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Simultaneous determination of selegiline-*N*-oxide, a new indicator for selegiline administration, and other metabolites in urine by high-performance liquid chromatography–electrospray ionization mass spectrometry

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Abstract

In order to discriminate selegiline (SG) use from methamphetamine (MA) use, the urinary metabolites of SG users have been investigated using high-performance liquid chromatography (HPLC)–electrospray ionization mass spectrometry (HPLC–ESI–MS). Selegiline-*N*-oxide (SGO), a specific metabolite of SG, was for the first time detected in the urine, in addition to other metabolites MA, amphetamine (AP) and desmethylselegiline (DM-SG). A combination of a Sep-pak C_{18} cartridge for the solid-phase extraction, a semi-micro SCX column (1.5 mm I.D.×150 mm) for HPLC separation and ESI–MS for detection provided a simple and sensitive procedure for the simultaneous determination of these analytes. Acetonitrile–10 m*M* ammonium formate buffer adjusted to pH 3.0 (70:30, v/v) at a flow-rate of 0.1 ml/min was found to be the most effective mobile phase. Linear calibration curves were obtained over the concentration range from 0.5 to 100 ng/ml for all the analytes by monitoring each protonated molecular ion in the selected ion monitoring (SIM) mode. The detection limits ranged from 0.1 to 0.5 ng/ml. Upon applying the scan mode, 10–20 ng/ml were the detection limits. Quantitative investigation utilizing this revealed that SGO was about three times more abundant (47 ng/ml, 79 ng/ml) than DM-SG in two SG users' urine samples tested here. This newly-detected, specific metabolite SGO was found to be an effective indicator for SG administration.

Keywords: Selegiline; Selegiline-*N*-oxide

1. Introduction

Selegiline [(*R*)-(−)-*N*-methyl-(1-phenyl-2-propyl)-*N*-propinylamine] (SG) is a potent, irreversible and selective inhibitor of monoamine oxidase type-B (MAO-B) [1,2]. It has been widely prescribed in many countries for the treatment of Parkinson's disease [3–5], and has also been sold as a prescription medicine in Japan since 1998 under the trade name FP. In 1996, Japanese authorities designated it as a precursor of the illicit stimulant methamphetamine (MA), and began to regulate its possession and use.

It is well established that SG is predominantly metabolized into MA and amphetamine (AP) [6–9].

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Therefore, the clear discrimination of legitimate therapeutic use of SG from illicit use of MA is indispensable in drug enforcement. However, the high metabolism of SG makes the detection of unchanged SG in urine usually difficult [10,11]. Since the presence of SG in urine cannot serve as an indicator for SG use, much attention is given to the development of a urinalysis procedure that can detect more specific metabolites to clearly distinguish SG use from MA use.

The discrimination of SG use from its counterpart has already been attempted by the enantiomeric determination of MA and AP based on the difference in their optical natures [10–16]; predominantly used illicit MA have been (+)-enantiomers or racemic isomers [15], whereas the metabolites of SG are (−)-enantiomers [10,11,17,18]. However, (−)-MA has often been seized in Japan since 1998 [19,20]. This makes the discrimination of SG use by such an enantiomeric analysis problematic. The detection of desmethyl selegiline (DM-SG), a specific metabolite of SG, has also been reported as an indicator for SG use [10,11,21]. It is also reported, however, that DM-SG was only detected in urine for less than 12 h after a single oral administration of 2.5–10 mg SG [10,11]. Thus, the presence of DM-SG can be employed as the indicator only for urine samples collected within a short time after the intake.

It is known that tertiary amine compounds are readily metabolized to their *N*-oxides in the human body by flavin-containing monooxygenase (FMO) [22,23]. The authors expected that SG would be appreciably metabolized to its *N*-oxide, or selegiline-*N*-oxide (SGO) (Fig. 1), and that this could serve as a novel indicator for SG administration. For the determination of amine-*N*-oxides, gas chromatography–mass spectrometry (GC–MS) has often been employed [24,25]. However, such GC–MS procedures require tedious pretreatments, including the reduction of the *N*-oxides with titanium trichloride [24] or acylation with an acid anhydride [25]. Also, such conversion spoils the identity of the analyte.

