Focusing Review

Development and application of highly sensitive high−performance liquid chromatographic methods for analysis of drugs of abuse

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Received for review December 8, 2005. Accepted January 13, 2006.

Abstract

In recent years, many kinds of drugs of abuse have been used worldwide and caused serious social problems. Among these, methamphetamine (MP) is the most popular drug of abuse in Japan. In addition to MP illegal designer drugs, 3,4−methylenedioxymethamphetamine and 3,4−methylendioxyamphetamine have become popular and spread in use with young people. On the other hand, anorectics such as fenfluramine, phentermine, *N*−nitorosofenfluramine and mazindol were adulterated in Chinese diet products and have caused serious side effects. For prediction of and protection from the risk to human health, sensitive and selective analysis of these drugs of abuse is very important. Thus, the authors have examined HPLC methods to determine these compounds with fluorescence detection and developed highly sensitive methods with the detection limits from amol to fmol levels. These methods have been successfully applied to analysis of abusers' or suspects' biological samples such as urine, blood and hair.

Keywords: HPLC with fluorescence detection, fluorescent labeling, drugs of abuse, biological samples, hair, forensic sciences

Introduction

Today, abuse of drugs causes serious social problems worldwide. Among the drugs of abuse, methamphetamine (MP), a stimulant, is the most common in Japan and the arrests for stimulant drug−related offences totaled 12,225 in 2004. Recently, the use of illegal designer drugs such as 4,5−methylenedioxymethamphetamine (MDMA) and 4,5−methylenedioxyamphetamine (MDA) is spreading among the young people. Although the arrests for MDMA−related offences numbered 418 in 2004 are very small compared with those for stimulants, the use of MDMAs is rapidly increasing in teenagers [1]. On the other hand, Chinese herbal medicines are now widely used in Western society, Asia, Africa, and the Middle East. The adulteration of such herbals with synthetic drugs has been reviewed [2]. In particular, many report the adulteration of Chinese diet products with western pharmaceuticals like ephedrine and mazindol. In fact, the Chinese diet products adulterated with anorectic compounds such as fenfluramine (Fen), phenterimine (Phen), *N*−nitrosofenfluramine (*N*−Fen) and mazindol have increased in Japan. DL−Fen and the active L−enantiomer, dexfenfluramine, cause serious side effects including valvular heart disease and primary pulmonary hypertension, and have been withdrawn from the drug market in USA in September 1997. In Japan Fen has never been approved, but it appears in Chinese diet products available either through the Internet or imported by individual across the border. More recently *N*−Fen, a kind of prodrug of Fen, has been adulterated in Chinese diet foods and caused serious liver failures. Unfortunately, some women have died by a long−term ingestion of these diet foods in Japan.

Analyses of drugs of abuse are very important for prediction of and protection from the risk to human health, especially for young people. With this in mind, we have developed simple and high sensitive methods for quantification of these drugs of abuse. The developed methods have been applied to the determination of drugs of abuse in preparations and biological samples such as urine, blood and hair samples from suspects. Furthermore, the hair analysis has been applied to elucidate a history of drug use as a

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practical forensic study.

In this paper, the results of our recent analytical research on drugs of abuse have been reviewed.

1. Analysis of stimulants

MP−related compounds have primary or secondary amino groups in their structures. We thus derivatized the amino groups using some fluorescent labeling reagents such as 4−(*N*, *N*−dimethylaminosulphonyl)−7−fluoro−2,1,3−benzoxadiazole (DBD−F), fluorescein isothiocyanate, 4−fluoro−7−nitrobenzo−2−oxa−1,3−diazole, dansyl chloride and 4−(4,5−diphenyl−1 *H*−imidazol−2−yl) benzoyl chloride (DIB−Cl), to increase selectivity and sensitivity of the detection in high performance liquid chromatography (HPLC). Among the reagents, DIB−Cl, the first of which was synthesized in our laboratory, gave the labeled MP related compounds with very strong fluorescence at the wavelengths of λex = 330 nm and λem = 440 nm, and thus it has been predominantly used for the labeling of analytes in the following studies [3].

Generally, the derivatization of MP related compounds with DIB−Cl could be performed in the mild conditions, i.e., at room temperature for 10 min at pH 9 as shown in Figure 1.

MP is known to be metabolized in the human body mainly to amphetamine (AP), *p*−hydroxymetamphetamine (*p*−HMP), *p*−hydroxyamphetamine (*p*−HAP), and norephedrine as shown in Figure 2. Determination of these metabolites is useful in the elucidation of the intake or the metabolic pathway of the drug. As the metabolites exist in very low concentration in biological samples such as urine, blood and hair, a highly sensitive and selective determination method for MP and its metabolites is requisite not only for pharmaceutical sciences but for forensic chemistry. To date, many analytical methods for MP and its related compounds have been developed [4,5]; gas chromatography (GC), gas−chromatography−mass spectrometry (GC−MS), thin layer chromatography, polarization fluoroimmunoassay, enzyme− or radio−immunoassay, and HPLC. Among these, GC has been used because of its convenience. However the sensitivity of this method is not enough to determine the trace amounts of MP related compounds. Although GC−MS is very sensitive and has been predominantly used in forensic sciences to determine stimulants, it requires an expensive apparatus. Therefore, we selected an HPLC with fluorescence detection to develop sensitive and selective methods for the determination of MP and its related compounds.

1−1) Urine sample

Urine is the most popular sample to clarify the drugs of the abuse. Generally, the drugs are extracted from urine by a liquid−

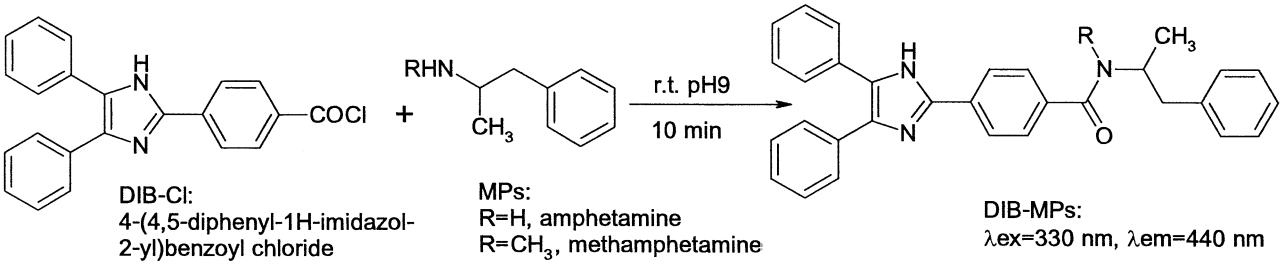

Figure 1. Derivatization reaction of MPs with DIB−Cl.

