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Screening of patients receiving selegiline from methamphetamine abusers using the urinary amphetamine/methamphetamine ratio

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Abstract

Methamphetamine (MA), the major metabolite of selegiline excreted in urine, can cause false positive interpretation of patient receiving selegiline as a MA user based on the routine non-chiral separation method. This study aims to compare the ratio of amphetamine (AM) to MA concentrations in urine of patients receiving selegiline and MA abusers. Urines were collected from fifteen patients at 2, 4, 6, 8 and 20 hours after selegiline administration. Urines from 97 MA abusers were collected at 2, 4, 6, 8 or 20 hours after the last exposure. AM and MA concentrations were determined by solid phase micro-extraction gas chromatography/ mass spectrometry. The results showed that urinary AM/MA ratios in the patients were significantly higher than those of the MA abusers at every time point. The lowest AM/MA ratio in the patients was 0.74 ± 0.07 and the highest AM/MA ratio in the MA abusers was 0.41 ± 0.05 at 6 hours. Thus, urinary AM/MA ratio could be used for preliminary differentiation of patients receiving selegiline from MA abusers with an accuracy of 84.88% when using a ratio of 0.40 as the cutoff value.

Keywords: Amphetamine, methamphetamine, amphetamine/methamphetamine ratio, selegiline

Introduction

Methamphetamine (MA) is a common illicit drug worldwide [1]. MA and its metabolite, amphetamine (AM), the dextrorotary (*d*-) form or (+) isomer, are frequently abused because they possess stronger psychostimulatory activity than the corresponding levorotatory (*l*-) form or (-) enantiomer [2]. MA and AM are both classified in the United States as schedule II controlled substances under the Controlled Substances Act 1970 [3]. Use of MA is normally detected by determination of MA and AM in urine. The cutoff concentrations of AM or MA in urine mandated by the Department of Health and Human Services, Substance Abuse and Mental Health Services Administration

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(SAMHSA) are 500 ng/mL of amphetamines in a preliminary screening immunoassay and 250 ng/mL of AM or MA using a GC-MS confirmatory assay [4].

Selegiline is a selective irreversible monoamine oxidase B inhibitor used in combination with levodopa for treatment of Parkinson's disease [5]. After administration, selegiline is rapidly metabolized in the liver via two reactions: (1) *N*-desmethylation yielding desmethylselegiline, which is further metabolized to (*R*)-(-)-amphetamine by *N*-despropynylation; and (2) *N*-despropynylation yielding (*R*)-(-)-methamphetamine, which is further metabolized to (*R*)-(-)-amphetamine by *N*-desmethylation. Both (*R*)-(-)-methamphetamine and (*R*)-(-)-amphetamine are converted to other minor metabolites by *p*-hydroxylation and *β*-hydroxylation. Thus, 9 metabolites of selegiline are found in urine: desmethylselegiline, (*R*)-(-)-methamphetamine, (*R*)-(-)-amphetamine, (1*S*, 2*R*)-norephedrine, (1*R*, 2*R*)-norpseudoephedrine, (1*S*, 2*R*)-(+)-ephedrine, (1*R*, 2*R*)-(-)-pseudoephedrine, (*R*)-(-)-*p*-hydroxyamphetamine, and (*R*)-(-)-*p*-hydroxymethamphetamine, as well as as well as very small amount of selegiline may be found excreted as the unchanged drug. Within 24 hours after selegiline administration, the major metabolite found in urine is MA, while AM is found in a lesser amount [6].

Since the main selegiline metabolites in urine are (-)-MA or (*l*-)-MA with a lesser amount of (-)-AM or *l*-AM, false positive interpretation of patients receiving selegiline as MA abusers can occur based on the routine non-chiral separation method using in forensic toxicological analysis. This may occur even though MA and AM in urine of MA abusers are found as (*d*-) forms [7]. To differentiate patients receiving selegiline from MA abusers, analysis of MA and AM in urine must be able to differentiate the compounds stereospecifically, which is not normally performed in the routine analyses.

