Balikova M. Nonfatal and fatal DOB (2,5-dimethoxy-4-bromoamphetamine) overdose. *Forensic Science International* 2005; **153**(1): 85–91.

Carrera V, Sabater E, Vilanova E and Sogorb MA. A simple and rapid HPLC–MS method for the simultaneous determination of epinephrine, norepinephrine, dopamine and 5-hydroxytryptamine: application to the secretion of bovine chromaffin cell cultures. *Journal of Chromatography B* 2007; **847**: 88–94.

Glennon RA and Rosecrans JA. Indolealkylamine and phenalkylamine hallucinogens: a brief overview. *Neuroscience and Biobehavioral Reviews* 1982; **6**: 489–497.

Gottardo R, Bortolotti F, De Paoli G, Pascali JP, Miksík I and Tagliaro F. Hair analysis for illicit drugs by using capillary zone electrophoresis–electrospray ionization–ion trap mass spectrometry. *Journal of Chromatography A* 2007; **1159**: 185–189.

Jacob III P and Shulgin AT. Structure–activity relationships of the classic hallucinogens and their analogs. *NIDA Research Monograph* 1994; **146**: 74–91.

Jimenez C, De la Torre R, Ventura M, Segura J and Ventura R. Stability studies of amphetamine and ephedrine derivatives in urine. *Journal of Chromatography B* 2006; **843**: 84–93.

Kanai K, Takekawa K, Kumamoto T, Ishikawa T and Ohmori T. Simultaneous analysis of six phenethylamine-type designer drugs by TLC, LC-MS, and GC-MS. *Forensic Toxicology* 2008; **26**(1): 6–12.

Kikura-Hanajiri R, Hayashi M, Saisho K and Goda Y. Simultaneous determination of nineteen hallucinogenic tryptamines/β-calbolines and phenethylamines using gas chromatography–mass spectrometry and liquid chromatography–electrospray ionisation–mass spectrometry. *Journal of Chromatography B* 2005; **825**: 29–37.

Kikura-Hanajiri R, Kawamura M, Saisho K, Kodama Y and Goda Y. The disposition into hair of new designer drugs; methylone, MBDB and methcathinone. *Journal of Chromatography B* 2007; **855**: 121–126.

Leung GNW, Leung DKK, Wan TSN and Wong CHF. High throughput screening of sub-ppb levels of basic drugs in equine plasma by liquid chromatography–tandem mass spectrometry. *Journal of chromatography A* 2007; **1156**: 271–279.

Lin L-C, Lin C-H, Liu J-T and Chou S-H. Identification of 2,5-dimethoxy-4-ethylthiophenethylamine and its metabolites in the urine of rats by gas chromatography–mass spectrometry. *Journal of Chromatography B* 2003; **798**: 241–247.

Marek GJ and Aghajanian GK. Indoleamine and the phenethylamine hallucinogens: mechanisms of psychotomimetic action. *Drug and Alcohol Dependence* 1998; **51**(1–2): 189–198.

Min JZ, Shimizu Y, Toyo'oka T, Inagaki S, Kikura-Hanajiri R and Goda Y. Simultaneous determination of 11 designated hallucinogenic phenethylamines by ultra-fast liquid chromatography with fluorescence detection. *Journal of Chromatography B* 2008; **873**: 187–194.

Min JZ, Hatanaka S, Toyo'oka T, Inagaki S, Kikura-Hanajiri R and Goda Y. 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. *Analytical and Bioanalytical Chemistry* 2009; **395**: 1411–1422.

Nagai F, Nonaka R, Satoh H and Kamimura K. The effects of non-medically used psychoactive drugs on monoamine neurotransmission in rat brain. *European Journal of Pharmacology* 2007; **559**(2–3): 132–137.

Nakamura S, Wada M, Crabtree BL, Reeves PM, Montgomery JH, Byrd HJ, Harada S, Kuroda N and Nakashima K. A sensitive semi-micro column HPLC method with peroxyoxalate chemiluminescence detection and column switching for determination of MDMA-related compounds in hair. *Analytical and Bioanalytical Chemistry* 2007; **387**: 1983–1990.

Nakashima K. High-performance liquid chromatographic analysis of drugs of abuse in biologic samples. *Journal Health Science* 2005; **51**: 272–277.

Nicholos DE. Structure–activity relationships of phenethylamine hallucinogens. *Journal of Pharmaceutical Sciences* 1981; **70**: 839–849.

Nishida M, Yashiki M, Namera A and Kimura K. Single hair analysis of methamphetamine and amphetamine by solid phase microextraction coupled with in matrix derivatization. *Journal of Chromatography B* 2006; **842**: 106–110.

Satou K. Rapid analysis of drug. *Bunseki* 2009; **8**: 428–429.

Spoerke DG, Hall AH. Plants and mushrooms of abuse. *Emergency Medicine Clinics of North America* 1990; **8**(3): 579–593.

Tanaka E, Kamata T, Katagi M, Tsuchihashi H and Honda K. A fatal poisoning with 5-methoxy-*N*,*N*-diisopropyltryptamine, Foxy. *Forensic Science International* 2006; **163**: 152–154.

Theobald DS and Maurer HH. Studies on the metabolism and toxicological detection of the designer drug 4-ethyl-2,5-dimethoxy-β-phenethylamine (2C-E) in rat urine using gas chromatographic–mass spectrometric techniques. *Journal of Chromatography B* 2006; **842**: 76–90.

Theobald DS, Staack RF, Puetz M and Maurer HH. New designer drug 2,5-dimethoxy-4-ethylthio-beta-phenethylamine (2C-T-2): studies on its metabolism and toxicological detection in rat urine using gas chromatography/mass spectrometry. *Journal of Mass Spectrometry* 2005; **40**: 1157–1172.

Tomita M, Nakashima MN, Wada M, Nakashima K. A simple and sensitive HPLC-fluorescence method for quantification of MDMA and MDA in blood with 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride (DIB-Cl) as a label. *Biomedical Chromatography* 2006; **20**: 1380–1385.

US National Institute of Justice. *Color Test Reagents/Kits for Preliminary Identification of Drugs of Abuse.* Available from: http://www.ncjrs.gov/pdffiles1/nij/183258.pdf.2000 (accessed 25 August 2009).

Wu Y-H, Lin K-L, Chen S-C, Chang Y-Z. Simultaneous quantitative determination of amphetamines, ketamine, opiates and metabolites in human hair by gas chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry* 2008; **22**: 887–897.

Yamamoto J. MHLW's countermeasures for uncontrolled substance and future tasks. *Japanese Journal of Toxicology* 2004; **17**: 245–250.

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Research Article

HPLC enantioseparation of α,α-diphenyl-2-pyrrolidinemethanol and methylphenidate using a chiral fluorescent derivatization reagent and its application to the analysis of rat plasma

Enantioseparation of α,α-diphenyl-2-pyrrolidinemethanol (D2PM) and methylphenidate (MPH; Ritalin®) using (*R*)-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole as the chiral derivatization reagent has been achieved for the first time, and a simple, reliable detection method using HPLC with fluorescence detection has been developed. D2PM and MPH have been derivatized with (*R*)-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole at 55°C for 15 min. The derivatives of D2PM and MPH have been separated, completely and rapidly, using a reversed-phase system within 16 min (resolution factor (R_s) = 1.60 and 2.53, respectively). The detection limits of (*R*)- and (*S*)-D2PM were found to be 6.8 and 13 ng/mL, respectively, and those of D- and L-*threo*-MPH were 61 and 66 ng/mL, respectively ($S/N = 3$). The proposed method was successfully applied to the analysis of rat plasma, where the rats were separately dosed with D2PM and MPH (Ritalin).

Keywords: α,α-Diphenyl-2-pyrrolidinemethanol / Enantioseparation / Fluorescence detection / HPLC / Methylphenidate
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1 Introduction

Many types of illegal drugs such as phenethylamines, tryptamines, and piperazines are currently easily obtainable *via* the Internet and on the streets. Considering the frequent occurrence of health hazards, including fatal accidents due to the increase in drug abuse, this wide illegal distribution has become a serious social problem. In recent years, increasing drug abuse, especially among young people, has also become a serious problem. The use of illegal drugs is alarming not only because they can be potentially addictive but also because it may lead to the abuse of narcotic drugs, stimulant drugs, and cannabis abuse. To avoid the widespread distribution of illegal drugs in Japan, a new regulatory system was introduced and many substances have been controlled as designated substances (Shitei-Yakubutsu) since April 2007 by the revision of the Pharmaceutical Affairs Law. This brought about a regulation/control on designated substances such as chemical agents and plants that cause central nervous system stimulants or suppressants, or are hallucinogens. However, structurally similar substances continually appear on the market, and this revision cannot deal with the increasing number of harmful substances already available in the market. α,α-Diphenyl-2-pyrrolidinemethanol (D2PM), which is a phenethylamine analog and was listed as a designated substance in October 2009 [1], is one such substance, and its widespread distribution as a new illegal drug is a matter of great concern. To prevent the distribution and spread of this substance, it is essential to establish an analytical method beforehand.

On the other hand, methylphenidate (methyl α-phenyl-2-piperidineacetate hydrochloride, MPH; Ritalin®) is another phenethylamine analog, which has a chemical structure similar to D2PM. MPH is a psychostimulant used for narcoleptic patients and is also prescribed for the treatment of attention-deficit hyperactivity disorder among children [2]. However, MPH is known to induce exhilaration and euphoria, and can be addictive because it is habit-forming. Because MPH is a prescription medicine, many drug abusers having the desire to obtain and use it engage in malingering,

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Abbreviations: CSP, chiral stationary phase; **D2PM,** α,α-diphenyl-2-pyrrolidinemethanol; **(*R*)-DBD-PyNCS,** (*R*)-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole; **FL,** fluorescence detection; **MPH,** methylphenidate hydrochloride; **PBH,** α-(4-piperidyl) benzhydrol; **R_s,** resolution factor

use forged prescriptions, steal, or obtain it illegally through the Internet. In Japan, Ritalin dependence and abuse emerged as a serious social issue in 2007. Since then, the regulations were changed so that the doctor who could prescribe Ritalin and the pharmacist who could dispense it were both registered and this data was classified.

D2PM has one chiral center in its structure, whereas MPH has two, and their enantiomers possibly exhibit distinct pharmacokinetic and pharmacodynamic properties in humans. It has been reported that D-*threo*-MPH exhibits 5–38 times greater pharmacological activity than L-*threo*-MPH [3, 4]. Moreover, enantiomeric separations of such substances are significant because the manufacturing method and the producer countries can be predicted from the ratio of enantiomers and impurities in case of illegally manufactured drugs.

In the past, several analytical methods were established to quantify illegal drugs, including phenethylamines and their analogs. Most of these were based on GC–MS, LC–MS, or CE–MS [5–10]. Although these methods, which enable us to confirm the molecular weight and to obtain structural information of the drugs, are excellent for the determination of phenethylamines, the analytical system, including the mass spectrometer, is extremely expensive, and cannot be used in many laboratories. Therefore, simple and highly sensitive analytical tools without MS are strongly desired.

