

Communication

A New Method for Enantiomeric Determination of 3,4-Methylenedioxyamphetamine and p-Methoxymethamphetamine in Human Urine

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Abstract: The abuse of paramethoxymethamphetamine (PMMA) and 3,4-methylenedioxyamphetamine (MDMA) among young people is increasingly serious and has become a public health problem. Since enantiomers of MDMA and PMMA are metabolized at different rates in the body and exhibit different neurotoxicity in tissues, we have developed a simple method for simultaneous enantiomeric determination of PMMA and MDMA, using parallel dual capillary immunoaffinity columns coupled with tandem mass spectrometry. Linear calibration curves were obtained in concentration ranges of 100–1000 ng/mL, with a limit of quantitation of <22 ng/mL. Good interday accuracy and precision were achieved with this method. Besides filtering the urine sample through a 0.45 µm MILLIPORE membrane, no other sample pretreatment was needed, and no toxic organic solvent was used. It is a rapid, environmentally friendly safe method, and could be applied for routine enantiomeric analysis of PMMA and MDMA in the pharmaceutical industry, forensic science, and environmental analysis.

Keywords: capillary immunoaffinity column; enantiomeric determination; LC-MS/MS; 3,4-methylenedioxyamphetamine; paramethoxymethamphetamine

1. Introduction

In recent years, 3,4-methylenedioxyamphetamine (MDMA) and paramethoxymethamphetamine (PMMA) have become prevalent abused drugs. These drugs are psychoactive and have potent stimulating effects on the central nervous system. They may lead to addiction and damage both the body and the mind. MDMA is one of the most popular emerging drugs among young people. PMMA is also a new psychoactive drug, used either alone or in combination with MDMA and sold as “ecstasy”. Because ecstasy is usually considered MDMA, drug abusers may inadvertently use PMMA and may become polydrug abuse victims. Compared with MDMA, PMMA has a slower onset and is more toxic, so it may cause overdose and lead to death. Thus, the US Drug Enforcement Administration placed PMMA into Schedule I of the Controlled Substance in the year 2021 [1]. In Taiwan, PMMA abuse has increased rapidly and the authorities have found it is difficult to prevent. From October to December 2019, 33 deaths related to the use of PMMA have been reported, and the average age of the victims was 26.7 years [2].

MDMA and PMMA are chiral compounds and exist as S-(+) (or D) and R-(−) (or L) enantiomers. Enantiomers of MDMA and PMMA are metabolized at different rates in the body and exhibit different neurotoxicity in tissues. S-(+)-MDMA is metabolized and eliminated faster [3], and is a more potent neurotoxin than the R-(−)-isomer [4]. PMMA can have long-term (possibly neurotoxic) effects on brain serotonin neurons [5]. The stimulus effects of PMMA are primarily associated with the S-(+) isomer of PMMA [6]. Therefore, the enantiomeric ratio of MDMA and PMMA in urine can be used to estimate the time a drug was abused. The enantiomeric ratio of MDMA in community wastewater has also been used to determine whether MDMA was ingested by humans before being discarded [7]. Recently, several studies have started to use enantioselective analysis along with wastewater-based epidemiology to investigate the origins of illicit drugs [7–10]. As a result, the enantiomeric analysis of MDMA and PMMA may be very helpful for forensic judgement.

Methods such as gas chromatography (GC) and liquid chromatography (HPLC) coupled with mass spectrometry (MS) have been reported for the chiral determination of MDMA [3,7,11–17]. However, to the best of our knowledge, chiral determination of PMMA has not been investigated so far. These methods suffer from elaborate sample preparation and expensive reagents, such as: liquid-liquid extraction or solid phase extraction, and chiral reagent for derivatization. Furthermore, most of the organic solvents used in these methods are toxic and hazardous to the operators and environment.

Previously, we successfully used an immunoaffinity column for directly separating MA enantiomers in human urine by adjusting the pH of the mobile phase without using any organic solvents [18,19]. Later, we also developed a rapid method for direct quantitation of morphine-like drugs in human urine samples using parallel dual immunoaffinity columns [20]. This study aims to develop a simple method for simultaneous enantioselective analysis of MDMA and PMMA in urine samples by applying parallel dual immunoaffinity columns along with tandem mass spectrometry.