Detection of MA and AM in urine samples in forensic toxicological analysis is generally divided into two processes. First, a preliminary screening test is performed using a color test or immunoassay. A sample with a positive result is further examined in a confirmatory test with techniques such as thin layer chromatography, gas chromatography, gas chromatography-mass spectrometry (GC-MS), liquid chromatography (LC) and liquid chromatography-mass spectrometry (LC-MS). These routinely used methods are normally not able to differentiate MA abusers and selegiline patients, false positive MA results in urines of selegiline patients sometimes occur in routine work. To differentiate selegiline patients from MA abusers, sensitive enantioselective methods of determination need to be developed with utilization of derivatizing reagents or chiral columns in confirmatory analysis using GC-MS [8-14], LC or LC-MS [15-18].

Previous studies of the concentrations of (*l*-)-MA and (*l*-)-AM in urine samples collected from decedents and patients receiving high doses of selegiline have found AM/MA ratios of 0.30 [19], 0.46 [20], 0.33 [21] and 0.40 [22], while the ratio of AM/MA concentrations in urine of MA abusers are <0.20 [21]. Hasegawa et al. [10] found that the AM/MA ratio gradually increased from 0.24 to 0.67 (r = 0.857) from 2-48 hours after selegiline administration but was <0.24 in 74% of 50 MA abusers. These findings suggest that the urinary AM/MA ratio may be useful to distinguish patients receiving selegiline from MA abusers before performance of a confirmatory enantioselective test.

To examine the possibility of using the AM/MA ratio to differentiate patients receiving selegiline from MA abusers, we determined the ratios of AM/MA in these two subject populations at various time points after administration of selegiline or MA. Relationships between the AM/MA ratios and time after administration were examined and the best cutoff AM/MA ratio for differentiating patients receiving selegiline from MA abusers was determined.

Materials and Methods

Materials

AM and MA hydrochloride (Lipomed, U.S.A.), diphenhydramine hydrochloride (Sigma Chemical Ltd., U.S.A.), potassium hydroxide (KOH) and sodium chloride (NaCl) (Sigma-Aldrich, U.S.A.) were used in the study.

Subjects

The subjects were 15 outpatients (11 men and 4 women, 45-76 years old) treated with selegiline at the Prasat Neurological Institute, Department of Medical Services, Ministry of Public Health, Bangkok, Thailand, and 97 MA abusers. The study protocol was approved by the Prasat Neurological Institute ethical committee for the protection of the rights of human subjects (Approval # 0310 (12500)/2.250, March 2, 2011). The patients were prescribed selegiline for medical purposes at doses of 2.5 or 5 mg once or twice daily. Urine collection was performed after at least 7 days of selegiline administration. Urine samples were collected at 2, 4, 6, 8 and 20 hours after selegiline administration. In the 97 MA abusers, urine samples were collected at 2, 4, 6, 8 or 20 hours after the last MA use. Urine collection was performed at only 1 time point for each abuser. Urine samples were collected from 17, 16, 21, 20 and 23 MA abusers at 2, 4, 6, 8 and 20 hours after the last MA use, respectively.

Analytical procedure

A urine sample (1 mL) was placed in a 20 mL vial and 300 µL of a mixture (1:1000 v/v) of the internal standard, diphenhydramine (4 mg/mL), and 200 mM KOH was added. After 3 g of NaCl was added, the vial was sealed with a silicone cap and an aluminum crimp seal. MA and AM in the sample were then analyzed by solid phase micro-extraction (SPME) and gas chromatography/mass spectrometry. Urine samples containing MA or AM concentrations greater than the linear range of 500-3000 ng/mL were diluted and the measurement was repeated.