There has been no report on the enantioseparation of D2PM in the past. However, a method using polysaccharide or α_1-acid glycoprotein as chiral stationary phase (CSP) of HPLC [11–14] and the CE method using cyclodextrins as running buffer additives [15] for the enantioseparation of MPH have been reported. These methods mainly use UV absorbance detection. Thus, they are less sensitive and are not practical for analyzing biological samples. On the other hand, the chiral derivatization method does not require the comparatively expensive analytical column containing CSP and it can be analyzed using a normal HPLC column such as an octadecyl silica column. Furthermore, using a fluorescence derivatization method such as HPLC–fluorescence detection (HPLC–FL) has the advantage that highly sensitive detection would be achieved.

In this study, we have developed a method for the enantioseparation of D2PM and MPH using a chiral derivatization reagent, (*R*)-(−)-4-(*N*,*N*-dimethylaminosulfonyl)-7-(3-isothiocyanatopyrrolidin-1-yl)-2,1,3-benzoxadiazole ((*R*)-DBD-PyNCS) having a benzofurazan (2,1,3-benzoxadiazole) fluorophore [16, 17], and established a detection method using HPLC–FL without MS detection. (*R*)- and (*S*)-DBD-PyNCS have proven to be very effective for total resolution of the racemic mixture of amino acids and thiol compounds by HPLC in the previous studies [18–21]. The aim of this study was to develop a simple, rapid, reliable, and sensitive method for the detection of D2PM and MPH as well as their simultaneous enantioseparations by using HPLC–FL. To the best of our knowledge, this is the first report of the enantiomeric determination of D2PM and MPH using a chiral derivatization method. Application to the analysis of rat plasma after the rats were given an oral dose of D2PM and MPH (Ritalin®) were also described in this paper.

2 Materials and methods

2.1 Materials and reagents

(*R*)-(+)-α,α-Diphenyl-2-pyrrolidinemethanol ((*R*)-D2PM), (*S*)-(−)-α,α-diphenyl-2-pyrrolidinemethanol ((*S*)-D2PM), α-(4-piperidyl)benzhydrol (PBH, internal standard 1; I.S.1), 2,2-diphenylethylamine (I.S.2), (*R*)-DBD-PyNCS, (*S*)-DBD-PyNCS, and triethylamine were purchased from Tokyo Kasei (Tokyo, Japan). Racemic methylphenidate hydrochloride (DL-*threo*-MPH), D-*threo*-[2R,2′R]-methylphenidate hydrochloride (D-*threo*-MPH), and L-*threo*-[2S,2′S]-methylphenidate hydrochloride (L-*threo*-MPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ritalin (powdered medicine, containing 1% racemic MPH) was purchased from Novartis Pharmaceuticals (Tokyo, Japan). Acetonitrile (CH_3CN), methanol (CH_3OH), and diethyl ether were obtained from Kanto Kagaku (Tokyo, Japan). Ethanol was purchased from Kishida Chemical (Osaka, Japan). Formic acid (HCOOH) was of HPLC grade (Wako Pure Chemicals). Saline was purchased from Otsuka Pharmaceutical Factory (Naruto, Japan) and heparin sodium injection (5000 units/5 mL), from Ajinomoto (Tokyo, Japan). All other reagents were of analytical-reagent grade and were used without further purification.

2.2 Experimental animals

Healthy male Wistar rats (7 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). The animal experiments were conducted according to the guidelines of the Ethical Committee for Animal Experimentation at University of Shizuoka. The rats were housed at a constant room temperature (24 ± 1°C) with an alternating 12 h light/dark cycle, and with free access to food and water. Before the experiment, they were made to fast for half a day. Racemic, (*R*)-, and (*S*)-D2PM (20 or 40 mg/kg) and Ritalin, D-, and L-*threo*-MPH dissolved in saline (10, 20, or 40 mg/kg as MPH) were orally administered to the rats. The control rats were orally administered with saline instead of D2PM or MPH. They were anesthetized with diethyl ether before collecting of blood samples. Blood was collected through a syringe from the descending aorta, transferred to a heparinized polyethylene tube and centrifuged at 3000 × *g* for 10 min to obtain plasma.

2.3 HPLC–FL conditions

A Shimadzu (Kyoto, Japan) HPLC system consisting of three LC-20AD pumps, a degasser (DGU-20A_3), an

auto-injector (SIL-20AC), a column oven (CTO-20AC), an RF-10A_{XL} fluorescence detector, and a CBM-20A system controller was used. An XBridge^{TM} C_{18} column (4.6 mm id × 150 mm, 3.5 μm; Waters, Milford, MA, USA) was used for reversed-phase HPLC analysis. The fluorescence detector was operated at excitation and emission wavelengths of 450 and 560 nm, respectively. The flow rate of the mobile phase was set at 1 mL/min, and the column temperature was 40°C. Isocratic separations were achieved using H_2O–CH_3OH–HCOOH (35:65:0.1, v/v/v) as the mobile phase, unless otherwise mentioned. The injection volume was fixed at 20 μL. The peak areas obtained from the fluorescence detector were calculated using LC Solution software ver. 1.21 SP1 (Shimadzu).

2.4 Derivatization of D2PM and MPH

One hundred microliters of 5.0 mM (*R*)-DBD-PyNCS in CH_3CN and 5 μL of triethylamine were added to 100 μL of the sample solution containing D2PM and MPH (0–500 μM) in H_2O–CH_3CN (1:1). The mixture was heated at 55°C for 15 min and then diluted three times by adding the mobile phase. The solution was cooled on ice and an aliquot (20 μL) was injected into the HPLC–FL system.

2.5 Analysis of rat plasma by HPLC–FL

Five microliters of 50 μM internal standard was added to the 50 μL of rat plasma. The solution was deproteinized by adding 250 μL of CH_3CN, vortex-mixed for 1 min and centrifuged at 14 000 rpm for 10 min at 4°C. The obtained supernatant (280 μL) was transferred to another tube and evaporated to dryness under a gentle stream of nitrogen. Subsequently, 50 μL of H_2O–CH_3CN (1:1) and 3.0 μL of triethylamine were serially added to the tube and reacted with 50 μL CH_3CN solutions of (*R*)-DBD-PyNCS. The reaction mixture was heated at 55°C for 15 min. The supernatant obtained was filtered using a Millex-LG filter (0.20 μm, 4 mm id; Nihon Millipore, Tokyo, Japan), and an aliquot of the filtrate (20 μL) was injected into the HPLC–FL system.

2.6 Method validation

The calibration curves were obtained by spiking a series of blank rat plasma with (*R*)- and (*S*)-D2PM to give concentrations of 0.50–50 μM, and with D- and L-*threo*-MPH to give concentrations of 12.5–250 μM. The curves were constructed by plotting the peak area ratios of (*R*)-D2PM, (*S*)-D2PM, D-*threo*-MPH, and L-*threo*-MPH relative to the internal standard against the injected amounts. The curves were plotted for five different concentrations. The determinations were repeated five times a day and between days, and the accuracy (%) and precision (CVs, %) of intra- and inter-day assays were evaluated.

3 Results and discussion

3.1 Enantioseparation of D2PM and MPH using chiral derivatization reagent

Figure 1 shows the chemical structures of D2PM and MPH. We have tried to achieve enantioseparation of D2PM and MPH using a chiral fluorescence derivatization reagent,

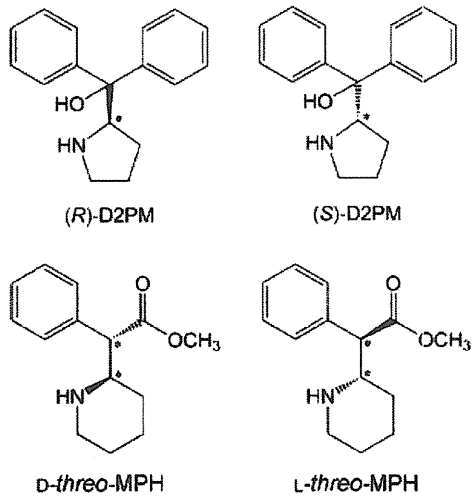

Figure 1. Chemical structures of (*R*)-D2PM, (*S*)-D2PM, D-*threo*-MPH, and L-*threo*-MPH.

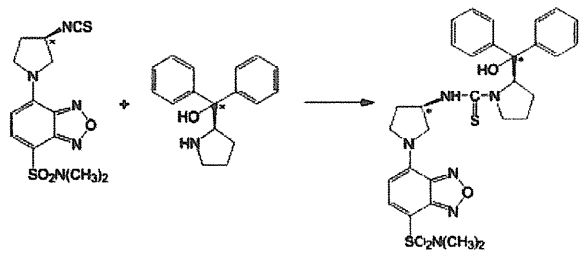

Figure 2. Derivatization scheme for (*R*)-D2PM with (*R*)-DBD-PyNCS.

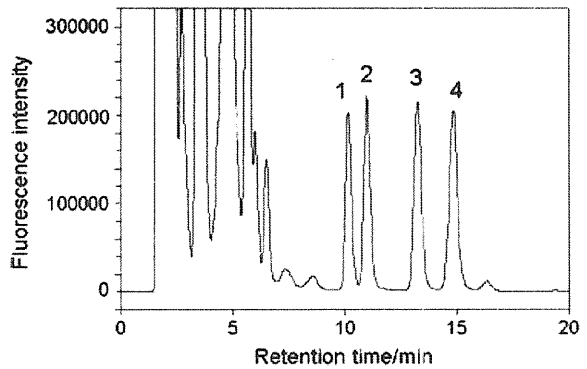

Figure 3. Enantioseparation of D2PM and MPH using (*R*)-DBD-PyNCS as chiral derivatization reagent. Peaks: (1) D-*threo*-MPH, (2) L-*threo*-MPH, (3) (*R*)-D2PM, (4) (*S*)-D2PM.

(*R*)-DBD-PyNCS [16–21]. The derivatization reaction of D2PM with (*R*)-DBD-PyNCS is shown in Fig. 2. It was difficult to achieve enantioseparation of both D2PM and MPH using H_2O–CH_3CN as the mobile phase. The resolution factor (R_s) for enantiomeric resolution of MPH was less than 1.35; thus, baseline separation could not be achieved. However, complete enantioseparations of D2PM and MPH was achieved using H_2O–CH_3OH as the mobile phase (Fig. 3). The R_s values of the diastereomers derived from MPH and D2PM were 3.05 and 4.75, respectively (mobile phase: H_2O–CH_3OH–HCOOH (40:60:0.1, v/v/v)). The value of MPH was preferable to that of the previous report using CSP method (R_s = 1.82) [12], even though the retention times of D- and L-*threo*-MPH for both methods are almost same. In addition, using H_2O–CH_3OH–HCOOH (35:65:0.1, v/v/v) as the mobile phase, baseline enantiomeric resolutions of both D2PM and MPH were achieved within 16 min (R_s = 2.53 and 1.60, respectively). Table 1 shows the separation factor (α) and R_s for enantiomeric resolution of D2PM and MPH using (*R*)-DBD-PyNCS as the chiral derivatization reagent. In addition to the improvement of the detection sensitivity because of fluorescence detection, the chiral derivatization method also proved to be beneficial for the enantioseparations of D2PM and MPH.