2. Materials and Methods

2.1. Chemicals

All chemicals were of analytical grade. Mouse monoclonal anti-D-MA antibody was purchased from Cashmere Scientific Company (Taiwan). Stock solutions of racemic PMMA (1.00 mg/mL), racemic MDMA (1.00 mg/mL), and deuterated racemic methamphetamine (MA-d₁₄; 0.10 mg/mL) dissolved in methanol were obtained from Cerilliant Corporation (USA). Their chemical structures are shown in Figure 1. Aqueous sodium phosphate buffer pH 7.0 (PB) prepared by mixing an appropriate volume ratio of Na₂HPO₄ (aq) (0.10 M) and NaH₂PO₄ (aq) (0.10 M) was employed as reaction solvent and column packing medium. Aqueous ammonium acetate buffer (AAB) solutions with different pH values were prepared by mixing ammonium acetate(aq) (50 mM) and acetic acid(aq) (50 mM) in an appropriate volume ratio and employed as a loading buffer (pH 6.7) and two elution buffers (pH 5.6 or 3.5).

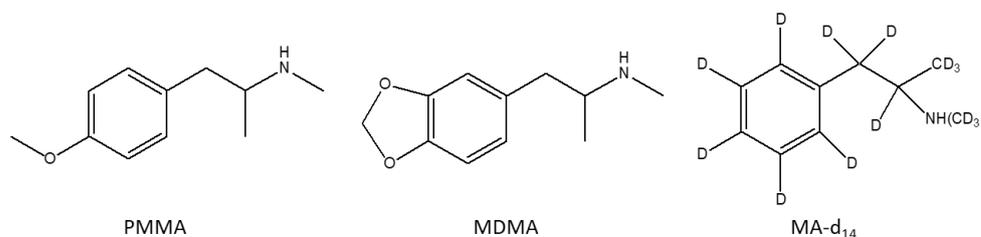


Figure 1. Chemical structures of the PMMA, MDMA, and MA-d₁₄.

2.2. Preparation of the Packing Material of Capillary Immunoaffinity Columns (CIACs)

The packing material of the CIACs was prepared using the method described in our previous paper [18]. Briefly, silica gel was first modified with (3-aminopropyl)triethoxysilane and then activated with glutardialdehyde. Next, the glutardialdehyde-activated silica

(40.0 mg) was reacted with a mouse monoclonal antibody (2.1 mg) against D-(+)-methamphetamine (D-MA) dissolved in 1.0 mL of PB. Absorbance of the antibody PB solution was measured at 280 nm both before and after the reaction, and it was estimated that 47 mg of the antibody was immobilized on 1.0 g of glutaraldehyde-activated silica gel. Finally, the antibody-immobilized silica gel was end-capped with 1.00 mL of 0.10 M glycine in PB.

2.3. Preparation of CIACs

A capillary-fused silica column (inner diameter, 250 μm ; outer diameter, 365 μm ; approximate length, 1 m; Polymicro Technologies Inc., Phoenix, AZ, USA) was capped with a 0.5- μm filter at the outlet. Packing material suspended in PB was introduced into the column and packed under a pressure of 35,000 kPa using PB as a delivery solvent overnight. A 13-cm-long segment from the column outlet was cut, and the inlet end of the obtained column was capped with another 0.5 μm filter.

2.4. Chromatographic and Mass Spectrometric Equipment and Conditions

A parallel dual column system similar to the one we used in our previous study [20] was designed as in Figure 2. The experiments were carried out with an Agilent 1100 Series LC/MSD-Trap-SL equipped with an electrospray ionization source (ESI) emitter. AAB (pH 3.5) in a syringe was delivered using a syringe pump (KD Scientific, Holliston, MA, USA) at a flow rate of 2.0 $\mu\text{L}/\text{min}$ for use as a sheath fluid for the ESI emitter. The loading buffer (AAB, pH 6.7) was delivered by a K-120 pump (Knauer, Berlin, Germany) through a sample injector, which had a sample loop volume of 100 nL. The flow rate of the loading buffer was regulated at 2.0 $\mu\text{L}/\text{min}$ by a splitter. The elution buffer was delivered by the LC-pump and the flow rate was regulated at 2.4 $\mu\text{L}/\text{min}$ by a splitter. After conditioning, the CIAC with the loading buffer (AAB, pH 6.7) for 10 min, 100 nL of a urine sample was injected into the CIAC, and washed with the loading buffer for another 10 min. Then, the ten-port valve was switched, the analyte retained in the CIAC was eluted step-by-step with pH 5.6 AAB, pH 3.5 AAB, and pH 5.6 AAB for 5, 5, and 10 min, respectively. The functions of column 1 and column 2 were alternated per 20 min.