Recently, high-performance liquid chromatography–mass spectrometry (LC–MS) has become a powerful analytical tool that allows us to confirm polar and/or thermolabile compounds without tedious pretreatment [15,26–31]. We successfully established an LC–MS procedure for the simultaneous determination of dimethylamphetamine (DMA) and its metabolites, including dimethylamphetamine-*N*-

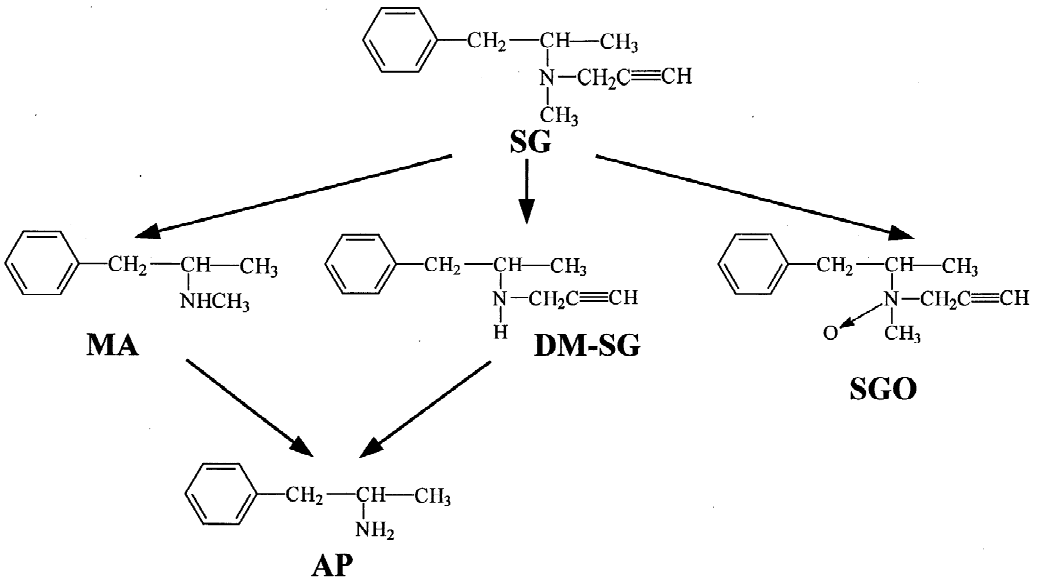

Fig. 1. Expected main metabolic pathways for selegiline (SG) in the human body.

oxide (DMAO), in urine by using LC–MS equipped with an electrospray ionization interface (LC–ESI–MS) [32].

In the present study, we have examined SG users' urine samples by LC–ESI–MS, and for the first time detected SGO in urine. By optimizing various analytical conditions, a rapid and sensitive procedure for the simultaneous LC–ESI–MS determination of SG and its metabolites SGO, DM-SG, MA and AP has been established. Applying the established method, urinary metabolites of long-term SG users are being further studied.

2. Experimental

2.1. Materials

SG hydrochloride and DM-SG hydrochloride were a kind gift from Fujimoto Pharmaceuticals (Osaka, Japan). MA hydrochloride and AP sulphate were purchased from Dainippon Pharmaceuticals (Osaka, Japan) and Takeda Pharmaceutical Industries (Osaka, Japan), respectively. SGO was prepared by oxidation of SG with *m*-chloroperbenzoic acid in our laboratory [33]. Standard stock solution of SGO was prepared in methanol, and those of SG, DM-SG, MA and AP in distilled water (each concentration being 1 mg/ml). They were diluted to appropriate concentrations with distilled water or human urine immediately prior to use. The internal standard (I.S.) ethylamphetamine (EAP) was synthesized according to the procedure described in a previous paper [34], and an I.S. solution was prepared in distilled water (concentration being 1 μg/ml). Acetonitrile and methanol were of HPLC-grade, and other chemicals used were of analytical grade. Sep-pak C_{18} cartridges were purchased from Waters Associates (Milford, MA, USA).

