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Reviews

Analytical Studies on the Development of High-Performance Liquid Chromatographic Methods with Fluorescence or Chemiluminescence Detections and Their Practical Applications

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This review describes a comprehensive investigation focusing on syntheses of analytical reagents, followed by their utilization in development of analytical methods, and leading to practical applications for rational use of medicaments centering on abused drugs. Many kinds of analytical reagents for fluorescence and chemiluminescence detections have been synthesized with the intention of improving sensitivity. By properly combining the developed analytical reagents with an HPLC separation technique, one could determine ultra-small amounts of abused drugs such as stimulants, narcotics and obesity drugs in various matrices. Furthermore, chiral analyses of some abused drugs and evaluation of their potential for drug-drug interaction were also performed. The developed methods might be useful for forensic and toxicological studies on drug abuse. Also, the results obtained in our study might contribute to the prediction of and the protection of human health from risks of abused drugs.

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1 Introduction

Medicaments, which are biologically active as internal physiologically active compounds, have been contributing to human health. However, they have sometimes showed a serious risk for human health, and thus the promotion of rational use of medicaments is very important. Developments of practical and precise analytical methods for medicaments could be the first steps to lead to such rational use. To date, numerous analytical methods for medicaments have been developed by using gas chromatography (GC), GC-mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) as summarized in the reviews.[1,2] GC has been commonly used for the analysis of volatile compounds, but it can not be applied to non-volatile compounds. Although GC-MS is sensitive and has been predominantly used in forensic sciences to determine medicaments such as abused drugs, it requires an expensive experimental apparatus. On the other hand, HPLC shows excellent capability for the analysis of aqueous samples. Most medicaments in biological samples are commonly non-volatile polar compounds, and thus HPLC is more suitable than GC for their analysis. In HPLC analysis, the choice of detection method is important and many kinds of detectors have been used in various cases. For the sensitive determination of medicaments, fluorescence (FL), chemiluminescence (CL) or MS detections have been adopted. Among these, an HPLC-FL method has been extensively employed for determination of trace amounts of compounds due to its high sensitivity and selectivity. Furthermore, its instruments are inexpensive compared to those for MS (or MS/MS) detection. CL is the emission of light from the electronically excited luminophores by a chemical reaction of precursors of those species. The CL reaction requires no exciting light source. And thus, this permits an increase in the detector's sensitivity and an attainment of a large signal-to-noise (*S*/*N*) ratio, resulting in a lower limit of detection (LOD). Therefore, several CL methods are of growing importance in forensic, pharmaceutical and biomedical analyses. Among

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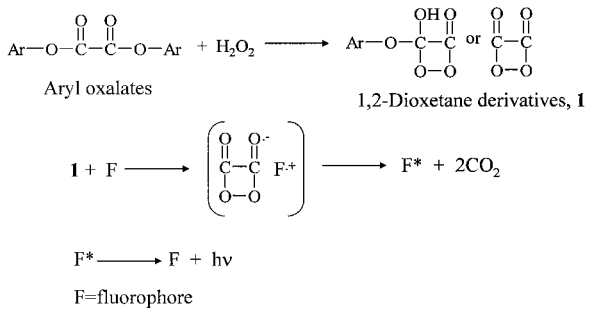

Fig. 1 Reaction mechanism of peroxyoxalate chemiluminescence.

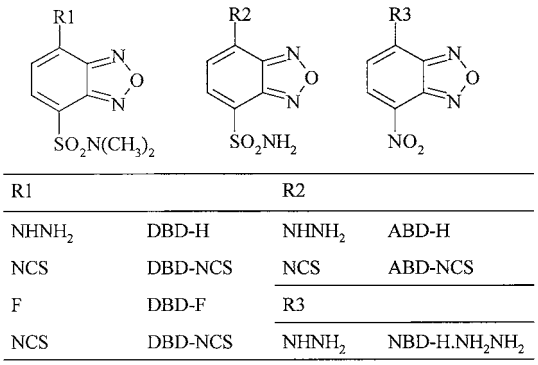

Fig. 2 Chemical structures of bezoxadiazole derivatives examined.

them, peroxyoxalate chemiluminescence (PO-CL) has been found to be one of the most effective CL methods for determination of ultra-small amounts of fluorophores. In a PO-CL system, oxalates or oxamides react with hydrogen peroxide in the presence of a fluorophore to produce an emission of light. The reaction is achieved by a chemically initiated electron exchange luminescence (CIEEL) mechanism *via* a high energy intermediate which forms a charge transfer complex with the co-existing fluorophore. The electron is transferred to the fluorophore, which transits to an excited state and returns to a ground state by emitting a photon ($h\nu$) (Fig. 1).[3] Hydrogen peroxide most effectively gives CL emission as an oxidant in PO-CL systems.

Furthermore, labeling is one of the powerful techniques to expand adaptation of the methods with FL and CL detections. For medicaments having no fluorescent property, a labeling is recommended in order to introduce desirable characteristics into the molecule. A labeling reaction is commonly achieved by a selective reaction between the functional group of the analyte and that of the labeling reagent. Labeling serves to improve sensitivity, selectivity and the chromatographic behavior. Therefore, many labeling reagents have been developed for each reactive functional group. To date, many reviews describing the labeling reagents for HPLC have been published.[4-8]

Among medicaments, many kinds of drugs have been widely abused and such abuse has caused serious social problems worldwide. For instance, methamphetamine (MP) is the most popular abused drug in Japan. In addition to MP, illegal designer drugs: 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA), have recently become popular and their use has spread among young people.[9] On the other hand, obesity drugs such as fenfluramine (Fen), phentermine (Phen), *N*-nitrosofenfluramine (N-Fen), mazindol (Maz) and phenylpropanolamine (PPA) were adulterated in commercially available diet products and have caused serious side effects.[10] Because of the growing seriousness of these situations, the prediction of and the protection of human health from the risks of abused drug are required. For these purposes, development of a sensitive and selective analytical method of drugs that may be abused is essential.

The aim of this review is to overview our recent studies on sensitive HPLC methods for determination of medicaments centered on drugs that may be abused. This review consists of three parts: 1) synthesis of original fluorescence and chemiluminescence reagents including their applications; 2) development of analytical methods for abusable drugs; and 3) practical applications of the developed methods.

Some sensitive detection methods of abusable drugs in various matrices by HPLC-FL and -PO-CL methods are presented. Furthermore, their practical applications for chiral analysis and evaluation of pharmacokinetic drug-drug interactions caused by some abusable drugs will be described. In each section, representative interesting results from our recent publications are summarized.

2 Synthesis and Evaluation of Fluorescence and Chemiluminescence Reagents with Their Applications

For development of analytical methods of medicaments, a choice of analytical reagent is very important, since it affects the following experiments. The design and synthesis of original analytical reagents in compliance with the purposes of analysis are the first step to promote analytical success. In this section, syntheses of analytical reagents for FL labeling and their applications to FL and PO-CL detections are described.

