Development and Application of Sensitive Methods with Luminescence Detections for Determination of Biologically Active Compounds

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This review describes a comprehensive investigation in our laboratory focusing on development of sensitive analytical methods by utilizing luminescence detections, and leading to practical applications for quantification of biologically related compounds, especially drugs of abuse. The developed methods mainly used a high-performance liquid chromatographic separation technique with fluorescence and chemiluminescence detections. The main targets of our investigation are ultra-small amounts of biologically active compounds, especially centered on abused drugs such as stimulants, narcotics and anorectics in various matrices. Furthermore, drug-drug interaction of these compounds was also examined. The results obtained in our study might contribute to the prediction of and the protection of human health from the risks of abused drugs. The developed methods might be useful for pharmaceutical, clinical and forensic studies on drugs of abuse as well as other important biologically active compounds.

Key words —— HPLC, luminescence detection, Drugs of abuse, biologically active compound

INTRODUCTION

The determination method for biologically active compounds including drugs of abuse is rapidly growing, because of advances in the sensitivity and selectivity of analytical methods. Recently, analysis of these compounds has primarily been conducted by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography (LC)-MS, because of their high selectivity and sensitivity. However, these methods require expensive equipments, and GC-MS also needs tedious sample preparation involving derivatization under anhydrous conditions. On the contrary, high-performance liquid chromatography (HPLC)-fluorescence (FL) and HPLC-chemiluminescence (CL) detections coupled with relatively simple and inexpensive apparatus are also sensitive and selective for determination of these biologically active compounds. To date, we have developed many sensitive HPLC-FL and -CL methods of biologically interesting compounds including bioactive components and drugs of abuse (e.g., stimulants, narcotics and anorectics) in various matrices such as urine, plasma and hair using FL labels such as 4-(4,5-diphenyl-1H-imidazol-2-yl)benzoyl chloride (DIB-Cl) and 4-(N,N-dimethylaminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F). Representative FL labeling reagents and target compounds discussed in this review are shown in Fig. 1. DIB-Cl reacts with both primary and secondary amines, and phenols under the mild conditions yielding intense FL derivatives. Therefore, in a series of our study, we have exclusively utilized DIB-Cl as a FL labeling reagent.

The aim of this review is to overview our recent studies on sensitive HPLC methods for determination of biologically active compounds centered on drugs that may be abused. This review consists of three parts: development of sensitive HPLC methods for biologically active compounds; hair analysis; and drug-drug interaction.
Analysis of biologically active compounds is very important for the basic and application studies in the fields of pharmaceutical sciences, clinical chemistry and forensic toxicology. We have been focusing on the development of HPLC methods for these compounds as follows.

Among the biologically active compounds, abused drugs such as stimulants [amphetamine (AP) and methamphetamine (MP)], narcotics [e.g., 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), cocaine and ketamine], and anorectics [e.g., fenfluramine (Fen), phentermine (Phen) and mazindol], have been illegally or illicitly used worldwide because of their strong effects on the central nervous system (CNS). Abusers sought the effects of these drugs as self-confidence and well-being, heightened awareness, loss of appetite, euphoria, and so on. As a result, this causes risks of human health and also serious social problems. Therefore, analysis of these compounds is very important for the prediction of and protection from the risk to human health, especially young peoples, and also reduction of the social problems such as incidents and accidents caused by abusers. Figure 2 shows the numbers of arrested due to the illegally use of drugs of abuse in Japan in 2009.

### Stimulants and Narcotics

AP and MP, classical phenethylamine derivatives, are powerful stimulants of the CNS. AP and MP are used as abused drugs or doping agents in sports other than legitimate usage. In Northeast Asia and South Africa, MP is the most prevalent drug of abuse. Analyses of amphetamine-type stimulants in biological samples are very important for clinical and criminal investigations and many methods have been developed. Forensic investigators have been utilizing biologic samples such as blood, urine, hair and sweat as valuable evidence in drugs of abuse. Commonly, urine and blood are often used
as a window to elucidate a short interval uptake of drugs and their metabolites, while hair is used for a long interval uptake. 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 have developed HPLC-UV and -FL methods of MP and its major metabolite, AP, in garments belong to known-abusers. DIB-Cl and 1-methyl-3-phenylpropylamine (MPPA) were used as a label and internal standard (IS), respectively. The reaction scheme of MP with DIB-Cl is shown in Fig. 3. DIB-Cl reacts with MPs under the mild conditions to yield intense FL derivatives. The limits of detection at signal-to-noise \((S/N)\) at 3 were less than or equal to 37.3 and 0.4 pg on column for HPLC-UV and -FL, respectively. The developed method was successfully applied to the determination of MP and AP in clothes belong to abusers.2)

