine conjugates were isolated in both reactions. The TSP mass spectrum of the adduct eluting at 11 min showed an $[M + H]^+$ ion at *m/z* 311 and a base peak at *m/z* 164 [(N-acetylcysteine + H)]$^+$, whereas the adduct eluting at 17 min showed an $[M + H]^+$ ion at *m/z* 315 and a base peak at *m/z* 164. Accurate mass measurement by peak matching of the protonated molecular ions of these conjugates by FAB gives the following compositions: for the peak eluting at 11 min, $C_{13}H_{15}N_2O_5S$ (calcd.: 311.0702, found: 311.0705); for the peak eluting at 17 min, $C_{13}H_{19}N_2O_5S$ (calcd.: 315.1015, found: 315.1019). The N-acetylcysteine conjugate of dopamine prepared from mushroom tyrosinase by the method of Rosengren et al. [28] gives an identical TSP mass spectrum and retention time which confirms that the conjugate eluting at 17 min is 5-S-(N-acetylcysteinyl)-3,4-dihydroxyphenethylamine. The peak eluting at 11 min is tentatively identified as 7-S-(N-acetylcysteinyl)-5,6-dihydroxyindole, but the formation of the 4-S-conjugate is also possible. Chemical synthesis of the 7-S and 4-S conjugates to confirm the structure is in progress.

Covalent binding of electrophilic metabolites to tissue macromolecules, and sulfhydryl nucleophiles has been suggested as a mechanism for cytotoxicity and the neuronal degeneration caused by dopamine, 6-OHDA, and related compounds [20,28–32]. Examples include conjugates resulting from dopamine or 6-OHDA autoxidation, such as 5-S-cysteinyldopamine in postmortem human brain tissue [28,29] and 4-S-glutathionyl-5,6-dihydroxyindole in rats after a stereotaxic injection of 6-OHDA [34,35]. In this

Table 2
High-resolution mass measurement of the TMS derivative of 2,4,5-trihydroxyphenehylamine (compound 4)

Formula	Calculated	found	Ion assignments
$C_{17}H_{35}NO_3Si_3$	385.1925	385.1928	$[M]^+$
$C_{16}H_{32}NO_3Si_3$	370.1690	370.1708	$[M - CH_3]^+$
$C_{16}H_{32}O_3Si_3$	356.1659	356.1662	$[M - CH_2 = NH]^+$
$C_{16}H_{31}O_3Si_3$	355.1581	355.1581	$[M - CH_2NH_2]^+$
$C_{13}H_{21}O_3Si_2$	281.1029	281.1033	$[M - (CH_2NH_2 + TMSH)]^+$
$C_{12}H_{19}O_3Si_2$	267.0873	267.0883	$[M - (CH_2NH_2 + TMSCH_3)]^+$
$C_{12}H_{21}O_2Si_2$	253.1080	253.1082	$[M - (CH_2NH_2 + TMSH + CO)]^+$
$C_{10}H_{13}O_2Si$	193.0685	193.0690	$[M - (CH_2NH_2 + TMSOTMS)]^+$
$C_9H_{11}O_2Si$	179.0528	179.0525	$[M - (CH_2 = NH + TMSCH_3 + TMSO)]^+$

study, the measured values for the covalent binding of the electrophilic products generated in the oxidation of dopamine by HRP/H_2O_2 to macromolecules were: DNA (5.82 ± 0.18 nmol/mg DNA), BSA (3.05 ± 0.11 nmol/mg/min) and tubulin protein (6.38 ± 0.20 nmol/mg/min). The blank values were: DNA (0.31 ± 0.15 nmol/mg DNA) and 0.21 ± 0.22 nmol/mg/min for both BSA and tubulin protein. The above results suggest that the electrophilic products which react with cellular nucleophiles are generated by the peroxidative oxidation of dopamine. This is consistent with the hypothesis that covalent binding and the depletion of sulfhydryl nucleophiles can result in neuronal degeneration and cell death [9,30,33,35].