2·1 Bezoxadiazole derivatives[11-21]

Among the various fluorescence labeling reagents, bezoxadiazole derivatives have desirable properties as an FL labeling reagent, because the labeling reagent has no fluorescence itself and the labeled compounds give an intense fluorescence at a longer wavelength region to compounds in biological sample. We synthesized several benzoxadiazole labeling reagents as shown in Fig. 2. 4-(*N*,*N*-Dimethylaminosulfonyl)-7-hydrazino-2,1,3-benzoxadiazole (DBD-H), 4-aminosulfonyl-7-hydrazino-2,1,3-benzoxadiazole and 4-hydrazino-7-nitro-2,1,3-benzoxadiazole hydrazine (NBD-H·NH_2NH_2) were synthesized for aldehydes and ketones.[11] The labeled compounds fluoresced at wavelengths from 548 to 580 nm with excitations from 450 to 470 nm. Among them, DBD-H was highly sensitive for short-chain aliphatic aldehydes with a sub-pmol level of LOD for an HPLC-FL method.[12] This reagent could be applied to monitor the acetaldehyde level in normal human plasma after an ingestion of ethanol. Furthermore, DBD moiety was found to be efficiently excited by PO-CL reaction.[13] Based on this fact, medroxyprogesterone acetate,[14] long-chain aliphatic aldehydes[15] and propentofylline[16] labeled with DBD-H could be sensitively determined by PO-CL detection. Figure 3 shows the chromatograms of DBD-hydrazones of 8 aliphatic aldehydes obtained by PO-CL detection with bis(2,4,6-trichlorophenyl)oxalate (TCPO) and hydrogen peroxide as CL reagents. The LODs of analytes were in the range of 86 – 152 fmol at an *S*/*N* ratio of 2.

Edman-type fluorescence labeling reagents, 7-*N*,*N*-dimethylaminosulfonyl-4-(2,1,3-benzoxadiazolyl)isothiocyanate (DBD-NCS) and 7-aminosulfonyl-4-(2,1,3-benzoxadiazole)-

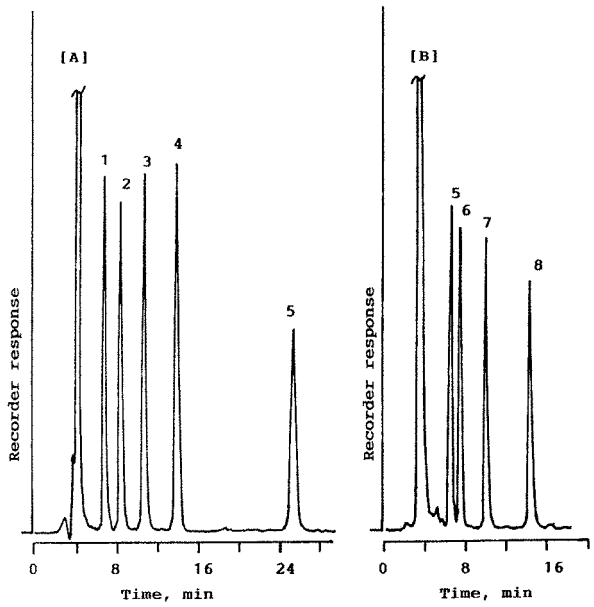

Fig. 3 Chromatograms of DBD-hydrazones of aliphatic aldehydes with PO-CL detection. Sample (concentration on column): [A] 1,1-hexanal (25.3 pmol); 2, 1-heptanal (26.9 pmol); 3, 1-octanal (29.0 pmol); 4, 1-nonanal (33.7 pmol); 5, 1-undecanal (29.3 pmol); detector sensitivity, 4 × 10. [B] 5, 1-undecanal (25.5 pmol); 6, *cis*-7-tetradecenal (25.6 pmol); 7, *cis*-9-hexadecenal (27.0 pmol); 8, *cis*-13-octadecenal (25.9 pmol); detector sensitivity, 32 × 10. Mobile phase: [A] 5 mM imidazole buffer (pH 7.5):acetonitrile (20:80, v/v); [B] 5 mM imidazole in acetonitorile. Printed from Ref. 15 with permission from John Wiley & Sons, Ltd.

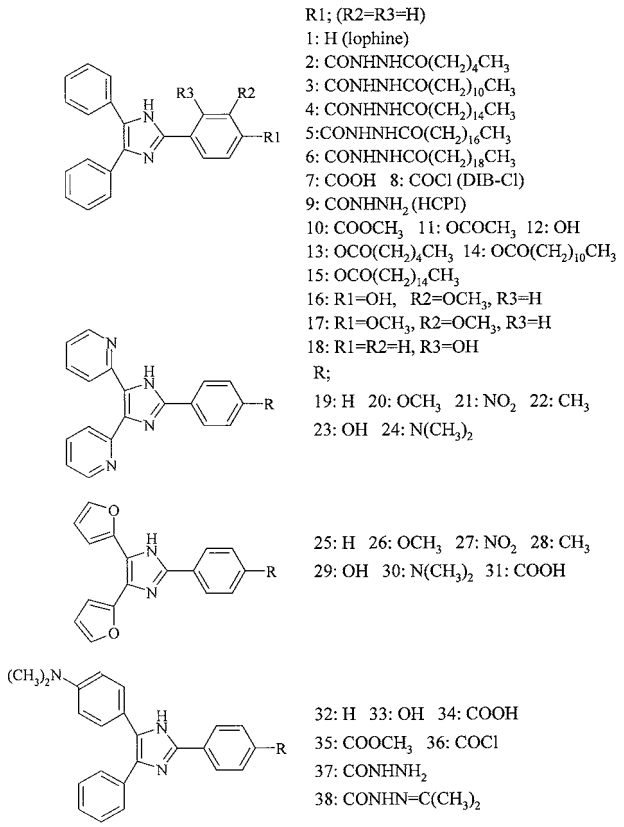

Fig. 4 Chemical structures of synthesized lophine derivatives.

isothiocyanate (ABD-NCS) were developed.[17] The DBD-labels of amino acids fluoresce at 505 nm with excitation at 385 nm. By using DBD-NCS, a novel analytical method for amino acid sequence and configuration determination of peptide containing D- or L-amino acids could be successfully developed.[18]

A DBD-type of labeling reagent, 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F), reacts with amino acids and biogenic amines; the resultant labels could be sensitively detected by PO-CL detection. The instrumental limit of detection (2 fmol) of authentic DBD-proline was comparable to that (1.3 fmol) of authentic dansyl-proline by the HPLC-PO-CL method.[13] β-Blockers having an isopropylamino group including alprenolol, metoprolol, oxprenolol or propranolol were detected with a few fmol levels of LOD.[19] Moreover, ebiratide, one of the synthetic analogues of ACTH4-9 for Alzheimer disease could also be determined and its time course in rat plasma could be traced with the lower levels of sub-pmol/mL.[20,21]

2·2 Lophine derivatives[22-34]

Lophine derivatives are well-known potential fluorescent or chemiluminescent compounds. Therefore, a variety of lophine derivatives were synthesized as shown in Fig. 4 and their FL and CL properties were evaluated. The lophine derivatives substituted by OCH_3 at the 3- and 4-positions (compound **17**), and COOH (**7**), COCl (**8**), $CONHNH_2$ (**9**) and $CONHNHCO(CH_2)_4CH_3$ (**2**) at the 4-position on the phenyl ring at the 2-position of imidazole skeleton showed strong chemiluminescence intensities which were comparable to that of lophine itself.[22] In 2-substituted-4,5-di(2-pyridyl)imidazoles, 2-substituted-4,5-di(2-furyl)imidazoles and 2-substituted-4(or 5)-(4-dimethylaminophenyl)-5(or 4)-phenylimidazoles, the 2-furyl groups showed intense fluorescence, while those having 2-pyridyl group gave weak intensities.[23] The relative chemiluminescence intensity (RCI) of 4-[4(or 5)-(4-dimethylaminophenyl)-5(or 4)phenyl-1*H*-imidazole-2-yl]benzoyl chloride obtained by a flow injection analysis (FIA) method showed the largest RCI, corresponding to 40% of that of lophine. Among them, 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole (HCPI, compound **9**) and 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride (DIB-Cl, compound **8**) were found to be available as fluorescence labeling reagents.