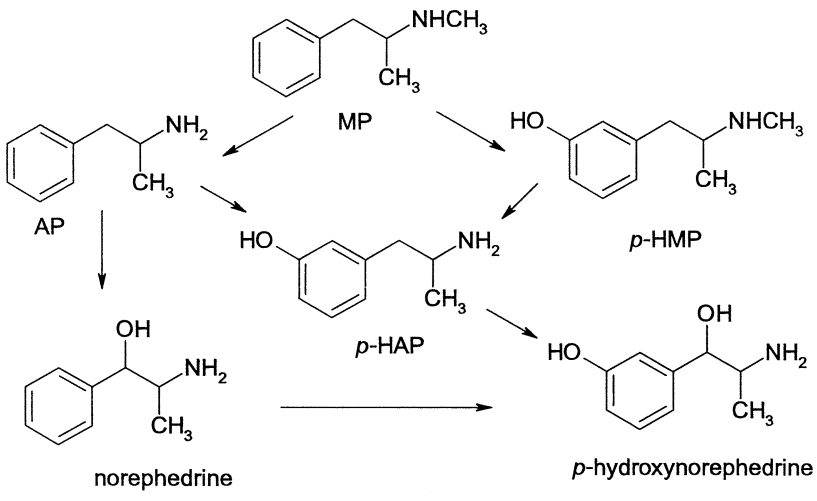

Figure 2. Metabolic pathway of MP in human body.

liquid extraction or a solid−phase extraction. However, these procedures are tedious and sometimes lower the recovery of the target compounds. Thus, we tried to use a direct condensation of urine samples under the acidic condition by nitrogen gas. The procedure is very simple, rapid and precise. Five DIB derivatives of MP, AP, *p*−HAP, ephedrine and 1−phenylethylamine as an internal standard (IS) could be isocratically separated within 33 min by an ODS column. The proposed method was highly sensitive with the detection limits of 0.6−5.2 fmol at a signal−to−noise (S/N) ratio of 3. The method was applied to 6 urine samples from MP addicts. MP and its major metabolite AP and *p*−HAP were quantified. Ephedrine was also detected in some samples, but it is believed to originate from cold medication rather than being a MP metabolite [6].

Not only is the enantiomeric discrimination of drugs important for evaluating their biological activities, it is also crucial for forensic toxicologists to confirm whether they were administered as a part of legitimate medical treatment or taken illicitly. The presence of (*S*)−(+)−MP and its corresponding metabolites, (*S*)−(+)−AP and (*S*)−(+)−*p*−HMP in biological samples indicates the illegal intake of this drug in Japan as (*S*)−(+)−MP is abused in more than 99% of cases, whereas the presence of their antipode might be due to a legitimate medical treatment as in the case of selegiline [(*R*)−(−)−*N*−methyl−*N*−(1−phenyl−2−propyl)−2−propinylamine; Deprenyl], a selective inhibitor of monoamine oxidase type B, that has been used in the treatment of Parkinson's disease and known to be metabolized in the human body to the corresponding enantiomers (*R*)−(−)−MP and (*R*)−(−)−AP and their conjugated *p*−hydroxy derivatives and also norselegiline metabolites. Therefore, a sensitive and simple enantiomer−specific method for the determination of MP enantiomers is necessary to differentiate between psyco−stimulant abusers and Parkinsonian.

Enantiomeric separation of MPs by HPLC has been performed either by derivatization with a chiral reagent followed by the separation of the diastereomers obtained on an achiral stationary phase, or by utilizing a chiral stationary phase (CSP) that can resolve the enantiomers of MPs with or without previous achiral derivatization. Methods that employ a CSP with a derivatization step might be preferred over diastereomeric derivatization since the enantiomeric purity of optically active reagents might prevent accurate analysis. Moreover, the enantiomers may have different rates when they react with another optically active compound. As the use of a CSP to separate the pairs of enantiomers after suitable derivatization with an achiral reagent is the alternative of choice to achieve a powerful separation with improved sensitivity, we selected this method.

For the separation of DIB derivatives of MPs' enantiomers in urine samples, we first used the two columns connected in series; a Daisopak SP−120−5−ODS column (250 x 4.6 mm i.d., 5 μm, Daiso, Osaka, Japan) and a Chiralcel OD−R column (250 x 4.6 mm, i.d., 10 μm, Daisel Chemical Ind., Tokyo). In this case, (*S*)−(+)−enantiomers of AP and MP co−eluted and were not separable by changing some conditions such as mobile phase composition, pH, flow rate and column temperature, and thus, a pre−separation step using a C 18 column was added. The proposed method was highly sensitive with the detection limits (S/N=3) of MPs enantiomers using spiked urine samples were 11, 12, 13, 32 and 35 nM in urine, which are equivalent to 2.8, 3.1, 3.2, 8.0 and 8.8 fmol on−column, for (*S*)−(+)−*p*−HMP, (*R*)−(−)−AP, (*S*)−(+)−AP, (*S*)−(+)−MP and (*R*)−(−)−MP. The method was applied to MP abusers' and a Parkinsonian's urine samples. As shown in Figure 3, the chromatogram of MP abuser's urine [Fig. 3(C)] showed three peaks considered to be DIB derivatives of (*S*)−(+)−*p*−HMP, (*S*)−(+)−AP and (*S*)−(+)−MP at 18, 37.5 and 40.4 min, respectively. In Fig. 3(D), which was obtained for urine from selegiline user, the expected two peaks corresponding to DIB−(*R*)−(−)−AP and −(*R*)−(−)−MP eluted at 35 and 41.8 min, respectively. Table 1 summa-

Table 1. Concentrations of MP enantiomers in MP abusers' (1−6) and selegiline user's (7) urine samples.

Sample	Age	Gender	Sample	Concentration in urine (μM)[a]					
No.	years		size (μL)	(*S*)−(+)−*p*−HMP	(*R*)−(−)−*p*−HMP	(*S*)−(+)−AP	(*R*)−(−)−AP	(*S*)−(+)−MP	(*R*)−(−)−MP
1	44	M	0.1	ND[b]	ND	135.8(5)	ND	2964.7(4)	ND
2	31	F	0.5	7.2(0)	ND	13.0(9)	ND	403.9(5)	ND
3	37	M	1.0	22.8(5)	ND	41.8(6)	ND	309.2(4)	ND
4	32	M	1.0	ND	ND	2.6(6)	ND	4.1(9)	ND
5	32	M	0.5	41.8(5)	ND	260.7(7)	ND	1959.5(5)	ND
6	44	F	1.0	6.3(6)	ND	10.3(10)	ND	74.5(10)	ND
7		F	10.0	ND	ND	ND	1.1(5)	ND	3.5(3)

[a] Average of triplicate determinations with RSD (%) in parentheses.
[b] Not detectable.

