Research report

Synthesis, in vitro formation, and behavioural effects of glutathione regioisomers of alpha-methyldopamine with relevance to MDA and MDMA (ecstasy)

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Abstract

Administration of 3,4-methylenedioxyamphetamine (MDMA) or 3,4-methylenedioxymethamphetamine (MDA) to rats produces serotonergic nerve terminal degeneration. However, they are not neurotoxic when injected directly into the brain, suggesting the requirement for peripheral metabolism of MDMA to a neurotoxic metabolite. Alpha-methyldopamine (α-MeDA) is a major metabolite of MDA. There are indications that a glutathione metabolite of α-MeDA and/or 3,4-dihydroxymethamphetamine may be responsible for the neurotoxicity and some of the behavioural effects produced by MDMA and/or MDA. The present study details the synthesis, purification and separation of the 5-(glutathion-S-yl)-α-MeDA and 6-(glutathion-S-yl)-α-MeDA regioisomers of α-MeDA. Incubation of MDA with human liver microsomes demonstrated that production of both glutathione adducts are related to cytochrome P450 2D6 isoform activity. Following intracerebroventricular administration (180 nmol) of either GSH adduct into Dark Agouti or Sprague–Dawley rats only 5-(glutathion-S-yl)-α-MeDA produced behavioural effects characterised by hyperactivity, teeth chattering, tremor/trembling, head weavining, splayed posture, clonus and wet dog shakes. Pre-treatment with a dopamine receptor antagonist (haloperidol, 0.25 mg/kg; i.p.) attenuated hyperactivity, teeth chattering, low posture and clonus and potentiated splayed postural effects. These results indicate that MDA can be converted into two glutathione regioisomers by human liver microsomes, but only the 5-(glutathion-S-yl)-α-MeDA adduct is behaviourally active in the rat.

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1. Introduction

3,4-(±)-Methylenedioxyamphetamine (MDA) and 3,4-(±)-methylenedioxymethamphetamine (MDMA or ‘Ecstasy’) are ring-substituted amphetamines used as recreational drugs with increasing popular abuse in Western Europe and North America [6]. The predominant adverse consequences of MDMA and MDA abuse in humans include convulsions, hyperthermia, rhabdomyolysis, and acute liver and renal failure [18]. MDMA is considered to be potentially toxic to serotonergic nerve terminals of rodents and non-human primate [16,41,42] and studies have shown that both MDA and MDMA produce an initial acute release of 5-hydroxytryptamine (5-HT) which is followed by prolonged depletion of its metabolite 5-hy-
droxyindoleacetic acid (5-HIAA) [50]. The prolonged depletion occurs due to tryptophan hydroxylase inhibition [21,49] and subsequent long-term structural damage to serotonergic nerve terminals. Serotonergic neurotoxicity occurs after systemic [14,36,37,45] but not central administration of MDA or MDMA [13,30,35,38,42] in rats and non-human primates. This implies an important role for systemic metabolism in the development of neurotoxicity [42]. Characterisation of MDMA metabolism is therefore essential to an understanding of its pharmacology and toxicology. MDMA can be demethylated in the liver to MDA and this metabolite and MDMA can be demethylated to α-methyl dopamine (α-MeDA) and N-methyl-α-methyl dopamine (N-Me-α-MeDA), respectively [5,28]. Intracerebroventricular (i.c.v.) administration of α-MeDA to rats fails to produce either the acute ‘serotonin syndrome’ [32] or the long-term 5-HT neurotoxicity [30]. Other products of MDMA derived from oxidative metabolism have been identified [25–27] but none produce a toxicity profile comparable with MDMA [12,20].

Miller et al. [31] demonstrated that α-MeDA readily oxidises and, in the presence of glutathione (GSH), produces 5-(glutathion-S-yl)-α-methyl dopamine (5-GSH-α-MeDA). This glutathione adduct of α-MeDA can, in the short-term, alter dopaminergic, serotonergic and noradrenergic systems [32] and can be further metabolised to products that can produce long term toxicity [3,34]. The fractional uptake of 5-GSH-α-MeDA into the brain is comparable to that of GSH and is reduced by its co-administration suggesting competition between 5-GSH-α-MeDA and GSH for a putative transporter [32]. Thus its uptake is reduced by GSH and rapidly increases following pre-treatment with acivicin, an inhibitor of γ-glutamyl transeptidase [32]. Additionally, pre-treatment of rats with acivicin has been shown to potentiate MDA and MDMA-mediated depletions of 5-HT and 5-HIAA, an effect thought to be attributed to an increase in the uptake of thioether metabolites of MDA and MDMA [2]. In contrast to the study by Miller et al. [31], Patel et al. [39] reported that 6-(glutathion-S-yl)-α-MeDA (6-GSH-α-MeDA) was formed from the reaction of α-MeDA with GSH. The reaction conditions used by Miller et al. [31] and Patel et al. [39] were almost identical and yet produced different GSH regioisomers of α-MeDA.