2.2. Sample preparation

Sep-pak C_{18} cartridges were prewashed successively with 10 ml distilled water, 10 ml methanol and 10 ml distilled water. Urine samples (1 ml) were loaded on the prewashed cartridges. Subsequently, the cartridges were rinsed with 1 ml of distilled water and then 5 ml 20% methanol aqueous solution. The retained analytes were eluted with 3 ml methanol. After acidification with acetic acid, the eluates were then evaporated to dryness under a gentle stream of nitrogen. The residue was reconstituted in 100 μl of the I.S. aqueous solution and filtrated through a 0.2 μm membrane filter. An aliquot of 10 μl was injected into the LC–MS system with an auto injector.

2.3. High-performance liquid chromatography–ESI mass spectrometry (LC–ESI–MS)

LC–MS was performed on an alliance-Platform LCZ system equipped with an ESI interface (Waters). The capillary voltage was 3 kV and the cone voltage was set at 20 V. The other ESI operating parameters were as follows: source temperature, 120°C; desolvation temperature, 350°C; nitrogen gas, 450 l/h; and multiplier voltage, 650 V. Under these conditions, full scan data acquisition was performed from m/z 100 to 500 in centroid mode and using a cycle time of 1.0 s and an interscan time of 0.1 s.

The chromatographic separation was carried out in the isocratic mode on a semi-micro SCX column (2.0 mm I.D.×150 mm, Shiseido, Tokyo, Japan). The mobile phase consisted of acetonitrile–10 m*M* ammonium formate (pH 3.0) (70:30, v/v) at the flow-rate of 0.1 ml/min. The effluent was directly introduced into the ESI source without a post-column split.

3. Results and discussion

3.1. Optimization of solid-phase extraction

For the solid-phase extraction of amphetamines in urine, either octadecylsilyl (ODS)-type or strong-cation exchanger (SCX)-type cartridges have often been employed. Especially, SCX-type cartridges have been reported effective for the extraction of DMA and its metabolites including MA, AP and DMAO [32]. In preliminary experiments, SCX-type cartridges were applied to extraction of SG and its metabolites. However, a high concentration of ammonium salt in the resultant samples extremely broadened the peak of SGO. Therefore, we have chosen Sep-pak C_{18} (ODS-type) cartridges for ex-

traction and optimized the extraction procedure by modifying the previously described method of Suzuki et al. [35] as described below.

In order to effectively elute the retained analytes from the cartridge, the methanol concentration in the eluent was varied between 50 and 100% and the recoveries and resultant chromatograms were compared by employing a urine sample spiked with SGO, SG, DM-SG, MA and AP at 100 ng/ml each. The higher methanol concentration produced the higher recoveries and the larger endogenous impurity peaks in the chromatograms. However, the impurity peaks caused no interference in the detection of all these analytes. We have, therefore, chosen 100% methanol as the eluent, which is also favorable in the subsequent evaporation.

3.2. Chromatographic conditions for LC–ESI–MS

It is well known that mobile phase with a higher organic solvent concentration is advantageous in achieving higher sensitivity in ESI–MS. In previous papers, simultaneous LC–MS determination of amines including opiates [36] and amphetamines [32] have been reported to be simultaneously analyzed with excellent separation on SCX-type column using high acetonitrile concentration in the mobile phase. Therefore, an SCX-type column was employed in combination with an acetonitrile–aqueous ammonium formate (NH_4OOCH) mobile phase, and the mobile phase was optimized by modifying the previously reported method of Katagi et al. [32].

The choice of pH of ammonium formate buffer in eluent, once optimized to be pH 4.0 in the separation of DMA and its metabolites, was reexamined for a standard aqueous solution of the five analytes. No notable pH influences were observed for MA and AP. The lower pH value produced the larger capacity factors (k' values) for SG, DM-SG and SGO. The optimum result was obtained at pH 3 (Fig. 2). This is most probably due to the dissociation of both N→O coordinate bond and propinyl moiety in neutral media; the anionized analytes and sulfonate moiety on SCX resin repel each other. An acidic pH restrains the ionization of SGO, as well as of SG and DM-SG, which contributed in achieving the appropriate retention. Thus, the pH of NH_4OOCH solution