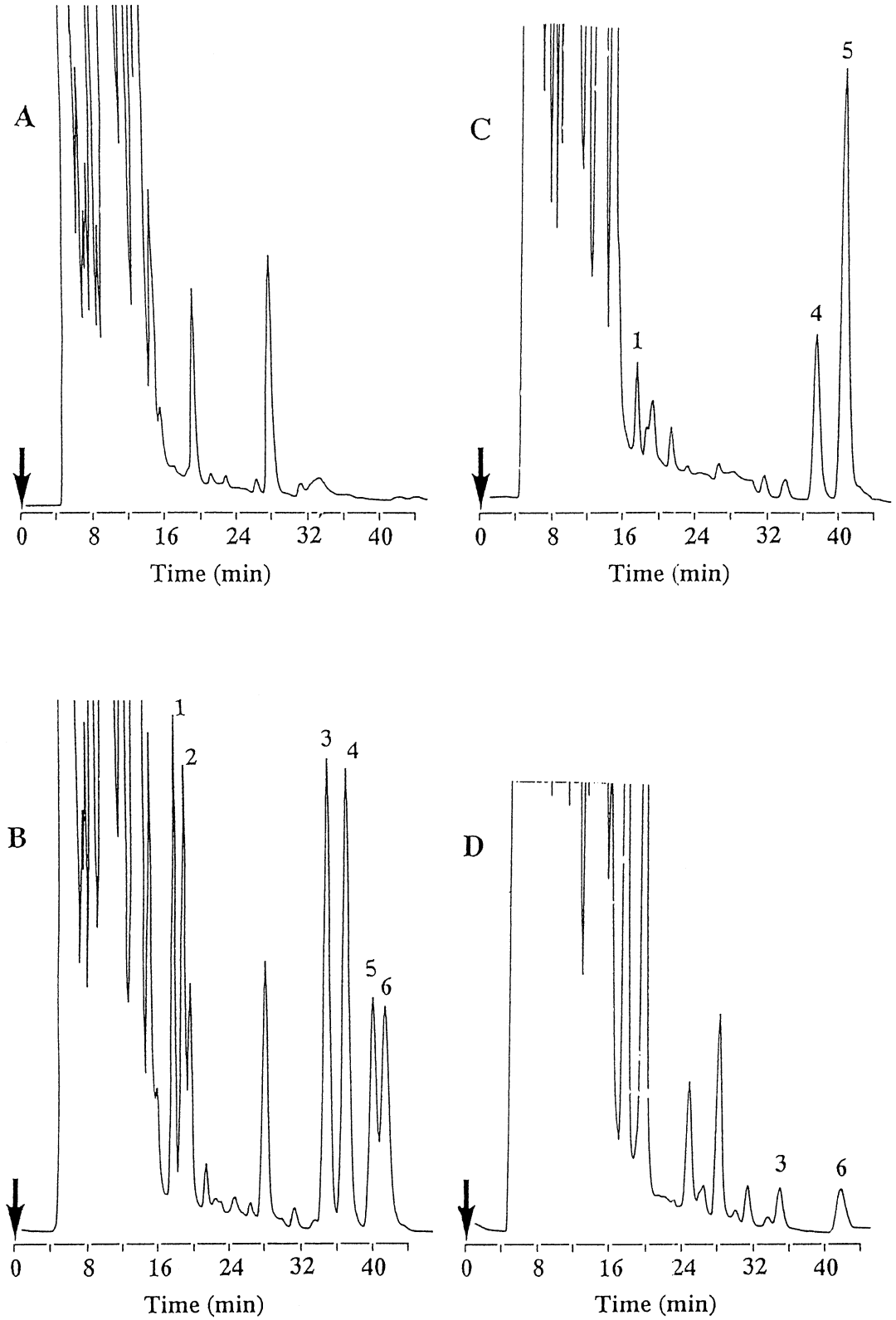

Figure 3. Typical chromatograms of DIB−Cl derivatives with fluorescence detection of (A) 10−fold diluted control human urine, (B) 10−fold diluted human urine spiked with MPs at a concentration of 10 μM per enantiomer, with 2.5 pmol of each compound injected on−column, (C) 10−fold diluted MP abuser's urine sample and (D) Parkinsonian's urine after selegiline intake. Peak numbers: 1, (*S*)−(+)−*p*−HMP; 2, (*R*)−(−)−*p*−HMP; 3, (*R*)−(−)−AP; 4, (*S*)−(+)−AP; 5, (*S*)−(+)−MP; 6, (*R*)−(−)−MP. Reproduced by permission of The Royal Society of Chemistry [7].

rizes the quantification results of MP and its major metabolites AP and *p*−HMP enantiomers detected in urine samples from six abusers (No. 1−6) and a urine sample (No. 7) from a Parkinsonian who uses selegiline as a lawful medication [7].

Miniaturized achiral and chiral HPLC methods were also developed for the determination of MP and AP in human urine. DIB−derivatives were separated on a semi−micro ODS column with Tris−HCl buffer (0.1 M, pH 7)−acetonitrile (45:55, v/v) at a flow rate of 0.2 ml/min or their enantiomers were separated on a semi−micro OD−RH column with sodium hexafluorophosphate (0.3 M aq.)−acetonitrile (44:56, v/v) at a flow rate of 0.1 ml/min as the mobile phase. The detection limits for the achiral and chiral analyses were 0.14−0.67 and 0.95−5.0 fmol on column (S/N=3), respectively. The methods were applied to the determination of MP and AP in human urine samples and the concentrations determined by the two methods were well correlated (r=0.994) [8].

1−2) Blood sample

It is more difficult to obtain a blood sample from the suspects compared to a urine sample from a legal point of view. We have developed a sensitive method for determination of MP and AP using one hundred μl of blood sample that is smaller than the volumes of 0.5−1.0 mL used in the methods reported. The pretreatment of the sample was performed by a liquid−liquid extraction method with ethyl acetate. DIB−Cl derivatives were separated isocratically using an ODS column, and quantified by an internal standard method using 1−methyl−3−phenylpropylamine (MPPA) as an I.S. The lower detection limits of the proposed method for MP and AP were 0.87 ng/mL and 0.46 ng/mL at S/N=3, respectively [9].