In animal studies, MDA is as effective as MDMA regarding its effects on the serotonergic system [36] and hence there is the potential for both GSH adducts of α-MeDA to produce neurotoxicity. It has been reported that approximately 1.6% of a dose of MDA (23 μmol; s.c.) in the rat is excreted in bile as 5-GSH-α-MeDA [32]. In this study 5-GSH-α-MeDA was shown to have a brain uptake index of ~7.5% and a minimum of 28 nmol can access the brain. As this adduct has been speculated to convert to the mercapturic acid 5-(N-acetylcysteine-S-yl)-α-MeDA which is reported to produce behavioural effects at only 7 nmol i.c.v. the metabolism data support the potential for 5-GSH-α-MeDA to produce behavioural effects or neurotoxicity similar to MDA.

Hiramatsu et al. [19] demonstrated the production of a GSH adduct of N-Me-α-MeDA following incubation of rat liver microsomes with MDMA and a NADPH generating system. However, the formation of GSH adducts from MDA in human liver microsomal incubations remains to be demonstrated. The relative adduct conjugation rates and potential variations of these rates in the human or rat liver are likely contributors to the susceptibility of humans to MDA/MDMA neurotoxicity and any toxic or behavioural responses elicited by them, but so far no data have been presented. In humans, demethylation of MDMA to the catechol is catalysed by CYP2D6 [50] which is known to exhibit genetic polymorphism, suggesting that there may be individual variations in the ability to produce GSH adducts. The possibility that the 6-GSH-α-MeDA adduct may produce a behavioural effect following central administration has not been investigated.

Previous studies examined GSH adducts of α-MeDA in the Sprague–Dawley rats without any comparison with another strain [2,3,31–33]. Dark Agouti and Sprague–Dawley male rats were used in this study as in MDMA research these two strains are the most regularly used strains. Dark Agouti rats were chosen as it has been well reported that a single dose of MDMA produces significant and consistent neurodegeneration of 5-HT pathways, bio-chemical changes and behavioural effects [8,10,11,37] in contrast to Sprague–Dawley, Lister Hooded and Wistar rats which require repeat dosing to produce the same effect [1,9,44]. Differences in brain concentrations of the GSH adducts may be responsible for these variations in dose possibly mediated by differing mercapturic acid pathways [23] which have been shown to modulate the redox properties of the quinol thiоethers [47] and may therefore exhibit differences in the severity of a behavioural effect. Female Dark Agouti rats have been reported to be deficient in CYP2D isoenzymes [7,11] and so male rats were used as they are only partially deficient in their capacity to metabolise CYP2D1 [51].

The behavioural profile of MDA when given subcutaneously to rats includes hyperactivity, forepaw treading, Straub tail, profuse salivation and low posture [33]. An acute release of dopamine following 5-GSH-α-MeDA central administration has been reported [32] and a role for the mesolimbic dopamine system in the psychostimulant actions of MDMA was reported by Gold et al. [15], however the contribution of the dopaminergic system to the behavioural effects produced by 5-GSH-α-MeDA have not been assessed.

The present study provides: (a) evidence for the formation of the two GSH adducts of α-MeDA by human liver microsomes following incubation with MDA; and (b) a direct comparison of behavioural effects of the two GSH adducts following i.c.v. administration in both the male Sprague–Dawley and Dark Agouti rats.
2. Materials and methods

2.1. Animals

Adult male Dark Agouti and Sprague–Dawley rats (Charles River, UK) weighing 180–200 g (aged 6–7 weeks) were chosen for these experiments and housed in groups of five at a constant temperature (21±2 °C) and a 12-h light/dark cycle (lights on: 07:00 h) with free access to food and water. After surgery animals were housed in single cages. Rat chow and water were available ad libitum. All animal procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 under Project Licence 40/1955.

2.2. Materials

α-Methyldopamine was kindly provided by Merck, Sharpe and Dohme (USA). Glutathione (reduced form), mushroom tyrosinase, β-nicotinamide adenine dinucleotide phosphate (NADP), isocitric acid (trisodium salt), isocitrate dehydrogenase, 1-octanesulfonic acid, angiotensin II and perchloric acid (60%) were obtained from Sigma (Poole, Dorset, UK). Sodium orthophosphate buffer, tris(hydroxymethyl)methylanine and Pontamine Sky Blue Dye were obtained from BDH Chemicals (UK). 3,4-Methylenedioxyamphetamine HCl was synthesised at the University of Dublin, Ireland. 5-GSH-α-MeDA and 6-GSH-α-MeDA were synthesised at School of Pharmaceutical Sciences, University of Nottingham (synthesis described in Section 2.3). Haloperidol (Haldol, 5 mg/ml ampoule) was obtained from Jennis-Cilag Ltd. All other chemicals were of the highest grade commercially available. Solid phase extraction cartridges (1000 mg sorbent dryness as separate products. Products were then analysed separately, combined, concentrated and lyophilised to the range of 5–10 mg.

