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ORIGINAL ARTICLE

Simultaneous analysis of mitragynine, 7-hydroxymitragynine, and other alkaloids in the psychotropic plant "kratom" (*Mitragyna speciosa*) by LC-ESI-MS

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Abstract The leaves of *Mitragyna speciosa*, a tropical plant known as "kratom," have been traditionally used as a substitute for opium in Thailand and Malaysia. Mitragynine, a major constituent of *M. speciosa*, has an opioid agonistic activity, and its derivative 7-hydroxymitragynine (7-OH-mitragynine) (a minor constituent) is much more potent than mitragynine or morphine. Recently, many products containing this plant have been distributed as "incense" on the drug market in Japan for their expected narcotic effects. Despite their potency and their wide distribution for abuse, there are no reports on the quantitative analysis of mitragynine and 7-OH-mitragynine in the raw materials or in the commercial products of kratom. In this study, a method for simultaneous analysis of mitragynine, 7-OH-mitragynine, and other indole alkaloids (speciogynine, speciociliatine, and paynantheine), present in the raw materials and commercial products of kratom, was developed using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS). By this method, mitragynine, 7-OH-mitragynine, and the other alkaloids were detected in 11 of the 13 products. The content of mitragynine in the products ranged from 1% to 6%, and that of 7-OH-mitragynine from 0.01% to 0.04%. Because 7-OH-mitragynine is much more potent than morphine, *M. speciosa* abuse is a matter of major concern. The present analytical method is considered useful for the screening of *M. speciosa* products in the drug market.

Keywords Mitragynine · 7-Hydroxymitragynine · *Mitragyna speciosa* · Kratom · Opioid agonist · LC-MS

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Introduction

In the past decade, many easily available psychotropic substances have been widely distributed in Japan as analogs of narcotics [1–5]. They are not controlled under the Narcotics and Psychotropics Control Law, because their pharmacological effects have not yet been proven scientifically. To control these substances, the Pharmaceutical Affairs Law of Japan was amended in 2006, and 39 compounds and one plant (*Salvia divinorum*) have been listed as "designated substances." However, various products of uncontrolled psychotropic plants have become popular substitutes for these compounds, and are causing serious concern in Japan [6,7]. Until recently, these substances were used only in limited areas or in groups for religious purposes, but are now easily available everywhere via the Internet. These products are believed by young people to be "nonchemical," "spiritual," or "mild" products, and are thought of a being safer than typical narcotic plant products such as opium.

The leaves of a plant endemic to tropical Southeast Asia, *Mitragyna speciosa* (Rubiaceae), have been traditionally used as a substitute for opium in Thailand and Malaysia. The leaves are known as "kratom" or "biak-biak" in these countries, and contain mitragynine and its

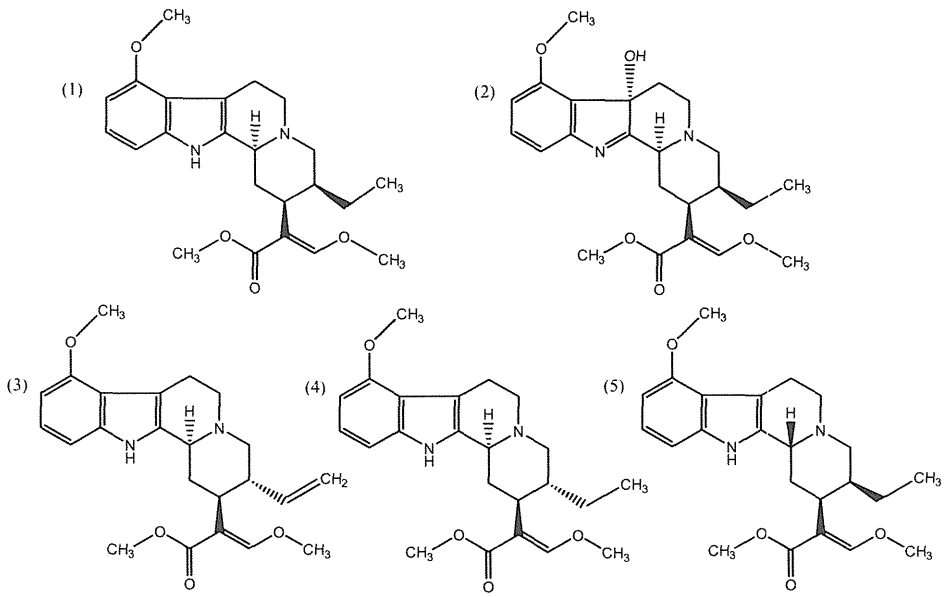

Fig. 1 Chemical structures of five alkaloids in *Mitragyna speciosa* investigated in this study. *1*, Mitragynine; *2*, 7-hydroxymitragynine (7-OH-mitragynine); *3*, paynantheine; *4*, speciogynine; *5*, speciociliatine

Table 1 Commercial kratom products examined in this study

Product	Labeled name	Form
1	Concentrated kratom (50%–60% pure)	Resin
2	Kratom crush leaf KR-L	Dried leaf (cutting)
3	Kratom gold KR-G	Powder
4	Kratom KR-1	Powder
5	Kratom	Dried leaf (cutting)
6	Kratom	Dried leaf
7	Mitragyna extract powder	Powder
8	Mitragyna	Dried leaf
9	Mitragyna 40x resin	Resin
10	Plant sample kratom	Dried leaf (cutting)
11	Kratom EX10	Dried leaf (cutting)
12	Concentrated kratom KR-XV	Resin
13	Concentrated kratom KR-1	Powder

related indole alkaloids such as speciogynine, speciociliatine, and paynantheine (Fig. 1) [8,9]. Mitragynine, a major constituent of *M. speciosa* (66.2%, based on the crude base from the young leaves of *M. speciosa* [10]), has an opioid agonistic activity [8,9,11,12]. Its derivative 7α-hydroxy-7*H*-mitragynine (7-OH-mitragynine) shows a much more potent antinociceptive effect in mice than does either mitragynine or morphine [8,9,13–16], although it is a minor constituent (2.0% based on the crude base [10]) of the leaves. Moreover, 7-OH-mitragynine exhibits morphine-like tolerance and withdrawal effects in mice [15]. Many kinds of products containing this plant have been recently distributed as "incense" in the drug market in Japan, because of their expected narcotic effects. Despite their great potency and wide distribution for abuse, no reports are available on quantitative analysis of mitragynine, 7-OH-mitragynine, and other related alkaloids in the raw materials and commercial products of kratom.

In this study, a method for simultaneous analysis of mitragynine, 7-OH-mitragynine, and other indole alkaloids (speciogynine, speciociliatine, and paynantheine), the active components of *M. speciosa*, was developed using liquid chromatography-electrospray ionization mass spectrometry (LC-ESI-MS). Moreover, the method was actually applied to quantitative analyses of mitragynine and 7-OH-mitragynine in raw materials and commercial products of kratom obtained from the drug market.

Materials and methods

Materials

Thirteen kinds of commercial products that claimed to contain kratom were purchased via the Internet in 2006 and 2007, as shown in Table 1. Of the 13 products, 6

were in the form of dried leaves, 4 in the form of powders, and 3 in the form of resins. The reference raw materials of *Mitragyna speciosa* (dried leaves) and *Mitragyna hirsuta* (dried leaves), identified by Dr. Sumphan Wongseripipatana, were collected at Chulalongkorn University, Thailand. The raw materials of *M. speciosa* were divided into big leaves and small leaves. *Mitragyna hirsuta* is a related species of *M. speciosa*.

Chemicals and reagents

Betamethasone valerate (internal standard, IS) was purchased from Wako (Osaka, Japan). Mitragynine (95.4% pure), 7-OH-mitragynine (95.1% pure), speciogynine, speciociliatine, and paynantheine, which had been isolated and identified from *M. speciosa* in the previous study [10], were used (Fig. 1). Centrifugal filter devices (Ultrafree-MC, 0.45 μm filter unit) were obtained from Millipore (Bedford, MA, USA). All other common chemicals and solvents were of analytical reagent grade or HPLC grade.

Instrumentation

For the qualitative and quantitative analyses of the substances, LC-ESI-MS consisting of an Agilent 1100 Series high-performance liquid chromatography (HPLC) system equipped with an 1100 Series LC/MSD SL (Agilent, Palo Alto, CA, USA) was used. Chromatographic separation was performed in the gradient mode using an Atlantis dC18 column (2.0 mm i.d. × 150 mm, 5 μm) protected by a Sentry guard column (2.0 mm i.d. × 10 mm, 5 μm) (Waters, Milford, MA, USA) at 40°C. The following gradient system was used with a mobile phase A (10 mM ammonium formate, pH 3.5) and a mobile phase B (methanol) delivered at 0.3 ml/min: 90% A /10% B (0 min) to 60% A /40% B (35 min, 20 min hold). The mobile phase composition was then brought back to the starting point in 1 min and the column equilibrated over 10 min. The injection volume was 1 μl. For the detection system, a tandem setting of a photodiode array (PDA) detector and a mass detector was adopted. The wavelength of the PDA detector was set from 190 to 400 nm, and chromatographic peaks were monitored at 254 nm.

MS analysis by ESI was conducted in the positive mode. Nitrogen gas was used for nebulization and was delivered at a flow rate of 13 l/min at 330°C. Other conditions were: nebulizer gauge pressure, 345 kPa; vaporizer temperature, 350°C; capillary voltage, 3500 V; fragmentation voltage, 100 V. MS data were recorded in the full scan mode (*m/z* 100 to 600). Quantitative analysis of mitragynine and 7-OH-mitragynine was carried out by monitoring areas of the protonated molecular peaks ($[M + H]^+$) of target compounds and IS in the scan mode. For mitragynine, the peak at 254 nm was also monitored for quantitative analysis without MS analysis. The monitoring ions were as follows: mitragynine (*m/z* 399), 7-OH-mitragynine (*m/z* 415 and 433), and betamethasone (IS) (*m/z* 477). Chromatographic peaks were detected and integrated by the ChemStation data analysis system (Agilent).

Standard solutions

Separate standard solutions at 0.1 mg/ml for each compound (mitragynine, 7-OH-mitragynine, speciogynine, speciociliatine, and paynantheine) were prepared in methanol and stored in darkness at –20°C. The solution of IS (betamethasone valerate) in methanol at 0.2 mg/ml was also prepared.