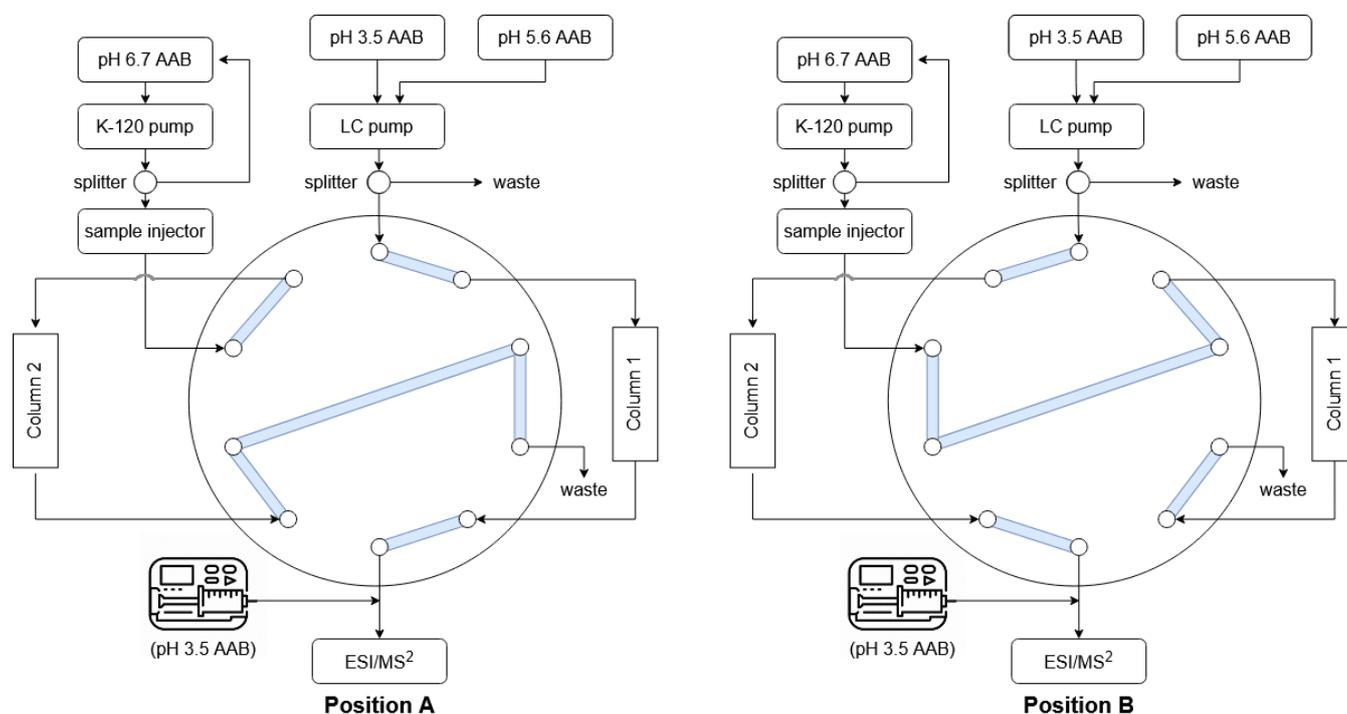


Figure 2. Schematic representation of the parallel dual column system.

The ESI and MSD parameters were as follows: ionization mode, positive; spray capillary voltage, 4500 V; nebulizer, 40 psi; dry gas flow comprising N₂, 5.5 L/min; dry gas temperature, 200 °C; spectra average, 3; ion current control, on; target 25,000; and dwell time, 250 ms. Mass spectra were obtained in scan mode with a scan range *m/z* of 80–200. To further identify the presence of analytes and the internal standard (IS), the protonated molecular ions of PMMA (*m/z* 180), MDMA (194), and MA-d₁₄ (*m/z* 164) were isolated and further fragmented to obtain mass–mass spectra (MS²). The major product ion in the MS² was used to calculate the concentration of these compounds.