2.3. Synthesis and purification of mono-GSH adducts of α-MeDA

Synthesis followed the procedure described by Miller et al. [31] except for minor changes. In brief, a mixture of α-MeDA (2 mM), GSH (10 mM) and mushroom tyrosinase (100 Units/ml) was stirred at 25 °C for 30 min in 200 ml of sodium phosphate buffer (50 mM at pH 7.4 adjusted using 5 M NaOH). The reaction mixture was then transferred directly into Sep-Pak C18 cartridges (approximately 12 ml/carbonate) that had been pre-treated sequentially with 10 ml of methanol, 10 ml water and 6 ml of sodium phosphate buffer (50 mM at pH 7.4). Extraction was performed using a Varian SPE positive pressure extraction manifold. The cartridges were then washed with 3 ml of water and α-MeDA-GSH adduct eluted with 6 ml of formic acid/water/methanol (1:49:50, v/v). The eluates were combined, concentrated by rotary evaporation, frozen using liquid nitrogen and freeze-dried overnight. An R200-basic Buchi rotary evaporator was used to evaporate eluent from the collected fractions. An Edwards Vacuum Pump with Modulyo 4K litre capacity freeze dryer (condenser capacity of 1.2 l over 12 h) was used for lyophilisation with the freezing chamber maintained at −45 °C with a vacuum of approximately 10⁻¹ atmospheres.

The resulting product was further purified by HPLC (Beckman Gold high-performance liquid chromatography system) by dissolving 20 mg product into 1 ml of 15% aqueous methanol and injecting 0.2-ml aliquots (4 mg load per injection) onto a Hichrom ‘Kromasil’ C₁₈ semi-preparative column (250×10 mm LD; 5 μm particle size). The product was eluted using 15% methanol in water at a flow-rate of 4.73 ml/min and the fractions exhibiting the greatest absorbing product at A=254 nm were collected. Fractions were analysed for glutathione mono-adducts by mass spectrometry electrospray ionization using a Micromass platform (cone voltage set at 19 kV, source temperature at 150 °C) and scanning for mass spectra in the range of m/z=472–474 (+H⁺). A gradient of 15–90% methanol over 10 min was run immediately following the disappearance of the major peaks to elute off any impurities from the column and to regenerate the column, thereby helping to prevent hydrophobic collapse. A short time (15 min) was then allowed for the baseline to re-equilibrate before further purification was performed. Following mass spectrometry the fractions containing the major absorbing products at A=254 nm were collected separately, combined, concentrated and lyophilised to dryness as separate products. Products were then analysed using ¹H NMR to confirm the chemical structures of collected fractions. Approximately 5 mg of material was dissolved in deuterium oxide (D₂O) and examined using a Bruker AMX-250 ¹H NMR machine operating at 250 MHz.

2.4. Human liver microsomal incubation of MDA

Liver tissue was obtained from patients that had died from conditions unrelated to liver failure (n=10; five female and five male). Liver microsomes were prepared as described previously [17] and protein content determined using the method of Lowry et al. [29]. The MDA incubation mixture contained 0.2 M Tris–HCl buffer (adjusted to pH 7.4 with 1 M HCl), magnesium sulphate (30 mM) and isocitrate dehydrogenase (10 Units/ml). Experiments were performed in the presence and absence of glutathione (1 mM) and presence and absence of cofactors (0.5 mM NADP; 5 mM isocitrate) in a shaking water bath at 37 °C. Incubates with and without liver microsomes (1 ml per 10 ml of incubation mixture) were used. MDA (30 μM) was added at the start of incubation.
and measurements of MDA metabolites made at 10, 20, 40, 60, 80 and 100 min intervals. At timepoints 40 and 80 min, additional incubations were set up to control for the absence of microsomes, glutathione or cofactors. Incubations were started by the addition of 1 ml of the microsomal suspension. Extracts of incubation mixtures (1 ml) were taken at appropriate time-points and were terminated by the addition of 25 µl of perchloric acid (60%), vortexed for 1 min, centrifuged at 10 000 g for 5 min and the supernatant (100 µl) analysed for each of the GSH adducts by HPLC–ECD.