Sample extraction procedure

A finely powdered sample of each product (10–50 mg) was extracted with 10 ml of 80% methanol aqueous solution including 100 μl of the IS solution (0.2 mg/ml) by ultrasonication for 1 h. Following storage at room temperature overnight, each sample mixture was centrifuged at 3000 rpm for 5 min and filtered through the centrifugal filter device prior to the injection. If necessary, the solution was diluted with methanol to a suitable concentration.

Results and discussion

Optimization of conditions

For successful chromatographic separation of standard solutions of the five compounds (mitragynine, 7-OH-mitragynine, speciogynine, speciociliatine, and paynantheine) and betamethasone (IS), the stationary phases from different column manufacturers were tested. The best result was obtained with an Atlantis dC18 column, which is a difunctionally bonded and silica-based reversed-phase HPLC column. With PDA detection (monitored at 254 nm), a relatively good separation was confirmed in 40 min when a gradient elution with 10 mM ammonium formate (pH 3.5) and methanol was adopted for the analytical column.

Figure 2 shows LC-PDA ultraviolet (UV) spectra and LC-ESI-MS mass spectra of the standard solutions of the five compounds (0.1 mg/ml each). The conditions for ionization of each drug were investigated using flow-injection analyses, and the optimum conditions were

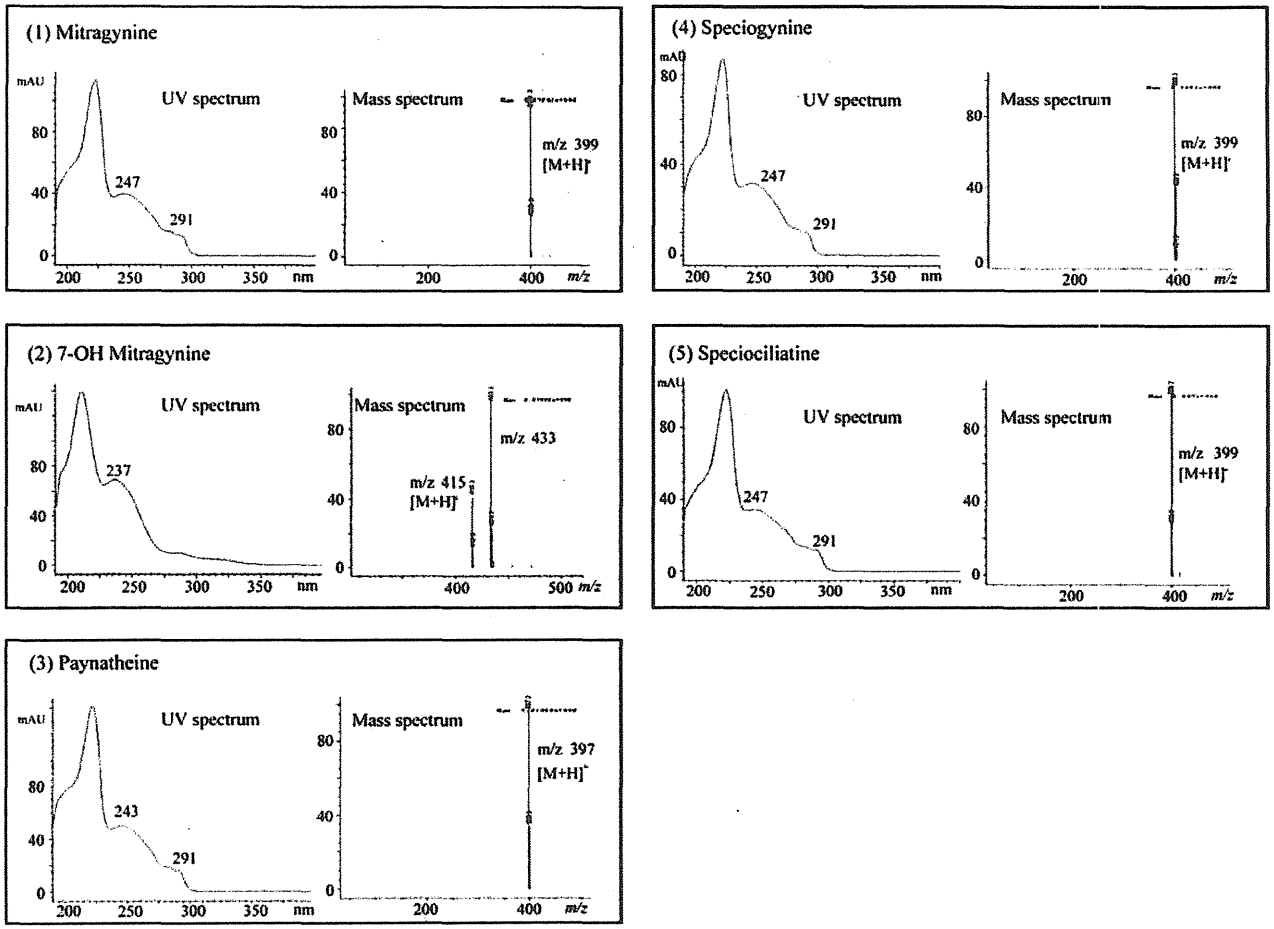

Fig. 2 Ultraviolet (UV) and mass spectra of the standard compounds of five alkaloids in *M. speciosa*. The retention times of mitragynine, 7-OH-mitragynine, paynantheine, speciogynine, and speciociliatine were 19.9, 21.5, 20.5, 19.9, and 23.3 min, respectively

determined as described in the Materials and methods section. The protonated molecular ions ($[M + H]^+$) were observed as base peaks for all compounds except for 7-OH-mitragynine. For 7-OH-mitragynine ($C_{23}H_{30}N_2O_4$), the ion corresponding to $[M + H + 18]^+$ (*m/z* 433) was the main peak in the spectrum together with the protonated molecular ion (*m/z* 415) (Fig. 2). The elemental composition of the former ion (*m/z* 433) was estimated as $C_{23}H_{33}N_2O_6$ through exact mass measurements using a time-of-flight mass spectrometer (AccuTOF JMS-T100, JEOL, Tokyo, Japan) (data not shown); it was found to be the protonated molecular ion with an added water molecule. Quantitative analysis of 7-OH-mitragynine was carried out using either ion at *m/z* 433 or *m/z* 415.

The optimum extraction conditions for mitragynine and 7-OH-mitragynine from the raw materials and the products were investigated using product No. 4 (Table 1) as a typical sample. To choose an extraction solvent and an ultrasonication time, the product sample was extracted with methanol, ethanol, or acetonitrile by ultrasonication for 1, 3, or 6 h. Extraction was most effective when the sample was extracted with methanol by ultrasonication for 1 h and kept at room temperature overnight, as shown in Figs. 3 and 4. Moreover, to determine the most suitable concentration of methanol, the sample was also extracted with various concentrations of methanol in water. An 80% methanol aqueous solution showed the most effective extraction of two compounds, especially for 7-OH-mitragynine (Fig. 5). Mitragynine and 7-OH-mitragynine were stable in the 80% methanol aqueous solution at least for the duration of the extraction procedure. More than 95% of each compound remained in the solution throughout the procedure.

Reliability of the method

Figure 6A shows LC-MS chromatograms of the extract from the reference raw material of *Mitragyna speciosa*

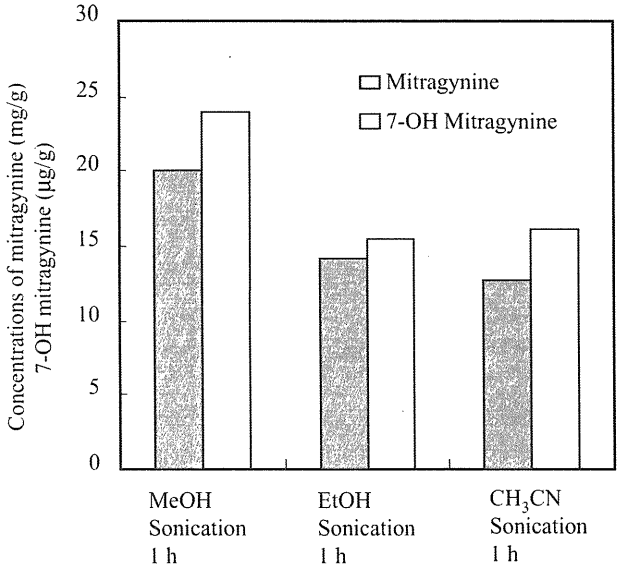

Fig. 3 Concentrations of mitragynine and 7-OH-mitragynine from product No. 4 using different organic solvents for extraction

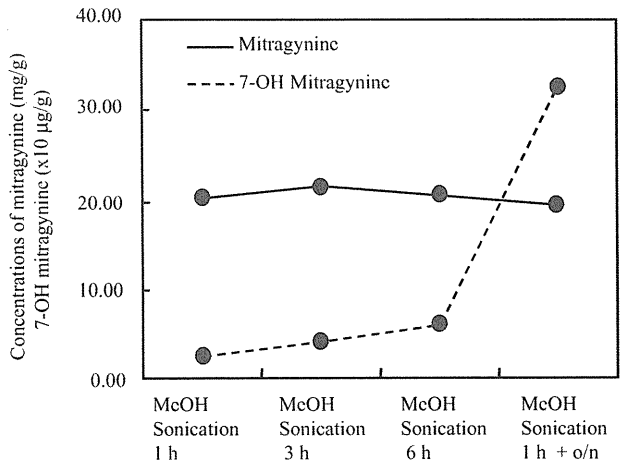

Fig. 4 Concentrations of mitragynine and 7-OH mitragynine from product No. 4 as a function of extraction time using methanol. *o/n*, Overnight storage at room temperature

(big leaves) using the optimum extraction procedure established above. Under the chromatographic conditions used, there was no interference with almost any of the target compounds by endogenous materials in the plant. The peak of speciociliatine overlapped with peak 6 (not identified) in the UV and total ion chromatograms; these two peaks showed different protonated molecular ions (*m/z* 399 for speciociliatine and *m/z* 397 for peak 6), and it was thus possible to differentiate them. The mitragynine content was much higher than that of 7-OH-mitragynine in the raw materials, and it was difficult to carry out a simultaneous quantitative analysis of both compounds in the same detection system.