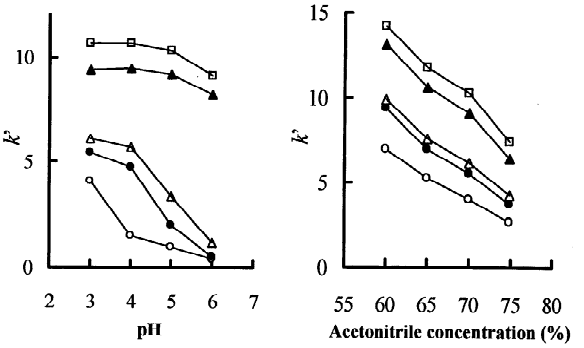

Fig. 2. Effect of buffer pH and acetonitrile concentration in the mobile phase on the capacity factor (k') for SGO (○), SG (●), DM-SG (△), MA (▲) and AP (□).

used for preparing the mobile phase was adjusted to 3.0 with formic acid in all the experiments.

It is well known that the organic solvent concentration in mobile phase affects the retention of amines on an SCX column [32]. Therefore, the acetonitrile concentration was varied between 60 and 75%, and the k' values for the analytes were measured. As shown in Fig. 2, the concentration of 65 or 70% led to sufficient separations. Additionally, a higher acetonitrile concentration is preferable in ESI-MS. Thus, acetonitrile–10 m*M* ammonium formate, adjusted to pH 3.0, (70:30, v/v), which provided both good separation and appropriate k' values, was finally selected as the mobile phase.

3.3. Instrumental parameters

Instrumental conditions were optimized employing an aqueous solution of SGO, DM-SG and MA at 100 ng/ml each as a sample with flow injection technique. The capillary voltage was varied between 1.0 and 4.0 kV. The abundance of base peaks, or protonated molecular ions of SGO (m/z 204), DM-SG (m/z 174) and MA (m/z 150), enlarged proportionally with the capillary voltage, and reached plateaus at 3.0 kV. The cone voltage was optimized between 10 and 50 V. The higher voltage produced the more extensive fragmentation that can help the identification. However, the sensitive detection of protonated molecular ions in full scan mode is essential in forensic drug analysis. The highest abundance, as well as the best level reproducibility

of each protonated molecular ion, was obtained at 20 V. Thus, the capillary and cone voltages were set at 3.0 kV and 20 V, respectively.

3.4. LC–ESI–MS identification

The confirmation of target analytes was performed by mass spectral identification in the scan mode. Figs. 3 and 4 show chromatograms and mass spectra of SGO, SG, DM-SG, MA and AP, both obtained from a spiked urine sample at 100 ng/ml each. All five analytes eluted within 20 min with excellent separation without any interference by endogenous urinary components. All of their mass spectra were characterized by predominant protonated molecular ions $[M+H]^+$ at m/z 204 for SGO, 188 for SG, 174 for DM-SG, 150 for MA and 136 for AP. In the mass spectra of DM-SG, MA and AP, a weak ion corresponding to $[M+CH_3CN+H]^+$ (m/z 215 for DM-SG, 191 for MA and 177 for AP) also appeared, while a dimerization ion $[2M+H]^+$ was observed for SGO at m/z 407. Urine samples basically gave similar results to those of the standard solutions, and chromatographic and spectral deterioration in peak profiles was hardly observed in this experiment.

3.5. Validation

3.5.1. Extraction recoveries

In order to evaluate the extraction recoveries for the solid-phase extraction, extracts obtained from spiked urine samples and standard aqueous solutions were each analyzed in the SIM mode. Peak areas of $[M+H]^+$ ions were compared for SGO, SG, DM-SG, MA and AP. The resultant data are summarized in Table 1. The recoveries ranged from 85 to 94% and from 85 to 90% at the concentrations of 100 ng/ml and 10 ng/ml, respectively ($n=5$).