The GC/MS system (QP-2010 plus, Shimadzu, Kyoto, Japan) was equipped with an AOC-5000 Auto injector and a 30 m length x 0.25 mm i.d. Rtx-1MS column (Restex, U.S.A). The column oven was set at 100˚C for 5 min and then programmed to increase from 100˚C to 150˚C at 15˚C/min for 3 min, held for 1 min, and finally increased to 250˚C at 15˚C/min for 6 min and held for 3 min. The total run time was 19 min. The injection

port and interface temperature were set at 240˚C and 220˚C, respectively. A splitless injection mode was used. Helium with a flow rate of 1.53 mL/min was used as the carrier gas. Quantification of the sample was done in the selective ion monitoring (SIM) method and the selected characteristic ions for AM and MA were *m/z* = 44 and 58, respectively. Supelco^{R} SPME Fast-Fit assembly with a replaceable extraction fiber, coated with 100 µm polydimethylsiloxane (Sigma-Aldrich, U.S.A.), was used. The samples were adsorbed for 10 min and the fiber was desorbed for 6 min.

Method validation

Linearity: MA or AM standard solutions of 500, 1000, 1500, 2000, 2500 and 3000 ng/mL were prepared in blank pooled urine samples and analyzed in triplicate by SPME-GC/MS, as described above. Linear regression and the coefficient of determination (R^2) between MA or AM concentrations and peak area ratios of the standard solution to internal standard were analyzed.

Accuracy: Three concentrations of MA or AM (500, 1500, and 2500 ng/mL) were analyzed by SPME-GC/MS, five times for each concentration (n = 5). The % accuracy was calculated using the mean measured MA or AM concentration and the corresponding actual MA or AM concentration.

Precision: Precision of the assay was evaluated as within-day and between-day precision and assessed from the percentage coefficient of variation (% CV) as follows: MA or AM concentrations of 500, 1500 and 2500 ng/mL were analyzed by SPME-GC/MS five times (n=5) for each concentration within 24 hours for evaluation of within-day precision. For between-day precision, MA or AM concentrations of 500, 1500 and 2500 ng/mL were analyzed by SPME-GC/MS for 5 days (n = 5). Each concentration was analyzed three times on each day of the analysis.

Statistical analysis

Data are presented as means ± standard deviation (SD) or standard error of the mean (SEM). Difference between AM/MA ratios in the urine samples of patients receiving selegiline and MA abusers were analyzed by Mann-Whitney test. Correlations between MA or AM concentrations and times after selegiline administration or MA use were assessed by Spearman correlation test. Correlations between MA or AM standard concentrations and peak area ratio of MA or AM to those of the internal standard were assessed by Pearson correlation test. Statistical analysis was performed using SPSS version 16. A difference was considered to be significant at $p < 0.05$.

Results

Method validation

The linearity of the procedure was shown by the close linear relationship between MA or AM concentrations and peak area ratio of MA or AM to internal standard (R^2 = 0.999, $p < 0.001$ for MA; R^2 = 0.999, $p < 0.001$ for AM). Accuracy and within-day and between-day precision of the method for determination of MA and AM concentrations in urine samples are shown in Table 1. The % CV of both within-day and between-day precision of all concentrations of MA and AM did not exceed 15%. Regarding accuracy, the mean measured values were all within 15% of the actual values.

Table 1. Accuracy, within- and between-day precision of the method for determination of AM and MA concentrations in urine samples

Substance	AM or MA Concentrations (ng/mL)	Accuracy (%)[a]	Precision (% CV)	
			Within-day[b]	Between-day[c]
AM	500	101.45 ± 3.99	3.94	1.99
	1500	101.29 ± 1.34	1.32	0.69
	2500	99.95 ± 1.49	1.49	0.62
MA	500	101.11 ± 4.59	4.54	0.33
	1500	99.64 ± 1.64	1.65	0.97
	2500	100.53 ± 1.69	1.68	0.58

[a] Data are shown as mean ± SD (n = 5).

[b] Data calculated from the mean and SD (n = 5 within one day).

[c] Data calculated from the mean and SD (n = 5, 5 days). Experiments were performed in triplicate each day.