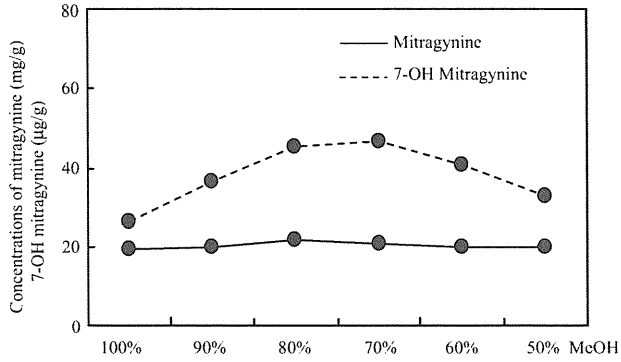

Fig. 5 Concentrations of mitragynine and 7-OH-mitragynine from product No. 4 as a function of different concentrations of methanol for extraction

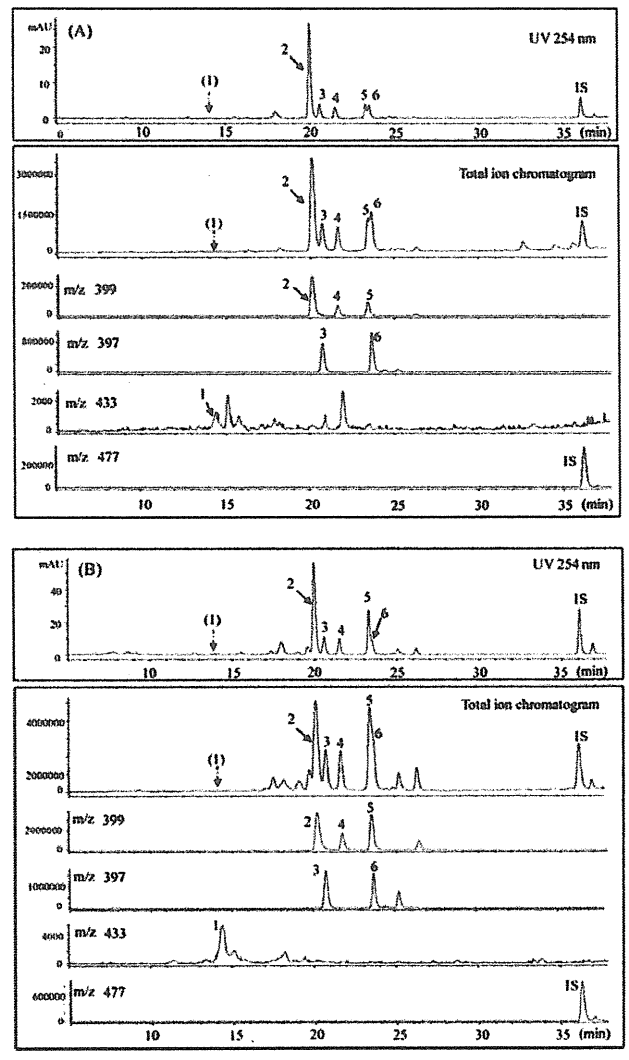

Fig. 6A,B UV chromatograms, total ion chromatograms, and mass chromatograms of the extracts from **A** a reference raw material of *M. speciosa* (big leaves) and **B** product No. 4. *1*, 7-OH-mitragynine; *2*, mitragynine; *3*, paynantheine; *4*, speciogynine; *5*, speciociliatine; *6*, unidentified

When a large amount of the sample for detection of 7-OH-mitragynine was used, the ionization of mitragynine became saturated because of its high concentration. Therefore, quantitative analysis of mitragynine was carried out by HPLC-UV detection using the peak at 254 nm.

Because we did not have blank product samples at the early stage of this study, we had to obtain validation data in the absence of matrices; thus, we prepared various concentrations of the standard mitragynine and 7-OH-mitragynine. After adding IS to each diluted standard solution, the mixtures were extracted according to the established procedure and analyzed by HPLC-UV for mitragynine, and by LC-MS for 7-OH-mitragynine. The concentrations of mitragynine and 7-OH-mitragynine in the samples were calculated using the peak area ratios of mitragynine versus IS at 254 nm and those of the protonated molecular ions ($[M + H]^+$) of 7-OH-mitragynine versus IS, respectively. The calibration curve for mitragynine was linear over the concentration range of 1.0–10.0 μg/ml; the equation and r^2 value were $y = 0.0682x + 0.028$ and 0.9996, respectively. The curve for 7-OH-mitragynine was also linear over the range of 10–1000 ng/ml; $y = 1.11x + 0.0010$ and $r^2 = 0.9984$. The intraday precision and accuracy data were tested after the same procedure as shown in Table 2. Both data were generally satisfactory.

Analyses of reference raw materials and commercial products of kratom

The proposed method was applied to analyze mitragynine, 7-OH-mitragynine, and other alkaloids (speciogynine, speciociliatine, and paynantheine) in the reference raw materials and the 13 products of kratom. From LC-ESI-MS analyses of the raw materials, all five target compounds were found in both big and small leaves of *M. speciosa* (Fig. 6A). The concentrations of mitragynine and 7-OH-mitragynine in the small leaves were significantly lower than those in the big leaves (Table 3). There were obvious differences in their leaf sizes, although they were both recognized as *M. speciosa* in the area of Bangkok, Thailand, where the samples were collected. We also previously investigated the botanical origins of the same raw materials and products used in this study, using an internal transcribed spacer (ITS) sequence analysis of rDNA [17]. The results of the DNA analyses suggested that the plants with small leaves were a hybrid between *M. speciosa* and *M. hirsutaldiversifolia*. This could be the reason why the small leaves had lower concentrations of the two compounds than the big leaves. On the other hand, mitragynine, 7-OH-mitragynine, and the other three target alkaloids were not detected in the raw material of *M. hirsuta* (Table 3). Although this plant is a related species of *M. speciosa*, it was reported not to contain the major narcotic compounds to be found in *M. speciosa* [18].

Table 2 Intraday precision and accuracy for analysis of mitragynine and 7-hydroxymitragynine

Compound	Concentration added (μg/ml or ng/ml)[a]	Precision (%)[b]	Accuracy (%)[b]
Mitragynine[c]	1.0	4.1	120
	10	4.2	88.8
	50	4.6	107
7-OH-Mitragynine[d]	10	6.6	116
	50	5.0	111
	500	6.0	96.2

[a] Mitragynine in μg/ml; 7-OH-mitragynine in ng/ml
[b] $n = 3$
[c] Analysis by high-performance liquid chromatography with ultraviolet detection
[d] Analysis by liquid chromatography-mass spectrometry

Thirteen products of kratom, which were advertised as having psychotropic/psychoactive effects, were obtained via the Internet. These products appeared in various forms, such as pieces of dried leaves, powders, or resins, and some of them were advertised as "concentrated extracts" (Table 1). By LC-ESI-MS analyses, mitragynine, 7-OH-mitragynine, and the other three indole alkaloids could be detected in 11 of the 13 products; the same compounds were also found in the raw materials (Fig. 6A, B). The contents of mitragynine in these products ranged from 1.2% to 6.3%, and those of 7-OH-mitragynine from 0.01% to 0.04% (Table 3). The concentrations of the target compounds in the resin samples were much higher than those in the dried leaves. Most samples of dried leaves contained the related alkaloids at the same levels as in the big leaves of the reference raw material. The highest concentration of mitragynine detected in the products was 62.6 mg/g (product No. 9, a resin form), followed by 47.4 mg/g (product No. 12, a powder form). The concentrations of 7-OH-mitragynine were less than 0.4 mg/g, and the sample forms might not be related to its concentrations. In product No. 11, 7-OH-mitragynine was not detected, but small amounts of mitragynine and other alkaloids were detected. Moreover, no alkaloids were detected in product No. 8. The shapes of the leaves in product Nos. 8 and 11 differed from those of other products, although all were sold as kratom.

Mitragynine, the major alkaloid of kratom, has been characterized as an opioid agonist, producing effects similar to those of morphine, but its weak potency cannot explain the kratom's potent opium-like effect. Horie et al. [14] reported that the opioid effect of *M. speciosa* was mostly based on the activity of the minor

Table 3 Concentrations of mitragynine and 7-hydroxymitragynine in the reference raw materials and the commercial kratom products

Sample	Form	Mitragynine concentration (mg/g)	7-OH-Mitragynine concentration (mg/g)
Reference raw materials			
Mitragyna speciosa			
Big leaves	Dried leaf	23.8 ± 0.2 (2.4)	0.124 ± 0.010 (0.01)
Small leaves	Dried leaf	1.6 ± 0.0 (0.2)	0.031 ± 0.004 (0.003)
Mitragyna hirsuta	Dried leaf	ND	ND
Kratom products			
1	Resin	35.6 ± 1.8 (3.6)	0.116 ± 0.007 (0.01)
2	Dried leaf	13.1 ± 0.1 (1.3)	0.168 ± 0.003 (0.02)
3	Powder	18.9 ± 0.2 (1.9)	0.267 ± 0.014 (0.03)
4	Powder	21.2 ± 0.4 (2.1)	0.393 ± 0.026 (0.04)
5	Dried leaf	19.6 ± 0.5 (2.0)	0.361 ± 0.046 (0.04)
6	Dried leaf	21.2 ± 0.6 (2.1)	0.213 ± 0.008 (0.03)
7	Powder	19.4 ± 0.2 (1.9)	0.159 ± 0.016 (0.02)
8	Dried leaf	ND	ND
9	Resin	62.6 ± 1.6 (6.3)	0.336 ± 0.041 (0.03)
10	Dried leaf	12.1 ± 0.2 (1.2)	0.114 ± 0.004 (0.01)
11	Dried leaf	0.8 ± 0.2 (0.08)	ND
12	Resin	47.4 ± 0.2 (4.7)	0.367 ± 0.016 (0.04)
13	Powder	21.4 ± 0.2 (2.1)	0.326 ± 0.023 (0.03)

Data given as mean ± standard deviation. $n = 3$. Values in parentheses are percentages
ND, Not detected

alkaloid 7-OH-mitragynine. This compound produced antinociceptive effects about 5.7 and 4.4 times more potent than those of morphine in the tail-flick [effective dose in 50% of subjects (ED_{50}) = 0.80 mg/kg] and hot-plate (ED_{50} = 0.93 mg/kg) tests, respectively [16], when administered subcutaneously (s.c.) to mice. Moreover, oral doses of 7-OH-mitragynine to mice at 5–10 mg/kg showed antinociceptive activities in these tests, whereas oral administration of morphine required a dose of 20 mg/kg to achieve similar activities [13]. The potent antinociceptive effect of 7-OH-mitragynine is reported to be based on activation of μ-opioid receptors, and it also exhibited morphine-like pharmacological characters, including tolerance and withdrawal [15]. In this study, 0.1–0.4 mg/g of 7-OH-mitragynine was found in the products (Table 3). As for mice (30 g weight), approximately 0.1 mg (s.c. administration) or 1 g (oral administration) of the products might be enough to show potent antinociceptive activities, possibly including other opium-like effects.