HCPI reacts with fatty acids to give intensely fluorescent labels at room temperature by using condensing reagents, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (ECD) and pyridine. When HCPI was used, 7 kinds of free fatty acids including medium- and long-chain saturated fatty acids (C_6, C_{10}, C_{12}, C_{14}, C_{16}, C_{18} and C_{20}) could be determined with margaric acid (C_{17}) as an internal standard (IS), as shown in Fig. 5. The LODs were 7 – 56 fmol (S/N = 3).[24] Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are major long-chain *n*-3 polyunsaturated fatty acids which are abundantly present in fish oils. The labeling of these compounds with HCPI was performed in 20 min at room temperature in the presence of ECD and pyridine.[25] EPA and DHA in human serum, dietary supplements and pharmaceutical preparations were successfully determined with LODs of 200 fmol (S/N = 3). Free carnitine and short- and medium-chain acylcarnitines in human plasma could be determined by HPLC with HCPI under the mild labeling conditions. This may have the advantage of preventing racemization during the labeling of enantiomers of carnitine and acylcarnitines.[26] Furthermore, HCPI was also a chemiluminogenic labeling reagent, and could be used for PO-CL detection of saturated free fatty acids (C_{16} and C_{18}).[27]

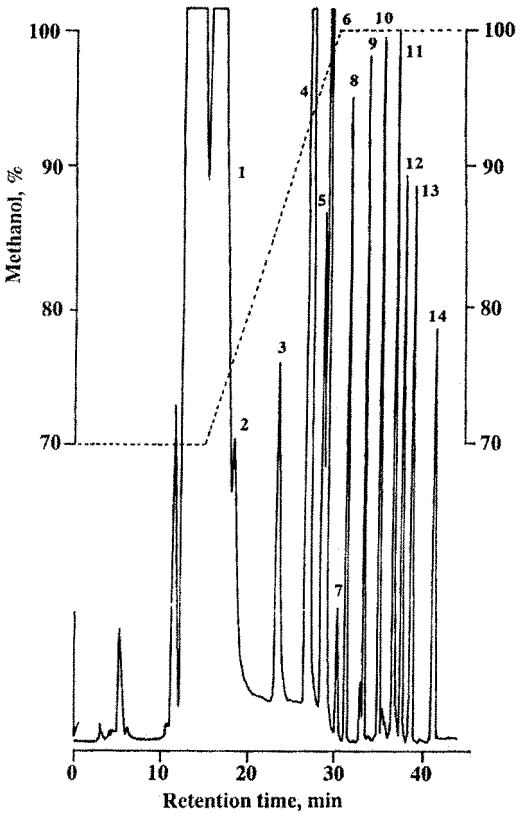

Fig. 5 Chromatogram of the HCPI-fatty acid derivatives. A 100-µl portion of a standard mixture of fatty acids (5 nmol/ml each) was treated according to the experimental procedure. Peaks: 1, 4, 6, 7, reagent blank; 2, C_5; 3, C_6; 5, C_8; 8, C_{10}; 9, C_{12}; 10, C_{14}; 11, C_{16}; 12, C_{17} (I. S.); 13, C_{18} and 14, C_{20}. Printed from Ref. 24 with permission from Elsevier Science Publishers B. V.

DIB-Cl is one of the useful labeling reagents because it exhibits high sensitivity for amines and phenolic compounds. This reagent rapidly reacts with amines or phenolic compounds in the presence of a basic catalyst or under an alkaline condition. For instance, urinary phenol, cresols and xylenols could be determined by an HPLC-FL method.[28,29] Bisphenol A (BPA), which has attracted considerable attention owing to its endocrine-disrupting effect, was labeled with DIB-Cl and determined in varied biological matrices. BPA that migrated from polycarbonate baby bottles was determined by an HPLC-PO-CL method.[30] Also, simultaneous determination of BPA, bisphenol B, bisphenol E, bisphenol F and 4-nonylphenol from polyvinyl chloride plastics were demonstrated by an HPLC-FL method.[31] Moreover, labeled BPA with DIB-Cl in biological fluids such as plasma,[32] microdialysates[33] and ascetic fluid[34] could be determined by an HPLC-FL method. The results obtained from these studies clarified the penetrating property of BPA through the blood-brain barrier[33] and the relationship of BPA concentrations in serum samples and in ascetic fluid samples.[34]

2·3 Reagents for PO-CL detection[35–42]

For PO-CL detection, the choice of an oxalate and a fluorophore is one of the most important factors to improve CL intensity. Several aryl oxalates and fluorophores were originally synthesized to increase the sensitivity of PO-CL detection. Representative aryl oxalates such as TCPO, bis(2,4-dinitrophenyl)oxalate (DNPO) and bis[2-(3,6,9-trioxadecyloxycarbonyl)-4-nitrophenyl]oxalate (TDPO) have been used in a PO-CL detection system. However, TCPO and DNPO show low solubility in acetonitrile and acetone which are common solvents for postcolumn CL reagents in HPLC. Improving the solubility of oxalates in these solvents is desirable for preparing proper concentrations of reagent solutions to produce an emission of light. For this aim, eight novel oxalates having an alkoxy moiety as well as TDPO, which shows high solubility on acetonitrile and acetone, were synthesized.[35]

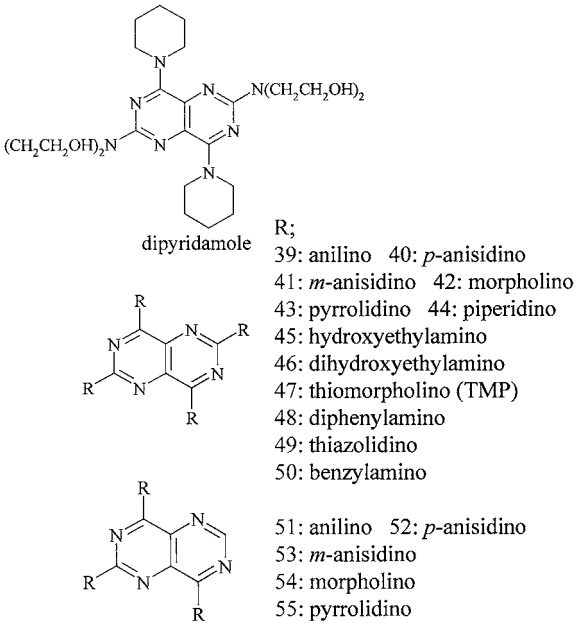

Fig. 6 Chemical structures of synthesized pyrimido[5,4-*d*]-pyrimidine derivatives.

Imai *et al.* reported that 2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido[5,4-*d*]pyrimidine (dipyridamole) showed the highest RCI in a study on estimation of sensitivity of fluorescent compounds by the PO-CL reaction system.[36] As shown in Fig. 6, pyrimido[5,4-*d*]pyrimidine derivatives (compounds **39** – **57**) synthesized could be anticipated to be useful fluorophores for PO-CL.[37] Evaluations of the derivatives as a PO-CL reagent by FIA and batch methods were performed, and the combinations of TCPO and 2,4,6,8-tetrathiomorpholinopyrimido[5,4-*d*]pyrimidine (compound **47**, TMP) gave more than 10 times larger RCI compared to those of representative fluorophores such as 9-aminoacridine, fluorescein, rhodamine B, 9,10-diphenylanthracene and perylene.[38,39] By using this combination, sensitive determination of hydrogen peroxide was achieved with an LOD of 10 nM at a signal-to-blank ratio of 3.[40] Furthermore, substrates for oxidase such as glucose, uric acid,[41] mono-acetyl polyamines and polyamines[42] could be determined. The method was based on the enzyme reaction to generate hydrogen peroxide, which was performed on-line using an immobilized enzyme column reactor. A typical chromatogram of the standard polyamines is shown in Fig. 7.