2.5. Sample Preparation

Urine samples were made by spiking various concentrations (100–1000 ng/mL) of each racemic PMMA, MDMA to drug-free urines collected from a healthy adult male. The urine samples were kept in a refrigerator (4 °C) when not in use. Prior to analysis, the urine samples were added 500 ng/mL of racemic MA-d₁₄ as IS, and then filtered through a 0.45-µm membrane.

3. Results

The analytical method described in Section 2.4 was used to analyze a urine sample spiked with 500 ng/mL of racemic MA-d₁₄ and 1000 ng/mL each of racemic PMMA and MDMA. The total ion chromatogram (TIC) exhibits two peaks around retention time (*t_R*) 6.0 and 11.1 min (Figure S1; Supplementary Materials). These peaks may be caused by the presence of analytes in the effluent eluted from the column. Therefore, we observed the MS² spectra (Figure 3) of protonated MA-d₁₄ (*m/z* 164), PMMA (*m/z* 180), and MDMA (*m/z* 194) at *t_R* around 6.0 or 11.1 min. The main fragment ions observed in these MS² spectra are *m/z*: 130, 149, and 163 for protonated MA-d₁₄, PMMA, MDMA, respectively. Based on differences in mass between the parent ion and the main product ion, it can be reasonably explained that the secondary amino group of these molecules is protonated by the acidic sheath liquid, and then, methylamine was removed from parent ions during the fragmentation process. Therefore, we used the extracted ion chromatograms (EIC) of *m/z* 164→130, 180→149, and 194→163 to demonstrate the presence of MA-d₁₄, PMMA, and MDMA in the effluent eluted from the column, respectively. The results obtained are shown in Figure 4.

Two peaks appeared in the EICs indicating that this method may completely separate the enantiomers of MA-d₁₄, PMMA, and MDMA in urine simultaneously, since the antibody's affinity toward its antigen is strongly affected by the pH of the mobile phase [18,19]. Therefore, two peaks that appeared in the EICs of PMMA and MDMA may be due to the variation of pH of the mobile phase. In order to confirm the two peaks appeared in the EICs of PMMA and MDMA responded respectively to two different compounds, the same experiment was performed and the effluent eluted from the column was fractionated by a collector with a fraction rate of one tube/min. The pH of each fraction was measured with a micro pH detector. The results are shown in Figure 5. Comparison of Figure 4 to Figure 5 reveals that the pH of the peak around 6.0 min and around 11.1 min is 5.6 and 4.2, respectively.

