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Research Article

Interspecies In Vitro Evaluation of Stereoselective Protein Binding for 3,4-Methylenedioxymethamphetamine

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Abuse of 3,4-methylenedioxymethamphetamine (MDMA) is becoming more common worldwide. To date, there is no information available on stereoselectivity of MDMA protein binding in humans, rats, and mice. Since stereoselectivity plays an important role in MDMA's pharmacokinetics and pharmacodynamics, in this study we investigated its stereoselectivity in protein binding. The stereoselective protein binding of *rac*-MDMA was investigated using two different concentrations (20 and 200 ng/mL) in human plasma and mouse and rat sera using an ultrafiltration technique. No significant stereoselectivity in protein binding was observed in both human plasma and rat serum; however, a significant stereoselective binding ($p < 0.05$) was observed in mouse serum. Since the protein binding of MDMA in mouse serum is considerably lower than in humans and rats, caution should be exercised when using mice for in vitro studies involving MDMA.

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA) is a commonly abused drug with estimated 18.8 million users worldwide in 2013 [1]. Acting as a sympathomimetic agent, MDMA causes acute and chronic side effects such as hyperthermia, neurotoxicity, respiratory arrest, and hepatotoxicity [2–4]. Metabolism of MDMA occurs primarily through hepatic cytochrome P450 enzymes, specifically via CYP2D6, CYP2B6, CYP2C19, and CYP3A4. Even though MDMA is ingested in its racemic form, metabolism of *rac*-MDMA is stereoselective with (+)-(S)-MDMA being preferentially metabolised [5, 6] and more potent in inducing euphoria and energy [7, 8], whereas the (−)-(R)-MDMA exert higher hallucinogen-like activity than the (+)-(S)-MDMA [9]. This is evident in the stereoselectivity observed in the concentrations of MDMA and related metabolites in urine and plasma samples [10–14].

MDMA enantiomers exhibit different pharmacodynamics due to differences in binding affinities to receptor sites. (+)-(S)-MDMA is a more effective releaser of dopamine and serotonin when compared to its antipode [15] and thus is more closely linked to the neurotoxic effects of MDMA. (−)-(R)-MDMA is associated with hallucinogenic properties whereas (+)-(S)-MDMA is more stimulant-like in its effects [9]. Furthermore, the rate of elimination of (+)-(S)-MDMA is higher than that of (−)-(R)-MDMA [4, 10, 13, 16, 17].

The binding of a drug to plasma or serum proteins is a major determinant of free drug concentrations in vivo and thus has a large impact on the drug's pharmacokinetics, pharmacodynamics, and toxicology [18]. For chiral drugs, especially those with different activities and effects associated with each enantiomer, stereoselectivity in protein binding is an important consideration. Among drugs that have exhibited considerable stereoselectivity in protein binding are warfarin [19], ibuprofen [20], and ketoprofen [21].

There is a significant lack of information regarding plasma protein binding of MDMA in humans or animals. To date, only one study has assessed protein binding of MDMA in dog plasma with the fraction of MDMA bound to protein

reported as 0.40 [22]. To our knowledge, no information is available regarding stereoselectivity of MDMA protein binding in humans, rats, and mice.

As stereoselectivity plays an important role in the pharmacokinetics and pharmacodynamics of MDMA, an assessment of stereoselectivity in protein binding was carried out. In this study, stereoselective protein binding of *rac*-MDMA using two different concentrations in human plasma and mouse and rat sera was determined using an ultrafiltration approach. Using a stereospecific gas chromatography-mass spectrometry (GC-MS) assay, the amounts of (−)-(R)-MDMA and (+)-(S)-MDMA bound to plasma or serum proteins were quantified.