2.5. HPLC–ECD analysis of glutathione adduct metabolites of MDA in microsomes

Separation of mono-GSH adducts of α-MeDA was accomplished using an HPLC Technology RR/066L solvent pump with an Antec EC controller CU-04AZ detector equipped with a VT03 glassy carbon working electrode set at a potential of +0.7 V with reference to a saturated KCl filled Ag/AgCl reference electrode. Separation was accomplished by use of a Hichrom ‘Kromasil’ C18 column (250×4.6 mm) with Phenomenex (UK) C18 pre-column (4×3 mm). Glutathione adducts were eluted isocratically using a mobile phase consisting of 0.1 M sodium citrate, 1 mM 1-octanesulfonic acid, methanol/acetonitrile/water (4:4:92, v/v/v), adjusted to pH 3.5 using o-phosphoric acid. The flow-rate was constant at 0.6 ml/min and the current produced was monitored by means of an integrator (Spectra-Physics Chromjet integrator, Spectra-Physics, Darmstadt, Germany). Using these conditions approximate retention times (in parenthesis) for the metabolites were as follows: 6-GSH-α-MeDA (39.77 min) and 5-GSH-α-MeDA (40.10 min) and α-MeDA (45.30 min).

2.6. CYP2D6 phenotyping by (+/-)-bufuralol 1’-hydroxylase assay

A 0.25-ml reaction mixture containing 0.8 mg/ml protein, 1.3 mM NADP+, 3.3 mM glucose-6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride and 25 µM (+/-)-bufuralol (delivered as a concentrated buffer solution) in 100 mM potassium phosphate (pH 7.4) was incubated at 37 °C for 20 min. After incubation, 25 µl of 70% perchoric acid was added and the mixture was centrifuged at 12 000×g to pellet the protein. A portion of the supernatant was injected into a 4.6×250 mm, 5-µm C18 HPLC column and separated at 45°C with a mobile phase of 1 mM perchloric acid in 30% aqueous acetonitrile at a flow-rate of 1.0 ml per min. The retention time of the product was approximately 6 min. The fluorescence of the product was measured in the flow cell of a spectrofluorometer with excitation at 252 nm and emission at 302 nm. The response was quantitated by comparing to a standard curve of product (carbinol). CYP2D6 activity was plotted against formation of each adduct and GraphPad prism (Software Version 3.00 for Windows, GraphPad, San Diego, CA, USA) used to calculate lines of best fit and linear regression.

2.7. Intracerebroventricular administration of 5-GSH-α-MeDA and 6-GSH-α-MeDA

A guide cannula was placed in the left lateral ventricle of male Dark Agouti and Sprague–Dawley rats to allow i.c.v. injections of mono-GSH adducts of α-MeDA. Rats were anaesthetised using isoflurane at 3–4% for induction and 1.5–2% for maintenance and placed in a stereotaxic apparatus. After shaving the skull a mid-sagittal incision was made in order to expose the skull which was flooded with Mercaine to help prevent excessive bleeding and to act as a local anaesthetic. The skull was leveled and an injection of carprofen at 0.25 µl s.c. administered (Rimadyl 5%, non steroidal anti-inflammatory drug) in the dorsal region at the level of the forepaws to manage post surgical pain. A small burr hole was made with a hand drill: (−) 1.5 mm lateral from bregma, and (−) 0.92 posterior. A 26G guide cannula was lowered (−) 2.6 mm ventral to the surface of the skull in order to guide an injection needle which extended 1.0 mm below the bottom of the guide cannula. Two other burr holes were made to place jewellers screws which together with dental cement+methylmethacrylate (curing liquid) were used to fix the guide cannula in place. A dummy cannula (stylet) was inserted into the guide cannula, and the incision covered with a thin layer of triple antibiotic ointment. The incision was closed with sutures, and the animals were allowed to recover for 5–7 days.

The accuracy of i.c.v. cannula placement was confirmed in a test group of rats which were infused with Pontamine Sky Blue dye (0.8 mg/ml water) and brain slices (200 µm thickness) made using a manually operated vibrotome and analysed for location of the dye (Camden Instruments). Angiotensin II (5.23 µg/µl in a volume of 2 µl; i.c.v.) was administered on days 5–7 to each rat in the experimental group and successful cannula placement was verified by a rapid (within 1 min) drinking response. The following day, animals were administered 10 µl of artificial cerebrospinal fluid (ACSF composed of: 147 mM NaCl, 4 mM KCl, 1.2 mM CaCl2 and 1.2 mM MgSO4), as the vehicle; either 5-GSH-α-MeDA or 6-GSH-α-MeDA adduct was then infused into the left lateral ventricle of the awake animals at a rate of 2 µl every 30 s (total volume 10 µl) using a 25-µl Hamilton syringe connected to an injection needle. The injection needle was left in place for a period of 1–2 min after the injection. A dummy cannula was then inserted into the guide cannula to close the injection site. Control animals received artificial cerebro-spinal fluid (ACSF; i.c.v.). Behaviours, including hyperactivity, wet dog shakes, head weaving, forepaw treading, splayed and low posture, teeth chattering, shaking/tremor and clonus,
were examined by visual observation for the first 30 min and were each given a score out of 10 (0= no incidence of behaviour, 5= moderate incidence and severity of behaviour, 10= high incidence and severity of behaviour) and rectal temperatures measured. Some rats were also pre-treated with the non-selective dopamine receptor antagonist haloperidol (0.25 mg/kg, i.p.) and 40 min later given 5-GSH-α-MeDA (180 nmol, i.c.v.) and behaviours scored both with and without antagonist pre-treatment. The scorer was blind with respect to treatment.