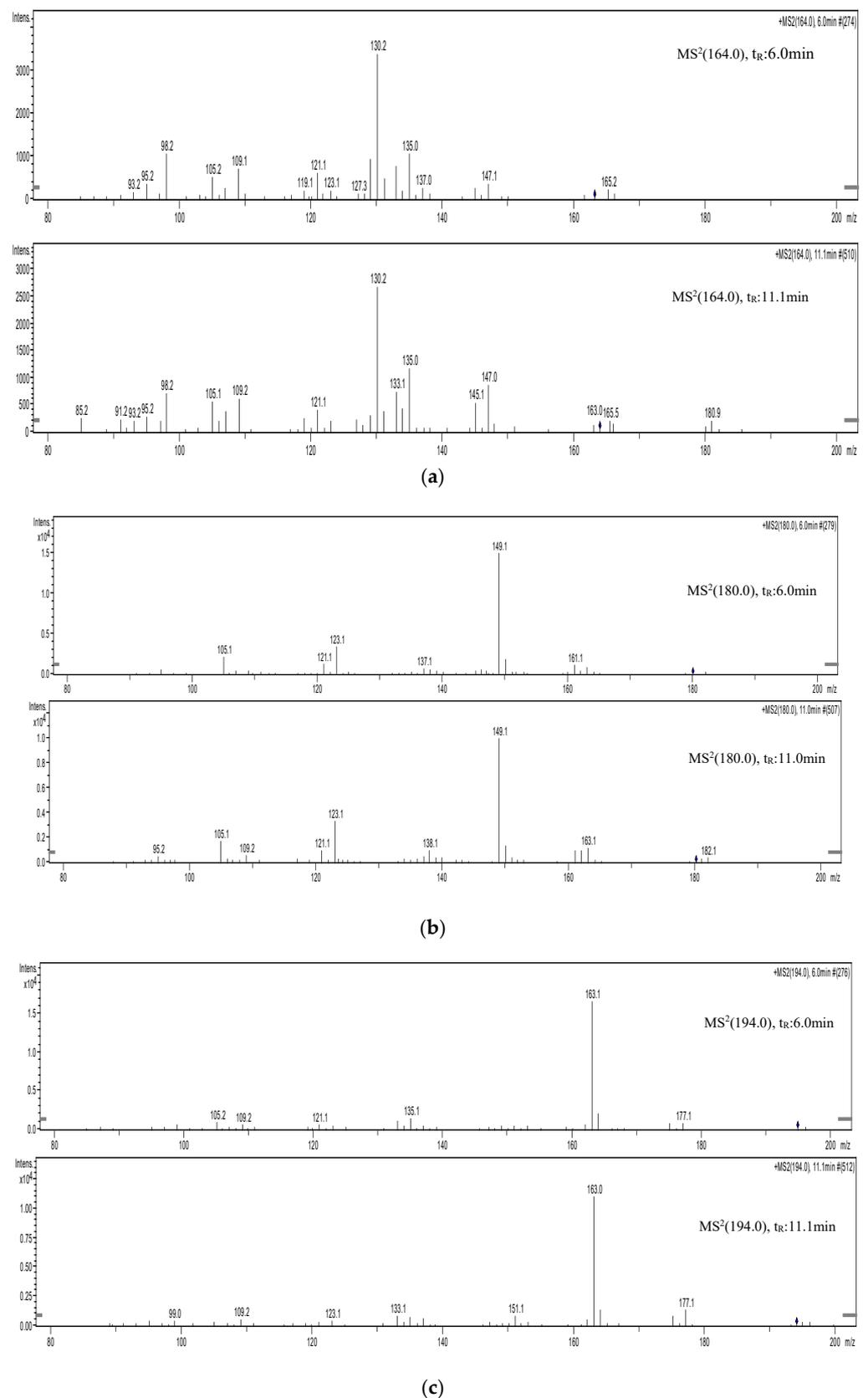


Figure 3. The MS² spectra of protonated MA-d₁₄ (m/z: 164) (a), PMMA (m/z: 180) (b), and MDMA (m/z: 194) (c) at retention time around 6.0 min and 11.1 min.