MDMA and MDA are generally known as “ecstasy” and “love drug”, respectively. Their illegal use are spreading in young generation and causing serious social problems worldwide. MDMA and MDA are used in tablet form and thus can be easily taken orally. This is the most likely reason why these drugs are widely used as recreational drugs among young people. MDMA has been known to have hallucinogenic and empathy-enhancing effects like MP because of its structurally similarity with MP. The mechanism of action of MDMA is believed to inhibit the reuptake of serotonin, to facilitate serotonin release and, to a lesser extent, to cause dopamine and noradrenaline release.

A simultaneous semi-micro column HPLC-FL method of MDMA, MDA, AP and MP in rat urine was proposed by using DBD-F as a labeling reagent and \(\alpha\)-phenylethylamine as IS. A good separation of DBD-derivatives was achieved within 45 min. The limits of detection at \(S/N = 3\) were ranged from 0.5 to 15 ng/ml. MDMA and MDA in rat urine could be monitored for 15 hr after a single administration of MDMA to rat, 2.0 mg/kg, intraperitoneal (i.p.).3)

A sensitive HPLC-FL method for determination of MDMA and MDA in blood was developed by using DIB-Cl and MPPA as a labeling reagent and an IS, respectively. The limits of detection were ranged from 0.36 to 0.83 ng/ml at \(S/N = 3\). MDMA and MDA in rat whole blood could be monitored for 6 hr after a single administration of MDMA (2.2 mg/kg, i.p.). The pharmacokinetic parameters for MDMA and MDA obtained by triplicate measurements were 426 ± 23 and 39 ± 6 ng/ml \((C_{\text{max}})\), 20 ± 5 and 100 ± 10 min \((T_{\text{max}})\), respectively.4)

Simultaneous determination of MDMA and MDA in rat blood and brain microdialysates by HPLC-FL was developed. Microdialysates were directly subjected to derivatization with DIB-Cl. MPPA was used as an IS. The limits of detection for MDMA and MDA were 1.2 and 4.2 ng/ml for blood microdialysate and 1.3 and 4.8 ng/ml for brain microdialysates, respectively. The proposed method was successfully applied for the monitoring of MDMA and its metabolite MDA in rat blood and brain microdialysates, and the pharmacokinetic parameters of MDMA and MDA in the microdialysates after administration of MDMA (5 mg/kg, i.p.) with or without caffeine (20 mg/kg, i.p.) were evaluated as described in the section of drug-drug interaction.5)

For analysis of MDMA’s in biological samples which contain relatively high concentration of analytes (e.g. urine), DBD-F might be suitable although its sensitivity is not so high in FL detection. Because DBD-F itself is non-fluorescent, and thus the pretreatment procedure could be expected to simplify. For highly sensitive determination of MDMA’s in blood or microdialysate sample, DIB-Cl is preferable due to the highly intense FL of its labels under the mild labeling condition. On the other hand, for the determination of MDMA’s in hair, DBD-F is desirable as a labeling reagent because it can provide ultra high sensitivity for determining MDMA’s with peroxyoxalate (PO)-CL detection.

Anorectics

Obesity is a chronic disease that requires lifelong management where drug use can play an important role in obesity treatment. Anti-obesity drugs promote weight loss either through acting
on the adrenergic receptors in the brain like Phen, norephedrine (NE), and mazindol, or by acting at the serotonergic receptors like Fen and fluoxetine (FLX). It has been clarified that using a combination therapy of two anorectics may result in better management for obesity; combination of Phen and Fen has been widely prescribed for a short-term treatment of obesity. No reports were available regarding the simultaneous determination of Phen and Fen in plasma. We thus developed the simultaneous determination of sympathomimetic amines by an HPLC-UV method using DIB-Cl as a label and cyclohexylamine as an IS. The limits of detection for Fen, Phen, ephedrine, NE, and 2-phenylethylamine (2-PEA) in plasma ranged from 0.32 to 22.9 pmol on column at $S/N = 3$. The method was successfully applied to the monitoring the time-concentration profile of Phen and Fen in rat plasma following a single administration of 5 mg/kg dose of each drug.