Table 1. Separation factor (α) and resolution factor (R_s) for enantiomeric resolution of D2PM and MPH using (*R*)-DBD-PyNCS as chiral derivatization reagent

Mobile phase [H_2O–CH_3OH–HCOOH (v/v/v)]	Samples	α	R_s
40:60:0.1	D2PM	1.18	4.75
	MPH	1.12	3.05
35:65:0.1	D2PM	1.60	2.53
	MPH	1.10	1.60

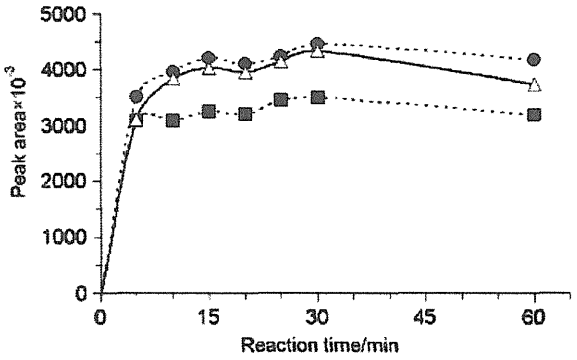

Figure 4. Time courses of the derivatization of (*R*)-D2PM (closed circles with dotted line), (*S*)-D2PM (open triangles with solid line), and PBH (closed squares with dotted line) with (*R*)-DBD-PyNCS.

Moreover, as might be expected, the elution orders of (*R*)- and (*S*)-D2PM as well as those of D- and L-*threo*-MPH were reversed using (*S*)-DBD-PyNCS as the chiral derivatization reagent instead of (*R*)-DBD-PyNCS. In addition, on analyzing underivatized MPH by reversed-phase HPLC, an extremely tailing peak was observed. The peak shapes significantly improved by the derivatization method applied in this study (data not shown), and this phenomenon was considered as one of the merits of using the chiral derivatization method.

3.2 Optimization of detection conditions

The reaction temperature, reaction period, and wavelength were investigated and optimized with respect to (*R*)-DBD-PyNCS with (*R*)- and (*S*)-D2PM. The time courses of the derivatization of (*R*)-, (*S*)-D2PM and PBH (I.S.1) with (*R*)-DBD-PyNCS are presented in Fig. 4. The derivatization reaction was examined in the range of 40–80°C. The

Table 2. Intra- and inter-day validations of the analysis of D2PM and MPH

Samples	Amount (μmol/L)	Intra-day assay			Inter-day assay		
		Mean ± SD[a]	CV%[b] (n = 5)	Accuracy (%)	Mean ± SD[a]	CV%[b] (n = 5)	Accuracy (%)
(*R*)-D2PM	0.5	0.596 ± 0.0345	5.74	119.2	0.661 ± 0.0326	4.93	132.2
	5.0	5.970 ± 0.185	3.09	119.4	6.420 ± 0.457	7.12	128.4
	10.0	10.70 ± 0.577	5.40	106.8	11.20 ± 0.196	1.75	111.8
(*S*)-D2PM	0.5	0.514 ± 0.0322	4.19	102.6	0.558 ± 0.0320	5.72	111.6
	5.0	4.740 ± 0.157	3.28	94.9	5.150 ± 0.588	11.4	102.9
	10.0	8.400 ± 0.406	4.83	84.0	8.610 ± 0.183	2.12	86.1
D-*threo*-MPH	12.5	12.4 ± 1.06	8.65	98.8	11.0 ± 0.86	7.78	88.0
	25.0	22.1 ± 0.641	2.89	88.5	25.5 ± 2.81	11.0	101.8
	50.0	49.2 ± 4.49	9.12	98.5	45.5 ± 3.44	7.56	90.9
L-*threo*-MPH	12.5	12.9 ± 1.08	8.30	103.8	12.8 ± 1.43	11.2	102.0
	25.0	25.5 ± 0.555	11.2	102.2	29.4 ± 2.88	9.81	117.5
	50.0	56.8 ± 4.74	8.27	113.7	52.4 ± 3.70	7.07	104.8

a) SD, standard deviation.
b) CV, coefficient of variation.

optimum reaction temperature was 55°C. The optimum reaction period was 15 min, as shown in Fig. 4. (*R*)-DBD-PyNCS reacted with (*R*)- and (*S*)-D2PM with high efficiency, and the peak of underivatized D2PM disappeared almost completely. Furthermore, the optimum excitation and emission wavelengths for fluorescence detection were 450 and 560 nm, respectively.

3.3 Method validation in HPLC–FL analysis of D2PM and MPH

The calibration curves were obtained using blank plasma spiked with (*R*)-D2PM, (*S*)-D2PM, D-*threo*-MPH, and L-*threo*-MPH. The curves obtained by plotting the peak area ratios of (*R*)-D2PM, (*S*)-D2PM, D-*threo*-MPH, and L-*threo*-MPH relative to the internal standard exhibited good linearity ($r^2 > 0.999$). The accuracy and precision of three different concentrations were also evaluated by intra- and inter-day assays. As shown in Table 2, the precision of the intra- and inter-day assays were 2.89–11.2% and 1.75–11.4%, respectively. Reasonable accuracy and precision was obtained for all the three concentrations.

3.4 Analysis of D2PM and MPH in rat plasma

The developed method was applied to the analysis of rat plasma, where the rats were administered an oral dose of 10–40 mg/kg of D2PM or MPH (Ritalin). As shown in Fig. 5A, no endogenous constituents of blank plasma eluted at the retention times of the peaks of (*R*)-D2PM, (*S*)-D2PM, D-*threo*-MPH, L-*threo*-MPH, PBH (I.S.1), or 2,2-diphenylethylamine (I.S.2). Adequate separation and detection was achieved under the current chromatographic conditions (Fig. 5B–D). The pretreatment of rat plasma is quite simple and does not involve complicated steps such as liquid–liquid or solid-phase extraction; only the deproteinization is required. Therefore, the developed method was found to

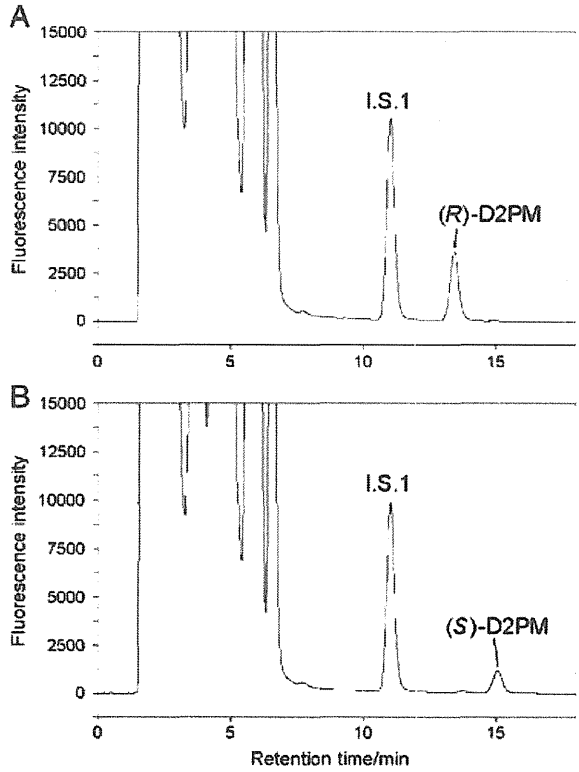

Figure 6. Typical chromatograms of rat plasma following a single oral dose of 20 mg/kg (*R*)-D2PM (A) and (*S*)-D2PM (B) at the time point of 15 min.

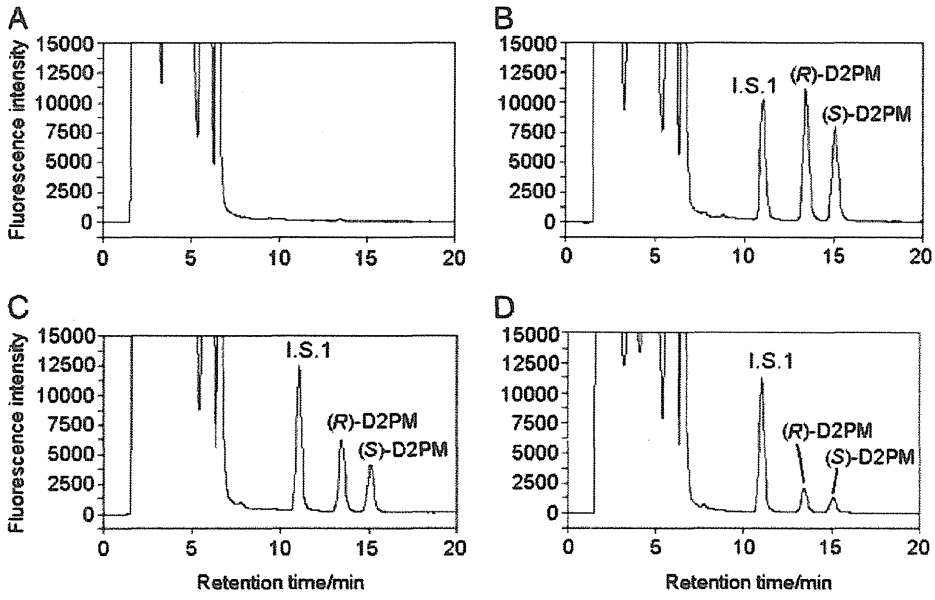

Figure 5. Typical chromatograms of blank rat plasma (A); rat plasma spiked with 1.3 μg/mL (*R*)-D2PM, (*S*)-D2PM, and PBH (B); rat plasma following a single oral dose of 40 mg/kg (*R,S*)-D2PM (C); and 20 mg/kg (*R,S*)-D2PM (D) at the time point of 15 min.

Table 3. Concentrations of (*R*)- and (*S*)-D2PM in rat plasma after 15 min of oral dosing

Amount of drug administered orally	Concentrations in rat plasma (ng/mL)	
	(*R*)-D2PM	(*S*)-D2PM
40 mg/kg (*R,S*)-D2PM	580	540
20 mg/kg (*R,S*)-D2PM	260	260
20 mg/kg (*R*)-D2PM	420	Not detected
20 mg/kg (*S*)-D2PM	Not detected	270

Table 4. Concentrations of D- and L-*threo*-MPH in rat plasma after 15 min of oral dosing

Amount of drug administered orally	Concentrations in rat plasma (ng/mL)	
	D-*threo*-MPH	L-*threo*-MPH
40 mg/kg DL-*threo*-MPH (Ritalin)	1100	890
20 mg/kg DL-*threo*-MPH (Ritalin)	650	590
10 mg/kg D-*threo*-MPH	187	Not detected
10 mg/kg L-*threo*-MPH	Not detected	312

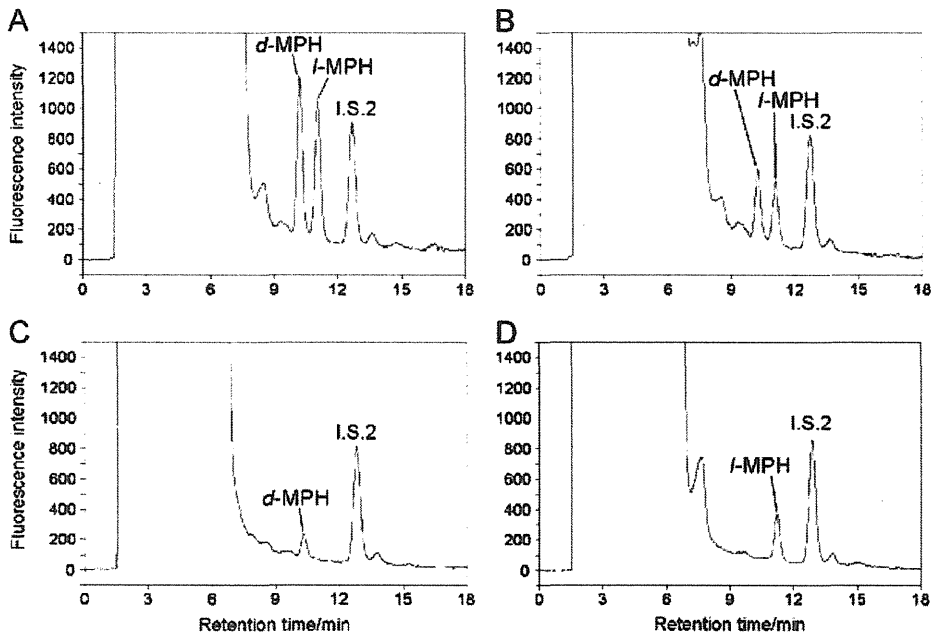

Figure 7. Typical chromatograms of rat plasma following a single oral dose of 40 mg/kg DL-*threo*-MPH (Ritalin) (A), 20 mg/kg DL-*threo*-MPH (B), 10 mg/kg D-*threo*-MPH (C), and 10 mg/kg L-*threo*-MPH (D) at the time point of 15 min.

be selective for D2PM and MPH in plasma without interferences from normal endogenous plasma constituents. The detection limits of (*R*)- and (*S*)-D2PM were found to be 6.8 ng/mL (27 nmol/L) and 13 ng/mL (51 nmol/L), respectively ($S/N = 3$). In addition, the detection limits of D- and L-*threo*-MPH were 61 ng/mL (230 nmol/L) and 66 ng/mL (240 nmol/L), respectively.