3 Development of Analytical Methods for Drugs of Abuse

Analysis of abusable drugs in forensic study including criminal justice requires sensitivity, selectivity and reliability. Also, a rapid and simple method is needed in clinical chemistry including emergency treatment. Therefore, for the development

of a new analytical method of such drugs, sophisticated analytical techniques must be used. In our studies, the combination of labeling technique using original reagents described in the above section and an HPLC method could provide the highly sensitive and selective determination of such drugs. In Fig. 8, the abusable drugs examined in our study are listed.

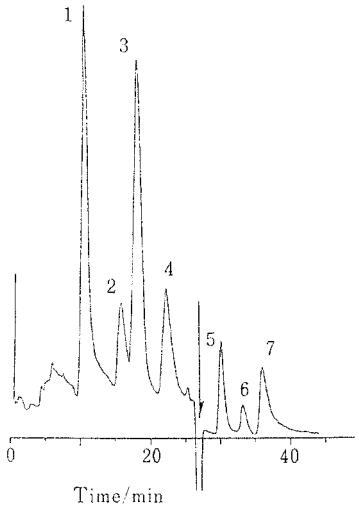

Fig. 7 Chromatograms of the standard polyamines. Sample: putrescine (Put), acetyl putrescine (AcPut), 250 pmol/injection; cadaverine (Cad), acetyl cadaverine (AcCad), spermidine (Spd), N_1-acetyl spermidine (N_1-AcSpd) and N_8-acetyl spermidine (N_8-AcSpd), 500 pmol/injection. Peaks: 1, AcPut; 2, AcCad; 3, Put; 4, Cad; 5, N_1-AcSpd; 6, N_8-AcSpd; 7, Spd. The arrow indicates the changing of the eluents. Sensitivity: 5.0 nAFS. Other HPLC conditions are as in the Experimental. Printed from Ref. 42 with permission from The Japan Society for Analytical Chemistry.

3·1 Stimulants[43–51]

Development of a sensitive and selective determination method of stimulants (MP and amphetamine (AP)) has been attracting considerable attention because one of these, MP, is the most widely abused drug in Japan.[9] Since stimulants and their related compounds have reactive functional groups such as primary or secondary amino groups, a labeling reagent of amines for sensitive determination could be applied with an HPLC-FL or a PO-CL detection method. In our reports, various labeling reagents such as fluorescein isothiocyanate,[43] 4-fluoro-7-nitro-2,1,3-benzoxadiazole[44,45] and DIB-Cl[46–49] for FL detection and DBD-F[50] and *N*-(4-aminobutyl)-*N*-ehylisoluminol[51] for PO-CL detection were examined. Especially, DIB-Cl was one of the most useful FL labeling reagents for MP and its related compounds owing to its high sensitivity and reactivity to amines. MP, AP, *p*-hydroxylmethamphetamine (*p*-HMP), *p*-hydroxyamphetamine (*p*-HAP) and ephedrine (EP) in human urine were determined with a simple solid-phase extraction (SPE).[46] The labeling reaction of DIB-Cl with amines easily proceeded at room temperature in the presence of triethylamine. The sensitivity of the proposed method with LOD of 0.6 – 5.2 fmol ($S/N = 3$) was higher than that of Dns-Cl. Using very small amounts of urine (about 10 μL), MP and its related compounds could be determined. The method was successfully applied to the urine samples collected from MP addicts. AP, *p*-HMP and *p*-HAP, which are major metabolite of MP, are simultaneously determined in addition to MP by this method. This result indicates the proposed method might be useful in forensic, pharmacokinetic and toxicologic studies.

Analysis of abusable drugs in blood and hair samples is important in forensic or clinical studies. However, its achievement may be more difficult compared to urine analysis, because the concentrations of analytes in matrices and the sizes of the usable sample are sometimes limited. When these limitations were overcome, the determination of MP and AP in human plasma and hair samples by an HPLC-FL method with

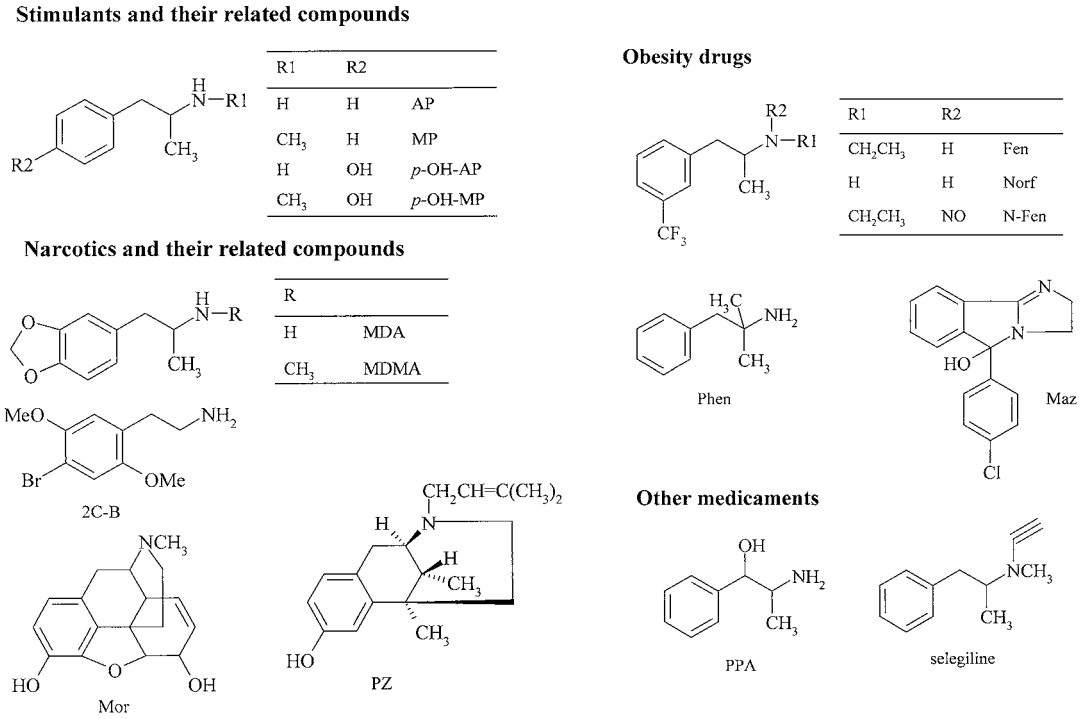

Fig. 8 Chemical structures of abused drugs examined.