An HPLC-FL method was developed for simultaneous determination of sympathomimetic amines including ephedrine, NE, 2-PEA, 4-bromo-2,5-dimethoxyphenylethylamine, Phen and D,L-Fen in spiked human plasma. Furthermore, an enantioselective HPLC method for dexfenfluramine (D-Fen) and levofenfluramine (L-Fen) in addition to their active metabolites D- and L-norfenfluramine (Norf), was developed by using DIB-Cl as a label. Especially, D,L-Fen and the active enantiomer D-Fen were withdrawn because many cases reported the appearance of serious side effects including valvular heart disease and primary pulmonary hypertension, and thus the chiral analysis of Fen and Norf might be useful. The chiral method based on a Chiralcel OD-R column separation was applied for the determination of D- and L-enantiomers of Fen and Norf, in addition to Phen in rat plasma after an i.p. administration of D,L-Fen and Phen, simultaneously. In this report, comparison of our method with known methods was described; Sensitive HPLC methods for determination of D-Fen and Fen enantiomers in plasma have been reported with dansyl chloride and 3,5-dinitrophenylisothiocyanate, but these methods needed longer derivatization time followed by a clean-up step (at least several hours). Also, compared to the improved dansyl chloride method proposed by us, the DIB-Cl method could improve the limits of detection (LOD) from 51 to 10 fmol on column for Phen and from 54 to 18 fmol on column for Fen.

Phenylpropanolamine (PPA) is one of the sympathomimetic amines, and structurally is an AP-related compound. A convenient HPLC-UV method to determine PPA in additional to phenylephedrine (PE) and chlorpheniramine (CPA) in commercially available over-the-counter (OTC) preparations was developed. The method was applied to the determination of PPA in pharmaceutical preparations including hard and soft capsules.

PPA determination by HPLC-FL using DIB-Cl as a label was developed and applied to determine PPA in human plasma and rat's blood and brain microdialysates. The limits of detection were 17, 48 and 40 fmol on column in plasma and blood and brain microdialysates, respectively. The method was applied for monitoring of PPA levels in rat's brain and blood microdialysates administered with a single oral dose of PPA (2.5 mg/kg).

Mazindol is a tricyclic imidazoisoindole compound, used as an anorectic for short term adjunct therapy in the treatment of obesity. A simple and sensitive HPLC-UV method for quantification of mazindol and its major metabolite, 2-(2-aminoethyl)-3-(p-chlorophenyl)-3-hydroxyphthalimidine (Met), in human plasma was developed. The limits of detection were 0.07 and 0.08 ng/ml of plasma for mazindol and Met, respectively. The method was successfully applied to the therapeutic monitoring of mazindol levels for patient (female, 34 years old) who had received a 0.5 mg tablet.

Mazindol and Met in mouse brain and plasma were also determined. The pretreatment involved the hydrolysis of mazindol to Met, by incubating the sample at 80°C for 15 min at pH 10.6 followed by liquid-liquid extraction while for the determination of Met, the hydrolysis step was omitted. Mazindol and Met concentrations were monitored in mouse plasma and brain tissue regions after a single i.p. administration of mazindol, 0.5 mg/kg.

Recently, a nitrosamine compound named N-nitrosofenfluramine (N-Fen) has been detected as a synthetic adulterant in Chinese herbal diet products. Because N-nitroso compounds have been linked with carcinogenesis in the liver, N-Fen could be a potential hepatotoxic compound. Over the past several years, seven women in Japan, Singapore and China have died from hepatotoxicity. The common thing between these women is that they were all on Chinese diet products and N-Fen was detected in these products.

Nitrosamines are known to be metabolized by liver microsomes via two mechanisms. One mechanism is considered as metabolic activation and the
second pathway is denitrosation which is catalyzed by cytochrome P450. Thus we were interested to identify Fen (the secondary amine) and Norf (the primary amine) as N-Fen metabolites. Also, to investigate N-Fen pharmacokinetics and its proposed metabolites levels in different tissues obtained from rats administered with a single i.p. dose of N-Fen (25 mg/kg). The tissues examined were blood, brain, kidney and liver. For the determination of Fen and Norf, HPLC-FL using DIB-Cl as the derivatizing reagent was utilized, while for the determination of N-Fen, an HPLC-UV method was developed.\textsuperscript{13}