Figure 6A and B show the chromatograms of rat plasma following an oral dose of (*R*)-D2PM or (*S*)-D2PM, respectively, 15 min after dosing. In this manner, simple and reliable detection of D2PM, including their simultaneous enantioseparation, was achieved using the developed method. As shown in the figure, racemization of D2PM from (*R*)-antipode to (*S*)-antipode or from (*S*)-antipode to (*R*)-antipode by enzymes present in the living body was not observed. The plasma concentration of both (*R*)-D2PM and (*S*)-D2PM is shown in Table 3.

Similarly, Fig. 7A–D shows the chromatograms of rat plasma following an oral dose of Ritalin (DL-*threo*-MPH), D-*threo*-MPH, and L-*threo*-MPH, respectively. Table 4 shows the concentrations of D-*threo*-MPH and L-*threo*-MPH in rat plasma. The proposed method is expected to be useful to prevent the wide distribution of D2PM as a new illegal drug, and it is also expected to be applicable for the analysis of not only rat plasma but also that of Ritalin abusers.

4 Concluding remarks

In this study, HPLC enantioseparation of the novel recreational drug D2PM and the psychostimulant MPH was accomplished using (*R*)-DBD-PyNCS as the chiral fluorescent derivatization reagent. A simple, reliable method for the detection of (*R*)-D2PM, (*S*)-D2PM, D-*threo*-MPH, and L-*threo*-MPH using HPLC–FL was developed. To the best of our knowledge, this is the first report on the enantioseparation of D2PM and MPH using the chiral derivatization method. The proposed method does not require a relatively expensive chiral column, is quite simple, and does not employ a complicated mass spectrometer. The

method was successfully applied to the analysis of rat plasma, where the rats were administered a single oral dose of D2PM or MPH (Ritalin). Moreover, the method does not involve complicated steps such as liquid–liquid or solid-phase extraction for the pretreatment of plasma samples; only the deproteinization is required. The detection limits of (*R*)- and (*S*)-D2PM were found to be 6.8 and 13 ng/mL, respectively, and those of D- and L-*threo*-MPH were found to be 61 and 66 ng/mL, respectively ($S/N = 3$). The calibration curves exhibited good linearity ($r^2 > 0.999$), and the relative standard deviations of intra- and inter-day variations were below 11.2 and 11.4%, respectively. The proposed method would be applicable in diverse ways not only for drug testing and confirmation of D2PM or MPH intake indicating drug abuse but also for weighing the pharmacokinetic differences between D2PM or MPH enantiomers. This method is expected to be beneficial to society in general.

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The authors have declared no conflict of interest.

5 References

[1] Wood, D. M., Buttun, J., Lidder, S., Ovska, H., Ramsey, J., Holt, D. W., Dargan, P. I., *Clin. Toxicol.* 2008, *46*, 393.

[2] Kimko, H. C., Cross, J. T., Abernethy, D. R., *Clin. Pharmacokinet.* 1999, *37*, 457–470.

[3] Challman, T. D., Lipsky, J. J., *Mayo Clin. Proc.* 2000, *75*, 711–721.

[4] Kula, N. S., Baldessarini, R. J., Tarazi, F. I., Fisser, R., Wang, S., Tromerter, J., Neumeyer, J. L., *Eur. J. Phamacol.* 1999, *385*, 291–294.

[5] Uchiyama, N., Miyazawa, N., Kawarnura, M., Kikura-Hanajiri, R., Goda, Y., *Yakugaku Zasshi* 2010, *130*, 263–270.

[6] Maher, H. M., Awad, T., DeRuiterc, J., Clark, C. R., *Forensic Sci. Int.* 2009, *192*, 115–125.

[7] Takahashi, M., Nagashima, M., Suzuki, J., Seto, T., Yasuda, I., Yoshida, T., *J. Health Sci.* 2008, *54*, 89–96.

[8] Boatto, G., Nieddu, M., Dessi, G., Manconi, P., Cerri, R., *J. Chromatogr. A* 2007, *1159*, 198–202.

[9] Theobald, D. S., Fritschi, G., Maurer, H. H., *J. Chromatogr. B Biomed. Sci. Appl.* 2007, *846*, 374–377.

[10] Theobald, D. S., Fehn, S., Maurer, H. H., *J. Mass Spectrom.* 2005, *40*, 105–116.

[11] Zang, J., Deng, Y., Fang, J., McKay, G., *Pharm. Res.* 2003, *20*, 1881–1884.

[12] Aboul-Enein, H. Y., Ali, I., *Chirality* 2002, *14*, 47–50.

[13] Bakhtiar, R., Ramos, L., Tse, F. L. S., *Rapid Commun. Mass Spectrom.* 2002, *16*, 81–83.

[14] Barbarin, N., Mawhinney, D. B., Black, R., Henion, J., *J. Chromatogr. B* 2003, *783*, 73–83.

[15] Denk, O. M., Watson, D. G., Skellern, G. G., *J. Chromatogr. B* 2001, *761*, 61–68.

[16] Toyo'oka, T., *Curr. Pharm. Anal.* 2005, *1*, 57–64.

[17] Toyo'oka, T., *Bunseki Kagaku* 2002, *51*, 339–358.

[18] Jin, D., Kumar, A. P., Song, G.-C., Lee, Y.-I., *Microchem. J.* 2008, *88*, 62–66.

[19] Jin, D., Zhang, M., Jin, S., Lee, M.-K., Song, G.-C., Back, G., Lee, Y.-I., *Chirality* 2007, *19*, 625–631.

[20] Toyo'oka, T., Liu, Y.-M., *Analyst* 1995, *120*, 385–390.

[21] Toyo'oka, T., Liu, Y.-M., *J. Chromatogr. A* 1995, *689*, 23–30.

無承認無許可医薬品及び違法ドラッグのスクリーニング分析を指向したLC/MSライブラリーの構築

髙橋美津子[*1]，桜井　克巳[*1]，渡部健二朗[*1]，花尻(木倉)瑠理[*2]，合田　幸広[*2]

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Establishment of LC/MS Library for Screening Analysis on "Non-approved or Unauthorized Pharmaceuticals" and "Designated Substances"

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緒　言

インターネットを中心に主に痩身や強壮に有効な医薬品成分やその類似化合物が添加された「痩身や強壮を標榜するいわゆる健康食品」が流通しており，一般市民がこれらを簡便に入手できるようになっている．2002年に中国産の痩身を標榜する健康食品による健康被害が出て以来[1] このような被害は後を絶たず，厚労省の集計では死亡事例を含め796件となった[2]．強壮を標榜する健康食品では2003年頃にED治療薬であるシルデナフィルを含有する製品が出回り，その後ED治療薬類似化合物が添加されている製品が続々と検出されるようになった[3,4]．

また，中枢神経作用を持つ蓋然性があり，社会的にも問題となる「違法ドラッグ」も2000年頃から乱用が広まっており，これらを規制するため2006年に薬事法が改正され，現在指定薬物として45化合物が定められている[5]．

「痩身や強壮を標榜するいわゆる健康食品」及び「違法ドラッグ」の場合，最近の傾向として，含有形態が多成分系である場合が多く，その組み合わせも多様である．したがって，成分が容易に特定出来ない場合が多く，特定できた場合でも同定用標品が市販されていないものがほとんどであるため，標品の入手が困難である場合も多い．また，これらの製品は，医師や薬剤師の関与なしに流通しているため，服用による健康被害の恐れが高く，より迅速で正確な検査結果が必要とされる．

「いわゆる健康食品」及び「違法ドラッグ」のような未知試料中の成分を推定する一つの手法として，分析機器等によるスクリーニング法が考えられる．一般的なスクリーニング法ではガスクロマトグラフ/質量分析計（GC/MS）のライブラリーを用いた手法が多い．しかし，医薬品成分の多くは水溶性で，熱に不安定な物質であるため，液体クロマトグラフ/質量分析計（LC/MS）や液体クロマトグラフ/多波長検出器（LC/PDA）等を用いた手法が有用である．LC/PDAを用いた保持時間及び吸収波長のライブラリーについてはいくつかの報告がある[6,7]．しかし，移動相にイオンペア試薬を加えた方法が多いため，最終的にMSで確認を行う際LC/MSに同じ分析条件を適用できない難点がある．

本研究では，上述の問題を解決する一手法としてLC/MSを用い，医薬品成分（類似成分も含む）及び指定薬

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Table 1-(1) Compounds Used for Building up the Spectral Library in This Study