Until now, no reports have detailed the amounts of the major narcotic alkaloids mitragyine and 7-OH-mitragynine in the kratom products, and also on their possible effects. Kratom is widespread and easily available via the Internet. These naturally occurring alkaloids give a useful clue for the development of new types of analgesic drugs having structures quite different from that of morphine; but the abuse of these plant products is a matter of serious concern.

Conclusions

In this study, we have established a detailed procedure for analysis of mitragynine, 7-OH-mitragynine, and other alkaloids in commercially available products. Using the method, we measured the actual contents of alkaloids in the kratom products; the content of mitragynine in the products ranged from 1% to 6%, and that of 7-OH-mitragynine ranged from 0.01% to 0.04%. Because 7-OH-mitragynine has a narcotic activity that is much more potent than that of morphine, the abuse of *M. speciosa* is a major concern. Our proposed analytical method will be useful for screening *M. speciosa* products in the drug market.

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ORIGINAL PAPER

Rapid, sensitive and simultaneous determination of fluorescence-labeled designated substances controlled by the Pharmaceutical Affairs Law in Japan by ultra-performance liquid chromatography coupled with electrospray-ionization time-of-flight mass spectrometry

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Abstract A simultaneous determination method based on ultra-performance liquid chromatography (UPLC) with fluorescence (FL) detection and electrospray-ionization time-of-flight mass spectrometry (ESI-TOF-MS) was developed for 16 "designated substances" (Shitei-Yakubutsu) controlled by the Pharmaceutical Affairs Law in Japan. These substances were first labeled with 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole at 60 °C for 2 h in 0.1 M borax (pH 9.3). The resulting fluorophores were well separated by reversed-phase chromatography using an Acquity UPLC™ BEH C_{18} column (1.7 μm, 100 mm×2.1 mm i.d.) by isocratic elution with a mixture of water and acetonitrile–methanol (20:80) containing 0.1% formic acid. The separated derivatives were sensitively detected by both FL and TOF-MS. However, the determination of several designated substances by FL detection showed interference from endogenous substances in biological samples. Therefore, the determination in real samples was carried out by a combination of UPLC separation and ESI-TOF-MS detection. The structures of the designated substances were identified from the protonated-molecular ions $[M+H]^+$ obtained from the TOF-MS measurement. The calibration curves obtained from the peak area ratios of the internal standard (I.S.), i.e., 3-phenyl-1-propylamine, and the designated substances versus the injection amounts showed good linearity. The limits of detection (S/N = 3) and the limits of quantification (S/N = 10) in 0.1 mL of human plasma and urine for the present method were 0.30–150 pmol and 1.0–500 pmol, respectively. Good accuracy and precision (according to intraday and interday assays) were also obtained with the present procedure. This method was applied to analyses of human plasma, urine and real products.

Keywords Designated substances (Shitei-Yakubutsu) · Fluorescence labeling · Piperazines · Phenethylamines · 4-(*N,N*-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole · Ultra-performance liquid chromatography · Time-of-flight mass spectrometry

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Introduction

Recently, the abuse of designer drugs has become a serious social problem around the world [1–8]. "Designer drugs" are a number of psychoactive substances that are especially designed to circumvent existing drug laws. This is achieved by modifying the molecular structures of existing drugs to

varying degrees. The term gained popularity in the 1980s when 3,4-methylenedioxymethamphetamine (MDMA) was first introduced onto the black market [9, 10]. 3,4-Methylenedioxyamphetamine (MDA) and MDMA are generally known as the "love drug" and "ecstasy," respectively. These new designer drugs generally present the structural features of phenethylamine and piperazine derivatives and are reported to produce hallucinogenic visual effects similar to those of LSD and mescaline as well as emotional empathic responses similar to those of MDMA [11, 12]. Special concerns relate to the lack of scientific knowledge about the pharmacotoxicology of these drugs in humans and the specific harmful effects of the substances when taken alone or in combination with other drugs. There are a number of reports of acute intoxications associated with the consumption of *m*-chlorophenylpiperazine (m-CPP) and 2,5-dimethoxy-4-*n*-propylthiophenethylamine (2C-T-7) that support the view that the consumption of these "hallucinogenic designer drugs" is likely to be a threat to human health [13–18]. Although several phenethylamines and piperazines are strictly controlled by the Narcotics and Psychotropic Control Law in Japan, various new psychotropic compounds possessing phenethylamine and piperazine structures have appeared and been distributed on the streets since the late 1990s and early 2000s, especially via the Internet. Their use, especially by young persons, is one of the most serious social problems in the world. To avoid their widespread use, some of these compounds were listed as psychotropic substances controlled as "designated substances" (Shitei-Yakubutsu) by the Pharmaceutical Affairs Law in Japan in April 2007 [19–24]. Furthermore, six kinds of substances, including 1-(4-iodo-2,5-dimethoxyphenyl)propan-2-amine (DOI), were listed as illegal drugs in 2008. Consequently, the development of qualitatitive and quantitative analyses that enable rapid screening for these substances is an important task.

Many separation methods, such as TLC [25], HPLC-UV [25–27], GC [28] and CE [29] have been reported for the qualitative analysis of phenethylamine analogs. Furthermore, GC-MS [30–33] and LC-MS [34–45] are used for preliminary studies and mass screening to discriminate these substances from other hallucinogens. It is well-known that fluorescence labeling is one of the most sensitive techniques for various compounds. Therefore, a rapid and simultaneous determination method for eleven hallucinogenic phenethylamines based on liquid chromatography (LC) with FL detection after fluorescence (FL) labeling with 4-(*N,N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) was reported [46]. Qualitative and quantitative determinations of phenethylamine in products that appear on the Japan market have been successfully performed using this method. However, the simultaneous determination of very low concentrations of designated substances in complex matrices, such as plasma and urine, appear to be rather difficult, even with highly sensitive FL detection, due to interference from endogenous substances in the samples. Therefore, a more sensitive and selective determination method is required for bioanalysis. Furthermore, this simultaneous determination method must be simple, selective and sensitive. However, no such method for the determination of various phenethylamine and piperazine analogs has been developed so far. Thus, we attempted the simultaneous determination of fourteen hallucinogenic phenethyamines and two piperazines by ultra-performance liquid chromatography (UPLC) separation coupled with both FL and ESI-TOF-MS detection.

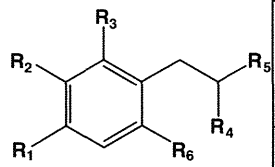
A UPLC system using an anti-pressurized column is a possible approach for rapid separation. On the other hand, the selective detection of the target compound is carried out by ESI-TOF-MS due to its excellent accuracy and the precision of the resulting *m/z* values. To increase the hydrophobicities of the substances, the primary and secondary amino functional groups in their structures were labeled with a fluorogenic reagent (DBD-F) [46, 47]. The resulting derivatives separated by UPLC were detected by both FL and ESI-TOF-MS connected to the outlet of the column in this order. The UPLC–FL and UPLC–ESI-TOF-MS methods were evaluated in terms of their selectivity and sensitivity. Furthermore, their application to human plasma, urine and phenethylamine-containing products are also described in this paper.

Experimental

Materials and reagents

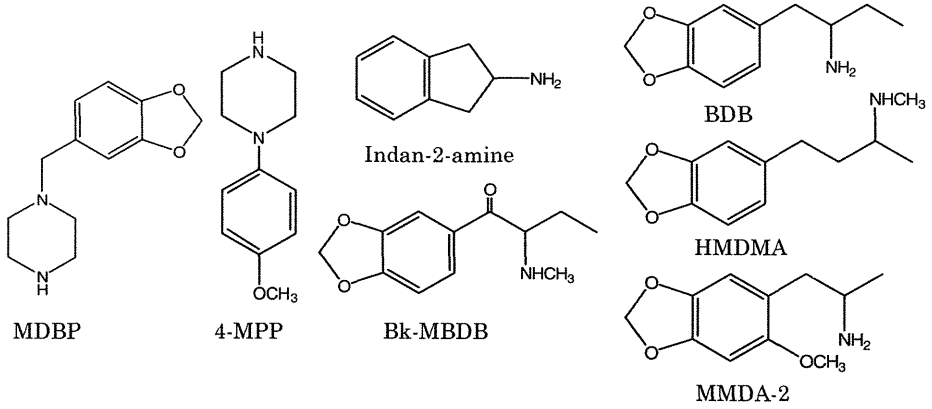
The hydrochloric acid salts of 16 designated substances, i.e., 1-(4-metho xyphenyl)piperazine (4-MPP), 1-(3,4-methylenedioxybenzyl)piperazine (MDBP), indan-2-amine, 1-(4-iodo-2,5-dimethoxyphenyl)propan-2-amine (DOI), 2-methylamino-1-(3,4-methylenedioxyphenyl) butan-1-one (Bk-MBDB), 4-iodo-2,5-dimethoxyphenethylamine (2C-I), 4-chloro-2,5-dimethoxyphenethylamine (2C-C), 4-ethyl-2,5-dimethoxyphenethylamine (2C-E), 4-ethylthio-2,5-dimethoxyphenethylamine (2C-T-2), 4-isopropylthio-2,5-dimethoxyphenethylamine (2C-T-4), 2,4,6-trimethoxyamphetamine (TMA-6), 4-fluoroamphetamine (4-FMP), 4-methoxymethamphetamine (PMMA), *N*-methyl-1-(3,4-methylenedioxyphenyl)butan-3-amine (HMDMA), 1-methyl-1-3,4-methylenedioxymethamphetamine (MMDA-2), and 1-(3,4-methylenedioxyphenyl)butan-2-amine (BDB), were obtained from the National Institutes of Health Sciences (NIHS, Tokyo, Japan) (Fig. 1). 3-Phenyl-1-propylamine (3-PPA) and 4-phenylbutylamine (4-PBA) were purchased from Sigma (St. Louis, MO, USA). 4-(*N,N*-Dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole

Fig. 1 Structures of hallucinogenic designated substances



Phenethylamines

Compounds	R_1	R_2	R_3	R_4	R_5	R_6
2C-I	I	OCH_3	H	H	NH_2	OCH_3
2C-C	Cl	OCH_3	H	H	NH_2	OCH_3
2C-E	CH_2CH_3	OCH_3	H	H	NH_2	OCH_3
2C-T-2	SCH_2CH_3	OCH_3	H	H	NH_2	OCH_3
2C-T-4	$SCH(CH_3)_2$	OCH_3	H	H	NH_2	OCH_3
TMA-6	OCH_3	H	OCH_3	CH_3	NH_2	OCH_3
4-FMP	F	H	H	CH_3	NH_2	H
DOI	I	OCH_3	H	NH_2	CH_3	OCH_3
PMMA	OCH_3	H	H	CH_3	$NHCH_3$	H



(DBD-F) was purchased from Tokyo Kasei Co. (Tokyo, Japan). Three products, Product 1 (pale yellow powder), Product 2 (mushroom-like substance) and Product 3 (a colorless liquid), which were sold on the Japanese market in 2006, were tested for phenethylamine(s). Formic acid (HCOOH), sodium tetraborate ($Na_2B_4O_7$, Borax), methanol (CH_3OH), and acetonitrile (CH_3CN) were of special reagent grade (Wako Pure Chemicals, Osaka, Japan). All other chemicals were of analytical reagent grade and were used without further purification. Deionized and distilled water (H_2O) was used throughout the study (Aquarius PWU-200 automatic water distillation apparatus, Advantec, Tokyo, Japan).

UPLC-FL-ESI-TOF-MS

The UPLC-ESI-TOF-MS system consisted of an Acquity™ ultra-performance liquid chromatography and a Micromass LCT Premier™ XE mass spectrometer (high-sensitivity orthogonal time-of-flight instrument; Waters, Milford, MA, USA). An FL detector (Acquity FLR, Waters) equipped with a 2-μL flow cell was also directly connected between the column outlet and the TOF-MS instrument. An Acquity UPLC™ BEH C_{18} column (1.7 μm, 100 mm×2.1 mm i.d., Waters) was used as the analytical column. The column was maintained at 40 °C. The flow rate of the mobile phase was 0.4 mL/min. The TOF-MS was operated in the positive ion mode using an electrospray-ionization source (ESI^+). The optimized conditions for the UPLC separation and FL and TOF-MS detection are shown in Table 1.

Derivatizing the designated substances with DBD-F

To 30 μL of aqueous solution containing each designated substance and 3-PPA (internal standard, I.S.) (100 μM each), 140 μL of DBD-F in CH_3CN (2.5 mM) and 120 μL of 100 mM borax (pH 9.3) were added and vigorously

Table 1 UPLC-FL and UPLC-ESI-TOF-MS conditions

UPLC (separation conditions)	
Column	ACQUITY UPLC™ BEH C_{18} (1.7 μm, 100 mm×2.1 mm i.d.)
Mobile phase A	0.1% HCOOH in H_2O
Mobile phase B	0.1% HCOOH in CH_3CN:CH_3OH (20:80)
Isocratic elution	A : B=40:60 (0–10 min)
Column temperature	40 °C
Flow rate	0.4 mL/min
Injection volume	10 μL
FL detector (FLR conditions)	550 nm (excitation: 450 nm)
TOF-MS (LCT Premier XE conditions)	
Polarity	ESI^+ (V mode)
Capillary voltage	3500 V
Sample cone voltage	10 V
Desolation gas flow	700 L/h
Cone gas flow	50 L/h
Source temperature	120 °C
Desolvation temperature	350 °C
MS range	100–1000 *m/z*

mixed. Each solution was heated at 60 °C for over 240 min. At a fixed time interval, 10 μL of 1% HCOOH in water was added to the reaction mixture to stop the derivatization reaction. The reaction solution was diluted with acetonitrile, and then a 10 μL portion of the diluted solution was separated by UPLC and fluorometrically detected. The peak areas were plotted versus the sampling times. The excitation and emission spectra for each derivative were also determined by the FL detector.

Recommended procedure for the determination of designated substances

One hundred forty microliters of 2.5 mM DBD-F in CH_3CN and 120 μL of 100 mM borax (pH 9.3) were added to 20 μL of an aqueous solution containing the designated substance (s). The solutions were mixed with 10 μL of 5 μM 3-PPA (I.S.) in water. The mixed solution was heated at 60 °C for 2 h. After the reaction, 10 μL of 1% HCOOH in water was added to the reaction mixture to stop the derivatization reaction. The acidic solution was suitably diluted with acetonitrile, and then an aliquot (10 μL) was injected into the UPLC-ESI-TOF-MS system. The mobile phases (A) and (B) were 0.1% HCOOH in H_2O and 0.1% HCOOH in CH_3CN:CH_3OH (20:80), respectively. The derivatives were separated by an Acquity UPLC BEH C_{18} (100×2.1 mm, i.d., 1.7 μm) with an isocratic elution of (A):(B) (40:60).

Validating the method

Calibration curves

Twenty microliters of the 16 designated substances in water (each 75 nM–18.75 μM) were mixed with 10 μL of 5 μM 3-PPA (I.S.) in water. The solution was reacted with 140 μL of 2.5 mM DBD-F at 60 °C for 2 h in 120 μL of 0.1 M borax (pH 9.3). After the reaction, 10 μL of 1% HCOOH in water were added to the reaction mixture to stop the derivatization reaction. Ten microliters of each solution were then subjected to the UPLC-ESI-TOF-MS system. The amounts corresponding to an injection of 10 μL were 0.05–12.5 pmol (n=5). The calibration curves were obtained by plotting the peak area ratios of the analytes relative to the I.S. (3-PPA) versus the injected amounts of the 16 designated substances. The CV (%) was calculated for each concentration (n=5).

Accuracy and precision determined by intraday and interday assays

The accuracy (%) and precision (CV) based on intraday and interday assays were determined by the proposed method. These parameters were evaluated using three different concentrations in the range of 0.05–0.5 nmol/mL for the 16 designated substances. The determinations were repeated five times within a day and between days. Each 30 μL solution was reacted with DBD-F and then subjected to UPLC-ESI-TOF-MS, as described in "Calibration curves." The accuracy (%) of each concentration was calculated from the calibration curves. The precision (CV%) of each concentration was also calculated from five replicate determinations.

Determining designated substances spiked into human plasma

Calibration curves for human plasma

One hundred fifty microliters each of aqueous solutions containing the 16 designated substances (corresponding to 0.2–2.0 nmol) and 20 μL of 5 μM I.S. were added to 200 μL of pooled plasma, obtained from healthy human volunteers, in glass vials (n=5). To precipitate the proteins in the plasma, 630 μL of acetonitrile was poured into each vial and the mixture was vigorously mixed. The mixed solution was centrifuged at 3000 rpm for 10 min, and then 500 μL of the supernatant fluids was collected and dried under a gentle stream of nitrogen gas. The resulting residues were redissolved with 0.1 M borax (pH 9.3) and reacted with DBD-F, as described in "Recommended procedure for the determination of designated substances." An aliquot (10 μL) of the solution was subjected to the UPLC-ESI-TOF-MS system. The calibration curves were obtained by plotting the peak area ratios of the analyte relative to the I.S. (3-PPA) versus the injected amounts of the 16 designated substances.

Determining the designated substances

Three concentrations of the 16 designated substances spiked into pooled plasma obtained from healthy human volunteers were analyzed by the proposed procedure. The spiked samples were treated using the procedures described in "Calibration curves in human plasma." The MS spectra and the selected ion chromatograms (SIC) were compared to those obtained from each standard substance. The determined amounts and assays (%) were calculated from the calibration curves. The assays at each concentration were also calculated as mean±SD values. The precision (CV%) of each assay value was also calculated from five replicate determinations.

Limits of detection (LODs) and limits of quantification (LOQs)

The limits of detection (LODs) and the limits of quantification (LOQs) were defined as the calculated concentrations at signal-to-noise ratios of 3 (S/N = 3) and 10 (S/N = 10), respectively. The standard solutions of the 16 designated

substances spiked in pooled plasma obtained from healthy human volunteers were diluted to a series of concentrations (0.007–4.0 μM). A 0.6 mL portion of CH_3CN was added to 0.2 mL of the spiked plasma and vigorously mixed. The mixture was then centrifuged at 3000 rpm for 10 min. A 0.4 mL portion of the supernatant solution was separated and dried under a gentle stream of nitrogen gas. The residues were redissolved with 0.58 mL of 0.1 M borax (pH 9.3) and then reacted with 5 mM DBD-F in 0.4 mL CH_3CN at 60 °C for 2 h. After the reaction, 20 μL of 1% HCOOH in water were added to the reaction mixture and suitably diluted with H_2O:CH_3CN:CH_3OH (40:12:48) if necessary. An aliquot (10 μL) was then injected into the UPLC-ESI-TOF-MS system (Table 1). The mobile-phases (A) and (B) were 0.1% HCOOH in H_2O and 0.1% HCOOH in CH_3CN:CH_3OH (20:80), respectively. The derivatives were separated by an Acquity UPLC BEH C_{18} (100×2.1 mm, i.d., 1.7 μm) with an isocratic elution of (A):(B) (40:60). The LODs and LOQs of the 16 designated substances in human plasma were calculated by comparing the noise levels and the peak heights in suitable chromatograms that detected trace amounts of the 16 designated substances. The LODs and LOQs in pooled urine obtained from healthy human volunteers were also determined according to the method described for human plasma.

Furthermore, determinations of the LODs and LOQs of the 16 designated substances spiked into human plasma and urine were carried out without DBD-F labeling. The human plasma and urine containing the designated substances were deproteinized and centrifuged, as described previously. The supernatant solution was dried and the resulting residues were redissolved with the mixture of 0.58 mL of 0.1 M borax (pH 9.3) and 0.4 mL CH_3CN. The solution was suitably diluted with H_2O:CH_3CN:CH_3OH (75:5:20). An aliquot (10 μL) was then injected into the UPLC-ESI-TOF-MS system. The mobile phases (A) and (B) were 0.1% HCOOH in H_2O and 0.1% HCOOH in CH_3CN:CH_3OH (20:80), respectively. The derivatives were separated by an Acquity UPLC BEH C_{18} (100×2.1 mm, i.d., 1.7 μm) with an isocratic elution of (A):(B) (75:25).