We have obtained two blood samples from the emergency hospital, which were collected from two abusers who had illegally ingested MP. One of the abusers (case Ⅰ) was treated with dialysis; samples before and after dialysis were analyzed for AP and MP whole the plasma levels of AP and MP of the second abuser (case Ⅱ) were monitored for three subsequent days including the day of digestion. In case I, the abuser's urine was screened for MPs with Triage 8 (Biosite Diagnostics Inc.), and positive results were obtained. After dialysis for 5 h, MP plasma levels decreased by 3.8−fold from 696 ± 33 to 184 ± 3 ng/mL, while AP decreased by 3.1−fold from 24.1 ± 1.0 to 7.9 ± 0.1 ng/mL. This result shows that dialysis is effective to eliminate MPs from blood. In case Ⅱ, on the day of MP digestion, AP concentration in plasma was 4.0 ± 0.3 ng/mL and declined within 2 days to 2.1 ± 0.2 ng/mL, while MP concentration decreased from 52.7 ± 1.6 to 8.5 ± 0.7 ng/mL. The results agree with the elimination half−lives of AP and MP where AP's half−live was reported to be in the range of 10−30 h, which is longer than that of MP from 4 to 5 h.

1−3) Hair samples

For forensic science laboratories and criminal justice agencies, hair might become the indispensable matrix for measuring exposure to xenobiotics, particularly drugs of abuse. Owing to its biological stability and physical state, hair is easy to collect and store until analysis can be carried out. Moreover, the slow growth rate and the absence of drug metabolism in hair allow investigations over a lengthy period in comparison with other biological samples. Hair analyses might disclose drug abuse history by providing long−term information on an individual's illegal drug consumption, in contrast to short−term information that blood or urine analysis provides. Although GC−MS is the most frequently utilized method to determine drugs in hair, other techniques such as radioimmunoassay and HPLC with chemiluminescence detection have also been reported.

A very sensitive and simple HPLC−FL detection method for the determination of MPs was developed with the detection limits of 74.6 pg/mg and 51.4 pg/mg for MP and AP at S/N=3, respectively. The method was applied to segmental analysis of some abusers' hair samples. Human scalp hair grows at an average rate of 1 cm per month. Therefore, segmental analysis of hair cut into sections of 1 cm long provides a monthly−based drug abuse time course as this information is stored in the keratinized hair. After marking their roots and tips, 10 strands of hair were struck in parallel on a commercially available double−faced sticker and cut into 1 cm long segments. After each segment was cut into 0.4−0.6 cm, the pieces were washed with 0.1% sodium dodecyl sulfate and ethanol, dried in a dish dryer, and extracted with HCl−methanol (1:20, v/v). The quantitative results for 6 hair samples from abusers clarified the illegal use of MP for several months [10].

As described above, MPs are enantioselective with the (*S*)−(+)−isomer being about five times more active than its antipode. In Japan, the (*S*)−(+)−enantiomer of MP is abused in more than 99% of cases. Therefore its presence, together with the corresponding enantiomers of its metabolites in biological sample indicates the illegal intake of this compound. However, the occurrence of (*R*)−(−)−MP and the respective enantiomers of its metabolites in body fluids or tissues might be due to a legitimate therapy, such as Vicks inhaler, which contains 50 mg of (*R*)−(−)−MP in the United States, or selegiline is known to be metabolized in human body into (*R*)−(−)−MPs.

We thus developed a sensitive enantio−selective method for determination of MPs in hair samples. The samples were segmentally analyzed based on 1 cm long segments. In four hair samples, only the (*S*)−(+)−enantiomers of MP and its *N*−desmethylated metabolite, (*S*)−(+)−AP were detected. The concentrations of (*S*)−(+)−MP and (*S*)−(+)−AP in MP abusers' 1 cm long hair segments are shown in Figure 4 [11].