Category	Compound name	MW	M/Z*	RT	Formula
Sexual enhancers	Aminotadalafil	390.4	391(389)	15.4	$C_{21}H_{18}N_4O_4$
	Chloropretadalafil	426.9	427(425)	18.4	$C_{22}H_{19}ClN_2O_5$
	Hongdenafil	466.6	467	13.3	$C_{25}H_{34}N_6O_3$
	Homothiodenafil	504.7	505	16.4	$C_{23}H_{32}N_6O_3S_2$
	Hydroxyacetildenafil	482.6	483(481)	13.1	$C_{25}H_{34}N_6O_4$
	Hydroxyhomothiodenafil	520.7	521	16.6	$C_{23}H_{32}N_6O_4S_2$
	Imidazosagatriazinone	312.4	313	19.2	$C_{17}H_{20}N_4O_2$
	Piperiacetildenafil	437.6	438	13.9	$C_{24}H_{31}N_5O_3$
	Sildenafil	474.6	475	14.0	$C_{22}H_{30}N_6O_4S$
	Tadalafil	389.4	390	15.9	$C_{22}H_{19}N_3O_4$
	Thioquinapiperifil	448.6	449	13.6	$C_{24}H_{28}N_6OS$
	Thioaildenafil	504.7	505	15.9	$C_{23}H_{32}N_6O_3S_2$
	Valdenafil	488.2	489	13.6	$C_{23}H_{32}N_6O_4S$
	Xanthoanthrafil	389.4	390	16.1	$C_{19}H_{23}N_3O_6$
	Yohinbine	354.5	355	12.6	$C_{21}H_{26}N_2O_3$
Hormones	Methyltestosterone	302.5	303	17.3	$C_{20}H_{30}O_2$
	3-3'-5-Triiodo-L-thyronine(T3)	651.0	652	14.4	$C_{15}H_{12}I_3NO_4$
Laxatives	Bisacodyl	361.4	362	17.5	$C_{22}H_{19}NO_4$
	Phenolphthalain	318.3	319	15.5	$C_{20}H_{14}O_4$
	Sennoside A	862.7	539	12.5	$C_{42}H_{38}O_{20}$
	Sennoside B	862.7	539	12.0	$C_{42}H_{38}O_{20}$
Diuretics	Acetazolamide	222.2	223(221)	8.7	$C_4H_6N_4O_3S_2$
	Furosemide	330.7	331(329)	15.0	$C_{12}H_{11}ClN_2O_5S$
	Hydrochlorothiazide	297.8	(296)	10.3	$C_7H_8ClN_3O_4S_2$
Anoretics	Diazepam	284.7	285	17.3	$C_{16}H_{13}ClN_2O$
	Fenfluramine	231.2	232	13.5	$C_{12}H_{16}F_3N$
	N-Nitrosofenfluramine	260.3	261	18.8	$C_{12}H_{15}F_3N_2O$
	Fluoxetine	309.3	310	15.1	$C_{17}H_{18}F_3NO$
	Mazindol	284.7	285	13.0	$C_{16}H_{13}ClN_2O$
	Nitrazepam	281.3	282(280)	15.2	$C_{15}H_{11}N_3O_3$
	Sibutramine	279.8	280	15.4	$C_{17}H_{26}ClN$
Hypoglycemics	Acetohexamide	324.4	325(323)	16.2	$C_{15}H_{20}N_2O_4S$
	Chlorpropamide	276.7	277(275)	15.8	$C_{10}H_{13}ClN_2O_3S$
	Glibenclamide	494.0	494(492)	18.3	$C_{23}H_{28}ClN_3O_5S$
	Gliclazide	323.4	324(322)	17.2	$C_{15}H_{21}N_3O_3S$
	Tolazamide	311.4	312(310)	16.6	$C_{14}H_{21}N_3O_3S$
	Tolbutamide	270.4	271(269)	16.1	$C_{12}H_{18}N_2O_3S$
Ephedrine derivatives	Ephedrine	165.2	166	8.1	$C_{10}H_{15}NO$
	Methylephedrine	179.2	180	9.5	$C_{11}H_{17}NO$
	Norephedrine	151.2	152	5.8	$C_9H_{13}NO$
	Pseudoephedrine	165.2	166	8.9	$C_{10}H_{15}NO$
Steroids	Amsinonide	502.6	503	18.9	$C_{28}H_{35}FO_7$
	Beclomethasone dipropionate	521.1	521	19.7	$C_{28}H_{37}ClO_7$
	Betamethasone	392.5	393	15.2	$C_{22}H_{29}FO_5$
	Betamethasone acetate	434.5	435	16.8	$C_{24}H_{31}FO_6$
	Betamethasone dipropionate	504.6	505	19.4	$C_{28}H_{37}FO_7$
	Betamethasone valerate	476.6	477	18.3	$C_{27}H_{37}FO_6$
	Clobetasole propionate	467.0	467	18.9	$C_{25}H_{32}ClFO_5$
	Dexamethasone	392.5	393	15.1	$C_{22}H_{29}FO_5$
	Dexamethasone 21-acetate	434.5	435	16.9	$C_{24}H_{31}FO_6$
	Fluocinolone acetonide	452.5	453	15.7	$C_{24}H_{30}F_2O_6$
	Flumethasone pivalate	494.6	495	19.1	$C_{27}H_{36}F_2O_6$
	Hydrocortisone	362.5	363	14.2	$C_{21}H_{30}O_5$
	Hydrocortisone acetate	404.5	405	16.0	$C_{23}H_{32}O_6$
	Hydrocortisone succinate	462.5	463	15.3	$C_{25}H_{34}O_8$
	Hydrocortisone valerate	446.6	447	17.7	$C_{26}H_{38}O_6$
	Halcinonide	455.0	455	18.7	$C_{24}H_{32}ClFO_5$
	Prednisolone	360.5	361	14.1	$C_{21}H_{28}O_5$
	Prednisolone succinate	460.5	461(459)	15.1	$C_{25}H_{32}O_8$
	Triamcinolone acetonide	434.5	435	15.5	$C_{24}H_{31}FO_6$

* Positive ion mode ( ): Negative ion mode

Table 1-(2) Compounds Used for Building up the Spectral Library in This Study

Category	Compound name	MW	M/Z*	RT	Formula
Designated substances	N-Isopropyl-5-methoxy-N-methyltryptamine (5-MeO-MIPT)	246.4	247	11.8	$C_{15}H_{22}N_2O$
	N,N-Diallyl-5-methoxytryptamine (5-MeO-DALT)	270.4	271	12.7	$C_{17}H_{22}N_2O$
	N,N-Diethyl-5-methoxytryptamine (5-MeO-DET)	246.4	247	12.0	$C_{15}H_{22}N_2O$
	5-Methoxy-N,N-dipropyltryptamine (5-MeO-DPT)	274.4	275	13.3	$C_{17}H_{26}N_2O$
	1-(5-Methoxy-1H-indol-3-yl)propan-2-amine (5-MeO-AMT)	204.3	205	11.1	$C_{12}H_{16}N_2O$
	N-Isopropyl-N-methyltryptamine (MIPT)	216.3	217	11.8	$C_{14}H_{20}N_2$
	N,N-Diisopropyltryptamine (DIPT)	244.4	245	12.7	$C_{16}H_{24}N_2$
	2-(4-Chloro-2,5-dimethoxyphenyl) ethanamine (2C-C)	215.7	216	12.1	$C_{10}H_{14}ClNO_2$
	2-(4-Ethyl-2,5-dimethoxyphenyl)ethanamine (2C-E)	209.3	210	13.0	$C_{12}H_{19}NO_2$
	2-(4-Fluorophenyl)propan-2-amine (4FMP)	153.2	154	10.8	$C_9H_{12}FN$
	1-Benzyl-4-methylpiperazine (MBZP)	190.3	191	7.0	$C_{12}H_{18}N_2$
	1-(4-Methoxyphenyl)piperazine (4MPP)	192.3	193	10.0	$C_{11}H_{16}N_2O$
	Indan-2-amine	133.2	134	6.8	$C_9H_{11}N$
	1-(4-Iodo-2,5-dimethoxyphenyl)propan-2-amine (DOI)	321.2	322	13.1	$C_{11}H_{16}INO_2$
	2-Ethylamino-1-(3,4-methylenedioxyphenyl) propane-1-one (Ethylone)	221.3	222	10.4	$C_{12}H_{15}NO_3$
	2-Methylamino-1-(3,4-methylenedioxy-phenyl)butan-1-one (bk-MBDB)	221.3	222	10.9	$C_{12}H_{15}NO_3$
	1-Piperonylpiperazine	220.3	221	4.7	$C_{12}H_{16}N_2O_2$
	Salvinorin A	432.5	433	17.5	$C_{23}H_{28}O_8$
Narcotics	2-(4-Iodo-2,5-dimethoxyphenyl)ethanamine (2C-I)	307.1	308	12.8	$C_{10}H_{14}INO_2$
	2-(4-Ethylsulfanyl-2,5-dimethoxyphenyl) ethanamine (2C-T-2)	241.4	242	12.8	$C_{12}H_{19}NO_2S$
	2-(2,5-Dimethoxy-4-isopropylsulfanylphenyl) ethanamine (2C-T-4)	255.4	256	13.4	$C_{13}H_{21}NO_2S$
	N-Methyl-α-ethyl-3,4-(methylenedioxy) phenethylamine (MBDB)	207.3	208	11.7	$C_{12}H_{17}NO_2$
	1-(3-Chlorophenyl)piperazine (3CPP)	196.7	197	12.2	$C_{10}H_{13}ClN_2$

* Positive ion mode

物について，保持時間とMSデータに関するライブラリーを構築した．このライブラリーを製品分析に適用し，良好な結果が得られたので報告する．

実験方法

1. 試　薬

市販されている医薬品成分の標品については購入し，市販されていない指定薬物や強壮系成分等の標品については，国立医薬品食品衛生研究所で指定薬物の標品等として合成等を行い準備したものを使用した．メタノール及びアセトニトリルは，関東化学㈱製HPLC用を用いた．その他の試薬は，和光純薬工業㈱製特級を用いた．

2. ライブラリー作成方法

Waters社製LC/MS制御ソフトMassLynxのオプションであり，ライブラリー検索ソフトのChromaLynxを用いた．ChromaLynxはLC/MSで検出した成分のMSデータと，既にライブラリーに登録済みのMSデータとを照合し，スペクトルの一致率が高い化合物を表示できる．また，新たにMSデータをライブラリーに登録できる機能を持っている．このソフトを使用して，過去の健康被害事例を参考に強壮系化合物や血糖降下薬等の高頻度で含有される成分，指定薬物等，目的とする化合物の標品を各々メタノールに溶解し，5～10 mg/Lの濃度に調製した．この溶液を下記の分析条件で測定し，MSデータを蓄積した．

分析条件

装置

LC/MS: Waters 2695/ZQ2000

ライブラリー検索ソフト：Waters ChromaLynx

条件

カラム：Waters Xterra（内径2.1 mm，長さ150 mm，3.5 μmのオクタデシルシリル化シリカゲル充填）

カラム温度：40℃

流　量：0.2 mL/min
注入量：5 μL
標準溶液濃度：5～10 mg/L
移動相：A液（0.05%ギ酸含有5 mmol/Lギ酸アンモニウム水溶液）
B液（0.05%ギ酸含有アセトニトリル溶液）
A：B＝95：5（2分間）—10：90（14分間）—95：5（4分間）—95：5（6分間hold）
イオン化法：エレクトロスプレーイオン化法（ESI）
コーン電圧：ポジティブ（+）は15 V～90 V（15 Vずつ変化させる）
ネガティブ（−）は15 V及び30 V
ESI（+，−）条件下，コーン電圧を15 V～90 Vまで15 Vずつ変化させた各々のMSクロマトグラムからMS