Determining the designated substances in real products

Three products, Product 1 (pale yellow powder), Product 2 (mushroom-like substance), and Product 3 (a colorless liquid), which were sold on the Japanese market in 2006, were tested for the designated substances. Product 1 (1 mg) was dissolved with 1.0 mL of 50% methanol and then centrifuged at 2000 rpm for 10 min. After centrifugation, the separated supernatant was filtered through a 0.45 μm membrane. Ten microliters of 5 μM 3-PPA (I.S.) added to 20 μL of the filtrate were reacted with DBD-F, as described in "Recommended procedure for the determination of designated substances." Similarly, 1 mg of Product 2 was treated as described above. Because Product 3 was a clear liquid sample, the solution was first diluted ten times with water. Ten microliters of 5 μM 3-PPA added to 30 μL of the filtrate were also labeled with DBD-F, separated by UPLC, and detected by mass spectrometry. The designated substances in the products were qualitatively and quantitatively determined by comparing the retention times and the ratios of the peak areas on the chromatograms.

Results and discussion

FL labeling of the designated substances with DBD-F

FL labeling is usually recommended for trace analyses of real samples due to its high sensitivity and selectivity. In a previous study, we developed a simultaneous determination method for phenethylamine-type designated substances by LC-FL [46]. The phenethylamines were labeled with DBD-F at 60 °C for 2 h in 0.1 M borax (pH 9.3). The labeling conditions required for piperazine-type designated substances were also tested in this study. The reaction scheme of DBD-F with MDBP, used as a representative designated substance, is shown in Fig. 2. Figure 3 shows the time courses of the labeling reactions of the designated substances with DBD-F at 60 °C in 0.1 M borax (pH 9.3). Although the maximal peak areas were different for each designated

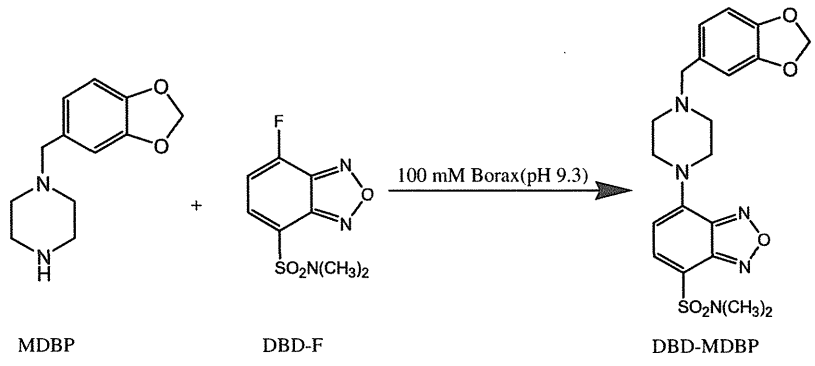


Fig. 2 Fluorescence labeling reaction of MDBP with DBD-F

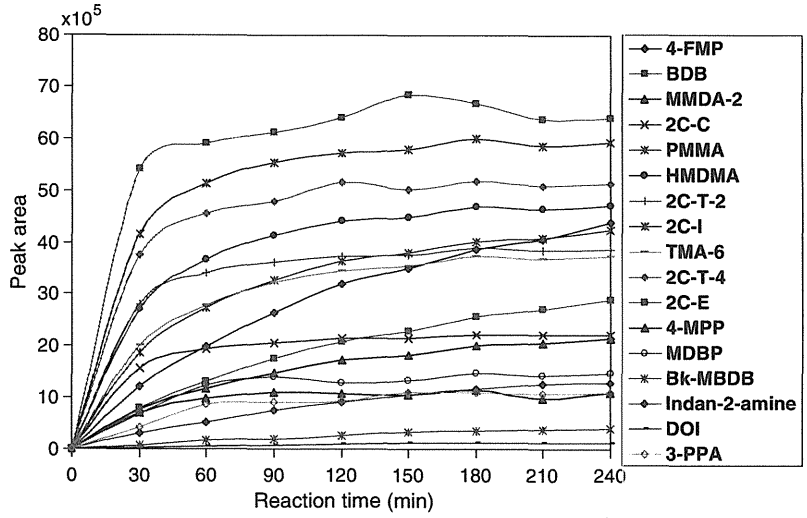


Fig. 3 Time courses of the reactions of the designated substances with DBD-F at 60 °C

substance, the reactions were almost complete after two hours of heating. Hence, reaction conditions of 60 °C for 2 h in 0.1 M borax (pH 9.3) were selected for the subsequent FL labeling of all of the designated substances.

Optimizing the separation and detection conditions

In a previous study, designated phenethylamine-type substances in real samples were successfully determined by LC separation and FL detection [46]. However, trace determinations in biological specimens, such as plasma and urine, appear to be rather difficult due to interference from endogenous substances. Furthermore, no structural information can be obtained from FL detection. On the other hand, MS has recently become a popular technique for the determination of trace quantities of chemicals in real samples, such as blood and urine. Among the various types of MS instrument available, ESI-TOF-MS is recommended for the selective determination of target compounds because of its excellent accuracy and the precision of the resulting *m/z* values [48]. Thus, the simultaneous determination of the designated substances with both FL and ESI-TOF-MS was attempted in this study.

An anti-pressurized column packed with a small porous resin, Acquity UPLC™ BEH C_{18} (100 mm×2.1 mm i.d., 1.7 μm), was used for the rapid separation of the DBD-labeled designated substances by UPLC, instead of conventional HPLC. To detect the derivatives, FL and ESI-TOF-MS instruments were directly connected to the outlet of the column in this order. The DBD-labeled designated substances are still basic compounds that have secondary and tertiary amino functional groups in their structures. Therefore, acidic and neutral mobile phases were evaluated for the simultaneous separation of these substances. The derivatives of the designated substances, except in a couple of cases, were simultaneously separated by isocratic elution conditions that used H_2O and CH_3CN containing 0.1% HCOOH as the mobile phase. However, the peak-to-peak separations of some derivatives, i.e., 2C-E/2C-T-4 and indan-2-amine/4-FMP, were incomplete in this mobile phase mixture. On the other hand, the separations improved with the addition of CH_3OH to the mobile phase, and the mutual separation of 2C-E and 2C-T-4 was performed using a mixture of H_2O and CH_3CN: CH_3OH (20:80) (40:60) containing 0.1% HCOOH. Thus, the existence of CH_3OH in the mobile phase is important for the efficient separation of the derivatives. Although the peak-to-peak separation of indan-2-amine and 4-FMP was still difficult, perfect separation does not seem to be necessary for MS determination. Based upon these observations, the conditions shown in Table 1 were finally selected for the simultaneous separation of the derivatives.

Figure 4 shows the FL chromatogram (A), total ion chromatogram (TIC) (B), selected-ion chromatograms (SIC) (C) and MS spectra (D) obtained for authentic DBD-labeled designated substances including 3-PPA (I.S.). Although the derivatives eluted after 2.0 min were well separated and detected by UPLC-FL, perfect separation and detection of all derivatives was very difficult, as shown in Fig. 4A. In contrast, total determination in a short run time (within 8.5 min) was possible using the selected-ion chromatograms (SIC) (Fig. 4C). Of course, the derivatives of all of the designated substances were identified from the protonated molecular ions $[M+H]^+$ in the mass spectra (Fig. 4D).

Validating the proposed method

Table 2 shows the calibration curves of the 16 designated substances. The calibration curves were obtained by plotting the peak area ratios of the designated substances relative to the I.S. versus the injected amounts of the designated substances. The calibration curves were obtained from five different

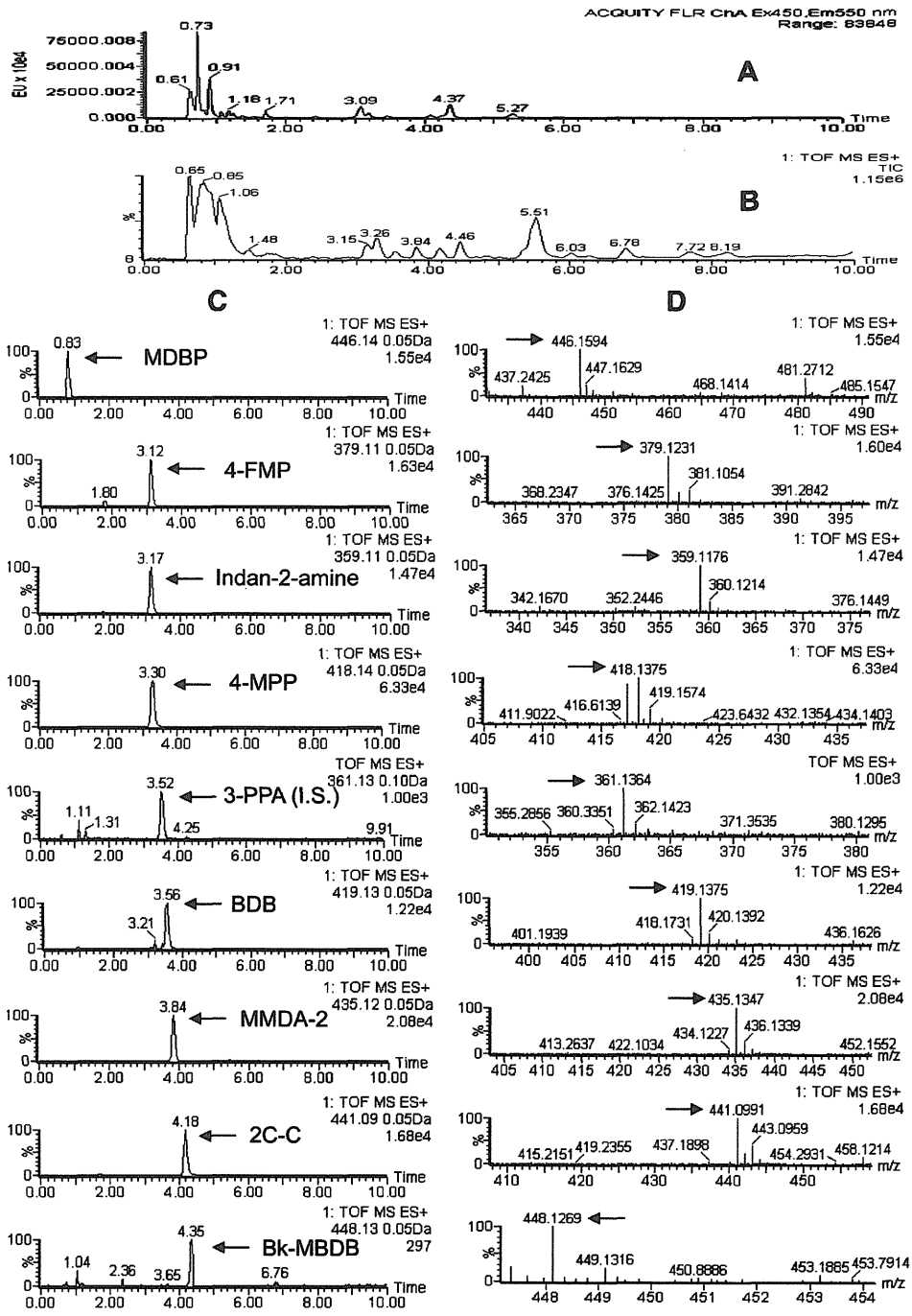


Fig. 4 Chromatograms obtained from FL and TOF-MS detection, and mass spectra for the derivatives of authentic designated substances. **A**, FL; **B**, TIC; **C**, SIC; **D**, mass spectra. The UPLC-FL-ESI-TOF-MS conditions used are described in Table 1

concentrations. The determination at each concentration was repeated five times. A good calibration curve was obtained for each designated substance.