2. Materials and Methods

2.1. Reagents and Materials. Methanolic drug standards of *rac*-MDMA (1000 μg/mL of free base) and *rac*-MDMA-d5 (100 μg/mL of free base) were purchased from Cerrilliant (Round Rock, TX, USA). Hexane was obtained from Fisher Scientific (Hampton, NH, USA). Ethyl acetate was purchased from Merck (Darmstadt, Germany). Triethylamine and phosphate-buffered saline (PBS) at pH 7.4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). The chiral derivatization reagent R-(−)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(R)-MTPCl] (ChiraSelect grade, 99% purity, 99.5 : 0.5 enantiomeric ratio) was obtained from Sigma-Aldrich (St. Louis, USA). All reagents and solvents were of analytical grade. Vivaspin 500 centrifugal ultrafiltration devices (polyether sulfone membrane, 10 kDa molecular weight cut-off) were purchased from Sartorius Stedim (Gottingen, Germany).

Human plasma was obtained from the Hospital Universiti Sains Malaysia blood bank. Rat (Sprague-Dawley) and mouse (BALB/c) serum were obtained from the School of Pharmaceutical Sciences, Universiti Sains Malaysia. The plasma and serum used in this study were sourced from three different donors, pooled according to species, and adjusted to pH 7.4 before being used in further experiments.

2.2. Preparation of Chiral Derivatization Reagent (R-MTPCl). Aliquots of 10 μL R-MTPCl were transferred to 4.0 mL amber glass vials, sealed with PTFE-lined screw caps, and stored at −20°C. When required, a vial was thawed to room temperature and diluted with 1 mL of hexane to give a 1% v/v solution of R-MTPCl in hexane.

2.3. Preparation of Working Solutions. A 20 μg/mL *rac*-MDMA solution was prepared. A second working solution of 2 μg/mL *rac*-MDMA was prepared from the 20 μg/mL working solution. An internal standard solution containing 10 ul μg/mL *rac*-MDMA-d5 was also prepared. All working and internal standard solutions were stored in the dark at 4°C until required.

2.4. Determination of Nonspecific Binding and Stereoselective Protein Binding Assay. The possibility of nonspecific binding of MDMA occurring in the ultrafiltration device and stereoselective binding were evaluated at two concentrations of *rac*-MDMA: 20 and 200 ng/mL. In this experiment, 0.01 M PBS at pH 7.4 was used as a surrogate matrix for plasma or serum. PBS (495 μL) was pipetted into 2 mL microcentrifuge tubes followed by the addition of 5 μL of the appropriate working solution. The final organic solvent concentration was maintained at less than 1% to avoid protein precipitation. Control samples with similar concentrations of *rac*-MDMA were also prepared. The samples were vortex-mixed thoroughly and incubated at 37°C in a shaking water bath for 20 min. Next, the samples were transferred to the upper portion of the ultrafiltration device using a glass Pasteur pipette. The device was closed and tapped gently in order to eliminate any air bubbles. Control samples were transferred to microcentrifuge tubes. The ultrafiltration devices and control samples were centrifuged in a fixed-rotor centrifuge at 1000 ×g, 37°C for 20 min. After centrifugation, the upper portion of the ultrafiltration device was removed and discarded. Separate aliquots of 300 μL each were removed from the ultrafiltrate in the lower portion of the ultrafiltration device as well as from the control samples and transferred to clean screw-cap glass tubes. Each assay was performed in triplicate. The concentrations of (−)-(R)-MDMA and (+)-(S)-MDMA in the ultrafiltrate from each sample were determined as described below.

2.5. Analytical Method. The samples were analysed using a previously validated method which enables the stereoselective analysis of chiral amphetamine-type substances using a GC-MS assay [23]. Using this method, the limit of detection for MDMA was 10 μg/L and recovery was more than 80%. In brief, ten microliters of internal standard working solution was added to the samples to be analysed. The pH of the samples was adjusted to pH 10 with 2 mL of 1.0 M sodium carbonate. A solution of 2 mL of hexane : ethyl acetate 1 : 1 (v/v) containing 0.03% triethylamine was added to the sample together with 150 μL of the chiral derivatization reagent (R-MTPCl). The samples were vortex-mixed for 1 min and then mixed on a rotary mixer at 40 rpm for 20 min. Subsequently, the samples were centrifuged at 3500 rpm for 5 min and the upper organic layer was transferred to another culture tube. The samples were dried on a heating block at 40°C under a gentle flow of nitrogen gas. The residue was dissolved in 50 μL of ethyl acetate before analysis using GC-MS.