AM and MA concentrations and the AM/MA ratio in urine of patients receiving selegiline

The study population included 11 males and 4 females and had a mean age (± SEM) of 63.53 ± 2.38 years (range: 45-76 years). Three different dosage regimens of selegiline were prescribed to these patients: 2.5 mg × 2 (4 patients), 5 mg × 1 (4 patients) and 5 mg × 2 (7 patients). The MA and AM concentrations detected in urine samples collected at 2, 4, 6, 8, and 20 hours after selegiline administration were >500 ng/mL in all patients (Table 2). The AM/MA ratios (mean ± SEM) in urine collected at 2, 4, 6, 8 and 20 hours after selegiline administration were 0.92 ± 0.10, 0.80 ± 0.08, 0.74 ± 0.07, 0.91 ± 0.10, 0.98 ± 0.14, respectively.

Table 2. Concentrations of AM and MA in urine of patients receiving selegiline and MA abusers collected at various times after administration

Time after administration and the metabolites in urine samples		Patients receiving selegiline			MA abusers		
		Range (ng/ml)	Median (ng/ml)	Sample size (n)	Range (ng/ml)	Median (ng/ml)	Sample size (n)
2hr	AM	1034.15 - 2541.82	1306.38	15	987.78 - 10148.08	1464.23	17
	MA	903.84 - 6171.15	1724.74	15	1781.55 - 64298.92	13411.01	17
4hr	AM	1036.45 - 4214.24	1346.94	15	985.27 - 5891.30	1365.11	16
	MA	993.58 - 10582.11	2011.36	15	1081.41 - 56558.51	9107.79	16
6hr	AM	1047.02 - 3010.64	1422.61	15	992.50 - 7284.37	1566.37	21
	MA	1133.26 - 5854.35	2014.99	15	1355.02 - 65637.52	5623.57	21
8hr	AM	1029.96 - 3081.68	1364.30	15	985.56 - 8635.58	1302.64	20
	MA	726.98 - 6809.67	1462.03	15	1102.05 - 69985.62	9866.00	20
20hr	AM	1025.30 - 5076.20	1181.88	15	1026.46 - 5260.40	1770.18	23
	MA	755.26 - 13509.34	1515.85	15	1415.46 - 72119.68	9691.09	23

The relationship between the AM/MA ratio and time after selegiline administration was examined by grouping the data according to the dosage regimen. For selegiline administered twice daily at 2.5 or 5 mg, the AM/MA ratio and time after administration were not linearly correlated (r = 0.100, p = 0.873 for 2.5 mg; r = - 0.200, p = 0.747 for 5 mg). In contrast, the AM/MA ratio was linearly correlated with the time after selegiline administration at 5 mg once daily (r = 0.926, p = 0.024).