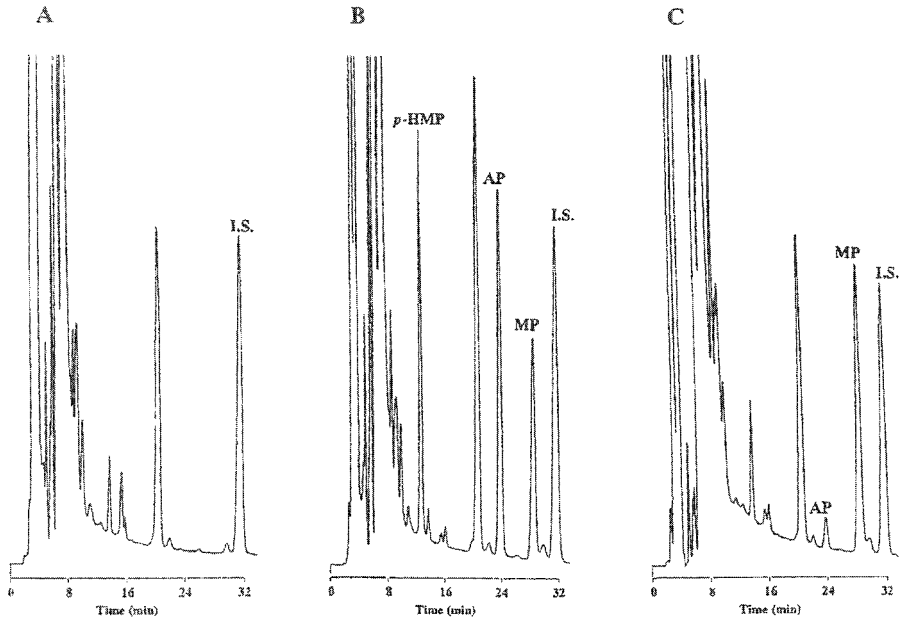

Fig. 9 Typical chromatograms obtained by the HPLC-FL method for a control undershirt (A), control undershirt spiked with standards MPs at 82.6, 67.6 and 74.6 ng/0.1 g of *p*-HMP, AP and MP, respectively (B), and an abuser undershirt (C, sample 1) extract. Each sample contains 74.6 ng/0.1 g of IS. For experimental details, refer to the text. Printed from Ref. 49 with permission from John Wiley & Sons, Ltd.

DIB-Cl could be performed.[47] The sample sizes used for plasma and hair were 100 μL and 1 mg, respectively. As an application, MP and AP in plasma and hair obtained from two cases of illegally ingested MP were determined. One of the advantages of hair analysis is to obtain historical information of the abuser by segmental analysis. However, an investigator encounters great difficulty to use the method successfully, because generally a trace amount of analytes is included in a small size of sample. An HPLC method with FL detection of MP and AP labeled with DIB-Cl was developed due to its excellent sensitivity and reactivity.[48] Abusers' hair cut into 1 cm long segments of 10 strands of hair was used for analysis. Six hair samples obtained from abusers were segmentally analyzed and the concentrations of MP determined in each segment were in the range of 1.8 – 170.7 ng/mg hair. Furthermore, unique samples such as parts of the garments of abusers were used for determination of MP and AP by an HPLC-FL method.[49] The analytes were completely extracted from textile with a mixture of chloroform and propan-2-ol. The LOD of MP was 0.4 pg (S/N = 3). Typical chromatograms obtained by the proposed method are shown in Fig. 9. Contents of MP and AP were higher in underwear compared to pants because the former might more easily contact with skin. This result supports the idea that MP and AP can be excreted through perspiration.

3·2 Narcotics[52-59]

Abusable drugs defined as narcotics include many types of compounds such as morphine (Mor), tryptamines and MDMAs. Among these, the compounds having amino group or hydroxyl group were the subjected to our studies. Recently, illegal uses of MDMA (called ecstasy) and MDA (called love drug) by the young are spreading and causing serious social problems worldwide. These drugs are used in a tablet form and can easily be taken orally. This is the most likely reason why these are widely used as recreational drugs among the young.[52] A simultaneous semi-micro column HPLC method with FL detection of MDMA, MDA, MP and AP in rat urine was examined by using DBD-F as a labeling reagent.[53] A semi-micro column HPLC can reduce solvent volume and sample size by miniaturizing the method. Thus, it is applicable for analysis of expensive chemicals and/or small volumes of biological samples. In this method only a small amount of urine sample (50 μL) was required to determine MDMA and MDA. To evaluate the applicability of the method, MDMA and its metabolite, MDA were monitored after a single administration of MDMA tablets (2.0 mg/kg). DBD-labels could be determined more sensitively by PO-CL detection than by FL detection as described in the above section. MDMA, MDA, MP and AP in hair labeled with DBD-F were determined by an HPLC-PO-CL system combined with a column switching technique.[54] The excess reagent blank peaks on the chromatogram were removed, and then the separation of the labeled compounds was achieved. After digestion of hair in an alkali solution, the analytes were extracted with *n*-heptane and labeled with DBD-F. A mixture of hydrogen peroxide and TCPO in acetonitrile was used as a postcolumn CL reagent. The proposed method was applied to determine MDMA and related compounds in hair samples obtained from patients treated in hospitals (Table 1). The concentration ratio of metabolite to parent compound (*e.g.* AP/MP or MDA/MDMA) is a useful parameter to indicate the use of metabolite in addition to the parent compound. A ratio of more than 1 suggests the use of AP or MDA. Typical chromatograms obtained from hair samples are shown in Fig. 10. DIB-Cl could be applied as a powerful labeling reagent to determine MDMA and MDA as well as MP and AP as described in the previous paragraph. Furthermore, HPLC-FL methods to determine these compounds in blood[55] and hair samples[56,57] could be developed.

Additionally, HPLC-FL determination of Mor[58] and pentazocine (PZ)[59] labeled with DIB-Cl were examined. Both medicaments are used as an analgesic for post-operative or cancer pain and are also illegally used as drugs. Mor in rat brain

Table 1 Amounts of MDMA and related compounds in patients' hair samples[55]

Sample No.	Amount (ng mg^{-1} hair)			
	MDMA	MDA	MP	AP
1	0.59	N.D.[a]	6.73	0.88
2	0.41	N.D.[a]	0.81	2.47
3	0.10	Trace[b]	4.13	Trace[b]
4	0.10	0.83	0.24	1.07

a. Not detected.
b. Trace: 0.16 ng mg^{-1} < trace < 0.50 ng mg^{-1} for MDA. 0.15 ng mg^{-1} < trace < 0.50 ng mg^{-1} for AP.

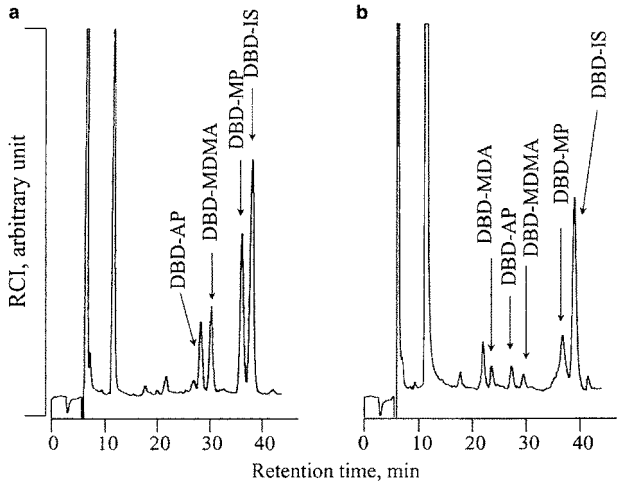

Fig. 10 Chromatograms obtained from samples of abusers' hair (a) sample No. 2 in Table 1, (b) sample No. 4 in Table 1. Printed from Ref. 54 with permission from Springer.