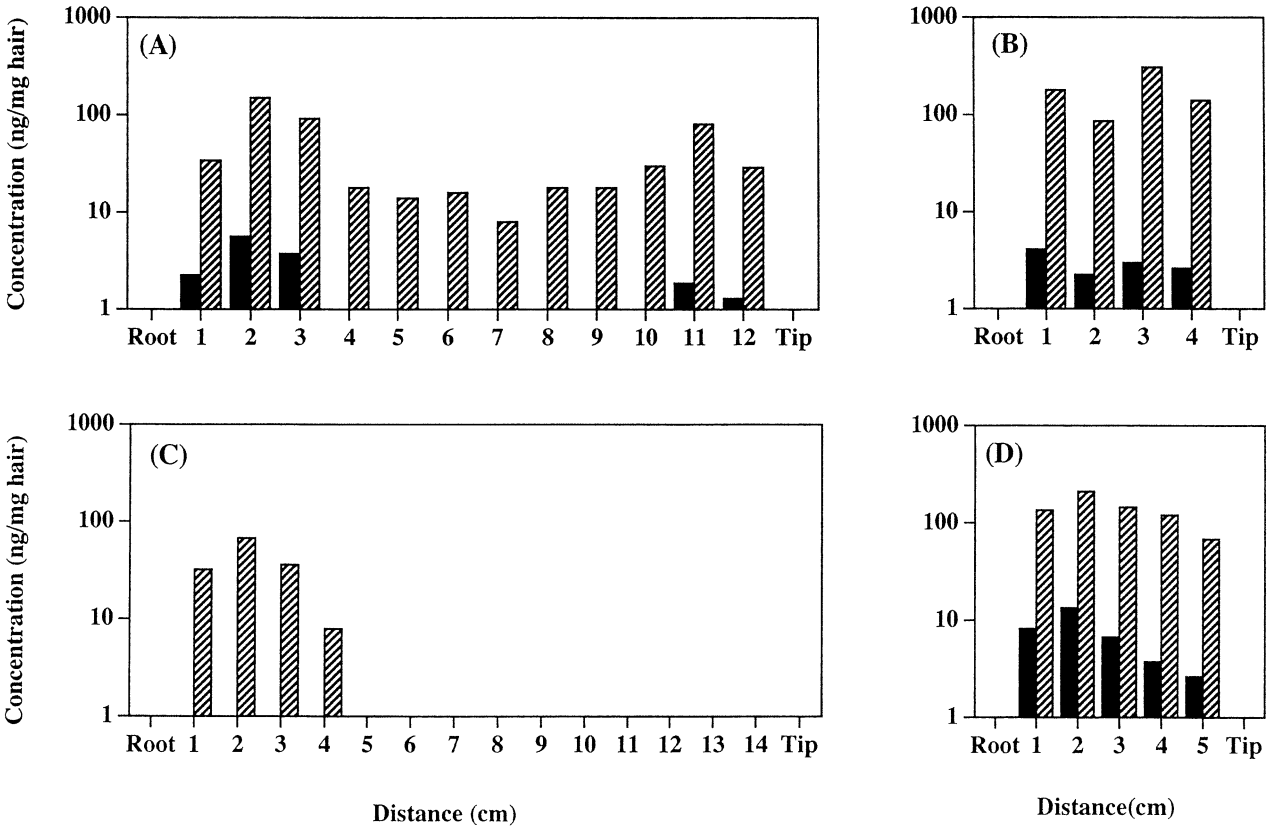

Figure 4. Concentrations of (*S*)−(+)−MP (hatched bars) and (*S*)−(+)−AP (solid bars) in MP abusers' 1 cm long hair segments. Reproduced by permission of John Wiley & Sons, Ltd. [11].

In an attempt to utilize the numerous advantages of semi−micro column HPLC, the determination of MP, AP and their enantiomers in abusers' hair was investigated. Theoretically, separations that are carried out on a conventional size HPLC are separable using miniaturized HPLC systems. The use of semi−micro column HPLC with a typical diameter of 1−2 mm is advantageous due to the reduction of sample size that enable the analyst to use the less available biological samples, whereas the noticeable reduction in chemicals and solvents used not only permits the use of rare, expensive chemicals without a significant increase in the analysis cost, but it also increases workplace safety. Moreover, the use of lower flow rate of mobile phase in comparison with conventional HPLC results in a significant improvement in the detection sensitivity due to less diluting by the eluent. For the analysis of drugs of abuse, the long detection window is the most important advantage of hair in comparison to other biological samples. However, the interpretation of its results and quantitative data is still debatable due to the incomplete understanding of the mechanisms that control drug incorporation into hair. Generally, a 20−50 mg amount of hair is considered enough material to answer analytical questions. However, in cases where the amount of sample available is limited or to study the biochemical aspects of drug incorporation into hair, a sensitive method with a minimal consumption of sample material is needed.

Therefore, we developed a highly sensitive and selective semi−mico column HPLC method for determining MP, AP and their enentiomers, and applied this analysis to sub−mg amounts of a single strand of hair to discuss the differences of MP and AP concentrations between physically similar hair strands that belong to the same subject. The Nanospace SI−1 series (Shiseido) were used to construct the HPLC system including a 250 x 1.5 mm i.d., 5 μm Capcell Pak C18 UG 120 S5 and a 150 x 2 mm i.d., 5 μm Chiralcel OD−RH for the achiral and chiral analyses, respectively. Extraction of MPs from hair was acheived with 5% TFA in methanol with a tentative recovery of 100%. The detection limits of MPs for both the achiral and chiral methods were 1.0−4.7 fmol/ 5 μL injection, and these are equivalent to 54.0−282 pg/mg hair.

Hair samples collected from eight Japanese who were suspected of abusing MP were analyzed using approximately 10 strands. Neither (*R*)−(−)−MP nor its corresponding metabolite, (*R*)−(−)−AP, were detectable in these samples. Ten hair strands of sample G were independently measured by the achiral method. As shown in Figure 5 c, the concentrations of AP and MP per hair that varied significantly were in the range of 0.05−0.24 ng/hair for the former and 2.5−10.5 ng/hair for the latter. As hair strands of the same length might differ in their weight due to diameter irregulari-

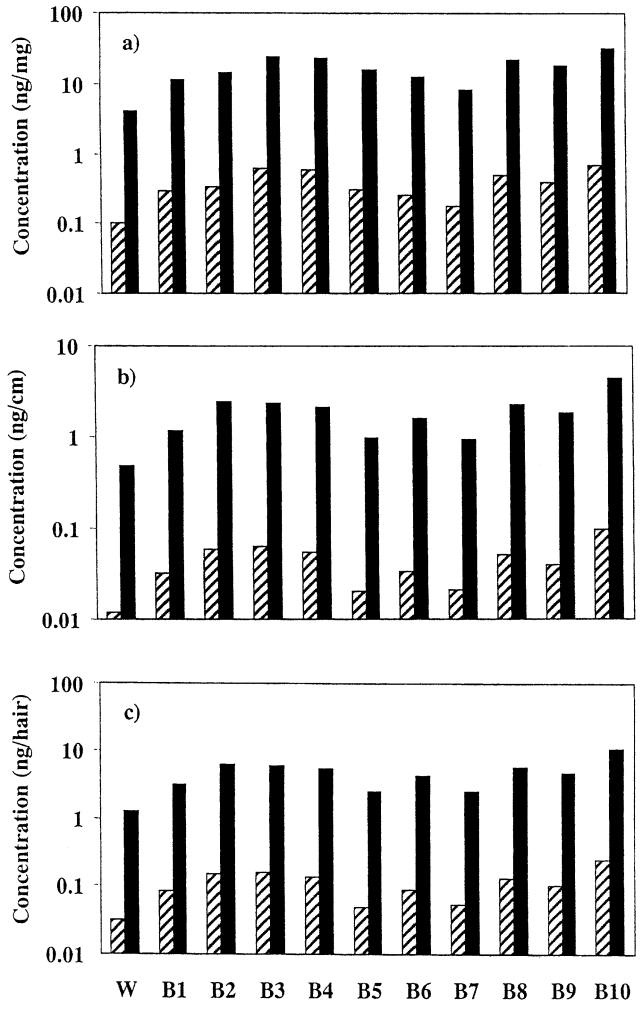

Figure 5. Concentrations of AP (hatched bars) and MP (solid bars) in individual hair strands of sample G expressed in (a) ng/mg, (b) ng/cm and (c) ng/hair. W and B designate the white and black hair strands, respectively. Reproduced by permission of John Wiley & Sons, Ltd. [12].

ties, we calculated the concentration of these compounds per unit weight (ng/mg hair). Figure 5a shows that concentrations of these compounds varied significantly among different hair strands. Therefore we assumed a constant growth rate for the analyzed hair strands and calculated the concentrations of AP and MP per unit length (ng/cm) regardless the weight or the diameter differences to reveal any difference in the amount of these compounds incorporated into hair within same period. However, as in Figure 5b, different concentrations that show a trend similar to those determined as ng/mg or ng/hair were obtained.