3.5.2. Quantitative analysis and detection limits

Calibration curves were constructed employing the internal standard EAP in the SIM mode by monitoring the $[M+H]^+$ ions (m/z 204 for SGO, m/z 188 for SG, m/z 174 for DM-SG, m/z 164 for EAP, m/z 150 for MA and m/z 136 for AP). As samples, spiked urine in which the concentrations of the added analytes were varied between 0.1 and 100 ng/ml. The analysis showed good linearity over the concentration range from 0.5 to 100 ng/ml for SGO ($y = 7.60\times10^{-3}x + 1.22\times10^{-2}$, $r^2 = 0.999$), SG ($y = 1.94\times10^{-2}x + 2.11\times10^{-2}$, $r^2 = 0.999$), DM-SG ($y = 3.57\times10^{-2}x + 2.81\times10^{-2}$, $r^2 = 0.999$), MA ($y = 3.60\times10^{-2}x + 6.34\times10^{-2}$, $r^2 = 0.999$) and AP ($y = 3.02\times10^{-2}x + 2.07\times10^{-2}$, $r^2 = 0.999$).

Precision and accuracy were evaluated by analyzing drug-free urine samples spiked with known concentrations of SGO, SG, DM-SG, MA and AP (quality control samples) in the SIM mode. The results are presented in Table 1. The within-day relative standard deviations (RSDs) ($n=5$) for all analytes ranged from 4.7 to 8.1% at the concentration of 100 ng/ml, and from 4.9 to 8.6% at 10 ng/ml. The accuracy was found to be between 97 and 100% at the concentration of 100 ng/ml, and between 91 and 103% at 10 ng/ml. The between-day RSDs determined from three batches over 3 days ($n=3$ for each batch) were calculated to be less than 7.7% for each analyte at the concentration of 100 ng/ml.

Also, the sensitivities were evaluated. The detection limits in the scan mode were 10 ng/ml for SGO, SG, DM-SG and MA, and 20 ng/ml for AP. The SIM technique by monitoring each protonated molecular ion lowered the detection limits further down to 0.1 ng/ml for SGO, 0.2 ng/ml for SG, DM-SG and MA, and 0.5 ng/ml for AP with a signal-to-noise ratio of 3:1. These results demonstrate high sensitivity and good reproducibility of the present procedure.

3.6. Analysis of SG patients' urine

Urine samples voluntarily submitted from two long-term SG patients (patients A and B) were analyzed by the present method: patient A has been receiving twice-daily administration (5 mg/day) of SG hydrochloride, and patient B once-daily administration (2.5 mg/day). Time after the last dosing was 13.5 h for patient A and 5.5 h for patient B.

Enantiomeric LC–MS analysis by our previously published method [15,28] indicated that MA and AP detected in their urine were all (−)-isomers. Fig. 5 depicts a total ion chromatogram and extracted mass chromatograms obtained from patient A's urine in the full scan mode. SGO was detected along with