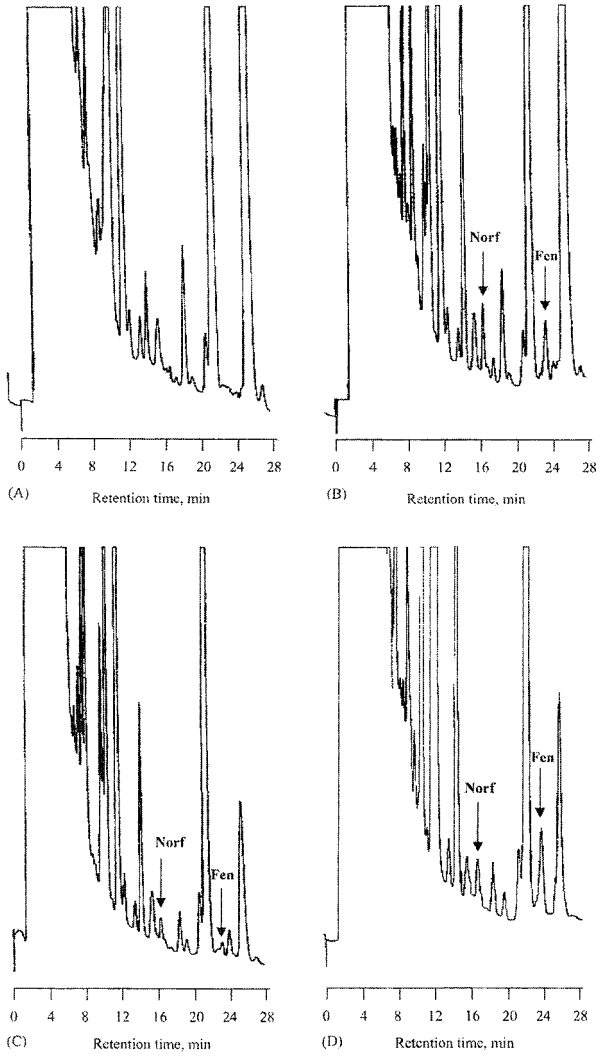

Fig. 11 Chromatograms obtained from (A) non-spiked control human hair, (B) spiked with 100 pg/mg Norf and 230 pg/mg Norf and 457 pg/mg Fen. Printed from Ref. 62 with permission from Elsevier Science Publishers B. V.

and blood microdialysates could be directly labeled with no pretreatment. DIB-Mor was separated within 14 min with nmol level of LOD, and the method was successfully applied to the preliminary study of potential pharmacokinetic interaction between Mor and diclofenac (Dic). For reliable PZ determination, cyclazocine was used as an IS, and PZ and IS in plasma or hair were extracted by ethyl acetate. This method was applied to monitor PZ in Zucker rat hair (black and white) and plasma samples after administration of 25 mg/kg PZ. As a result, PZ concentration in black hair was about eight-fold higher than that in white hair. Incorporation rate values of PZ (= PZ concentration in black or white hair/area under the curve ($AUC_{0\text{-inf}}$) of PZ in plasma) were 0.053 and 0.007 for black and white hair, respectively.

3·3 Obesity drugs[60-64]

Obesity drugs such as Fen, Phen and PPA, which can be adulterated in diet products, have amino groups in their chemical structures to react with labeling reagents. At first, simultaneous determination of dansylated Fen and Phen, in addition to sympathomimetic amines including norephedrine, ephedrine, 2-phenylethylamine and 4-bromo-2,5-dimethoxyphenethylamine was performed by an HPLC-FL method.[60] The labels were well separated within 45 min with LOD ranging from 16 to 255 fmol (S/N = 3). The proposed method was applied to the determination of Fen and Phen after an oral administration of these compounds to rats. The analytes labeled with DIB-Cl were also determined by an HPLC-UV method.[61] However, since the sensitivity of these methods was not enough to determine Fen in hair, an HPLC-FL method with DIB-Cl labeling was examined. In this study, Fen and its active metabolite of norfenfluramine (Norf) were measured as target compounds.[62] The DIB-labels were monitored at 430 nm with excitation at 325 nm. The method was sensitive to LOD of 36 pg/mg hair for Fen and to LOD of 16 pg/mg hair for Norf. Furthermore, the availability of Fen and Norf as biomarkers for N-Fen ingestion was examined. N-Fen, which was also found in diet products, was believed to associate with liver failure. However, sensitive determination of N-Fen is difficult because of its weak UV absorption. Additionally, N-Fen is a tertiary amine and for it to undergo labeling for FL-detection is not feasible. Therefore the proposed method could be applied to determine Fen and Norf in hair samples obtained from patients who were on diets and were suspected to have ingested products containing N-Fen and to suffer from hepatotoxicity. As a result, Fen and Norf were detected in patients' hair samples; their typical chromatograms are shown in Fig. 11.

PPA has been used as an over-the-counter (OTC) drug,

a prescription medicament for cough and cold and an appetite suppressant due to its sympathomimetic activity. Recently, some serious side effects such as hemorrhagic stroke, arrhythmias and hypertension were reported. For pharmacokinetic study of PPA, a sensitive determination method with HPLC-FL detection was developed.[63] PPA in plasma, and blood and brain microdialysates was extracted by a liquid-liquid extraction with ethyl acetate followed by labeling with DIB-Cl. The LODs of PPA in plasma and blood and brain dialysates were 17, 48 and 40 fmol on column, respectively. The method was applied for monitoring of PPA in rat's brain and blood microdialysates administered with a single oral dose of PPA.

Maz, having no functional group to react with a labeling reagent, is also adulterated in diet products. Therefore, for Maz determination, hydrolysis of Maz to its metabolite, 2-(2-aminoethyl)-3-(*p*-chlorophenyl)-3-hydroxyphthalimidine (Met), was performed. This was followed by labeling with DIB-Cl. Maz and Met in plasma and brain were determined by an HPLC-FL method measuring Met after two different sample treatments.[64] The LOD of Met in plasma and brain were 2.8 and 3.5 fmol at a *S*/*N* ratio of 3. The developed method was successfully applied to monitor Maz and Met after a single administration of Maz (0.5 mg/kg).

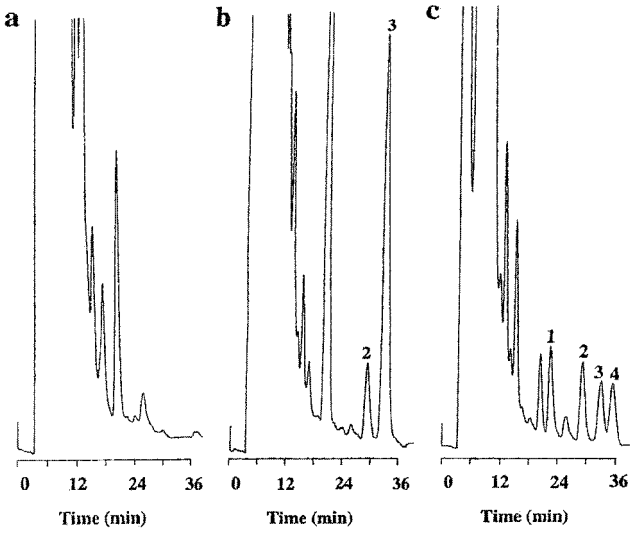

Fig. 12 Typical chromatograms with fluorescence detection obtained by the chiral method for a control human hair (a), abuser hair sample A (b), and a standard solution of 0.5 mM per enantiomer of AP (125 fmol on column) and 1.0 mM per enantiomer of MP (250 fmol on column) (c). Peaks: 1, DIB-*R*(–)-AP; 2, –*S*(+)-AP; 3, –*S*(+)-MP; 4, –*R*(–)-MP. For experimental details, refer to the text. Printed from Ref. 66 with permission from John Wiley & Sons, Ltd.