In some extreme cases such as at the crime spot, the sample material available for forensic science or toxicological analyses might not exceed a single 1 cm−long hair. We cut a 3 cm−long hair strand of samples A and G into 3 pieces of 1 cm, and analyzed them individually. The mean concentrations of MP in sample A and D were 77.43 ng/mg and 21.20 ng/mg, respectively [12].

1−4) clothes

Sweat is a liquid secreted from sweat glands and known to contain trace elements, waste products and any substances and/or xenobiotics present in the blood. We developed two HPLC methods with fluorescence and UV detections to determine MPs in abusers' garments to serve as a further evidence that strengthens the results obtained from other biological samples such as urine, blood and hair. Table 2 shows the concentrations of AP and MP in MP abusers' clothes samples. MPs were detected in rather high concentrations in three kinds of clothes. The higher concentration in underwear compared to pants might due to direct contact with skin [13].

2. Analysis of MDMA and MDA

Recently, MDMA and MDA are becoming increasingly popular and its abuse is spreading among young people due to their entactogen properties. Therefore mental disease caused by MDMAs has become very serious. This made us to develop new methods for determining MDMA related compounds. When MDMA is suspected to be ingested by MP's abusers, a simultaneous determination method is useful to identify which drug was abused. The proposed HPLC method with fluorescence detection showed high sen-

Table 2. Concentrations of MP and AP in abusers clothes as determined by the HPLC−UV and HPLC−FL methods.

Sample	Description	Amount used (mg)	Concentration[a] (ng/0.1μg)					
			p−HMP		AP		MP	
			UV	FL	UV	FL	UV	FL
1	White cotton undershirt	10	ND[b]	ND	113.1	109.2	1173.2	1063.4
2	Colored cotton underpants	10	ND	ND	87.9	73.3	1729.8	1679.9
3	Gray synthetic fiber pants of a training suit	50	ND	ND	4.8	3.4	127.7	91.4

[a] Average of 6 measurements.
[b] Not detectable.

sitivity, and successfully applied to hair analysis with the detection limits of 50 pg/mL and 200 pg/mL for MDMA and MDA at S/N= 3, respectively. The results of segmental analysis using 2 strands of abusers hair is presented in Figure 6, from which it can be determined that the abuser has continuously ingested for at least one year [14, 15].

Special attention has been focused on seized tablets of MDMA containing other drugs, such as MDA, caffeine, ephedrine, MP and ketamine. Some compounds may be impurities in the formulation, where others may be added with an expectation of an additional effect. The consumption of these tablets has a potential possibility for multi−drug abuse. Therefore, pharmacokinetic and pharmacodynamic studies on drug−drug interactions among these drugs are very important, as well as forensic studies. Hence, a sensitive and selective determination method of MDMA and its related compounds in urine as well as blood is required. For this purpose, we developed a simultaneous semi−micro column HPLC method with fluorescence detection of MDMA and its related compounds

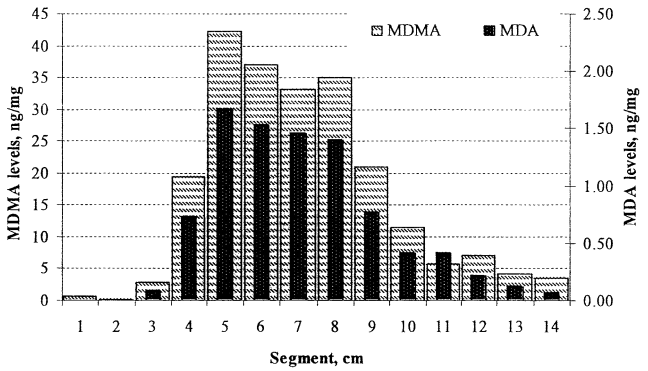

Figure 6. Concentrations of MDMA and MDA in MDMA abuser's hair strands sectioned into 1 cm length segments. Reproduced by permission of John Wiley & Sons, Ltd. [14].

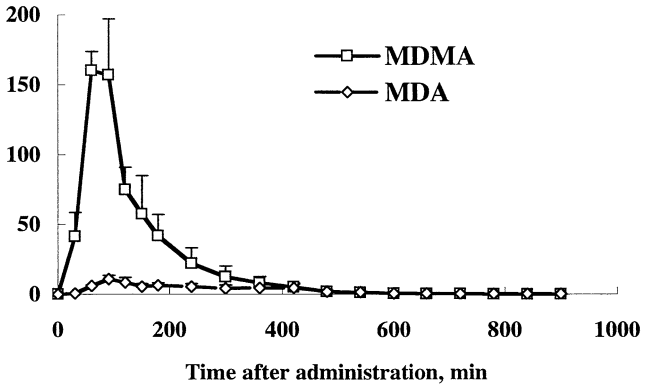

Figure 7. MDMA and MDA concentration−time profile in rat urine after administration of MDMA. Dose, 2 mg/kg; n =3. Reproduced by permission of John & Wiley Sons, Lts. [16].

such as MDA, MP and AP in rat urine by using DBD−F as a label and 1−phenylethylamine as an I.S. MDMA and its related compounds were extracted from urine with n−hexane, which was evaporated under the acidic condition with N_2 gas. The residue was derivatized with DBD−F at pH 8.5 at 80℃ for 20 min. The detection limits for MDMA and related compounds were 7.8−195 fmol on column at S/N=3.

To evaluate the applicability of the proposed method for practical samples, MDMA and its metabolite MDA were monitored after a single administration of MDMA tablets. Figure 7 shows the time course of MDMA and MDA after administration of MDMA at a dose of 2 mg/kg. This result demonstrates that the proposed method can be applied to pharmacokinetic studies of MDMA [16].

3. Analysis of obesity drug

1) Fen, Phen and *N*−Fen

Although Fen and Phen have been withdrawn from the market, they are still being prescribed and widely abused, which suggests the development of a method for their simultaneous determination in biological fluids will be helpful in monitoring their levels. We first developed a simultaneous HPLC−FL method with dansyl chloride as a label to determine Fen and Phen in addition to four other sympathomimetic amines, namely, norephedrine (NE), ephedrine (E), 2−phenylethylamine (2−PEA), and 4−bromo−2,5−dimethoxyphenylethylamine (2−CB), using fluoxetine (FLX) as an I.S. The dansyl derivatives of these compounds could be well separated from the components of human plasma within 45 min, and sensitively detected with detection limits from 16 to 255 fmol on column at S/N=3. This method could be successfully applied to the determination of Fen and Phen in rat plasma after a single intraperitoneal administration of each drug, individually and combination [17].