2.8. Statistical analysis

Differences in behavioural rated values between strains and the effects of pre-treatment with haloperidol were compared by Mann–Whitney non-parametric test. Correlation between CYP2D6 activity and GSH adduct formation was calculated using regression analysis. Prism statistical analysis software was used for this purpose (GraphPad Prism Software, version 3.00 for Windows, GraphPad, San Diego, CA, USA).

3. Results

3.1. HPLC purification of α-MeDA-GSH adducts

Following solid-phase extraction and freeze drying of the reaction mixture a pale tan coloured product was produced. HPLC purification of the synthesis reaction mixture resulted in the appearance of two major peaks detected at a UV wavelength of 254 nm and a mobile phase methanol concentration of 10% (Fig. 1). Both peaks exhibited similar chromatographic profiles, having similar retention times (13.4 and 14.3 min), peak shape and peak areas, and the compounds responsible for each peak were then analysed by mass spectrometry.

3.2. Mass spectrometry of α-MeDA–GSH adducts

Using HPLC–UV separation the two major peaks of interest were collected individually following elution off the C18 column and were analysed by mass spectrometry to detect which of the peaks contained the mass ion for the mono-α-MeDA adduct of GSH. It was established that both of the major peaks contained the protonated mass ion for GSH–α-MeDA adduct of m/z 472.9 (471.9+H+). Protonated (M+H)+ and sodiated (M+Na)+ molecular ions at m/z=472.9 and 494.9, respectively, were present for peak 1 and m/z=472.8 and 494.9 for peak 2. The double charged ions (M+2H)2+ exhibiting a m/z of 237.0 and 236.8 and other breakdown components of the adduct were also detected. There was no evidence of the bis-adduct at m/z 778 (777+H2+) under these synthesis conditions.

3.3. 1H NMR analysis of α-MeDA–GSH adducts

The 1H NMR spectrum for peak 1 suggests that this compound can be confirmed as 6-GSH-α-MeDA. 1H NMR spectra (D2O) δ 6.77 (1H, s, J=1.75 Hz, H3), 6.67 (1H, s, H2), 4.29 (1H, m, Cys-α), 3.61 (1H, t, J=6.25 Hz, Glu-α), 3.46 (2H, s, Gly-α), 3.43 (1H, m, CH–α-MeDA), 3.26 (1H, dd, J=13.8, 7.5 Hz, Cys-β), 3.10 (1H, dd, J=13.8, 7.5 Hz, Cys-β), 2.68 (2H, m, CH2–α-MeDA), 2.34 (2H, t, J=7.5 Hz, Glu-λ), 2.01 (1H, d, J=7.5 Hz, Glu-β), 1.95 (1H, d, J=7.5 Hz, Glu-β), 1.17 (3H, d, J=5.0 Hz, CH3-α-MeDA). The aromatic region of the spectrum on closer analysis shows two singlet peaks (6.67 and 6.77 ppm). This is consistent with the two remaining aromatic protons having a para orientation, free protons being located at positions 2 and 5 concluding that the GSH was attached at position 6.

The 1H NMR spectrum for peak 2 suggests that this compound can be confirmed as 5-GSH-α-MeDA. 1H NMR spectra (D2O) δ 6.77 (1H, d, J=1.75 Hz, H3), 6.67 (1H, d, J=1.75 Hz, H2), 4.29 (1H, dd, J=7.6, 5.0 Hz, Cys-α), 3.61 (1H, t, J=6.25 Hz, Glu-α), 3.46 (2H, s, Gly-α), 3.43 (1H, m, CH–α-MeDA), 3.26 (1H, dd, J=13.8, 7.5 Hz, Cys-β), 3.09 (1H, dd, J=13.8, 7.5 Hz, Cys-β), 2.67 (2H, m, CH2–α-MeDA), 2.34 (2H, t, J=7.5 Hz, Glu-λ), 2.01 (1H, d, J=7.5 Hz, Glu-β), 1.95 (1H, d, J=7.5 Hz, Glu-β),
3.5. HPLC–ECD analysis of microsomal incubates for GSH adduct formation