All samples were analysed using an Agilent (Santa Clara, CA, USA) 6890 series gas chromatograph equipped with a model 5973 mass selective detector. Injections were performed using an Agilent autosampler (model 7893) and injector. Separations were achieved using an Agilent HP-5ms column (5% Phenyl-95% methylpolysiloxane stationary phase; column dimensions: 15 m, 250 μm i.d., and 0.25 μm film thickness). The GC was operated with helium as carrier gas with a flow of 1.0 mL/min. The column oven was operated as follows: being held at 150°C for 1 min, to 240°C at 10°C/min, and held for 5 min, to 290°C at 40°C/min, and held for 1 min for a total run time of 17.25 min. Samples were introduced using a 4 μL pulsed splitless injection with the injector port at 270°C, 20 psi pressure pulse at 2 min, and a purge

Table 1: Percentage of nonspecific binding of *rac*-MDMA to ultrafiltration devices.

Concentration of *rac*-MDMA (ng/mL)	Compound	Mean peak area ratios		Nonspecific binding (%)
		Sample ($n = 3$)	Control ($n = 1$)	
20	(−)-(R)-MDMA	0.093 ± 0.019	0.091	−2.3
	(+)-(S)-MDMA	0.098 ± 0.003	0.095	−3.1
200	(−)-(R)-MDMA	0.926 ± 0.076	0.884	−4.6
	(+)-(S)-MDMA	0.940 ± 0.073	0.890	−5.6

time of 2 min. The MS parameters were electron impact ionization with an ion source temperature of 230°C, transfer line temperature of 280°C, and a solvent delay of 5 min. The analysis was performed in selected ion monitoring (SIM) mode. The m/z values monitored are as follows: m/z 274, 162, and 136 for MDMA and m/z 278, 164, and 136 for MDMA-d5.

2.6. Determination of Nonspecific Binding and Protein Binding. Equal volumes of ultrafiltrate and control sample (300 μL) were aspirated and analysed as described above. The amount of each MDMA enantiomer in the samples was represented by the peak area ratio of analyte [(−)-(R)-MDMA or (+)-(S)-MDMA] to the respective internal standard [(−)-(R)-MDMA-d5 or (+)-(S)-MDMA-d5].

The percentage of bound MDMA for both nonspecific binding and protein binding was calculated according to the following formula [24]:

$$\text{Percent MDMA bound (\%)} = 100 - \left[\frac{(\text{Peak area ratio in ultrafiltrate})}{(\text{Peak area ratio in control sample})} \times 100\right]. \quad (1)$$

Stereospecificity in protein binding was determined by calculating the R- to S-enantiomer ratio. Statistical significance was determined using a paired t-test at a significance level of $p < 0.05$.

3. Results and Discussion

3.1. GC-MS Analysis. Under the analytical conditions used, the enantiomers of MDMA were separated with good resolution and sensitivity. The order of elution for the enantiomers had been identified in a previous study to be (−)-(R)-MDMA followed by (+)-(S)-MDMA [23, 25]. In the present study, using comparison with pure R- and S-MDMA standards, it was determined that the order of elution was the same.

3.2. Determination of Nonspecific Binding. Nonspecific binding of MDMA enantiomers to the ultrafiltration device was estimated using 20 and 200 ng/mL of *rac*-MDMA. The extracted MRM chromatograms obtained for MDMA-d5 and MDMA at 20 ng/mL are shown in Figure 1. The choice of 20 and 200 ng/mL in the present study was based on a previous study [26] to represent low and high concentrations, respectively. For each of the concentrations assessed, it was observed that the calculated percentages of nonspecific binding all gave negative values (Table 1) indicating that MDMA enantiomers do not exhibit nonspecific binding to the walls of the ultrafiltration device. Thus, it can be concluded that the ultrafiltration device is suitable for determining protein binding of MDMA.