Reports concerning the chiral separation of D−Fen, L−Fen, in addition to their metabolites D−norfenfluramine (D−Norf) and L−norfenfluramine (L−Norf) in biological fluids are few. Thus, we improved the detection limits of Fen and DL−Fen in addition to some amines such as NE, E, 2−PEA, 2−CB by using DIB−Cl as a label. By an achiral method, the DIB derivatives of 6 standard compounds including FLX as an I.S. could be separated within 55 min (Figure 8), and sensitively detected with detection limits of 1.2 −100 fmol on column at S/N=3. Furthermore, the detection limits for D−Fen, L−Phen and Phen in spiked human plasma and rat plasma were over the range of 1.4−3.5 and 1.4−4.3 fmol on column, respectively. On the other hand, Phen and enantiomers of Fen and Norf in rat plasma were determined by the chiral method. After a single intraperitoneal administration of 1 mg/kg of Phen and DL−Fen, the time course of these compounds could be successfully monitored for 10 h [18].

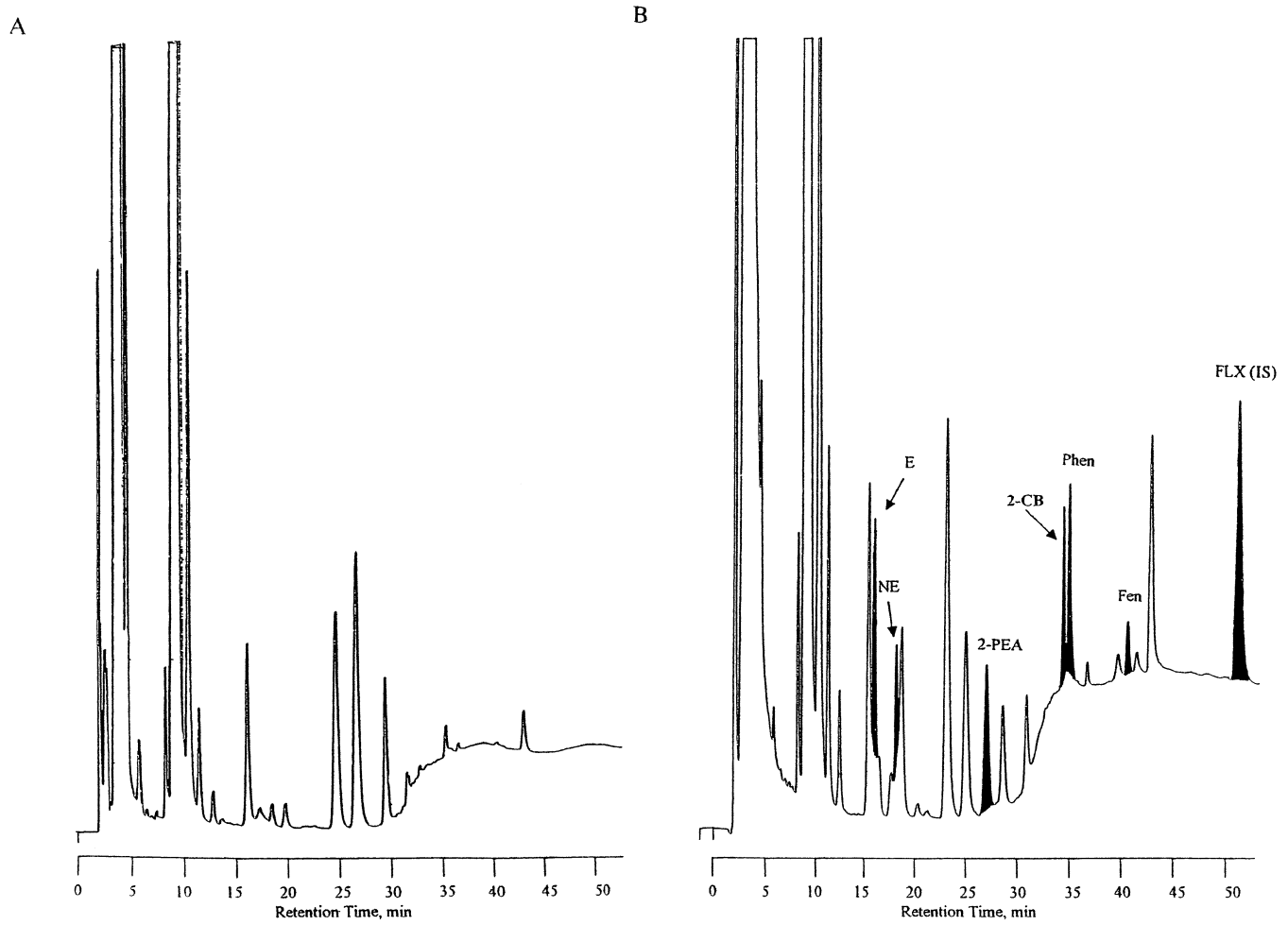

Figure 8. Typical chromatograms of human plasma, (A) free and (B) spiked with known concentration of the sympathomimetic amines by the achiral system. The peak represent 250 nM for E, NE and 2−PEA, and 100 nM for 2−CB, Phen and DL−Fen. Reproduced by permission of Elsevier [18].

Phen−Fen combination affords superior appetite control when compared to either drug alone. The anorectic properties of Fen are related to enhanced serotonergic neurotransmission; it facilitates the release of serotonin (5−HT) and inhibits its uptake by serotonergic nerve endings, and its metabolite Norf releases 5−HT and stimulates postsynaptic 5−HT 2 receptors. On the other hand, Phen stimulates the release of dopamine. Therefore, it is of interest to resolve the mechanism by which the combination Phen−Fen enhances weight loss at lower doses as well as the neurotoxic effect. To study the mechanism, we developed the method for determining Phen, Fen and Norf simultaneously in rat brain and blood microdialysates, and applied this to a drug−drug interaction study of Phen and Fen. We also applied this method to examine the effect of the drug combination on the protein binding of Phen and Fen in rat plasma ultrafiltrate after intraperitoneal administration of 1 and 5 mg/kg of Phen and Fen, respectively. Consequently, the results showed that Fen and/or Norf significantly altered the pharmacokinetic parameters of Phen in both blood and brain, but did not alter its protein binding. On the other hand, there were no significant differences in pharmacokinetics of Fen when administered with Phen [19].