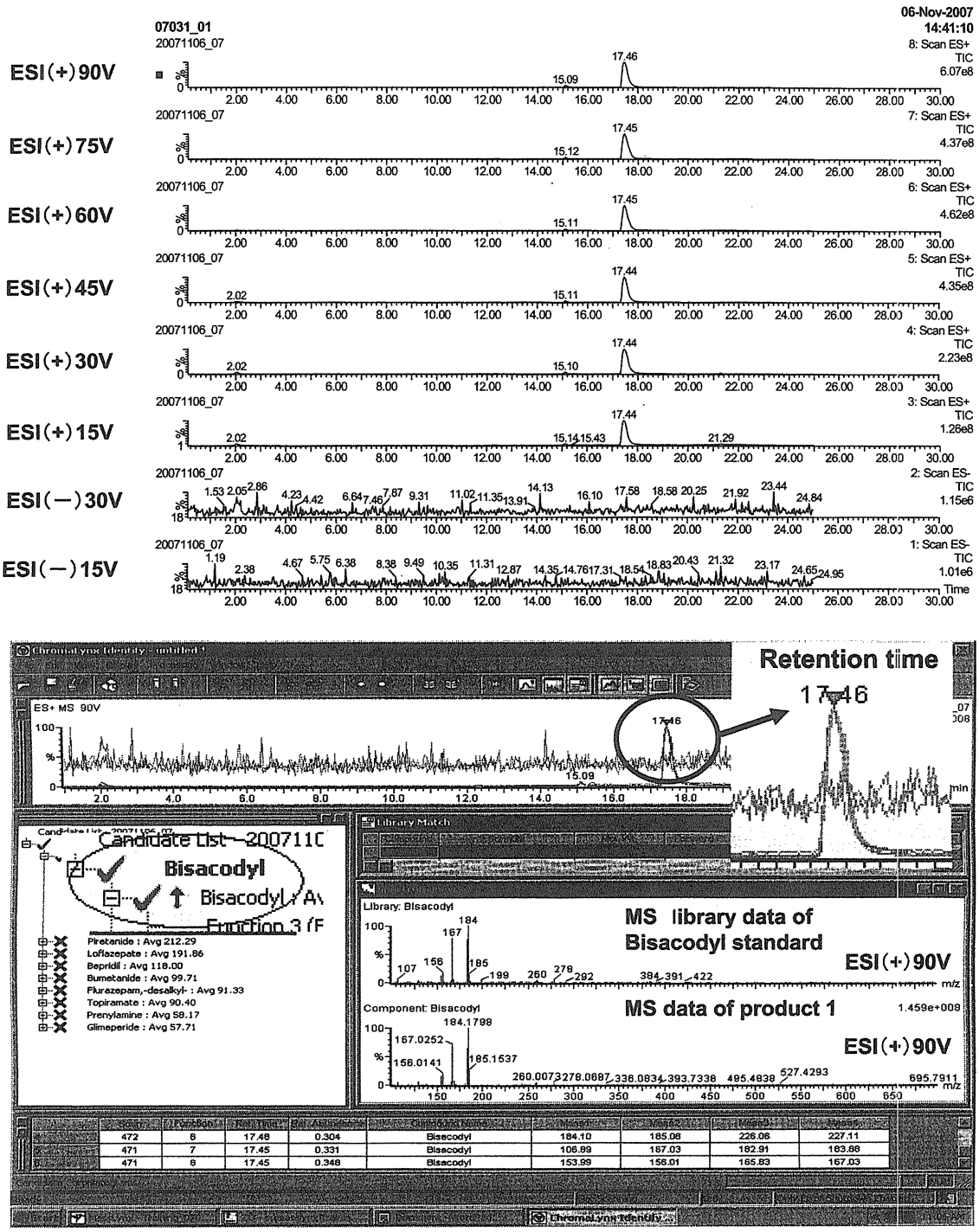

Fig. 1 Scan Chromatograms of LC/MS Analysis Using Eight Different Cone Voltages and Illustration of Spectral Library Searching for Product 1 (Dietary supplement for weight reducing)

スペクトルを抽出して，ライブラリー検索ソフトに登録した．更に保持時間，分子量，電圧の強さ，CAS No. 等を登録してライブラリーを作成した．

3. 未知試料のLC/MSライブラリー適用によるスクリーニング方法

固体試料の場合は約0.1 gを精密に量り取り，液体の場合は1 mLを正確に量り取って，メタノールを加えて10 mLとする，これを10分間超音波抽出して上澄を取

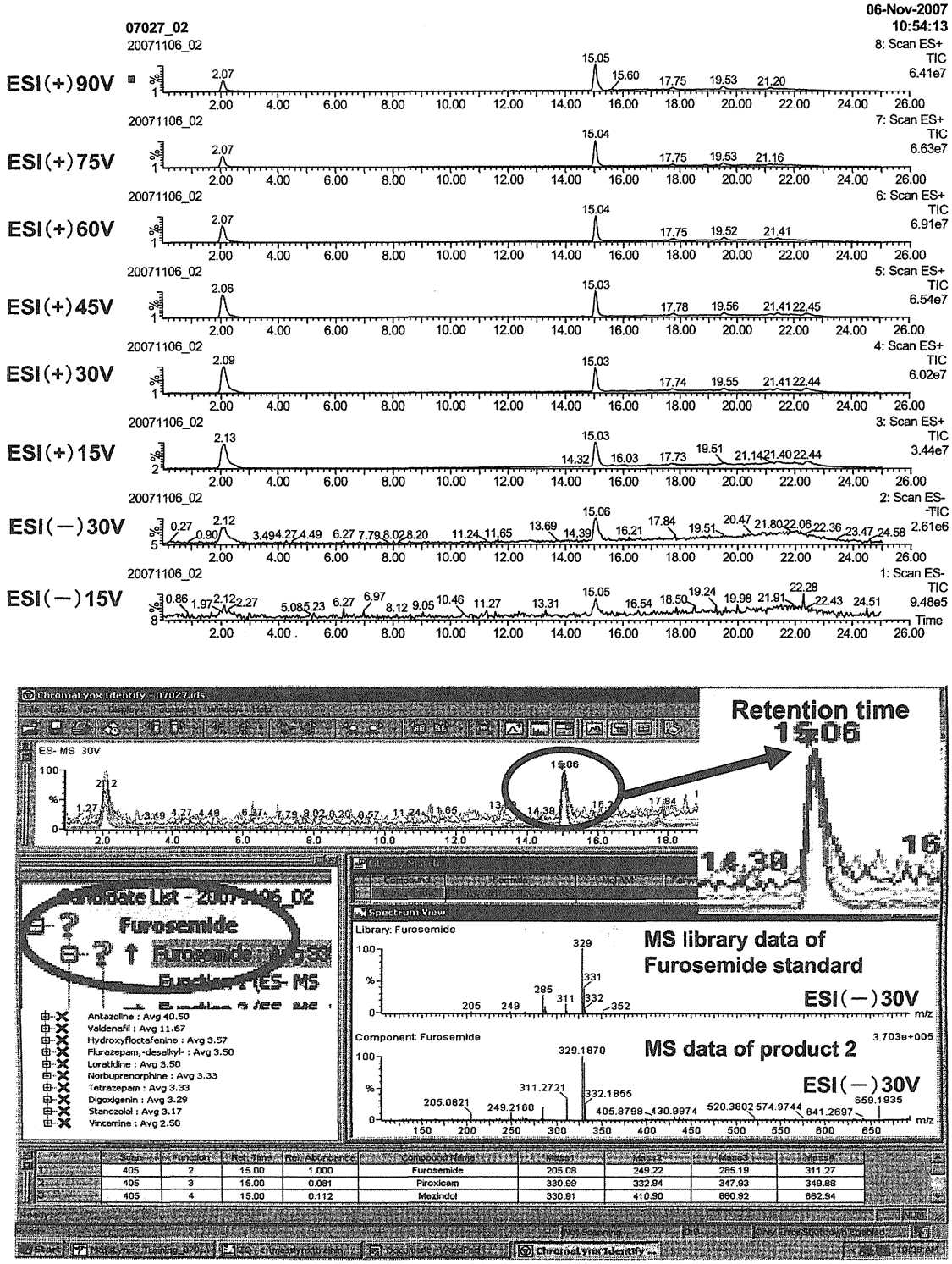

Fig. 2 Scan Chromatograms of LC/MS Analysis Using Eight Different Cone Voltages and Illustration of Spectral Library Searching for Product 2 (Dietary supplement for weight reducing)

り，メタノールで10倍希釈し試料溶液とした．

上述のLC/MS分析条件により測定し，ライブラリー検索ソフトにより化合物のMSデータ及び保持時間と照合した．高い確率でライブラリーと合致しているものは，「√」のマーク，確率が低い場合は「×」マーク，両者の中間でライブラリーの合致率が曖昧である場合「？」マークで検索結果が示されるシステムとなっている．

結　果

ライブラリー構築の結果，Table 1に示したとおり，強壮系成分15化合物をはじめ，ホルモン成分2，下剤及び利尿剤成分7，食欲抑制薬7，血糖降下薬6，エフェドリン類4，及びステロイド成分19，指定薬物18及び麻薬5物質の合計83物質が検索可能となった．これら化合物は，過去に薬事法違反事例のあったもの及びその関連化合物である．更にChromaLynxのライブラリーに登録されている医薬品成分473物質を含めると，556物質の検索が可能となった．このライブラリーを適用した事例について以下に示した．

1. 痩身用健康食品のライブラリー適用によるスクリーニング結果

2007年11月にタイ産の「ホスピタルダイエット」と称した痩身用健康食品を服用したことにより微熱や動悸等の健康被害事例が発生し，医療機関から検査依頼を受けた．当該製品は錠剤2種類及び硬カプセル2種類からなり，この4検体を各々メタノールで抽出しLC/MSのライブラリーによるスクリーニング法で分析した結果，各検体から1成分ずつシブトラミン，フルオキセチン，ビサコジル，フロセミドがライブラリーと合致した．そのうち2検体のマスクロマトグラムとライブラリー検索結果の画面をFig. 1及びFig. 2に示す．検体1（Product 1）はESI（＋）でよくイオン化され，良好な擬似分子イオンピークが得られた．リテンション及びESI（＋）のMSデータがライブラリーのデータと高確率で合致したビサコジルに「√」が付いたため，標品を用いて再測定し，ビサコジルと同定した．検体2（Product 2）以外の検体についても該当する物質に「√」が付き同様に標品で確認した．

検体2（Product 2）はESI（＋）の他，ESI（－）においてもイオン化され，フロセミドのライブラリーと合致したが，曖昧な検索結果のマーク「？」が表示された．この様な現象が起こった原因として，一つはフロセミドのESI（＋）におけるMSデータは，ライブラリー検索としては好ましくない付加イオンや二量体のフラグメントが検出されたこと，二つ目としてESI（－）のMSデータは2つのみ登録されており，データ数が少ないため

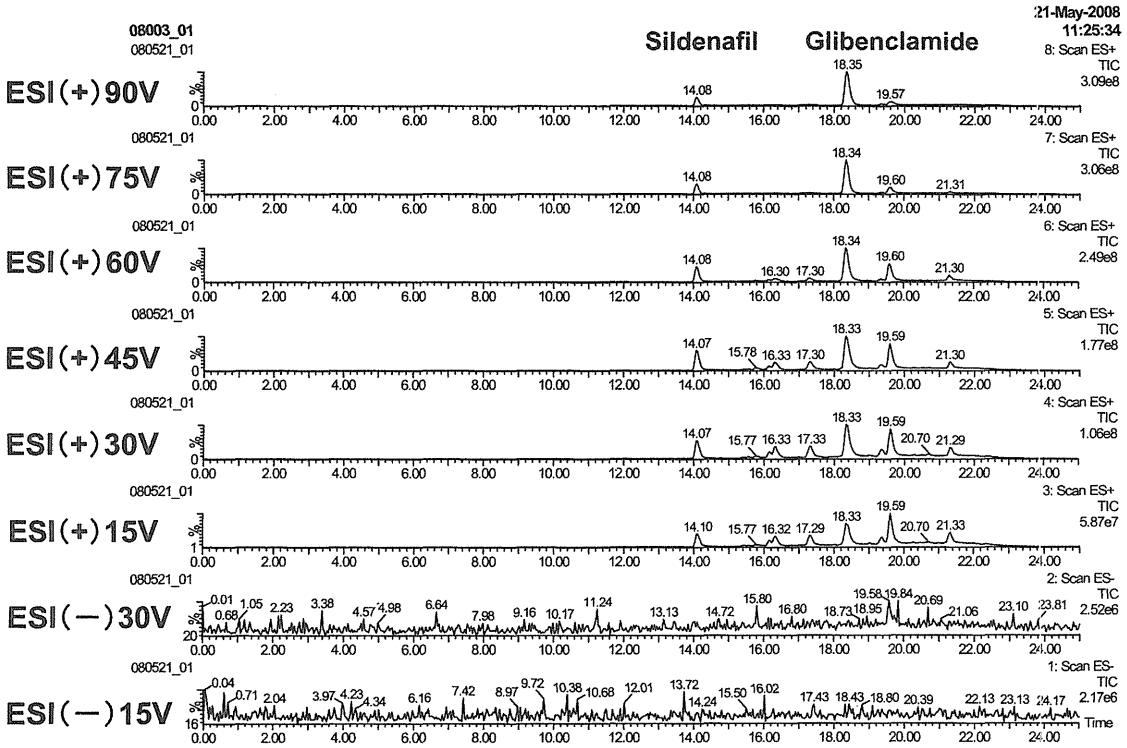

Fig. 3　Scan Chromatograms of LC/MS Analysis Using Eight Different Cone Voltages for Product 3 (Supplement for sexual enhancement)