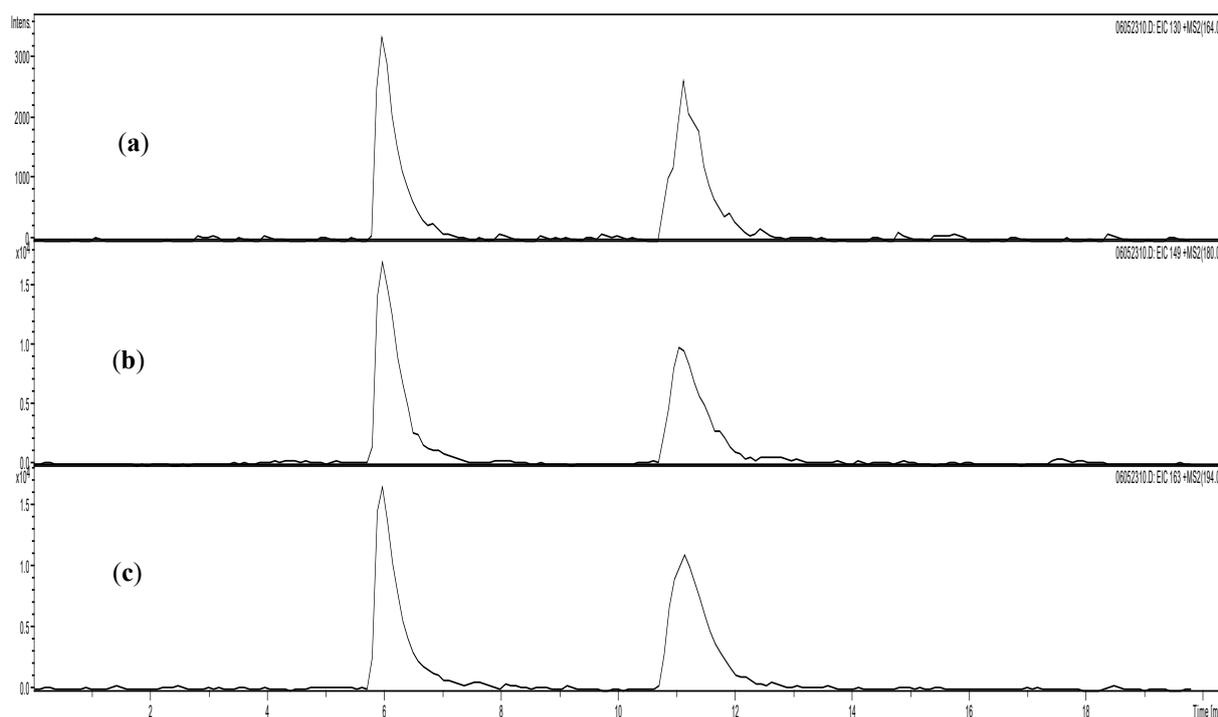


Figure 4. The extracted ion chromatograms of 164→130 (a), 180→149 (b), and 194→163 (c) obtained by analyzing a urine sample spiked with 500 ng/mL of racemic MA-d₁₄ and 1000 ng/mL of each of racemic PMMA and MDMA.

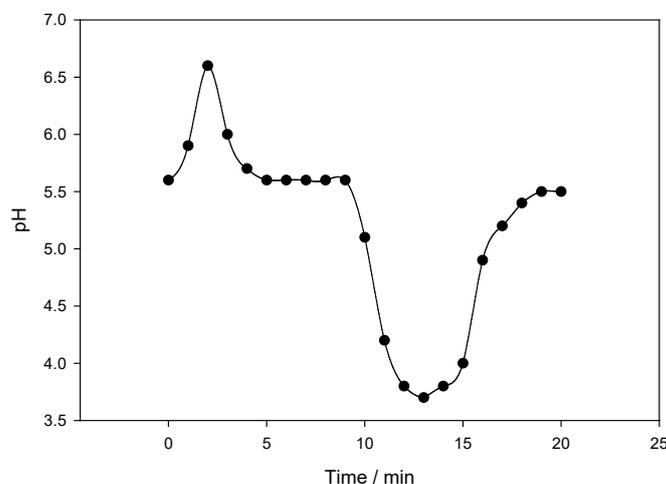


Figure 5. The variation in pH of the effluent eluted from the CIAC.

On the other hand, a sample of 0.10 mg/mL of racemic PMMA (or MDMA) in methanol was chromatographed with the immunoaffinity columns employed in our previous work [19] by using AAB solutions with different pH (6.7, 5.6 and 3.5) as mobile phase. The chromatographic parameters (Table S1) and EIC of MDMA (Figure S2) are shown in the Supplementary Materials. The same experiment was performed and the effluent was fractionated by a collector with a fraction rate of one tube/min and the pH of each fraction was measured with a micro pH meter. The fractions with pH 5.6 and 4.2 were further analyzed respectively by our proposed analytical method. However, to enhance the signal and verify the extraction function of the CIAC toward PMMA or MDMA enantiomers, the sample was injected thrice sequentially. The EICs obtained for MDMA are shown in Figure 6. Only one peak appears at t_R of approximately 6.0 and 11.1 min for the fraction with pH 5.6 and 4.2, respectively. Similar results were also observed for PMMA.

Because the configurations of enantiomers of MDMA (or PMMA) are similar to those of MA, it is reasonable to conclude that the peaks at around t_R 6.0 min and 11.1 min in the EIC of 194→163 (or 180→149) in Figure 4 represented the L-MDMA (or L-PMMA) and D-MDMA (or D-PMMA), respectively. This evidences that the method developed in this study can simultaneously extract and separate the enantiomers of PMMA and MDMA in human urine.