The formation of electrochemically active metabolites in the microsomal incubates was assayed by HPLC with electrochemical detection (HPLC–ECD) and quantified at each time point. The chromatogram obtained for MDA incubation is shown in Fig. 2. An authentic mixed standard of 6-GSH-α-MeDA and 5-GSH-α-MeDA produced peaks with retention times of 39.77 and 40.10 min, respectively (Fig. 2a). Two time-dependent peaks (Fig. 3) were detected in incubates exposed to MDA with glutathione and cofactors (Fig. 2b). Peak A was shown to be 6-GSH-α-MeDA by cochromatography with authentic standard (Fig. 2d). Peak B was identified as 5-GSH-α-MeDA by cochromatography with authentic standard (Fig. 2c). Peaks A and B disappeared when microsomes were incubated without cofactors (Fig. 2e). Peaks A and B could not be detected following incubation in the absence of glutathione (Fig. 2f). To demonstrate that a biological component was required for the formation of the adducts for their formation, microsomes were omitted from the incubation mixture and respective peaks were again not detected (not shown). The generation of both GSH regioisomers from incubation of 30 μM MDA ranged from 22 to 440 pmol/mg of protein. (up to 1.5% of MDA is converted to each metabolite). Almost identical amounts of each GSH adduct were produced, both overall (Fig. 3) and on an individual sample basis (Fig. 4). Note that in separate experiments rat liver microsomes incubated with MDA showed very little difference both in the rate of formation or the ratio of the two regioisomers when compared to human liver microsomes data (data not shown).

3.5. CYP2D6 activity

The bufuralol assay showed a variation in CYP2D6 activity between the human liver samples used for the microsomal assay, with hydroxylation ranging from between 0.02 and 0.3 nmol/min/mg. Regression analysis demonstrated a significant positive linear relationship of cytochrome P450 2D6 activity with the level of glutathione adduct formation for both adduct isomers (Fig. 4).

3.6. Behavioural analysis of mono-glutathione adducts of α-MeDA

In contrast to the behavioural studies on 5-GSH-α-MeDA performed previously [31] which reported that Sprague–Dawley rats required an i.c.v. dose of 720 nmol to produce a behavioural effect without lethal effects, initial dose finding studies showed that a dose of 360 nmol i.c.v. of 5-GSH-α-MeDA in both strains of rats resulted in approximately 50% lethality whilst doses of 720 nmol i.c.v. were 100% lethal in both rat strains tested (data not shown). Initial dose range finding study indicated that an i.c.v. dose of 180 nmol for 5-GSH-α-MeDA adduct would be suitable to explore the behavioural effects as this was a sub-maximal dose for behavioural effects in both strains of rat. The behaviours observed were: hyperactivity (characterised by very rapid running, jumping, rolling along the head-tail axis), wet dog shakes, head weaving, forepaw treading, splayed and low posture, teeth chattering, shaking, tremor and clonus, all of which began within the first 2 min and continued for up to 90 min. There were no significant differences in 5-GSH-α-MeDA induced behavioural effects between Sprague–Dawley and Dark Agouti rats (Fig. 5). Pre-treatment with the dopamine antagonist haloperidol (0.25 mg/kg; i.p. 40 min pre-test) in the Sprague–Dawley rats resulted in attenuation of hyperactivity, low posture and clonus-like behaviour and decreases in teeth chattering and shaking, together with a significant increase in splayed posture behaviour (Fig. 6). Haloperidol produced no behavioural effect when administered alone. Administration of 6-GSH-α-MeDA at doses of up to 1000 nmol i.c.v. failed to produce any behavioural effects and neither of the adducts altered rectal temperatures (data not shown).

4. Discussion

Attempts to synthesise a purified form of the 5-GSH-α-MeDA adduct for use in behavioural studies resulted in the additional unexpected synthesis of the 6-GSH-α-MeDA adduct. This adduct was produced by use of the same synthesis protocol used for the 5-GSH-α-MeDA adduct described by Miller et al. [31] with only slight modification. During purification HPLC column end contamination occurred by either a breakdown product of the adduct or a starting material component which resulted in a rapid loss of chromatographic resolution. Without the use of adequate column protection the selectivity was decreased and peak broadening occurred to an extent where the two peaks appeared as one. Separation of the two peaks was greatly affected by very small changes in the organic content of the mobile phase. Utilising the lower wave-
Fig. 2. HPLC–ECD identification of metabolites in human liver microsomes following incubation with MDA (30 μM) for 80 min. HPLC chromatograms: (a) injection of $1 \times 10^{-5}$ M mixed 5-GSH-α-MeDA and 6-GSH-α-MeDA standard; (b) extract from microsomes incubated with MDA (30 μM) in the presence of GSH and cofactors; (c) coinjection of MDA incubation extract with authentic 5-GSH-α-MeDA; (d) coinjection of MDA incubation extract with authentic 6-GSH-α-MeDA; (e) extract from microsomes incubated with MDA in the absence of cofactors; (f) extract from MDA incubated in the absence of glutathione. Note the absence of GSH adduct formation without substrates. Substrate (MDA) present in all.
Fig. 3. Accumulated amount of glutathione adduct formed (nmol/mg protein) over time (min) in human liver microsomes (n=10) incubated with MDA (30 μM). Data shown are total values with S.E.M.