**Other Compounds**

Triazolum (TZ) is a short-acting hypnotic that is indicated for the treatment of insomnia. A semimicro column HPLC-UV method to determine TZ was developed. The method was applied to determine plasma and brain microdialysate concentrations of TZ after a single intravenous bolus administration of 2.5 mg/kg to rat. The limits of detection at $S/N = 3$ were 2.1 and 0.7 ng/ml for spiked plasma and artificial cerebrospinal fluid, respectively. The developed method was applied to study the disposition of TZ in rat by analyzing plasma and brain microdialysate samples.\textsuperscript{14}

Bisphenol A (BPA) has been used in the chemical industry for production of polycarbonate plastic, epoxy resins and other resins. Since a weak estrogen-like activity of BPA was reported, its effects on human health have become of growing concern. We developed a simultaneous determination method by HPLC-FL for phenolic xenoestrogens such as BPA, bisphenol B, bisphenol E, bisphenol F and 4-nonylphenol (4-NP). DIB-Cl and 4,4′-cyclohexylidenebisphenol were used as a label and an IS. BPA was found in both polycarbonate and polyvinyl chloride plastics, while 4-NP was found in plastics made of polyvinyl chloride and another polymer.\textsuperscript{15}

BPA levels in human blood serum and ascetic fluid were also achieved. The mean concentrations of BPA ($n = 9$) in maternal and umbilical cord sera obtained from healthy pregnant women were $0.46 \pm 0.20$ and $0.62 \pm 0.13$ ppb, respectively. Relationships of BPA concentrations were observed between maternal and umbilical cord blood serum samples ($r = 0.626$), as well as blood serum and ascites fluid samples ($r = 0.785$).\textsuperscript{16}

BPA in human breast milk was determined by HPLC with column-switching and FL detection. After a two-step liquid-liquid extraction, BPA was labeled with DIB-Cl. The excess labeling reagent was removed effectively using a column-switching system. The limit of detection at $S/N = 3$ was 0.11 ng/ml. Twenty-three breast milk samples of healthy lactating women were analyzed; the mean value was $0.61 \pm 0.20$ ng/ml.\textsuperscript{17}

HPLC analysis of BPA in biological and environmental samples were reviewed focusing on the detection methods such as UV, FL, CL, electrochemical detection (ECD) and MS.\textsuperscript{18}

Donepezil hydrochloride, [(±)-2-{[(1-benzyl-piperidine-4-yl)methyl]-5,6-dimethoxyindan-1-one hydrochloride, DP], which is commercially available as Aricept, is a potent, selective and reversible inhibitor of acetylcholinesterase and has been prescribed worldwide for treatment of Alzheimer’s disease. DP is thought to exert a therapeutic effect by increasing the concentration of acetylcholine in the brain. More recently, DP treatment for Down syndrome showed potential improvement of the symptom in a nonrandomized-controlled trial.

A stereoselective HPLC-UV determination of DP enantiomers in tablets and plasma was proposed. The method had enough sensitivity to follow the DP pharmacokinetics in rats up to 12 hr after a single oral dosing. Enantiometric resolution was achieved on a cellulose tris(3,5-dimethylphenyl carbamate) column known as Chiralcel OD with UV detection at 268 nm. The proposed method was found to be suitable and accurate for the quantitative determination of DP enantiomers in tablets. The method also showed good specificity to DP enantiomers, and it could be successfully applied to its pharmacokinetic studies and therapeutic drug monitoring.\textsuperscript{19}

A simple and sensitive HPLC-FL method for DP in plasma and microdialysate samples was developed. A rapid isocratic separation of DP was achieved by a short C$_{30}$ column. The limits of detection of DP in human plasma, rat plasma and rat brain or blood microdialysates were 0.2, 1.0 and 2.1 ng/ml, respectively. The method was successfully applied to monitor DP levels in rat plasma, blood and brain microdialysates and patient plasma.\textsuperscript{20}

Paclitaxel (TXL) has been adapted to various human solid tumors such as non-small cell lung cancer, breast cancer and stomach cancer. A simple and sensitive HPLC-UV method was developed for determination of TXL in human and rat blood sample. 4-Hydroxybenzoic acid n-hexyl ester was used as an IS. The eluent was monitored at 230 nm. The lim-
its of detection of TXL for human plasma, serum and rat plasma samples were 10, 9.5 and 7.5 ng/ml at S/N = 3. The proposed methods were applicable to the determination of TXL in human patients’ plasma ranging from 15 to 27 ng/ml.\(^{21}\)