3.3. Protein Binding of MDMA Enantiomers. In all experiments, protein binding of *rac*-MDMA at concentrations of 20 and 200 ng/mL was evaluated and the percentage protein binding for each species was calculated (Table 2). In human plasma, at a concentration of 20 ng/mL MDMA, it was found that 41% and 44% of (−)-(R)-MDMA and (+)-(S)-MDMA, respectively, were bound to plasma proteins. A tenfold increase of MDMA to 200 ng/mL resulted in a slight increase in binding to 51% for both (−)-(R)-MDMA and (+)-(S)-MDMA.

In rat serum, it was found that, for both concentrations, MDMA enantiomers were bound to serum proteins to a similar extent as that of human plasma proteins. At 20 ng/mL of *rac*-MDMA, 38% of (−)-(R)-MDMA and 41% of (+)-(S)-MDMA were bound to serum proteins. Also, similar to human plasma proteins, these values increased slightly to 47% and 50% for (−)-(R)-MDMA and (+)-(S)-MDMA, respectively, for 200 ng/mL of MDMA in rat serum. These values are comparable to the bound fraction of 40% reported for dog plasma [22].

In mouse serum, MDMA enantiomers were bound to serum proteins at a lesser extent when compared to human plasma or rat serum. At a concentration of 20 ng/mL, the percentage of (−)-(R)-MDMA and (+)-(S)-MDMA bound to serum proteins was 28% and 36%, respectively. As opposed to the observations in human plasma and rat serum which demonstrated a slight increase in the amount of protein-bound MDMA with an increase in MDMA concentration, mouse serum demonstrated a decrease in the percentage of bound MDMA when the concentration of MDMA was increased to 200 ng/mL, with only 11% (−)-(R)-MDMA and 15% of (+)-(S)-MDMA being bound to serum proteins. This decrease indicates that it is likely that the binding sites for MDMA on mouse serum proteins are saturated at lower concentrations of MDMA when compared to that of humans and rats. Therefore, in mice, a tenfold increase in MDMA concentration did not result in an increase in protein-bound MDMA.

Stereoselectivity in protein binding was estimated by calculating the ratio of bound (−)-(R)-MDMA versus (+)-(S)-MDMA for each matrix tested. A ratio of <1 indicates