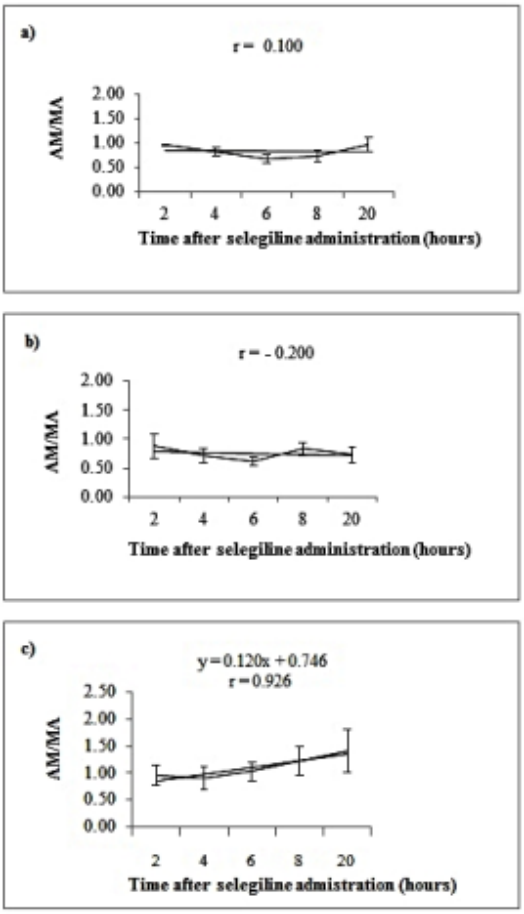

Figure 1 Correlations between ratios of AM/MA concentrations in urine of patients receiving selegiline 2.5 mg twice daily (a), 5 mg twice daily (b), 5 mg once daily (c) and times (2, 4, 6, 8 and 20 hours) after selegiline administration. The correlation was assessed by Spearman correlation test using SPSS version 16. Data are shown as the mean ± SEM of n = 4 (a), 7 (b) and 4 (c).

Concentrations of MA and AM in urine of MA abusers collected after MA use

Urine samples were collected from 97 MA abusers at 2, 4, 6, 8 or 20 hours after MA use (Table 2). The MA abusers included 89 males and 8 females, and had a mean age of 28.46 ± 0.72 years (range: 16-48 years). All MA and AM concentrations in the urine of MA abusers were >500 ng/mL. There was no significant correlation between the AM/MA ratio in urine of MA abusers and the time after MA use (r = 0.300, p = 0.624).

Comparison of the urinary AM/MA ratio in MA abusers and patients receiving selegiline

There were significant differences between the AM/MA ratios in urine of MA abusers and patients receiving selegiline at 2, 4, 6, 8 and 20 hours after MA use or selegiline administration (Figure 2).

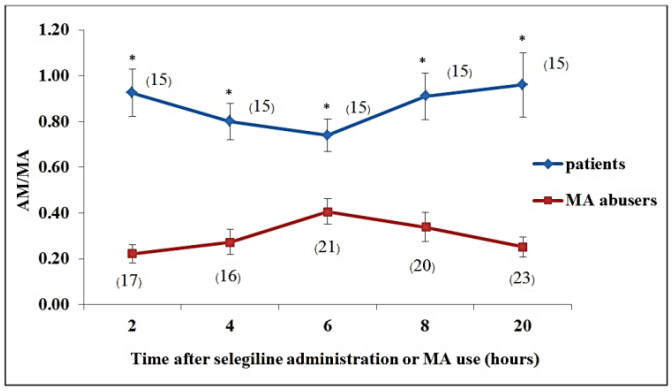

Figure 2 Comparison of AM/MA ratio between MA abusers and patients receiving selegiline at 2, 4, 6, 8 and 20 hours after MA use or selegiline administration. Data are shown as the mean ± SEM with the sample size (n) shown in parentheses. * p < 0.01; MA abusers vs patients receiving selegiline at the same time point after selegiline administration or MA use. Statistical analysis was performed using a Mann-Whitney test.

The urinary AM/MA ratios of the patients were significantly higher than those of the MA abusers at every time point. The AM/MA ratio in patients was lowest (0.74 ± 0.07) and that in MA abusers was highest (0.41 ± 0.05) at 6 hours after exposure to the compounds. To identify the most reliable cutoff AM/MA ratio for differentiating patients receiving selegiline from MA abusers, AM/MA ratios from 0.40 to 0.75 were tested using the 75 samples from selegiline patients (15 patients × 5 time points of urine collection) and 97 samples from MA abusers (1 sample from each subjects). Using an AM/MA ratio of 0.40 as the cutoff value, 72 samples from selegiline patients were predicted to be patients and 3 samples were falsely predicted to be MA abusers; while 74 samples from MA abusers were predicted to be MA abusers and 23 samples were falsely predicted to be patients. Thus, using an AM/MA ratio of 0.40 gave an accuracy of prediction of 84.88% (Table 3). This cutoff value gave the highest sensitivity (96%) or the highest probability (96%) that patients receiving selegiline would be predicted to be patients and the lowest probability (4%) of patients being predicted to be abusers (Table 4).