The accuracies (%) and precisions (CVs, %) for three different concentrations were also evaluated using intraday and interday assays. As shown in Table 3, the accuracies of the intraday and interday determinations were 89.6–113.1% and 92.2–115.2%, respectively. The CVs of the intraday and interday determinations were 1.69–12.97% and 1.94–10.87%, respectively. Acceptable accuracies and precisions were obtained at the three different concentrations for all of the designated substances.

Application of the method to real products containing the designated substances

The designated substances in some products obtained from an adult shop and via the Internet were determined by the

Fig. 4 (continued)

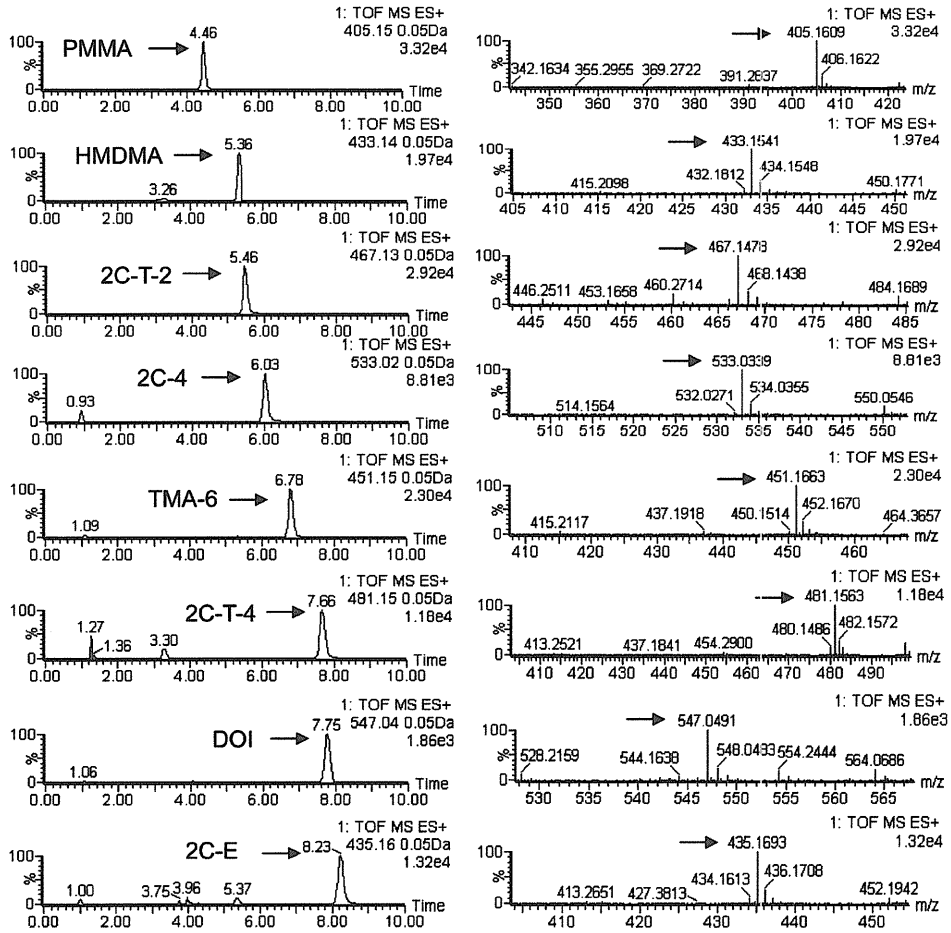


Table 2 Calibration curves for DBD-designated substances using the proposed method

Designated substance	Calibration range (pmol)	Linear equation	Linearity (R^2)
MDBP	0.05–5	y = 1.33x + 0.162	0.9985
4-FMP	0.3–5	y = 0.693x + 0.104	0.9999
Indan-2-amine	0.05–5	y = 0.772x + 0.124	0.9994
4-MPP	0.05–5	y = 13.2x + 1.04	0.9996
BDB	0.05–5	y = 3.62x + 0.469	0.9992
MMDA-2	0.1–5	y = 1.74x + 0.200	0.9996
2C-C	0.05–5	y = 2.55x + 0.278	0.9994
Bk-MBDB	3–12.5	y = 0.00880x − 0.0170	0.9834
PMMA	0.05–5	y = 2.42x + 0.251	0.9993
HMDMA	0.05–5	y = 2.68x − 0.0920	0.9997
2C-T-2	0.05–5	y = 3.21x + 0.0600	0.9999
2C-I	0.05–5	y = 3.29x + 0.383	0.9996
TMA-6	0.05–5	y = 4.30x + 0.592	0.9995
2C-T-4	0.05–5	y = 3.89x + 0.600	0.9996
DOI	0.2–5	y = 0.652x + 0.0580	0.9998
2C-E	0.1–5	y = 4.56x + 0.756	0.9995

proposed procedure. The designated substances in a pale yellow powder (Product 1), a mushroom-like substance (Product 2), and a colorless liquid (Product 3) were simply extracted with water-miscible solvents, labeled with DBD-F, and determined by UPLC-ESI-TOF-MS, as described in the "Experimental" section. The typical chromatograms obtained from these three products are shown in Fig. 5. BDB, MMDA-2, and 2C-I were clearly identified in Products 1, 2, and 3, respectively. The results show that the proposed method seems to be applicable to various products.

Determining designated substances spiked into healthy human plasma and urine

Designated substances spiked into healthy human plasma and urine were determined to test the applicability of the present method to biological samples. After deproteinization with acetonitrile, the designated substances in the biological samples were labeled with DBD-F and determined by UPLC-ESI-TOF-MS. As expected from the results obtained for authentic substances (see Fig. 4A), FL determination appears to be rather difficult for complex

Table 3 Accuracies and precisions of the proposed method (evaluated by performing intraday and interday assays) for the designated substances at three different concentrations

Designated substance	Amount (pmol/mL)	Intraday assay			Interday assay		
		Mean±SD	CV% (n=5)	Accuracy (%)	Mean±SD	CV% (n=5)	Accuracy (%)
MDBP	50	45±4.0	7.41	89.60	47±6.0	3.07	94.40
	100	113±3.0	8.81	113.1	113±6.0	1.96	113.3
	500	498±10	3.59	99.64	516±6.0	2.73	103.1
4-FMP	50	49±1.0	5.79	97.00	49±9.0	8.28	97.80
	100	104±4.0	8.68	103.8	108±4.0	7.64	108.4
	500	500±14	5.38	99.90	510±1.0	2.48	102.0
Indan-2-amine	50	47±9.0	7.04	94.60	48±8.0	7.24	95.40
	100	109±5.0	6.11	108.7	108±8.0	3.58	107.7
	500	499±11	4.67	99.76	534±17	5.47	106.7
4-MPP	50	48±3.0	2.57	96.60	52±1.0	5.55	104.8
	100	108±5.0	8.54	107.8	115±3.0	5.92	115.2
	500	499±8.0	2.43	99.78	545±31	6.53	108.9
BDB	50	48±5.0	5.79	95.60	52±5.0	4.72	103.2
	100	111±2.0	5.14	111.0	115±4.0	3.13	115.2
	500	498±13	1.69	99.68	537±22	5.46	107.4
MMDA-2	50	48±5.0	5.47	96.60	50±5.0	5.13	100.2
	100	108±1.0	7.84	108.1	111±4.0	3.50	111.0
	500	499±13	4.06	99.76	519±7.0	2.72	103.7
2C-C	50	48±3.0	7.02	95.00	49±4.0	5.89	99.20
	100	109±2.0	8.06	109.2	111±2.0	4.14	110.6
	500	499±13	3.90	99.74	513±4.0	2.18	102.6
Bk-MBDB	750	732±265	12.97	97.64	704±249	10.59	93.87
	1000	956±294	12.91	95.60	922±269	10.06	92.23
	1250	1296±263	6.17	103.6	1226±308	10.87	98.08
PMMA	50	47±4.0	4.78	94.20	48±4.0	4.75	95.60
	100	109±2.0	7.59	109.2	109±4.0	2.85	108.7
	500	499±16	4.56	99.74	515±5.0	2.22	103.0
HMDMA	50	46±7.0	6.16	92.60	49±7.0	6.04	97.40
	100	105±11	6.62	105.2	108±9.0	4.29	108.1
	500	499±28	4.89	99.88	536±31	5.03	107.1
2C-T-2	50	49±3.0	6.88	97.60	49±1.0	4.28	97.80
	100	103±7.0	6.94	103.4	106±3.0	3.58	106.1
	500	499±11	2.22	99.90	516±9.0	1.94	103.1
2C-I	50	48±6.0	4.14	96.20	52±5.0	5.01	103.6
	100	108±1.0	6.22	107.8	113±3.0	4.42	112.5
	500	499±13	4.23	99.78	521±8.0	3.14	104.1
TMA-6	50	48±6.0	5.91	96.00	50±8.0	2.48	100.0
	100	109±1.0	7.98	109.0	111±5.0	4.24	111.2
	500	499±16	5.10	99.74	519±6.0	2.99	103.8
2C-T-4	50	48±8.0	5.52	96.60	50±9.0	5.49	100.6
	100	108±2.0	5.15	107.7	109±6.0	4.79	109.4
	500	499±17	3.58	99.78	513±1.0	2.54	102.6
DOI	50	50±2.0	6.89	99.40	51±2.0	7.16	102.6
	100	106±4.0	8.86	106.0	109±2.0	6.32	108.7
	500	499±11	3.30	99.80	523±13	3.48	104.6
2C-E	50	48±9.0	6.31	96.40	52±9.0	4.72	104.2
	100	108±4.0	7.45	108.4	110±8.0	3.63	109.7
	500	499±9.0	4.19	99.76	525±4.0	3.18	104.9