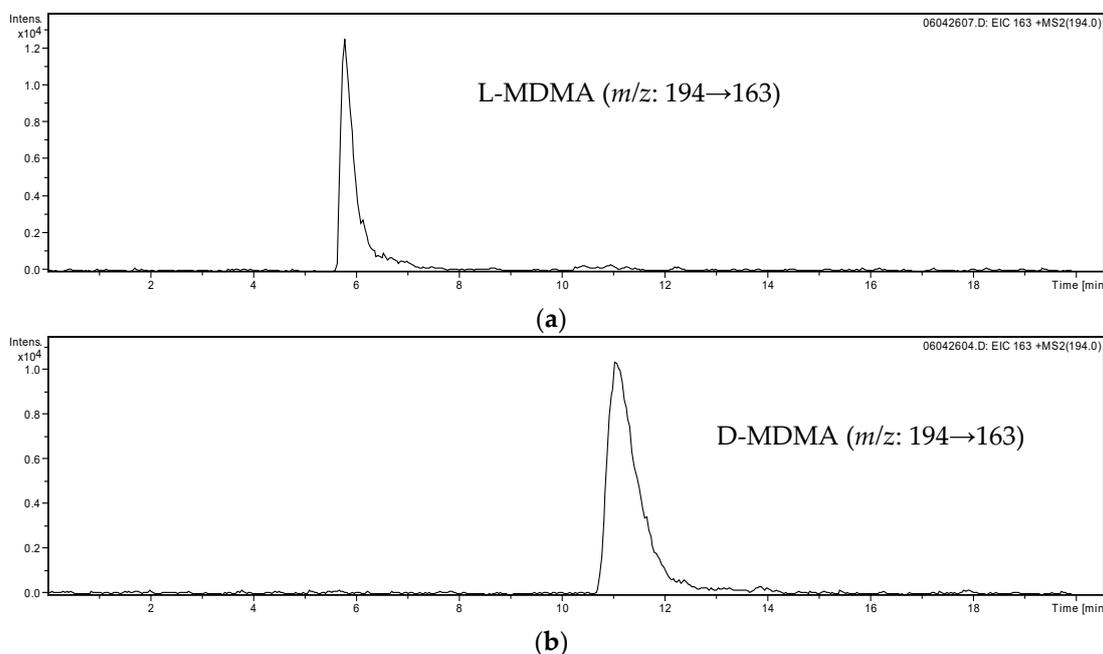


Figure 6. The extracted ion chromatograms obtained from the analysis of the fraction with pH 5.6 (a) and 4.2 (b).

Finally, the proposed method was applied for the enantiomeric quantification of PMMA and MDMA in urine samples. Urine samples containing different concentrations (50–500 ng/mL) of racemic PMMA and racemic MDMA and a fixed concentration (500 ng/mL) of IS were used to establish the calibration curve under the assumption of 1:1 ratio of each form in the racemic mixture. Peak area ratios of the analytes (EICs of m/z : 180→149, and 194→163) to the respective configuration of IS (EICs of m/z : 164→130) were calculated as a function of the concentrations of analytes. Least-squares regression analysis was used to obtain the calibration curve. As summarized in Table 1, the correlation coefficients (R^2) were >0.99 and the lowest concentration of detection (LOD) was <12 ng/mL for all analytes. Urine samples spiked with five different concentrations of analytes (with the assumption of equal concentration of each configuration in the racemic mixture) were used as quality control, and the results obtained are shown in Table 2. The repeatability and accuracy shown in Table 2 demonstrate that the method is suitable for simultaneously enantiomeric determination of PMMA and MDMA in urine. Table 3 summarizes the comparison between our method and previous analytical methods for PMMA and MDMA. The LODs of our method are worse than the published methods, especially the one proposed by Gonçalves et al., which is probably due to the enrichment resulting from solid phase extraction [11,13,17,21]. However, as the cutoff level for initial/confirmatory test of MDMA is 250/500 ng/mL according to Mandatory Guidelines for Federal Workplace Drug Testing Programs for urine testing set by the Substance Abuse and Mental Health Services Administration (SAMHSA) [22], this method is suitable for direct enantioselective analysis of PMMA and MDMA in urine. Besides, as shown in Table 3, the published methods suffer from elaborate sample preparation and expensive reagents, such as chiral reagent for derivatization and solid phase extraction. Our method has the advantage of being simple and time saving.