Table 3. Assessment of the accuracy of an AM/MA cutoff value of 0.40 for differentiating patients receiving selegiline from MA abusers

Predicted status	Actual status	
	Patient	Abuse
Number of subjects predicted to be patients	72	23
Number of subjects predicted to be abusers	3	74
Total numbers of subjects	75	97

$$\text{Sensitivity of prediction (\%)} = \frac{72}{75} \times 100 = 96\%$$

$$\text{Specificity of prediction (\%)} = \frac{74}{97} \times 100 = 76.28\%$$

$$\text{Accuracy of prediction (\%)} = \frac{72+74}{75+97} \times 100 = 84.88\ \%$$

Sensitivity = probability that the test indicates that patients received selegiline when in fact they did receive selegiline
Specificity = probability that the test indicates that persons were abusers when in fact they were abusers
Accuracy/efficiency = efficiency of the test to give true results for patients receiving selegiline and true abusers

Table 4. Summary of assessment of the reliability of AM/MA cutoff values for differentiating patients receiving selegiline from MA abusers

AM/MA	Sensitivity (%)	Specificity (%)	Accuracy (%)
0.40	96.00	76.28	84.88
0.45	93.33	78.00	84.88
0.50	90.66	80.41	84.88
0.51	89.33	81.44	84.88
0.52	86.66	82.47	84.30
0.53	86.66	82.47	84.30
0.54	85.38	82.45	83.72
0.55	82.66	82.47	82.55
0.56	80.00	83.50	81.97
0.57	78.66	84.53	84.53
0.58	78.66	84.53	81.97
0.59	78.66	84.53	81.97
0.60	77.33	84.53	81.39
0.65	70.66	87.62	87.62
0.70	61.33	90.72	77.90
0.75	49.33	91.75	73.25

Sensitivity = probability that the test indicates that patients received selegiline when in fact they did receive selegiline
Specificity = probability that the test indicates that persons were abusers when in fact they were abusers
Accuracy/efficiency = efficiency of the test to give true results for patients receiving selegiline and true abusers

Discussion

In this study, MA and AM concentrations in urines were determined using headspace SPME-GC/MS using methods modified from other studies [23, 24]. GC/MS is accepted as the specific method for identification of substances, interference of urine analysis of either MA or AM by other substance is rarely occurred, especially in SIM mode, the mass spectrometer is set to measure only the specified m/z 44 and 58 for AM and MA respectively, along with specific reference ion ratio to identify each substance. Moreover, we used headspace-SPME technique to avoid matrix interference.

This technique has been used for analysis of amphetamines and related compounds, but cannot differentiate enantiomers such as *l*-MA and *l*-AM, which are metabolites of selegiline, from *d*-MA and *d*-AM, which are excreted in urines of MA abusers. Before performing urinary MA and AM analysis, the assay was validated based on guidance for analysis of compounds in biological samples [25]. Because the method used in this study is well-established and routinely used at the Institute of Forensic Medicine, Police General Hospital, we used this method according to the standard of procedure of the laboratory by which method validation has been performed. Thus, we performed verification on only some parameters such as linearity, accuracy and precision. Linearity, within-day and between-day precision, and accuracy were tested. Urinary MA or AM concentrations were shown to be linearly correlated with the peak area ratio of MA or AM to internal standard. Within-day and between-day precision were shown by a % CV of <15%. Likewise, the accuracy of the method was shown by the closeness of the mean measured values of MA and AM within 15% of the actual value. These results are within the acceptable ranges for bioanalytical method validation [25].

MA and AM concentrations and the AM/MA ratio in urine of patients receiving selegiline