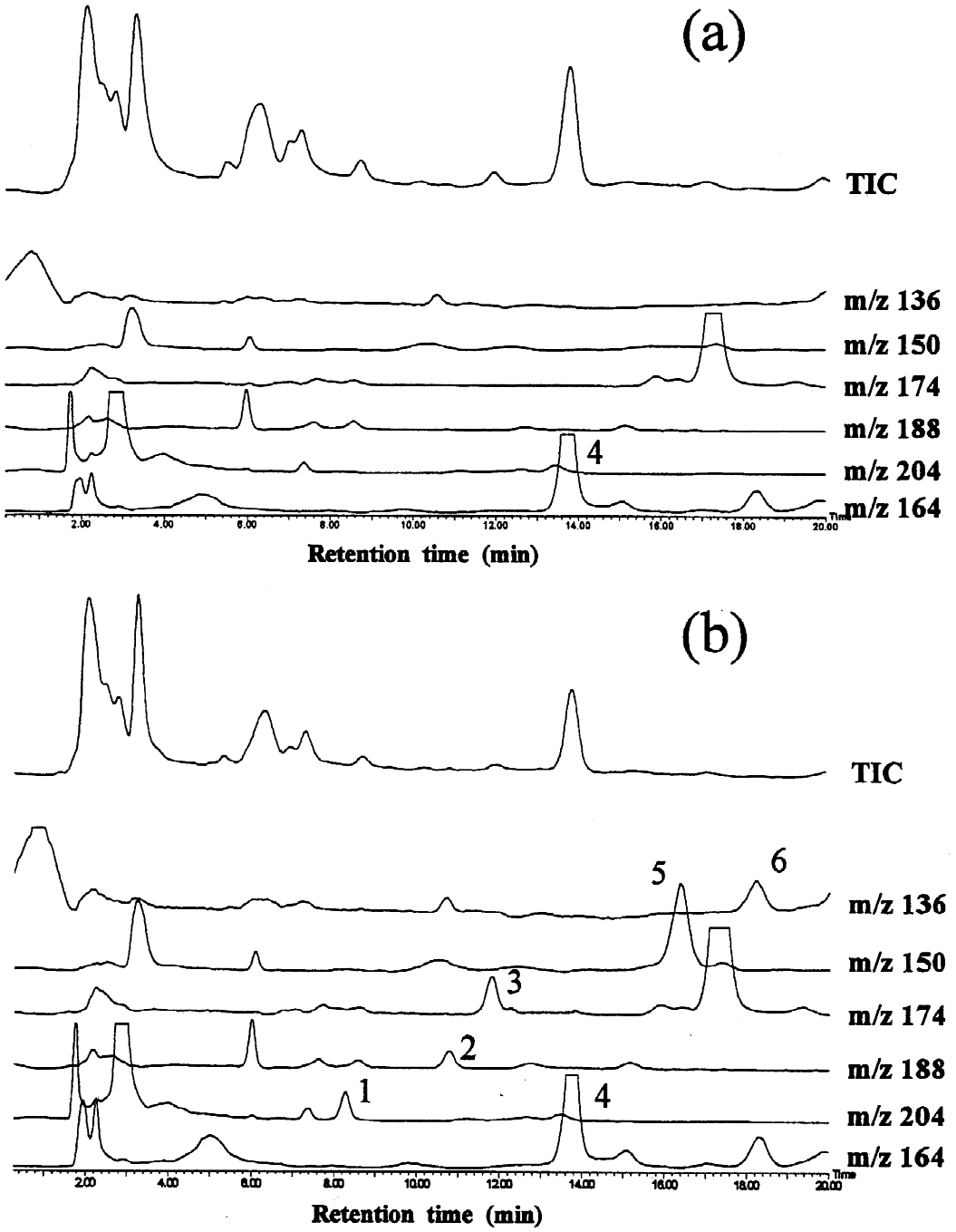

Fig. 3. Total ion chromatograms and extracted mass chromatograms obtained from (a) a blank urine and (b) a spiked urine sample. Eluent: acetonitrile–10 m*M* ammonium formate (pH 3.0) (70:30, v/v). The concentration of all the analytes in the sample was 20 ng/ml each. Other chromatographic conditions appear in Section 2. Peaks: 1, SGO; 2, SG; 3, DM-SG; 4, EAP (I.S.); 5, MA; 6, AP.