4 Advanced Application for Determination of Drugs of Abuse

Indicating applications of the developed methods is very important for researchers who are concerned with analytical chemistry to avoid falling into self-satisfaction. In this section the advanced applications based on sensitive determination of abusable drugs are described.

4·1 Chiral analysis of abusable drugs[65-68]

Some medicaments having stereogenic center in their structures are used as racemic mixtures. Chiral separation of medicaments is very important when one enantiomer shows main pharmacological effect and the other shows inactivity, a qualitatively different effect, or severe toxicity. In forensic studies, chiral separation of drugs of abuse sometimes provides helpful information. For instance, the *S*(+)-enantiomer or racemic mixture of MP is mainly abused in Japan. On the other hand, selegiline, an anti-Parkinson's disease agent is metabolized in human body into *R*(–)-MP, *R*(–)-AP and their corresponding *p*-hydroxy derivatives. To distinguish patients from abusers, precise determination of enantiomers is required. Thereupon, HPLC-FL methods using DIB-Cl for the chiral quantification of MP and AP in urine and hair were developed.[65-67] The enantio-separation of DIB-labels was achieved on a Chiralcel OD-RH column with a mixture of acetonitrile and phosphate-citrate buffer containing $NaPF_6$ as a mobile phase. Chiral analyses of MP and AP by using urine samples collected from a patient who received selegiline as an anti-Parkinson's disease medicament and from suspected MP abusers were performed.[65] *R*(–)-Enantiomers of MP and AP were only detected in the Parkinson sample at concentrations of 1.2 and 0.7 μM, respectively, whereas the *R*(–)- and *S*(+)-enantiomers of MP and AP were found in abuser's samples, which suggests that the racemic MP was ingested by the abuser.

Representative chromatograms of hair samples are shown in Fig. 12. Resolution values for AP and MP enantiomers were 3.4 and 1.1, respectively. The LODs for *R*(–)- and *S*(+)-AP, *R*(–)- and *S*(+)-MP were 75.6, 86.4, 282.0 and 228.0 pg/mg hair, respectively.[66] Furthermore, hair samples could be segmentally analyzed based on 1-cm-long segments.[67] In four hair samples only the *S*(+)-enantiomers of MP and AP were detected.

Moreover, an HPLC-FL method was developed for the simultaneous determination of Phen, D-Fen and L-Fen in addition to their metabolites, D-Norf and L-Norf after labeling with DIB-Cl.[68] A gradient separation of DIB-labels which were monitored at 430 nm with excitation at 325 nm was achieved on a Chiralcel OD-R column. The LODs at a *S*/*N* ratio of 3 were 27, 19 and 47 fmol for D-Fen, Phen and L-Fen in human plasma, respectively. The method was applied for the determination of Phen, D- and L-enantiomers of Fen and Norf in rat plasma after simultaneous administration of a mixture of Phen and DL-Fen. The average concentrations of Phen, D-Norf, L-Norf, D-Fen and L-Fen were 159, 420, 497, 23 and 20 nM, respectively. The mean ratio between L-and D-Fen was 0.76 with a range of 0.59 – 0.89 (n = 3). This suggests a more rapid metabolism of L-enantiomer compared to the D-enantiomer, which is evidenced by the higher plasma level of L-Norf compared to D-Norf.

4·2 Evaluation of drug-drug interaction potential for abusable drugs[69-72]

Drug-drug interaction causes an adverse drug response produced by the co-exposure of the drug and another substance, which modifies the patient's response to some drugs. In forensic cases, abusable drugs might interact with each other by multi-drug abuse, and this can lead to serious toxic symptoms. Drug-drug interactions include pharmacokinetic and/or pharmacodynamic interaction. Pharmacokinetic interaction causes the extreme change of free-form (unbound to protein) drugs' concentration in blood and/or target tissue. Consequently, determination of free-form drugs is helpful to assess the pharmacokinetic interaction. Many pharmacokinetic approaches of drug-drug interaction are already well established in the measurements of total drug concentration rather than the free-form drug and pharmacokinetic parameters in plasma. Some studies have focused on free-form drugs in target tissue (*e.g.* brain and bile). Determination of free-form drugs in target tissue is expected to be very useful to

predict more particular information about interactions.

Microdialysis has been found to be an important application for *in vivo* studies of drug pharmacokinetics. This approach makes it possible to sample free-form drugs in extracellular fluids of essentially any tissue by passive diffusion through the microdialysis probe that consists of a small semi-permeable membrane. In this technique, the typical distribution of free-form drug in target tissue can be estimated with minimal damage to experimental animals.[69] However, sensitive analytical methods are required to determine the analytes because concentrations of the analytes in microdialysates fluids are generally low and quite small sizes of sample can be obtained. In our study, the combination of HPLC separation with fluorescence detection using our original labeling reagent (DIB-Cl) and microdialysis sampling were used for estimation for drug-drug interactions of abusable drugs.[58,70-72]

MDMA tablets often contain other active components having hallucinogenic and/or stimulant effect such as caffeine, ketamine, ephedrine or methamphetamine. After administration of MDMA (5 mg/kg, *i.p.*) with caffeine (20 mg/kg, *i.p.*) to rats, the $AUC_{0\text{-}300}$ of MDMA in blood significantly increased to 1.7 times higher (91 ± 5 μg min mL^{-1}), and the clearance of MDMA decreased by half to 51 ± 4 mL min^{-1} kg^{-1}, such results suggested that caffeine might interfere with renal clearance of MDMA. On the other hand, the C_{max} of MDMA and MDA (a metabolite of MDMA) in brain significantly decreased by half (496 ± 80 and 93 ± 21 ng/mL, $P < 0.01$ and $P < 0.05$, respectively). Moreover, the T_{max} and the mean residence time (MRT) of MDMA and MDA were prolonged (2.1 and 1.3 times, respectively), but the elimination half-lives ($T_{1/2}$) of MDMA and MDA were unchanged. These results showed that caffeine inhibits the transportation of MDMA and MDA to brain *via* the blood-brain barrier, although the exclusions of MDMA and MDA from brain to blood were not influenced by caffeine.[70] Such results suggested that ingredients in MDMA tablets might influence the pharmacokinetics of MDMA, and this interaction could lead abusers to take higher doses of MDMA and to cause poisonously high levels of MDMA in blood.

Mor is frequently prescribed in combination with non-steroidal anti-inflammatory drugs like Dic for pain relief. Mor was a low-transitive compound for the blood-brain barrier, since the AUC ratio (AUC_{brain}/AUC_{blood}), which indicates transitivity of Mor to brain, was 0.38 after a single administration (10 mg/kg, *i.p.*) to rats. Following co-administration of Mor with Dic (5 mg/kg, *i.p.*), the AUC_{brain}/AUC_{blood} ratio was not changed (0.31), and no pharmacokinetic parameters of Mor in either brain or blood were significantly altered. These results suggested that the clinical benefit of combined use of Mor and Dic might be caused by synergism of their different pharmacodynamic effects and/or by pharmacokinetic effects of Mor glucuronide.[58]

Appetite suppressants have been used as Phen-Fen combinations; however, such use was found to enhance weight loss at lower doses as well as the neurotoxic effect. The profiles of pharmacokinetic parameters of Phen in rats, both blood and brain, following Phen (1 or 5 mg/kg) in the absence or presence of Fen (1 or 5 mg/kg) were significantly altered. In rat brains, Fen significantly increased Phen levels, C_{max} and AUC of Phen increased 2.0 – 2.2 fold and 3.4 fold ($P < 0.05$ and $P < 0.01$, respectively). The T_{max} and MRT of Phen were prolonged about 2 times ($P < 0.05$ respectively), and clearances of Phen were notably decreased by 0.2 – 0.3 times ($P < 0.05$). In blood, similar results were shown, where the AUC of Phen was significantly increased ($P < 0.05$). Interestingly, protein binding % of Phen (35%) was not altered by co-administration of Fen. On the other hand, there was no significant effect on