Based on the cutoff concentration of MA in GC/MS confirmatory test of 250 ng/mL [4], the results from this study demonstrated 100% incidence of false positive interpretation of MA consumption in patients receiving selegiline at therapeutic doses. The generally higher ratio of AM to MA in urine of patients receiving selegiline than in MA users found in previous studies [10, 19-22] may be a helpful marker to distinguish selegiline patients and MA abusers. However, these studies have analyzed urine from deceased patients due to selegiline overdose or healthy volunteers, whereas the subjects in our study were patients who were prescribed selegiline for therapeutic purposes. The mean age of the patients (63.53% ± 2.38 years) was consistent with that of patients who are typically prescribed selegiline for Parkinson's disease. The sample size of selegiline patients of 15 was obtained from statistical calculation based on the information of a similar previous study [10]. Urine samples were collected at 5 time points after selegiline administration to assess the correlation between the AM/MA ratio and times after selegiline administration. There was no significant correlation when selegiline was given twice daily, but a significant correlation emerged for selegiline given once daily. This result is consistent with the similar correlation found by Hasegawa et al. [10]. For twice (after breakfast and lunch) daily dosage regimens, the AM/MA ratio seemed to be lower at 6 hours than at other times after selegiline ingestion, which could be due to the effect of the second (after lunch) dose of selegiline producing a higher concentration of the MA metabolite.

Concentrations of MA and AM in urine of MA abusers collected after MA use

In contrast to the elderly population of patients receiving selegiline, the MA abusers were mostly younger, with a mean age of 28.46 ± 0.72 years. Even though *d*-MA is the active compound in some medication prescribed in some countries for narcolepsy, attention deficit disorder, etc., abuse of MA is illegal. There were limitations to perform a controlled study of MA in volunteers or addicted persons. Therefore, the MA abusers used in this study were accused/suspected persons who were arrested by the police and all information was given by the abusers. Such information included purity of MA used, time since last use, and route of administration. We also had the limitation that urine collection was performed at only 1 time point after the last exposure for each abuser. Thus, the data for MA and AM concentrations and the corresponding AM/MA ratios at 2, 4, 6, 8 or 20 hours after MA use were not obtained from the same person, unlike for the patients. MA concentrations in urine samples of most MA abusers were far higher than those of the patients and all were >250 ng/mL. Thus, false negative interpretations of MA use were not found in any MA abusers in this study. There was no correlation between AM/MA ratios and times after MA use in MA abusers (data not shown). Urinary AM/MA ratios of patients receiving selegiline were significantly higher than those of MA abusers at every time point after ingestion of selegiline or MA. This difference may be explained by the pharmacokinetics of the compounds. Selegiline is metabolized to AM via two pathways: one yields AM and the other yields MA, which is further metabolized to AM [6]. Thus, a higher AM/MA ratio is found in patientsreceiving selegiline. In contrast, in MA abusers, MA is excreted mainly unchanged in urine (up to 43-45% of the dose in a 24-hour period) while less AM (5-7%) is excreted in urine [26, 27]. Thus, a lower AM/MA ratio is found in MA users.

An attempt to find the most reliable AM/MA cutoff value to differentiate patients receiving selegiline from MA abusers was performed using AM/MA ratios of 0.40-0.75. This range included the highest AM/MA ratio in MA abusers (0.41 ± 0.05) and the lowest in patients (0.74 ± 0.07), found at 6 hours after administration. An AM/MA ratio of 0.40 gave the highest accuracy (84.88%, Table 4), highest sensitivity (96%, Table 4) or the highest probability (96%) that patients receiving selegiline were predicted as patients, and the lowest probability (4%) that patients would be predicted as MA abusers. Since abuse of MA is illegal, reporting patients who are prescribed selegiline as MA abusers (MA false positive) has a more negative effect on patients than the outcome caused by identifying MA abusers as patients. Thus, in this study,

we chose an AM/MA ratio cutoff with high sensitivity rather than high specificity (Table 4). False positive results in patients using the AM/MA cutoff can be further analyzed by an assay that is more specific and can differentiate between the *d*- and *l*-enantiomers or is able to detect desmethylselegiline, another metabolite of selegiline. This approach can attenuate the 4% of false positive results in selegiline patients. In contrast, using an AM/MA ratio of 0.40 gave the specificity of 76.28% (Table 4) meaning that an approximately 24% of MA abusers may be interpreted as selegiline patients. In this situation, medical history, previous use of drugs of abuse, young age, and other information can be used to justify performing a more specific test. Thus, based on the results of this study, an AM/MA ratio of 0.40 can be preliminarily used as the cutoff value to differentiate selegiline patients from MA abusers. However, this ratio is not an absolute marker. The result requires confirmation using detection of desmethylselegiline or a sensitive enantioselective method based on utilization of a derivatizing reagent or a chiral column in GC-MS, LC or LC-MS.