The length of 254 nm instead of 280 nm [31] during purification enabled optimal separation to distinguish the two peaks from one another and from residual impurities. Mass spectrometry analysis for each of the collected peaks produced a molecular ion peak of m/z=473 and 495 (protonated and sodiated forms, respectively). The similarity in molecular ions suggested that the peaks were regiosomers. 1H NMR analysis (D2O) for peak 1 confirms this as 6-GSH-α-MeDA. 1H NMR analysis (D2O) for peak 2 confirms this as 5-GSH-α-MeDA. The 1H NMR data for peak 1 were consistent with the data produced by Patel et al. [39] for 6-GSH-α-MeDA and the results for peak 2 were consistent with the data produced by Miller et al. [31] for 5-GSH-α-MeDA. These results suggest that the purification of glutathione adducts synthesized from α-MeDA may be more complicated than had previously been thought, and that two glutathione regiosomers of α-MeDA are readily formed. As a consequence, the metabolic pathway leading to formation of the 5-GSH-α-MeDA adduct proposed in previous studies may not be the only initial conjugation involving GSH available from the

Fig. 4. CYP2D6 activity (nmol/min/mg) against amount of glutathione adduct formed (nmol/mg protein) at the 80-min timepoint in human liver microsomes incubated with MDA (30 μM). 5-GSH-α-MeDA: r²= 0.9023, P<0.0001, F=119.1; 6-GSH-α-MeDA: r²=0.9371, P<0.0001, F=73.92.

Fig. 5. Behavioural analysis following administration of ACSF or 5-GSH-α-MeDA (180 nmol; i.c.v.) in Sprague–Dawley (SD) and Dark Agouti (DA) strain of rats (n=4). Behavioural scoring was assessed over a 30-min period from administration and scored on a scale of 0–10 for each rat (0=no behaviour and 10=behaviour readily observed) and the median rating was calculated. Variation between strains of rat (DA vs. SD) was analysed by Mann–Whitney non-parametric test and was found to be non-significant. Piloerection occurred in all treated animals.

Fig. 6. Behavioural analysis following administration of ACSF or 5-GSH-α-MeDA (180 nmol; i.c.v.) in Sprague–Dawley rats (n=4) with and without haloperidol antagonist pre-treatment (0.25 mg/kg, i.p.; 40 min pre-test). Behavioural scoring was assessed over a 30-min period from administration and scored on a scale of 0–10 for each rat (0=no behaviour and 10=behaviour readily observed) and the median rating was calculated. Effects of haloperidol pre-treatment scores were analysed by Mann–Whitney non-parametric test. * P<0.05 different from animals with no antagonist pre-treatment. Haloperidol alone produced none of the scored behaviours. Piloerection occurred in all treated animals.
metabolism of α-MeDA. The metabolism of α-MeDA to the 6-GSH-α-MeDA metabolite and its neurotoxic or behaviour has not previously been considered.

N-Methyl-α-MeDA can be produced from MDMA in humans [43], and in rat liver microsomes [19]. The formation of MDA from MDMA in rat liver microsomes is substantially lower than that of N-Me-α-MeDA and the formation of the catechol metabolite α-MeDA is not readily detected after incubation of MDMA but is present in incubations containing MDA [5]. In our microsomal incubation system two products are formed, which require the presence of GSH, cofactors, and microsomes for their formation. This suggests that the formation of the metabolites is P450-mediated and requires GSH as a substrate. The products could be identified as the 5-GSH-α-MeDA and 6-GSH-α-MeDA adducts by co-injection with previously synthesised authentic adduct. The fact that the GSH adducts were produced in large quantities (up to 1.5% of the available MDA), and also require α-MeDA as a substrate for their formation would indicate that α-MeDA is readily formed from MDA by human liver microsomes and in our HPLC system α-MeDA eluted later than the GSH adducts. The HPLC data are consistent with those produced by Patel et al. [39] and the retention times for the GSH adduct formed are similar to that reported by Hiramatsu et al. [19] for the N-Me-α-MeDA glutathione adduct. Separation of each peak by HPLC following microsomal incubations was again complicated due to the components of the incubation mixture exhibiting very similar retention characteristics. The human livers used in this study showed a large variation in CYP2D6 activity. Livers with higher levels of CYP2D6 activity produced up to four times as much of the GSH adducts than those of lower activity and this relationship was highly correlated.