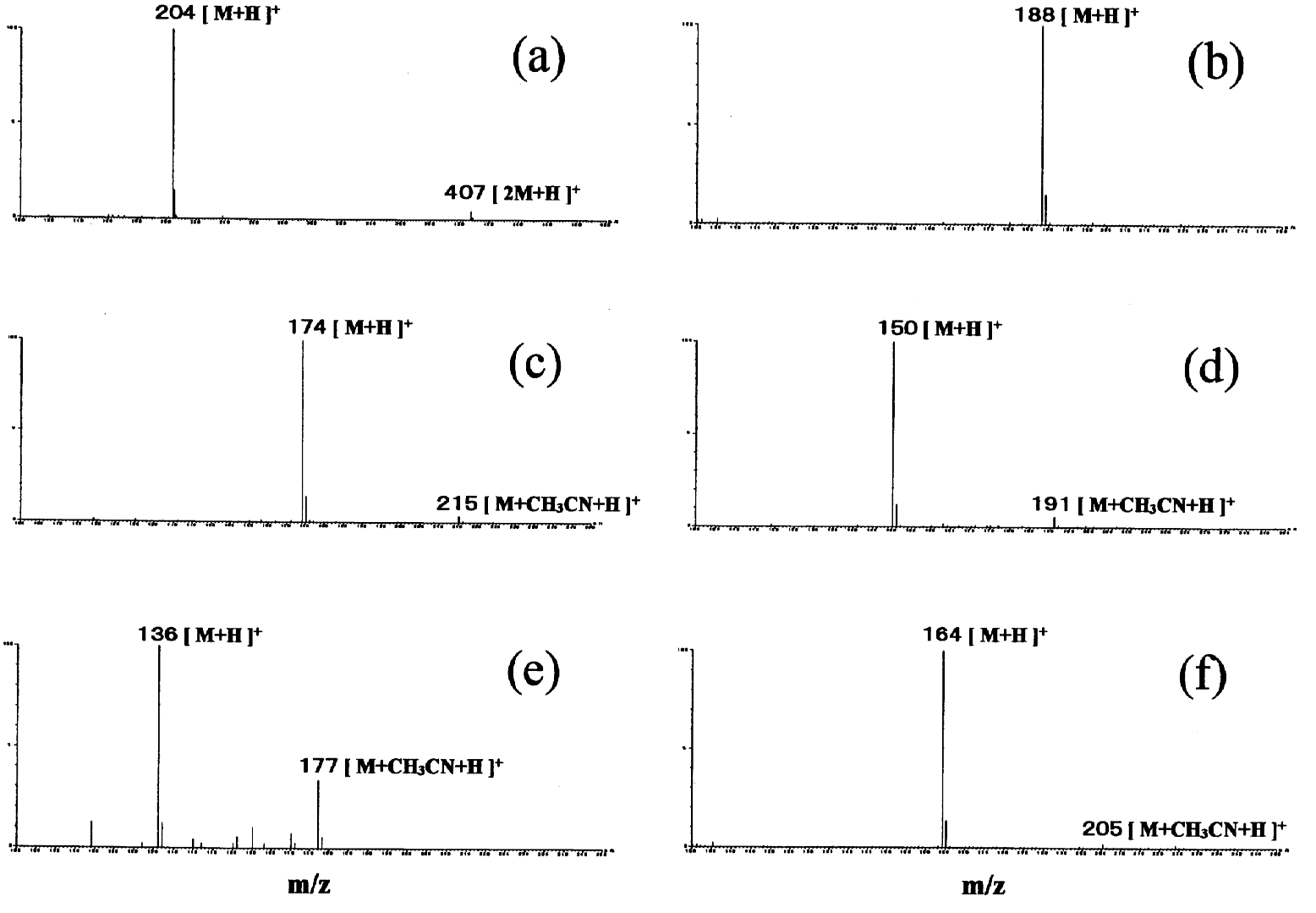

Fig. 4. Mass spectra produced from peaks of SGO (a), SG (b), DM-SG (c), MA (d), AP (e) and EAP (I.S.) (f). The concentration of all the analytes in the sample was 100 ng/ml each.

Table 1
Extraction recoveries, precision and accuracy

	10 ng/ml					100 ng/ml				
	SGO	SG	DM-SG	MA	AP	SGO	SG	DM-SG	MA	AP
Extraction recoveries (%)	89	86	90	85	87	93	89	94	85	88
Within-day	10 ng/ml					100 ng/ml				
	SGO	SG	DM-SG	MA	AP	SGO	SG	DM-SG	MA	AP
Mean	9.2	10.3	9.1	9.1	9.5	97	99	97	100	98
SD	0.79	0.51	0.49	0.77	0.70	4.8	4.8	7.0	4.7	8.0
RSD (%)	8.6	4.9	5.4	8.5	7.4	5.0	4.8	7.2	4.7	8.1
Accuracy (%)	92	103	91	91	95	97	99	97	100	98

$n=5$.

DM-SG, MA and AP, though SG was not detectable. For patient B, SGO as well as MA and AP was detected, but neither DM-SG nor SG was detectable in the full scan mode. For the sake of further research on the metabolites by more sensitive technique, the concentrations of each metabolite were also measured in the SIM mode. The results are summarized in Table 2. Although DM-SG was not detected for user B in the full scan mode, SGO was detected from both urine samples, and its level was

Table 2
Quantitative analysis of urine samples collected from selegiline patients by ESI–LC–MS in the SIM mode

	Concentration (ng/ml)				
	SGO	SG	DM-SG	MA	AP
Patient A	47	n.d.[a]	15	680[b]	210[b]
Patient B	79	n.d.	8.0	1100[b]	350[b]

[a] n.d., not detected.
[b] Data obtained by sample dilution with drug-free urine.