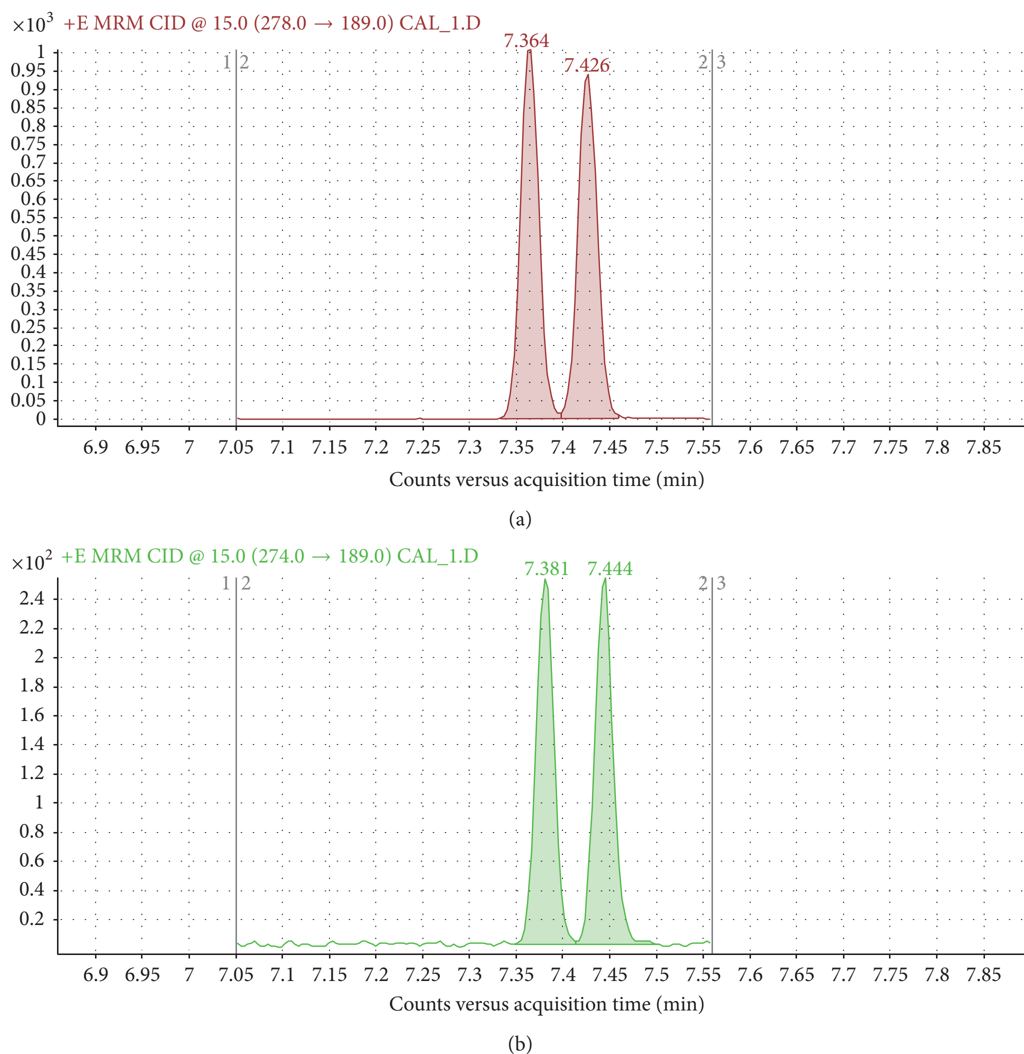

Figure 1: Extracted MRM chromatograms for (a) MDMA-d5 and (b) MDMA at 20 μg/mL.

that (+)-(S)-MDMA is favoured during protein binding and a ratio of >1 indicates that (−)-(R)-MDMA is favoured instead. A ratio of 1 signifies that both (+)-(S)-MDMA and (−)-(R)-MDMA are equally bound to plasma or serum proteins and, thus, stereoselectivity in protein binding does not occur. The stereoselectivity of protein binding for each species is reported in Table 2.

For both human plasma and rat serum, the enantiomer ratio was close to 1, with values ranging from 0.92 to 0.99 at both concentrations of *rac*-MDMA tested. This indicates that stereoselectivity in protein binding in both human plasma and rat serum is negligible and both enantiomers of MDMA are almost equally bound to plasma or serum proteins.

However, in mouse serum, there was significant stereoselective binding (paired t-test, $p < 0.05$). The enantiomeric ratios were 0.78 and 0.74 for 20 ng/mL and 200 ng/mL of *rac*-MDMA, respectively. These ratios indicate that, in mouse serum, stereoselective protein binding occurs with (+)-(S)-MDMA being preferentially favoured over (−)-(R)-MDMA.

Our data clearly shows that even though there is a marked stereoselectivity in protein binding in the mice samples when compared to that of human and rat samples, the total protein binding for mice was very low (11–36%). Therefore it is unlikely that the stereoselectivity of protein binding in mice plays a significant role in the observed stereoselective metabolism of MDMA in these species. This indicates that even though mice can be used as animal model to study stereoselective metabolism of MDMA in vitro, it should not be used in studies that investigate protein binding.

4. Conclusion

Protein bindings of MDMA enantiomers in human plasma and rat serum were found to be similar with percentages between 38 and 51%. In mouse serum, however, protein binding values were considerably lower at 11–36%. Stereoselectivity was not evident in both human and rat serum, whereas mouse serum exhibited considerable stereoselectivity in protein binding with (+)-(S)-MDMA being bound

Table 2: Percentage of MDMA enantiomers bound to protein in serum or plasma according to species.