It can be speculated that the 6-GSH-α-MeDA adduct possesses the ability to penetrate the brain by the same transporter that carries other GSH adducts from the circulation into the brain [31], suggesting that this adduct has the potential to also contribute to the behavioural effects of MDA. 5-GSH-α-MeDA produced distinct behavioural effects at a dose of only 120 nmol when given i.c.v. In contrast, a 1000 nmol dose of 6-GSH-α-MeDA had no effect on rat behaviour. 5-GSH-α-MeDA given i.c.v. is therefore at least eight times more potent in its ability to produce a behavioural response than the 6-GSH-α-MeDA adduct, the latter adduct being behaviourally inactive. 5-GSH-α-MeDA produced a lethal response at just 360 nmol when administered i.c.v. in both strains of rat which is half the dose previously reported to produce a moderate behavioural effect in the Sprague–Dawley strain of rat [32]. 5-GSH-α-MeDA at doses above 180 nmol, i.c.v. resulted in clonus (within 15 min) followed by death (within 45 min) in both strains of rat. One explanation for the increased potency of our 5-GSH-α-MeDA sample may be that the compound used for behavioural and toxicity testing in the study by Miller and co-workers [31,32] was a mixture of the 5 and 6-GSH-α-MeDA adducts. The human liver microsome studies indicated that the GSH adducts are formed in similar proportions. Therefore previous synthesis of the 5-GSH-α-MeDA adduct may have produced a compound that contained only approximately 50% of the 5-GSH-α-MeDA adduct, the rest being the behaviourally inactive 6-GSH-α-MeDA adduct.

The behavioural effects of 5-GSH-α-MeDA appeared to be more typical of dopaminergic activity, with very few serotonin-like behaviours observed. For example, behaviours such as wet dog shakes, head weaving and forepaw treading (typical of serotonin release) were not scored as highly as other behaviours such as teeth chattering, generalised shaking and locomotor disturbances including hyperlocomotion and moderate to severe clonus behaviour. To investigate the role of dopamine in the behavioural response we gave the dopamine receptor antagonist, haloperidol in the presence and absence of the 5-GSH-α-MeDA adduct and re-examined the behaviours in both strains of rats. Haloperidol abolished the hyperactivity, low posture and clonus behaviours seen with the 5-GSH-α-MeDA adduct when administered alone. Splayed posture was observed with haloperidol pre-treatment but in the absence of the antagonist this behavioural effect was not observed. Splayed posture in which the limbs are abducted and extended and the body lies flat on the ground is considered a serotonergic behaviour [46] and it is possible that this is uncovered due to the blockade of dopamine receptor function. The finding that the behavioural effects of 5-GSH-α-MeDA adduct are attenuated by haloperidol would suggest involvement of the dopaminergic system, at least in part either by release of dopamine or by direct action of the metabolite at dopamine receptors.

MDMA is a potent releaser of both dopamine and 5-HT [48] and therefore it is not surprising that a number of different receptors (including D₂ receptors) may underlie the behavioural effects of MDMA. The present results indicate that the parent compounds MDMA/MDA may produce some of their locomotor and motor disturbances via production of the 5-GSH-α-MeDA adduct which we have demonstrated to be formed in human liver microsomes, and the effects of which may be related to activation of the dopaminergic system. An involvement of dopamine is also consistent with the findings of Callaway et al. [4] and Kehne et al. [22] showing that in humans D₂ receptor antagonism appears to diminish MDMA-induced locomotor stimulation. Liechti and Vollenweider [24] also demonstrated that haloperidol pretreatment in humans blocks MDMA-induced positive and mania-like mood (the euphoric effects), further implying a role for D₂ receptors in the mediation of MDMA behaviour. The present results indicate that dopamine system modulation by 5-GSH-α-MeDA may be important in specific motor and postural control.

This study indicates that 5-GSH-α-MeDA and its downstream metabolites may contribute towards the overall
behavioural profile produced by MDA. It is reasonable to assume that the 6-GSH-α-MeDA adduct can be metabolised to other compounds in the mercapturic acid pathway not previously considered, for example 6-(N-acetylcysteine-S-y1)-α-MeDA, based upon evidence that 5-GSH-α-MeDA adduct can form 5-(N-acetylcysteine-S-y1)-α-MeDA and other downstream metabolites. The possibility that GSH adducts of the N-methyl-α-MeDA metabolic pathway could also exhibit a similar positional shift of GSH around the aromatic ring cannot be excluded. Other metabolites of MDMA and MDA such as 2,4,5-trihydroxymethamphetamine, 4-hydroxy-3-methoxyamphetamine and 4-hydroxy-3-methoxymethamphetamine may conjugate with GSH and therefore have altered behavioural or neurotoxic properties and this area requires further investigation.

To summarise, the results demonstrate that α-MeDA can conjugate with glutathione to form both 5- and 6-GSH-α-MeDA regioisomers, and that this conjugation can also occur in human liver microsomes. Furthermore, production of the 5- and 6-adducts from MDA is mediated, at least in part, by human liver CYP2D6 in catalysing the conversion of MDA to α-MeDA. In turn this determines the extent to which both GSH adducts are formed. The 5-GSH-α-MeDA but not the 6-GSH-α-MeDA regioisomer is behaviourally active. Most of the behaviours induced by 5-GSH-α-MeDA were abolished by the dopamine antagonist haloperidol implicating a role of the dopaminergic system in some of the behaviours produced by the parent compounds MDA and MDMA.

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