確率的に曖昧な検索となったことが考えられる．しかし，少ないESI（－）データからでもライブラリー検索により第一候補でフロセミドがライブラリーと合致したことが明らかとなった．フロセミドについても標品を用いて同定した．

標品を用いて当該製品中の成分をHPLCで定量した結果，シブトラミンが10 mg/Cap.，フルオキセチンが16 mg/Cap.，ビサコジルが4.6 mg/Tab，フロセミドが，8.2 mg/Tab.で，十分薬用量に相当する値だった．

2. 強壮用健康食品のライブラリー適用によるスクリーニング結果

2008年5月に中国製強壮用健康食品を服用したことにより意識不明，低血糖等の健康被害事例が発生し，医

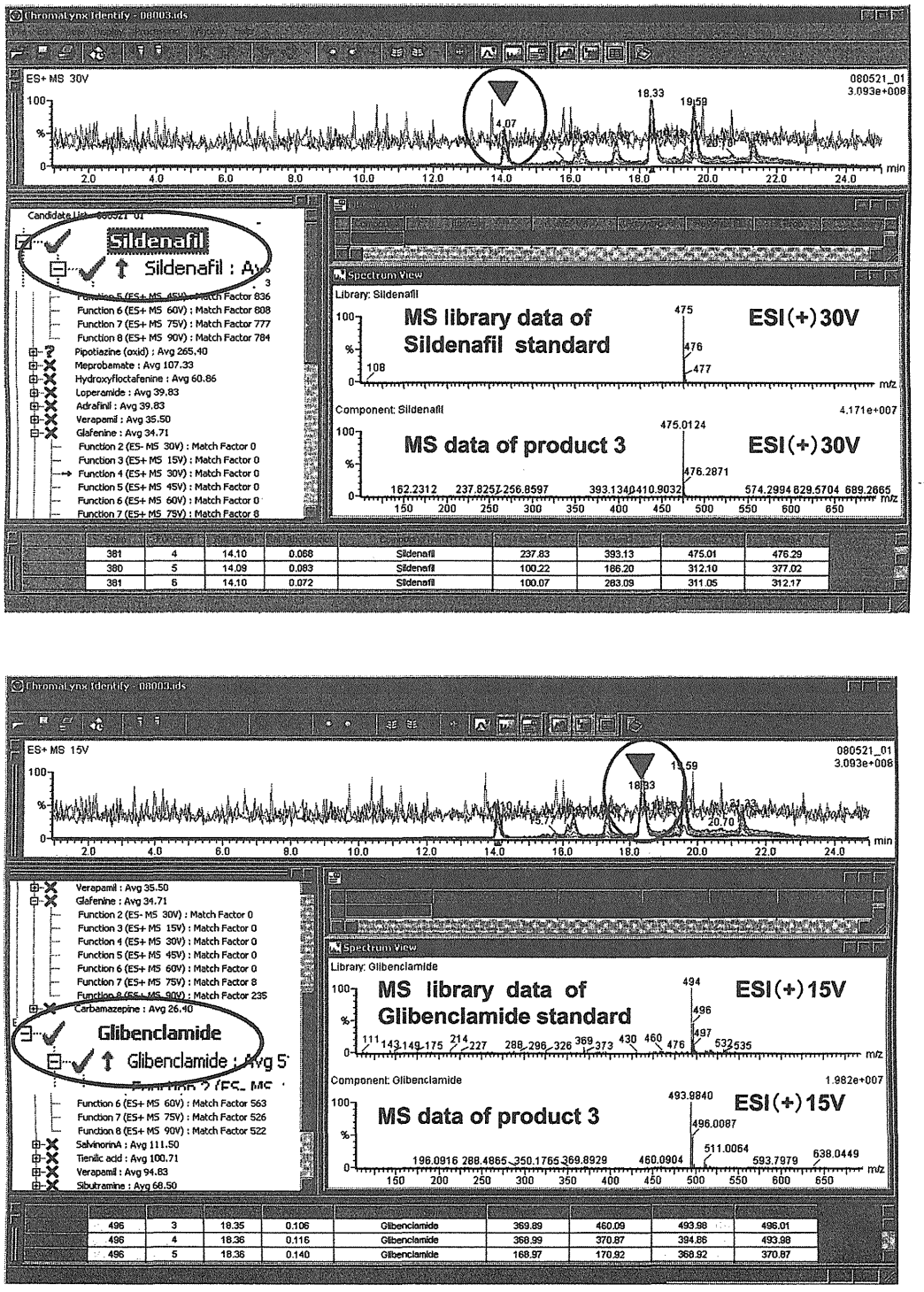

Fig. 4 Illustration of Spectral Library Searching for Product 3

療機関から相談を受けた．当該製品は青色の菱形の錠剤だった．この検体（Product 3）を今回のスクリーニング方法で成分検索した結果，シルデナフィルとグリベンクラミドの2成分がライブラリーと合致した．当該製品のMSクロマトグラムと各成分のライブラリーが合致した画面をFig. 3及びFig. 4に示した．Fig. 4のとおり2成分共に高確率でライブラリーと合致し「√」が付いた．これらの結果から，複数の成分を含有している場合でも化合物の検索が可能であることが明らかとなった．

標品を用いて当該製品中の成分をLC/MSで定量した結果，シルデナフィル48 mg/Tab.及びグリベンクラミド67 mg/Tab.を含有していた．グリベンクラミドについては通常使用量[8]の20倍以上含有していた．

まとめ

LC/MSのライブラリー検索用ソフトを使用して，薬事法違反事例のある医薬品成分及び指定薬物のLC/MSデータ及び保持時間を登録し，スクリーニング分析を指向とした検索ライブラリーを構築した．その結果，ライブラリーデータと照合することで含有成分の絞込みが可能となり，実際に健康被害の疑われる当該製品からシブトラミン，フルオキセチン，ビサコジル，フロセミド，シルデナフィル，グリベンクラミドが検出された．今回の事例でみられた強壮用健康食品でありながら血糖降下薬という想定外の成分が混入されている場合においても，ライブラリー検索が可能であることが明らかとなった．

GC/MSと比較してLC/MSライブラリーは，メーカーごとにMSスペクトルが異なるため共通化ができない等の難点はあるが，データを蓄積することで医薬品等が含まれる未知試料のスクリーニングには十分有用であると考えられた．また，入手が困難な指定薬物等の化合物についてはそれらのMSデータを追加することにより物質の推定が可能となり，違法ドラッグ等への対応が迅速になると考えられた．

なお，今回構築されたLC/MSライブラリーは，公的研究・検査機関には，無償で提供される予定である．

文　献

1) 厚生労働省：個人輸入した未承認医薬品等の服用後に発生した健康被害事例について，報道発表資料，2002年7月12日．
2) 厚生労働省:中国製ダイエット用健康食品等関連情報 http://www.mhlw.go.jp/kinkyu/diet/index.html
3) 合田幸広，鎌倉浩之：*Functional. Food*，**2**(2)，198-202 (2008)．
4) 厚生労働省:無承認無許可医薬品情報 http://www.mhlw.go.jp/kinkyu/diet/musyounin.html
5) 薬事法第2条第14項に規定する指定薬物及び同法第76条の4に規定する医療等の用途を定める省令，厚生労働省令第149号，2009年10月21日．
6) 守安貴子，蓑輪佳子，岸本清子，重岡捨身，門井秀郎，安田一郎：*Ann. Report of Tokyo Metropolitan Institute of Public Health*，**56**，81-89 (2005)．
7) Saint-Marcoux, F., Lachatre, G., and Marquet, P.: *J. Am. Soc. Mass Spectrometry*，**14**，14-22 (2003)．
8) (財)日本医薬情報センター編，JAPIC医療用医薬品集2006，丸善，東京，723-725．

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ORIGINAL ARTICLE

Chemical constituents and DNA sequence analysis of a psychotropic herbal product

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Abstract In recent years, the distribution of a variety of psychotropic products, especially "spice" and "herbal blends," which are advertised to have narcotic-like effects, has become more widespread in the Japanese illegal drug market. We recently found two synthetic cannabinoids, cannabicyclohexanol and JWH-018, that serve as adulterants in herbal products purchased via the Internet. In this study, we focused on a herbal product being sold as incense, which showed unknown components by liquid chromatography-mass spectrometry (LC-MS). The product did not show any peak corresponding to the above synthetic cannabinoids, but seven other peaks were identified by high-performance liquid chromatography and LC-MS. We identified them as *N*-methyltyramine (**1**), (*R*)-normacromerine (**2**), (*R*)-macromerine (**3**), (*S*)-vasicine (**4**), mescaline (**5**), harmaline (**6**), and harmine (**7**) by polarimetry, LC-MS, gas chromatography-mass spectrometry, high-resolution mass spectrometry, and nuclear magnetic resonance spectroscopy. We also used DNA sequence analyses to identify the plant species of the product. As a result of the sequencing of *trnL-F*, internal transcribed spacer (ITS), and *rpl16* intron regions, three sequences derived from *Coryphantha macromeris* (Cactaceae), *Peganum harmala* (Zygophyllaceae), and *Turnera diffusa* (Turneraceae) were observed. Compounds **2** and **3**, both phenethylamines, were reported to cause hallucinogenic effects and are frequently found in *Coryphantha* genus (Cactaceae). Therefore, the plant source of these compounds was considered to be *C. macromeris*. Compound **5** is known to be a psychoactive phenethylamine found in peyote (*Lophophora williamsii*) and San Pedro cactus (*Trichocereus pachanoi*). The β-carboline alkaloids **6** and **7** are known to be found in the seeds of *P. harmala*. Therefore, there seems to be no contradiction between the chemical constituents and the plant species estimated by DNA analyses, except for compound **5**. This is the first report dealing with identification of the psychoactive cactus *C. macromeris* and its constituent compounds in a herbal product distributed in the illegal drug market.