In the real situation, toxicology laboratories mostly focus on determination of AM and MA in urine samples of suspected AM or MA abusers so as to be used as an evidence in court, or in other situations for individual benefits such as insurance benefit, work's compensation benefit, work's enrollment benefit, etc. Thus, a lot numbers of urine samples are analyzed for AM and MA routinely every day in the toxicology laboratories. The methods used to determine AM and MA are varied among laboratories, generally immunoassay then confirmed by chromatography such as TLC, GC, LC. Most of the toxicology laboratories use GC/MS for the confirmation test and the protocols are mostly not able to differentiate the isomer form of AM and MA, thus the false positive results sometimes (rarely) occur with the urine samples of patients receiving selegiline. Selegiline can also be directly measured in case to confirm the receiving of selegiline. However, selegiline is found unchanged in a very less extent while larger amount of *l*-MA , *l*-AM, desmethylselegiline and very less amount of other metabolites of selegiline are found in urine, thus, determination of *l*-MA , *l*-AM, desmethylselegiline are more appropriated. In the situation that most laboratories have limitations to use the methods which are able to differentiate isomer form of AM and MA with a lot numbers of urine samples, this AM/MA ratio can be used to rule out at least 96% of the patients (who are prescribed medicines that are metabolized yielding *l*-isoform of AM or MA as selegiline) not to be interpreted as AM or MA abusers. Even though 4% of patients may be predicted as MA abusers (false positive) and 24% of MA abuser may be interpreted as selegiline-treated patients (false negative), then, further specific test can be used to confirm these smaller groups of samples.

Because selegiline and MA are metabolized via CYP enzyme system in the liver and the metabolites are mainly excreted in urine [6], thus, hepatic and renal function as well as CYP modulation by other drugs/xenobiotics may or may not significantly affect the results. Different age of subjects used in this study (young MA abusers and elderly selegiline patients) may raise another concern regarding age-related effect on the AM/MA ratio obtained from this study. So far, there is no available data regarding age-related modulation of systemic clearance of selegiline [28] as well as MA. As mentioned earlier, selegiline is metabolized via two reactions: (1) *N*-desmethylation yielding desmethylselegiline, which is further metabolized to *l*-AM; and (2) *N*-despropynylation yielding *l*-MA, which is further metabolized to *l*-AM [6]. CYP2B6 is the main enzyme involved in the metabolism of selegiline to desmethylselegiline, *l*-AM and *l*-MA with a possible minor contribution of CYP3A4 and CYP2A6 [29]. In MA abusers, *d*-MA is mainly excreted unchanged in urine with the smaller amount of *d*-MA is metabolized via CYP2D6, 2B6 and 3A4 yielding *d*-AM [30] which is excreted in urine [26, 27]. Age-associated reductions in functions/activities of some CYPs have been reported such as CYP1A2, 2C while other CYP isoforms such as CYP2D6, 3A4 (reduced in some study but not all) are not affected. Effect of age on some other CYP isoforms are not known (CYP1A1, 2B6) or inconclusive (CYP2A, 2E1) [31]. Whether or not age difference affects metabolism of selegiline and MA, is still unclear. Therefore, results of this study are suggested to be further validated before implementation using unrelated groups of selegiline patients and MA abusers which comprise appropriate sample size with different age, possess different pathological conditions, receive various CYP inducers/inhibitors, etc.

Conclusion

The results of this study showed that the AM/MA ratio in urine of patients receiving selegiline therapy was significantly higher than that in urine of MA abusers. This ratio could be used for preliminary differentiation of patients receiving selegiline from MA abusers with an accuracy of 84.88% based on an AM/MA ratio of 0.40 as the cutoff value.

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