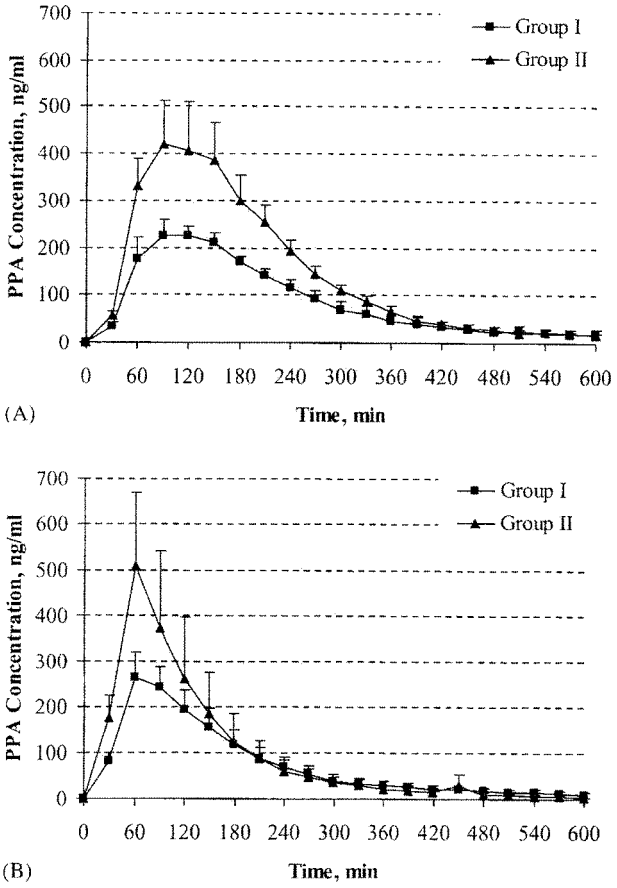

Fig. 13 Mean concentration of PPA in rat (A) brain and (B) blood microdialysis after the administration of single *i.p.* doses of PPA in saline (2.5 mg/kg, group I) and a mixture of PPA (2.5 mg/kg) and caffeine (5 mg/kg) in saline (group II). Printed from Ref. 72 with permission from Elsevier Science Publishers B. V.

pharmacokinetic parameters of either Fen or its metabolite Norf. These results suggested Fen and/or Norf may have a similar inhibiting effect on the transporters, which is responsible for Phen elimination, thus inhibiting its efflux from brain to the blood. This would lead to higher brain distribution, and hence alter its pharmacokinetic parameters. The consequence of such alteration in the pharmacokinetics of a CNS drug may play a part in the enhancing effect as well as in the neurotoxicity of Phen and Fen.[71]

PPA is a synthetic sympathomimetic amine available as a nasal decongestant and is also used as an appetite suppressant in the USA. In Japan, PPA was only available as a decongestant of OTC products by combination with caffeine and/or chlorphenylamine. Co-administration of caffeine and/or chlorphenylamine significantly affected the pharmacokinetic parameters of PPA in brains. Combinations of caffeine (5 mg/kg, *i.v.*) with PPA (2.5 mg/kg, *i.p.*) caused a 1.6-fold increase in the AUC in brain compared to single administration of PPA (Fig. 13), and combination of chlorphenylamine (0.4 mg/kg, *i.v.*) caused a 1.8-fold increase ($P < 0.05$) (Fig. 14). The multiple combinations caused an increase in the AUC by 1.9-fold ($P < 0.05$) similar to that in the AUC of PPA in brain compared to the single administration of the OTC product including PPA, chlorphenylamine, caffeine, belladonna alkaloids and lysozyme chloride (2.2-fold, $P < 0.05$). The C_{max} of PPA in brain was significantly increased by co-administration of caffeine, chlorphenylamine or both of them (1.8-, 1.8- and 1.9-fold;

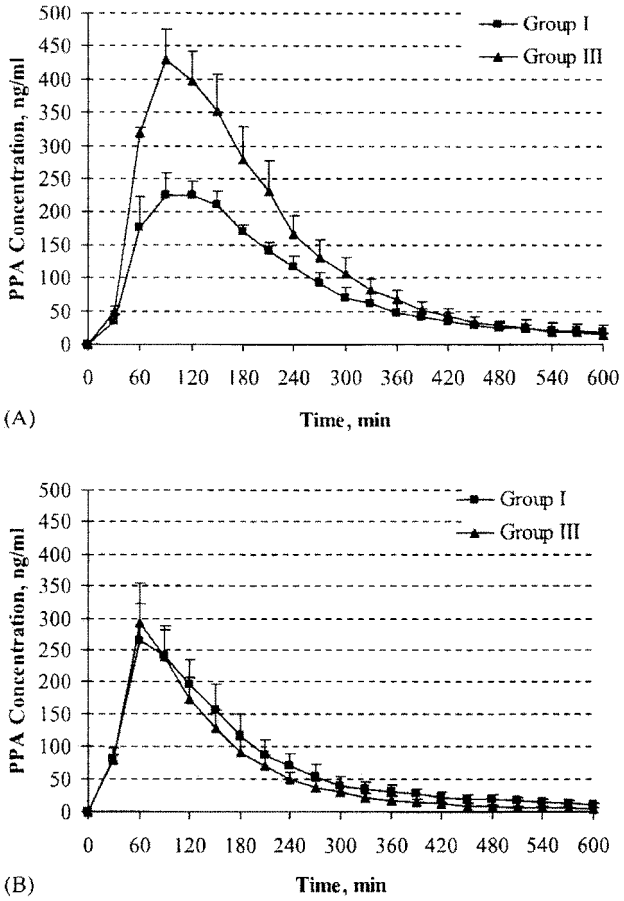

Fig. 14 Mean concentration of PPA in rat (A) brain and (B) blood microdialysis after the administration of single *i.p.* doses of PPA in saline (2.5 mg/kg, group I) and a mixture of PPA (2.5 mg/kg) and chlorphenylamine (0.4 mg/kg) in saline (group III). Printed from Ref. 72 with permission from Elsevier Science Publishers B. V.

$P < 0.05$, respectively), and the clearance of PPA in brain was significantly decreased by co-administration of them (0.6-, 0.7- and 0.6-fold; $P < 0.05$, respectively). Contrary to these results in brain pharmacokinetics, combined administration did not affect the pharmacokinetics of PPA in blood. In August 2002, the manufacturers voluntary removed PPA from OTC products because of the mega-study, which shows an increased risk of hemorrhage stroke related to intake of PPA. Our results showed that the observed adverse effects associated with PPA use could be related to the significant increase in its levels in the brain.[72]

These results revealed the importance of monitoring of pharmacokinetic parameters in target tissue in addition to these in blood to evaluate the pharmacokinetic drug-drug interactions.

5 Conclusions

In this review, we have presented our research results obtained over several years. The significant points can be summarized as follows;

- Synthesis and application of analytical reagents, complying with the requests
- Combination of suitable analytical tools on purpose: especially of luminometric detection and LC separation components
- Indication of advanced applicabilities of the developed methods

We believe that this research style will be highly helpful for young researchers not only scientifically, but also educationally, and we would like to continue to make progress from now on.

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