In the present study we demonstrated the peroxidative oxidation of dopamine in vitro, using an enzyme that mimics the hydroperoxidase component of PHS. We isolated and characterized products previously suggested to arise from dopamine autoxidation. Dopaminochrome has been suggested as the active alkylating agent which disrupts catecholamine metabolism by irreversibly deactivating catechol-O-methyltransferase [36]. The identification of 6-OHDA as a reduction product of compound 3 suggests an alternative pathway for its formation.

MDA is one of the end products of lipid peroxidation. Dexter et al. [37] have demonstrated increased levels of MDA formation in the substantia nigra in Parkinsonian brain tissue. Therefore, adducts of MDA with catecholamines can be formed. Indeed, we were able to isolate the 1:1 MDA adducts of serotonin, norepinephrine and dopamine which gave TSP mass spectra (MH^+ ion) at m/z 231, m/z 224 and m/z 208, respectively (Fig. 3). The observation of the MDA–catecholamine adducts may evidence a possible mechanism leading to a reduced level of neurotransmitter in the neuronal cell body. Further confirmation of the structures of these adducts is in progress.

We also demonstrated that the oxidation products derived from peroxidative metabolism of dopamine can react with sulfhydryl nucleophiles and lead to the formation of sulfhydryl conjugates. The sulfhydryl conjugates identified in this study are being investigated as markers in cerebrospinal fluid for early diagnosis of Parkinson's

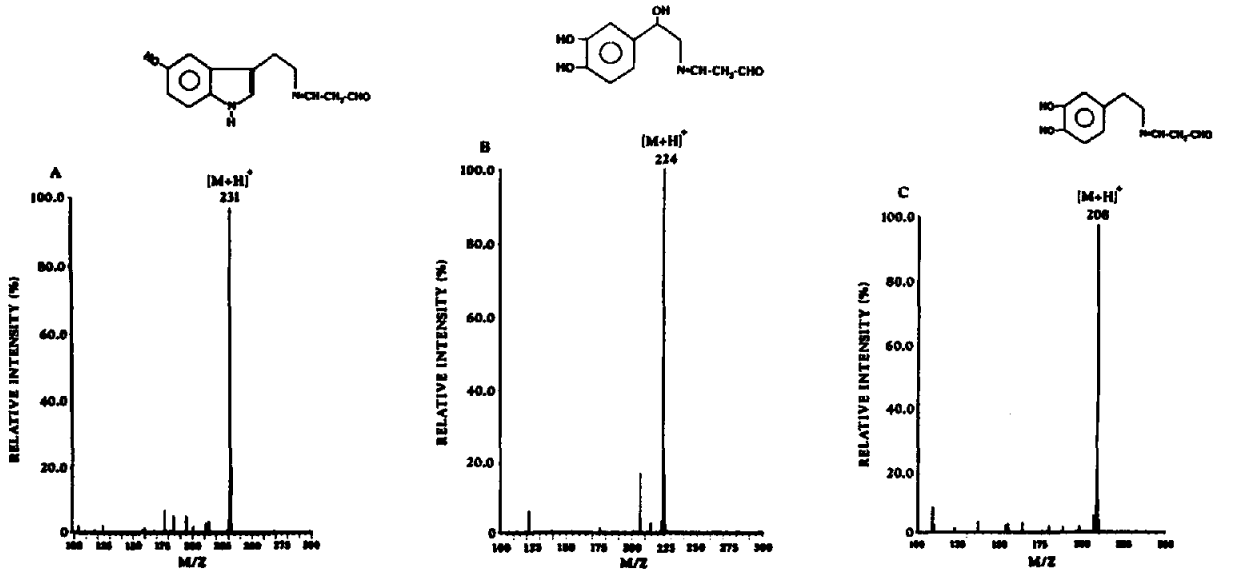


Fig. 3. The thermospray mass spectra of the malonaldehyde adducts of serotonin (A), norepinephrine (B) and dopamine (C).

disease. The mass spectra reported in this study could be useful for in vivo investigation in the future.

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