Table 1. Linearity results, limit of detection (LOD), and limit of quantification (LOQ) of D-PMMA, L-PMMA, D-MDMA, and L-MDMA in urine.

Analytes	Regression Line	Correlation Coefficient (R ²)	LOD ^a (ng/mL)	LOQ ^a (ng/mL)
D-PMMA	$y = 4.87 \times 10^{-3}x + 3.33 \times 10^{-3}$	0.9994	11.5	38.2
L-PMMA	$y = 4.03 \times 10^{-3}x + 8.33 \times 10^{-3}$	0.9999	11.9	39.5
D-MDMA	$y = 8.91 \times 10^{-3}x - 3.64 \times 10^{-2}$	1.0000	9.3	30.9
L-MDMA	$y = 5.96 \times 10^{-3}x - 7.05 \times 10^{-3}$	0.9995	10.4	34.6

^a The standard deviation (S) of the Y values of five blank urine samples was used to define the LOD (3S/m) and LOQ (10S/m), where m represents the slope of the calibration curve.

Table 2. Accuracy and precision of D-PMMA, L-PMMA, D-MDMA, and L-MDMA in urine ^a.

Analytes	Concentration (ng/mL)									
	50	100	250	400	500	50	100	250	400	500
	Repeatability (RSD ^b %)					Accuracy (%)				
D-PMMA	9.7	8.4	7.3	6.7	5.3	17.4	4.5	−3.6	−5.6	−9.8
L-PMMA	4.0	13.1	8.9	9.0	7.9	−9.4	−5.1	−0.2	−2.9	−8.8
D-MDMA	10.7	5.8	4.4	2.3	2.3	−3.6	9.6	−1.9	0.0	2.2
L-MDMA	8.3	7.2	5.3	8.1	7.9	12.4	19.7	−3.9	3.8	2.2

^a Five replicate analyses were performed on five different days to validate the method. ^b Relative standard deviation.

Table 3. Comparison between analytical methodologies for PMMA and MDMA.

	Analysis Technique	Sample Treatment	Linea Range (ng/mL)	LOD (ng/mL)	Ref
PMMA	LC-MS/MS	-	50–500 (D)	11.5 (D)	This work
			50–500 (L)	11.9 (L)	
	GC-MS	derivatization	2–250	0.6	[21]
MDMA	LC-MS/MS	-	50–500 (D)	9.3 (D)	This work
			50–500 (L)	10.4 (L)	
	GC-MS	solid phase extraction and derivatization	0.006–60 (D) 0.005–60 (L)	1.7 (D) 1.5 (L)	[11]
	GC-MS	derivatization	5–500	1.7	[13]
	GC-MS	solid phase extraction and derivatization	0.0063–4 (D) 0.00156–4 (L)	0.0013 (D) 0.0021 (L)	[17]

4. Conclusions

This research has developed a simple, convenient, and pollution-free method that can be used to quantitatively determine both PMMA and MDMA enantiomers in urine. The method does not require any complicated sample preparation or derivatization of the analyte, and does not require toxic organic solvents. The detection time for each urine sample is about 20 min. Because this method could extract PMMA and MDMA enantiomers from a complex matrix, it is also suitable for quantitative detection of PMMA and MDMA enantiomers in emerging drugs containing multiple components. Although the LODs of our methods are not as good as the published methods, they are lower than the cutoff values regulated by SAMHSA and could be used for drug testing in urine. In addition, this method can provide enantiomeric ratios of PMMA and MDMA in urine; it helps predict when the drug was abused. Therefore, this method will be of great help for preventing the PMMA and MDMA abuse that is currently very prevalent.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/chemosensors10020050/s1>, Figure S1: The TIC of a urine sample spiked with 500 ng/mL of racemic MA-d14 and 1000 ng/mL each of racemic PMMA and MDMA;

Figure S2: The immunoaffinity column and LC-MS equipment used in our previous research [19] was used to analyze a sample of racemic MDMA (0.10 mg/mL) in methanol. The EIC (m/z :194→163) obtained is shown in (A) and the MS2 spectrum at the retention time of 52.3 min is shown in (B). Sample volume injected was 0.50 μ L. The flow rate of mobile phase is 0.40 mL/min. Table S1: Mobile phase gradient for HPLC.

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Conflicts of Interest: The authors declare no conflict of interest.

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