SD, standard deviation; *CV*, coefficient of variation

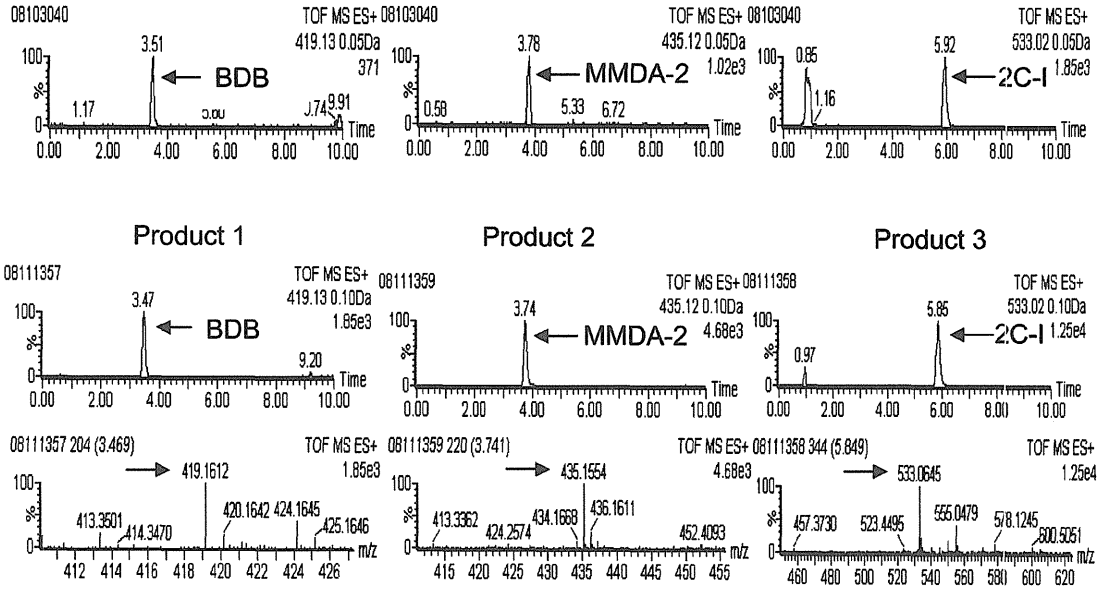


Fig. 5 Mass chromatograms and mass spectra obtained from real products containing the designated substances. The UPLC-ESI-TOF-MS conditions used are described in Table 1

matrices. Indeed, the determinations of several designated substances in the human plasma and urine showed interference from peaks due to endogenous substances (data not shown). This interference mainly occurred for the hydrophilic substances that eluted earlier than 2.0 min. Although it may be possible to avoid this interference by optimizing the elution conditions, the determination based on a short run time failed.

The interference from endogenous substances was essentially the same for TOF-MS detection. However, because in this case the technique involves the selected-ion monitoring of each $[M+H]^+$, the interference was generally negligible. Indeed, the peaks corresponding to the 16 designated substances were completely detected by ESI-TOF-MS without any interference from the endogenous substances in the samples. The derivatized peaks eluted from 0.82 min to 8.2 min, and the sensitivity was higher than that obtained with FL detection. Based upon these observations, the UPLC-ESI-TOF-MS system was adopted for the simultaneous determination of the designated substances in human plasma and urine. All of the designated substances were identified using the TOF-MS system. Table 4 shows the accuracies (%) and the precisions (CV%) for three different concentrations of each substance spiked into human plasma. As shown in Table 4, good accuracies (87.7–107.4 %) and precisions (0.36–7.81 %) were obtained using the proposed method.

The limits of detection (LODs) and limits of quantification (LOQs) in human plasma and urine are shown in Table 5. Good sensitivities were obtained with the present method. However, wide variations were also observed. The LODs and LOQs in 0.1 mL of the biological samples were 0.30–150 pmol and 1.0–500 pmol, respectively, and the detection sensitivities were almost comparable for both human plasma and urine.

Separation and ESI-TOF-MS detection of the designated substances without DBD-F labeling was also possible using the present UPLC-ESI-TOF-MS system. However, the chromatographic run time required approximately doubled; the first substance eluted was MDBP at 0.9 min, and the last to be eluted was 2C-T-4 at 14.0 min. Furthermore, the detection sensitivities in this case were 3–20 times lower than those obtained with DBD labeling, except in the cases of Bk-MBDB and 4-FMP. The LODs for native Bk-MBDB and 4-FMP were almost comparable to those of the derivatives. The relatively low sensitivities of these derivatives may be due to low chemical reaction yields. However, the exact reason is not obvious.

Compared with the GC-MS method [49], the present UPLC-ESI-TOF-MS method coupled with FL derivatization of the designated substances is superior in terms of sensitivity, selectivity, ease of sample pretreatment, and chromatographic run time. Because the high efficiency of the proposed method was identified based on an analysis of human plasma and urine, the method appears to be promising for determining designated substances in not only human plasma and urine but also in various biological specimens.

Table 4 Determination of designated substances spiked into human plasma (n=5)

Designated substance	Spiked amount (pmol/0.1mL)	Detection amount (pmol/0.1mL)	Accuracy (%) mean±SD	CV (%) (n=5)
MDBP	100	88±4.0	87.7±3.7	4.24
	300	316±16	105.5±5.4	5.19
	500	495±12	98.9±2.4	2.46
4-FMP	100	103±1.0	103.5±0.41	0.39
	300	287±18	95.5±6.2	6.46
	500	516±37	103.2±7.3	7.09
Indan-2-amine	100	102±6.0	102.1±5.5	5.42
	300	292±24	97.3±7.9	8.11
	500	507±24	101.5±4.9	4.81
4-MPP	100	99±2.0	99.0±2.3	2.33
	300	307±10	102.4±3.4	3.30
	500	501±13	100.2±2.6	2.56
BDB	100	107±2.0	107.4±2.4	2.22
	300	298±20	99.3±6.7	6.78
	500	506±2.0	101.2±0.4	0.36
MMDA-2	100	106±2.0	106.3±2.0	1.90
	300	298±23	99.4±7.8	7.81
	500	504±15	100.9±3.1	3.06
2C-C	100	100±4.0	100.4±3.6	3.57
	300	293±10	97.8±3.1	3.13
	500	505±13	101.0±2.6	2.55
Bk-MBDB	500	497±3.0	99.4±0.67	0.68
	750	762±16	101.6±2.1	2.04
	1000	986±17	98.6±1.7	1.75
PMMA	100	105±3.0	105.2±2.9	2.81
	300	293±5.0	97.5±1.7	1.70
	500	507±15	101.5±3.0	2.95
HMDMA	100	107±7.0	106.6±6.5	6.07
	300	288±4.0	96.1±1.4	1.44
	500	507±3.0	101.4±0.60	0.59
2C-T-2	100	99±5.0	99.0±4.5	4.53
	300	301±7.0	100.4±2.5	2.46
	500	505±21	101.0±4.2	4.13
2C-I	100	98±6.0	98.0±6.1	6.21
	300	304±5.0	101.5±1.8	1.77
	500	504±19	100.7±3.9	3.86
TMA-6	100	101±4.0	100.6±3.7	3.70
	300	304±15	101.4±5.1	5.02
	500	500±17	100.1±3.3	3.34
2C-T-4	100	102±2.0	101.8±2.2	2.18
	300	296±6.0	98.8±1.9	1.96
	500	500±12	99.9±2.3	2.31
DOI	100	96±6.0	96.1±5.9	6.17
	300	309±8.0	102.9±2.5	2.45
	500	501±14	100.2±2.7	2.73
2C-E	100	105±1.0	104.8±1.2	1.18
	300	293±23	97.6±7.7	7.92
	500	506±10	101.2±2.0	1.96

SD, standard deviation; CV, coefficient of variation

Table 5 Limits of detection and limits of quantification for designated substances spiked into human plasma and urine

Designated substance	Plasma		Urine	
	LOD (pmol/0.1mL)	LOQ (pmol/0.1mL)	LOD (pmol/0.1mL)	LOQ (pmol/0.1mL)
MDBP	2.5	8.0	15	50
4-FMP	30	0.11*	15	50
Indan-2-amine	5.0	17	3.0	10
4-MPP	0.40	1.3	0.30	1.0
BDB	3.0	10	3.0	10
MMDA-2	5.0	17	5.0	15
2C-C	2.0	7.0	2.0	7.0
Bk-MBDB	0.15*	0.50*	0.15*	0.50*
PMMA	2.2	7.5	3.0	9.0
HMDMA	3.0	10	2.0	6.0
2C-T-2	3.0	10	3.0	10
2C-I	2.0	8.0	1.5	5.0
TMA-6	2.0	7.0	1.5	5.0
2C-T-4	2.0	7.0	3.0	10
DOI	10	35	8.0	25
2C-E	5.0	15	6.0	20

LOD, limit of detection (S/N = 3); LOQ, limit of quantification (S/N = 10).

* nmol/0.1 mL

Conclusion

This paper describes the simultaneous determination of phenethylamine-type and piperazine-type designated substances after labeling with DBD-F. The resulting derivatives were simultaneously separated within 8.5 min by UPLC and sensitively determined by both FL and ESI-TOF-MS. Although individual determinations were possible using the FL detection method, the determination of all of the designated substances in human plasma and urine using FL detection failed due to interference from endogenous substances. On the other hand, a sensitive and reliable determination was carried out by ESI-TOF-MS. Because the proposed method allows trace detection of the designated substances in human plasma and urine, it seems to be applicable to various biological specimens. Consequently, we believe that the present method is useful for the qualitative and quantitative analysis of various designated substances in biological specimens.

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