Fen itself is not normally associated with liver damage, but it has been found to be present in a carcinogenic and hepatotoxic form called *N*−Fen and has been identified in some Chinese diet products. Unfortunately some Japanese women were died by ingesting these products. The addition of *N*−Fen to diet products was done under the assumption that it would to aid weight loss like Fen. This, however, could be an attempt to evade the Chinese drugs regulations since its use as Fen are not approved in China. *N*−Fen is a new compound and can be easily prepared from Fen. Sensitive determination of *N*−Fen is difficult because of its very weak UV absorption. Also, as it has no reactive amino function in its structure, fluorescent derivatization with DIB−Cl cannot be used. Therefore, we tried to determine its metabolites, Fen and Norf as biomerkers for *N*−Fen ingestion.

We have developed a very sensitive method for determining

Fen and Norf in hair samples. The detection limits were 36 and 16 pg/mg hair for Fen and Norf at S/N=3, respectively. The method was successfully applied to the segmental analysis of Fen and Norf in hair sample obtained from hospitalized patients diagnosed with hepatotoxicity and suspected to have ingested *N*−Fen. Both Fen and Norf could be detected in 6 patients' hair samples in the ranges of 43−1389 pg/mg for Fen and 18−680 pg/mg for Norf, and the results showed that the patients might have ingested *N*−Fen for a period of not less than 5 months. Furthermore, in addition to the historical recording of *N*−Fen ingestion, the determination of Fen and Norf in a single hair was performed. The results for the hair strands of patient B (four black strands, length ranged from 8 to 10 cm) and patient C (three black strands, length ranged from 7 to 8 cm) were shown in Table 3. Figure 9 shows concentration of Fen in pg/mg determined in single strands of hair of patient C. The white hair with the weight of 0.57 mg contained 110 pg/mg of Fen, which was comparable to the mean of three strands of C (1−3). Additionally, the method was applied to the determination of Fen and Norf in rats that possess pigmented and non−pigmented hair after an intraperitoneal administration of Fen. The concentrations of both compounds were determined in black as well as in white hair. These results were different from those of amphetamines, which can be more easily incorporated into black hair than white hair. Further investigation will be required to clarify the reason of this difference [20].

Table 3. Concentration of Fen in single hair strand of patients B and C

Patient	Weight (mg)	Concentration (pg/mg)
B−1	1.59	Trace[a]
B−2	1.1	75
B−3	1.96	108
B−4	0.41	46
C−1	0.41	99
C−2	0.33	49
C−3	0.65	158

[a] Lower than the quantitation limit.

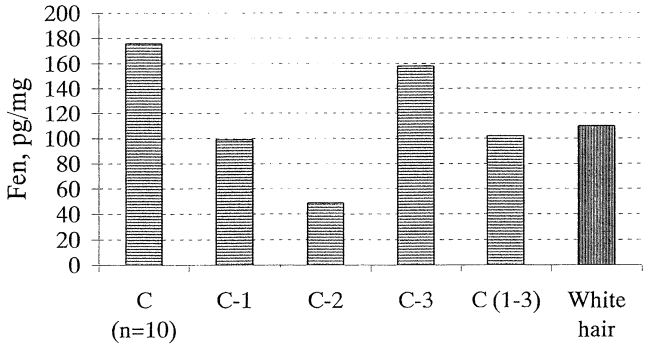

Figure 9. Concentrations of Fen in pg/mg determined in single strands of hair of patient C. The first bar represents the average of Fen concentration obtained from the segmental analysis of 10 strands, C (1−3) bar represents the average of C−1, C−2, and C−3. Reproduced by permission of Elsevier [20].

2) Other obesity drugs

Some other obesity drugs such as mazindol and phenylpropanolamine also have been abused in society. Mazindol is a tricyclic imidazoisoindole compound, and used as an anorexant for short−term adjunct therapy in the treatment of obesity. We have developed an HPLC method using DIB−Cl as a label for the analysis of mazindol and its metabolite 2−(2−aminoethyl)−3−(*p*−chlorophenyl)−3−hydroxyphtalimidine (Met) in mouse brain and plasma. The two compounds were quantified by measuring Met after two different sample pretreatments. For mazindol determination, the treatment involved its hydrolysis to Met, while for Met determination the hydrolysis was omitted. The method was highly sensitive with the detection limits for Met on column of 2.8 fmol and 3.5 fmol in plasma and brain tissue, respectively, at S/N=3. The developed method was successfully applied for the monitoring of mazindol and Met levels in mouse plasma and brain tissue regions after a single intraperitoneal administration of 0.5 mg/kg of mazindol [21].

Phenylpropanolamine (PPA) is a sympathomimetic compound, and has been widely used as over−the−counter and prescription medications for cough and cold, nasal decongestant and as an appetite suppressant. Recently, its serious side effects such as hemorrhagic stroke, arrhythmias and hypertension were reported. We developed an HPLC method with DIB−Cl as a label for determining PPA in human plasma and rat brain and blood microdialysates. The method was sensitive with the detection limits of 17, 48 and 40 fmol on column in plasma, and blood and brain microdialysates, respectively. The method was successfully applied for monitoring of PPA levels in rat plasma and blood microdialysates administered with a single oral dose of 2.5 mg/kg of PPA [22]. Most PPA products including cough−cold and slimming preparation contain PPA along with added ingredients which may indicate that drug−drug interaction effects are likely and may involve various side effects associated with PPA use. The most common ingredient present in PPA preparations is caffeine. In addition to caffeine, hypnotics such as chlorpheniramine and clemastine are also included. We thus examined pharmacokinetic interactions between PPA, caffeine and chlorpheniramine in rats. The single i.p. administration of caffeine (5 mg/kg) with PPA (2.5 mg/kg) to rats caused a 1.6−fold increase in the area under the curve (AUC) of PPA in brain compared to the single administration of PPA, and

was comparable to the 1.5−fold increase in the AUC caused by chlorpheniramine (0.4 mg/kg). The multiple combinations caused an increase in the AUC by 1.9−fold [23].

Conclusion

We have studied on the development of highly sensitive determination methods for many kinds of drugs of abuse. The main purpose of this study is to protect from and prevent for risks of human health, which have been caused by a serious drug abuse. We hope the developed methods will be useful to develop sensitive methods for analysis of new illicit designer drugs, and will contribute to resolving social problems caused by a drug abuse.

Acknowledgement

The author is very grateful to Prof. Kazuhiro Imai, Musashino University, for his valuable suggestion to our studies. All works have been performed with many co−workers. The author would also like to thank them for their assistance and discussions, especially to Dr. Osama Y. Al−Dirbashi and Dr. Amal Kaddoumi. The part of this work was supported by Grants from the Ministry of Health, Labor and Welfare of Japan and from the Ministry of Education, Science, Sports and Culture of Japan.

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