Species	Concentration of *rac*-MDMA (ng/mL)	Compound	Mean peak area ratios		Percentage of enantiomer bound to protein (%)	Enantiomer ratio (R) versus (S)	Total MDMA bound to protein (%)
			Sample ($n = 3$)	Control ($n = 1$)			
Human (serum)	20	(−)-(R)-MDMA	0.052 ± 0.011	0.089	41	0.94	43
		(+)-(S)-MDMA	0.054 ± 0.017	0.096	44		
	200	(−)-(R)-MDMA	0.423 ± 0.161	0.865	51	0.99	51
		(+)-(S)-MDMA	0.410 ± 0.154	0.847	51		
Rat (plasma)	20	(−)-(R)-MDMA	0.045 ± 0.005	0.073	38	0.92	39
		(+)-(S)-MDMA	0.046 ± 0.005	0.078	41		
	200	(−)-(R)-MDMA	0.444 ± 0.068	0.843	47	0.93	49
		(+)-(S)-MDMA	0.423 ± 0.070	0.861	50		
Mouse (plasma)	20	(−)-(R)-MDMA	0.063 ± 0.017	0.088	28	0.78	32
		(+)-(S)-MDMA	0.053 ± 0.014	0.084	36		
	200	(−)-(R)-MDMA	0.836 ± 0.122	0.836	11	0.74	13
		(+)-(S)-MDMA	0.720 ± 0.085	0.850	15		

to a higher extent compared to (−)-(R)-MDMA. However, as protein binding for mice was rather low, it is unlikely that the observed stereoselectivity would have a significant effect on MDMA disposition in mice. Based on these results, it can be concluded that protein binding of MDMA does not contribute to stereoselective disposition of MDMA in humans, rats, and mice. It should also be pointed out that protein binding of MDMA in mouse serum is considerably lower than in humans and rats and this factor should be taken into account if mice are used for in vitro studies involving MDMA.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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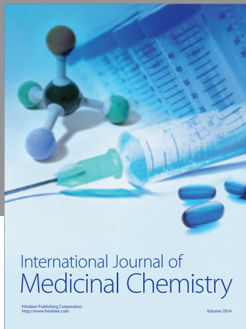
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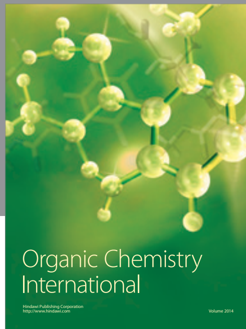
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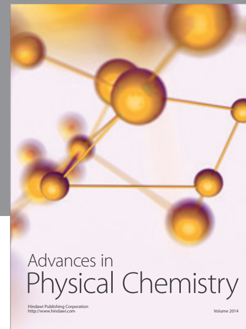
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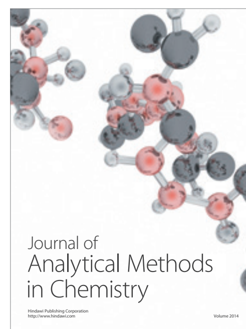
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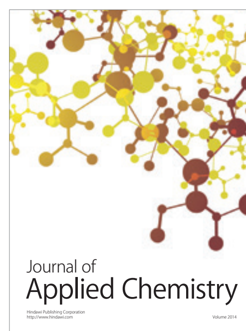
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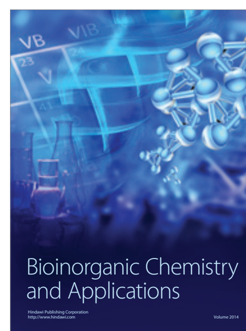
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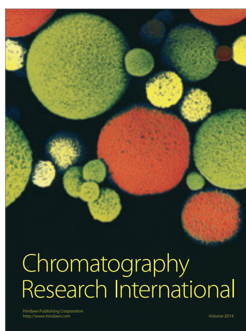
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