Keywords (*R*)-Normacromerine · (*R*)-Macromerine · Herbal product · *Coryphantha macromeris* · LC-MS · DNA analysis

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Introduction

A variety of psychotropic substances are widely distributed and easily obtainable worldwide, mainly via the Internet. Some of these compounds occur as natural substances, but most of them are chemically synthesized as designer drugs [1–3]. Many of them are advertised to have narcotic pharmacological effects and are sold as incense and chemical reagents. Although in Japan, the distribution of these products has been dramatically decreased by implementation of the designated substances (Shitei-Yakubutsu) system starting in April 2007, the popularity of uncontrolled psychotropic plants and their blends, such as "spice," is becoming apparent in the illegal drug market [4–7]. These products are being sold in the form of dried plants, powders, seeds, resins, and their mixtures. As a result of a 2008 market survey of illegal drugs, we identified two synthetic cannabinoids, namely cannabicyclohexanol and JWH-018, as

adulterants present in a spice-type herbal product and reported our findings early in 2009 [6,7]. At the same time, several other groups reported the detection of synthetic cannabinoid analogues from such herbal products [8,9]. By the prompt action of regulatory authorities, these synthetic chemicals have been strictly regulated in many countries including Japan.

In our continuing survey of illegal drugs, we recently discovered a herbal product containing unknown components using liquid chromatography-mass spectrometry (LC-MS). The product showed no peaks corresponding to the known synthetic cannabinoids by the LC-MS analysis. In this article, we report identification and quantitation of the unknown components detected in a herbal product by gas chromatography-mass spectrometry (GC-MS), LC-MS, high-resolution MS, and nuclear magnetic resonance (NMR) spectroscopy. Furthermore, we performed genetic analyses of the plant species found in the product to clarify the relationship between the chemical constituents and the plant species in the product.

Materials and methods

Plant materials

The herbal product used in this study was purchased via the Internet in June 2008. The product was in the form of a dried plant in a 3-g package. The whole plant of putative *Coryphantha macromeris* was also purchased via the Internet in June 2009.

Chemicals and reagents

Lidocaine (internal standard, IS), harmaline (compound **6**), and harmine (compound **7**) were purchased from Wako (Osaka, Japan). Mescaline (compound **5**) sulfate was prepared according to our reported method [10]. The authentic standards for compounds **1**–**4** to be identified in this study were either unavailable commercially or difficult to obtain. Centrifugal filter devices (Ultrafree-MC, 0.45 μm filter unit) were obtained from Millipore (Bedford, MA, USA). All reagents and chemicals used were of analytical reagent grade or high-performance liquid chromatography (HPLC) grade.

Instrumentation

Optical rotations were measured on a Jasco DIP-370 digital polarimeter (Jasco, Tokyo, Japan). NMR spectra were recorded on an ECA-600 spectrometer (JEOL, Tokyo, Japan) with a deuterated solvent, the chemical shifts of which were used as IS (CD_2HOD, δ_H = 3.30; CD_3OD, δ_C = 49.0 ppm). Accurate mass spectra were measured using a direct-analysis-in-real-time (DART) ion source coupled to a time-of-flight (TOF) mass spectrometer (AccuTOF JMS-100LC, JEOL, Tokyo, Japan).

LC-MS conditions

LC-MS analysis was conducted on an ultra-performance liquid chromatography-electrospray ionization-mass spectrometer (UPLC-ESI-MS), consisting of an ACQUITY UPLC system, a single-stage quadrupole mass detector and a photodiode array (PDA) detector (Waters, Milford, MA). The sample solutions were separated using an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm i.d., particle size 1.8 μm; Waters) protected by a Van Guard column (5 mm × 2.1 mm i.d., 1.8 μm; Waters) at 40°C. The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The LC flow program was: initially 95% A, linearly changed to 80% A in 5 min, then changed to 20% A at 25 min, and held for 25 min. The flow rate of the mobile phase was 0.3 ml/min and the injection volume was 1 μl. The wavelength of the PDA detector was set from 190 to 500 nm. Quantitative analysis was carried out on the basis of the area ratios of the protonated molecular peaks ($[M+H]^+$) of the test compounds to that of IS. The ion peaks were monitored at *m/z* 212, 226, 212, 215, and 213 for compounds **2**, **3**, **5**, **6**, and **7**, respectively.

GC-MS conditions

The instrument used was a Hewlett-Packard (Palo Alto, CA, USA) 6890N GC with a 5975 mass-selective detector using a capillary column (HP1-MS capillary, Hewlett-Packard; 30 m × 0.25 mm i.d., 0.25 μm film thickness) and helium gas as a carrier. The GC-MS conditions were: initial column temperature, 80°C; temperature program, 5°C/min to 190°C, followed by a 10°C/min increase up to 310°C; MS detection, full-scan mode (scanning range *m/z* 40–550). The analysis was performed using the established method and under conditions described in our previous report [11].

Extraction and isolation by preparative HPLC

The dried plant (9 g, three packages) was extracted with 1000 ml of methanol by ultrasonication for 1 h. After removal of the solvent under reduced pressure, the residue (3.9 g) was made alkaline (pH 10) with 25% NH_4OH solution and then extracted with ethyl acetate (EtOAc) (300 ml × 3). A portion of the EtOAc-soluble

fraction was subjected to reversed-phase preparative HPLC (Inertsil ODS-3, 250 mm × 20 mm, GL Sciences, Tokyo, Japan) at a flow rate of 5.0 ml/min with 0.1% trifluoroacetic acid (TFA) /acetonitrile (CH_3CN) (70:30, v/v) to obtain three fractions (1A–1C). Fraction 1A (213.9 mg) was applied again to the reversed-phase HPLC (Inertsil ODS-3, 250 mm × 20 mm) at a flow rate of 5.0 ml/min with 0.1% TFA/CH_3CN (88:12, v/v) to separate compounds **1** (7.7 mg, retention time 17 min), **2** (9.8 mg, 25 min), **3** (8.2 mg, 27 min), and **4** (8.0 mg, 32 min).

Sample extraction procedure before LC-MS analysis

Five milligrams of each product sample was extracted with 10 ml of methanol containing 0.5 μg/ml of IS (lidocaine) by ultrasonication for 10 min ($n = 5$). After filtration though a centrifugal filter device, 1 μl of the sample solution, without further purification, was injected into the LC-MS system.

DNA extraction, amplification, and sequencing

The plant sample (20 mg) was crushed in a mixer mill, MM-300 (Qiagen, Hilden, Germany), under liquid N_2. Genomic DNA was extracted from the powdered sample using a DNeasy Plant Mini Kit (Qiagen) following the manufacturer's protocols.

Polymerase chain reaction (PCR) amplification was performed for three regions: *trnL-F* (the *trnL* intron and its 3′ exon, and the *trnL-trnF* spacer), the *rpl16* intron from the chloroplast genome and the internal transcribed spacer (ITS) region (small subunit rDNA-ITS1-5.8S rDNA-ITS2-large subunit rDNA) from nuclear rDNA using different sets of universal primers. The PCR reaction mixture included 0.1 μl of Ex Taq (Takara, Ootsu, Japan), 10 μl of Ampdirect (Shimadzu, Kyoto, Japan), 0.5 μl of each 10-μM primer, 1 μl of DNA template, and H_2O for a final volume of 20 μl. PCR was performed by preheating at 95°C for 3 min, 30 cycles at 95°C for 30 s, 52°C for 30 s, 72°C for 2 min, and then a final extension at 72°C for 10 min. The primers were designed for the conserved sequence among plant genes. The sequence data are: trn-S, 5′-CGAAATCGGTAGAC GCTACG-3′; trn-AS, 5′-ATTTGAACTGGTGACAC GAG-3′; rpl16-S, 5′-GCTATGCTTAGTTGTTGAC TCGTT-3′; rpl16-AS, 5′-CGTACCCATATTTTTCCA CCACGA-3′; ITS-S, 5′-TCCTCCGCTTATTGATA TGC-3′; ITS-AS, 5′-GGAAGTAAAAGTCGTAAC AAGG-3′.

The amplified PCR products were purified and concentrated using polyethylene glycol precipitation. The purified DNA fragments were fused into the pMD20-T vector of a Mighty TA-cloning Kit (Takara), and insert DNA was amplified using M13 primers by colony PCR. Sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) with an ABI Prism 3100-Avant Genetic Analyzer (Applied Biosystems). Sequence data were aligned using the computer program GENETYX (Tokyo, Japan). Then, by means of three-region nucleotide sequence analysis, the plant species were identified based on BLAST searches (DNA Data Bank of Japan, Mishima, Japan).

Results and discussion

Identification and quantitation of unknown peaks

We first isolated compounds appearing as the unknown peaks **1**–**4** by preparative HPLC to identify them. A large amount of the plant sample was extracted with methanol by ultrasonication for 1 h. The extract was dissolved in NH_4OH solution (pH 10) and reextracted with EtOAc. The alkaloid extract was again subjected to the same reversed-phase HPLC to obtain four compounds, **1**–**4**. Unfortunately, none of them was identical to any authentic compound in our collection. Therefore, we had to obtain data by polarimetry, NMR spectroscopy, and accurate MS for each compound. These compounds were finally identified as *N*-methyltyramine (**1**), (*R*)-normacromerine (**2**), (*R*)-macromerine (**3**), and (*S*)-vasicine (**4**) by comparing the data with those in the literature [12–15] (Fig. 1). Our data obtained from the four isolated compounds were as follows.

Compound **1** (*N*-methyltyramine): ^{1}H NMR (600 MHz, CD_3OD) δ_H 2.69 (3H, s, *N*-Me), 2.87 (2H, t, J = 7.4 Hz, H_2-1), 3.18 (2H, t, J = 7.4 Hz, H_2-2), 6.76 (2H, d, J = 8.2 Hz, H-3′, 5′), 7.08 (2H, d, J = 8.2 Hz, H-2′, 6′); ^{13}C NMR (125 MHz, CD_3OD) δ_C 32.5 (C-1), 33.7 (*N*-Me), 51.8 (C-2), 116.8 (C-3′, 5′), 128.0 (C-1′), 130.8 (C-2′, 6′), 157.9 (C-4′); DART-TOFMS *m/z* 152.1054 $[M+H]^+$ (calculated for $C_9H_{14}NO$, 152.1075).

Compound **2** [(*R*)-normacromerine]: $[\alpha]_D^{20}$ −18° (*c* 1.0, ethanol); ^{1}H NMR (600 MHz, CD_3OD) δ_H 2.73 (3H, s, *N*-Me), 3.15 (2H, m, CH_2-2), 3.82 (3H, s, OMe-4′), 3.84 (3H, s, OMe-3′), 4.87 (1H, overlapped), 6.96 (2H, br s, H-5′, 6′), 7.03 (1H, br s, H-2′); ^{13}C NMR (125 MHz, CD_3OD) δ_C 33.6 (*N*-Me), 56.4 (OMe-3′), 56.5 (OMe-4′), 56.6 (C-2), 69.8 (C-1), 110.6 (C-2′), 112.9 (C-5′), 119.6 (C-6′), 134.8 (C-1′), 150.6 (C-4′), 150.7 (C-3′); DART-TOFMS *m/z* 212.1291 $[M+H]^+$ (calculated for $C_{11}H_{18}NO_3$, 212.1287).

Compound **3** [(*R*)-macromerine]: $[\alpha]_D^{20}$ −44° (*c* 2.0, ethanol); ^{1}H NMR (600 MHz, $CDCl_3$) δ_H 2.98 (6H, s,