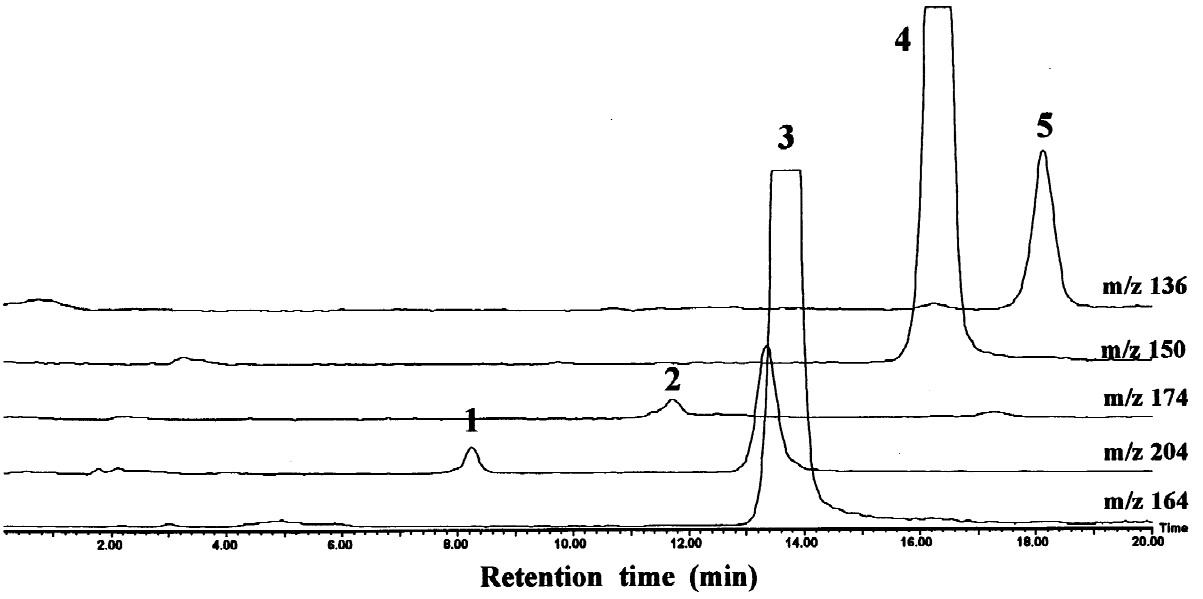

Fig. 5. Extracted mass chromatograms obtained from a urine sample taken from a selegiline (SG) patient (patient A). Peaks: 1, SGO; 2, DM-SG; 3, EAP (I.S.); 4, MA; 5, AP. The estimated concentrations appear in Table 2.

~3 times higher than DM-SG. According to a previous report [10], the less abundant metabolite DM-SG was not detectable in all the urine samples from long-term SG patients who took the 10 mg/day dosages (the number of urine samples was not described). Thus, it is concluded that the more abundant metabolite SGO can serve as a much more reliable indicator for distinguishing therapeutic SG use from MA use.

4. Conclusion

The presence of SGO was for the first time detected in the urine samples of therapeutic SG users by using LC–ESI–MS. A simple, yet sensitive LC–ESI–MS procedure was established for the simultaneous determination of SG and its metabolites SGO, DM-SG, MA and AP in urine. Since this procedure was able to detect SGO, a newly-found metabolite, more abundantly than DM-SG from the SG patients' urine tested here, this can serve as a powerful method to distinguish therapeutic SG use from MA use. In spite of its negligible effects as a psychotropic or stimulant drug, SG has recently been sold as a 'smart drug' via the internet. SG may also be used as a kind of abused drug. Since the detectable period of SGO after SG intake is of forensic interest, a further human metabolism study is currently being carried out at our laboratory under various dosage conditions.

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