A simultaneous determination of aspirin and its metabolite, salicylic acid in human serum by a semimicro column HPLC-UV was developed. The limits of detection for aspirin and salicylic acid were 114 and 38 ng/ml at S/N = 3. These compounds in patients’ sera administered with low-dose enteric-coated aspirin were determined, and the concentration ranges obtained for aspirin and salicylic acid were 1.2–2.2 and 0.5–57.3 µg/ml, respectively.\(^{22}\)

Mildly to severely elevated plasma concentrations of homocyste in (HCY) are positively associated with an increased risk of atherosclerosis, independent of traditional vascular disease risk factors. The determinants of HCY and carotid intima-media thickness, a clinical marker for the detection of atherosclerosis in Japanese were evaluated. In 289 Japanese adults (age 37–86 years), we screened plasma total HCY by HPLC-FL and evaluated maximum carotid intima-media thickness by ultrasound. Total HCY levels were higher in men than women and increased with age. Maximum carotid intima-media thickness was higher in men than in women and increased with age.\(^{23}\)

A simple and rapid semi-micro column HPLC-FL method for the determination of medium-chain fatty acids (MCFAs) including C\(_6\), C\(_8\), C\(_{10}\), C\(_{12}\), C\(_{14}\) and C\(_{16}\) was developed. 2-(4-Hydrazinocarbonylphenyl)-4,5-diphenylimidazole (HCPI) was used as a labeling reagent. A simple deproteinization with a mixture of CH\(_3\)CN and dimethylformamide was employed for the extraction of MCFAs. The limits of detection of fatty acids at S/N = 3 were in the range of 0.15–0.26 pmol/5 µl injection. The proposed method was applied to determine MCFAs in human plasma. Furthermore, the relation between the levels of MCFAs and HCY could be estimated.\(^{24}\)

Stimulants and Narcotics

Hair analysis is an excellent tool for confirmation of drug abuse. Hair has been used as an alternative specimen to blood or urine for documentation of use or exposure to drugs, because hair analysis can provide the information of drug intake for a long time after the drug was eliminated from the body. Hair has a preferable feature as a biological sample because of its stability and easiness for sampling and storing compared to the conventional biological samples such as blood and urine. This gives valuable and preferable information in forensic toxicological and drug abuse studies.\(^{26}\)

**HAIR ANALYSIS**

Hair analysis of stimulants and narcotics is rapidly growing because it is very useful for forensic and clinical toxicology: it can furnish information about drug-uptake history and time-line of use. In general, hair analysis requires high sensitivity and selectivity, and thus GC-MS (or GC-MS/MS) and LC-MS (or LC-MS/MS) are most frequently used. HPLC with FL or CL detection involving FL derivatization increases the option for hair analysis because of its high sensitivity.

Highly sensitive determination of AP and MP in abuser’s plasma and hair samples with HPLC-FL was achieved by using DIB-Cl as a label. The limits of detection were less than 0.87 ng/ml and 0.12 ng/mg in plasma and hair, respectively, for both AP and MP. The method was successfully applied to the determination of MP and its metabolite AP in plasma obtained from two cases of illegally ingested MP and in one of the cases hair received later. Case I was treated with dialysis; samples before and after dialysis were analyzed by the developed method. After dialysis for 5 hr, the total plasma levels of AP and MP decreased from 720 to 190 ng/ml. For case II, MP and AP levels were monitored for 3 days after ingestion. Total plasma levels decreased from 57 ng/ml in the day of digestion to 11 ng/ml after 3 days. In hair samples, AP and MP could also be detected in very low concentrations.\(^{27}\)
Recently, ecstasy tablets containing other drugs such as MDA, MP, AP, caffeine, aspirin, paracetamol and ketamine were found. Taking these tablets may cause unexpected adverse effects.

To elucidate uptake of MDMA related compounds (i.e., MDMA, MDA, MP, and AP), a highly sensitive simultaneous HPLC-FL determination method of these in human hair samples was developed by using DIB-Cl as a labeling reagent. The limits of detection for the four compounds ranged from 11 to 200 pg/mg. By the method, MDMA and its metabolite MDA in hair samples from MDMA abuser were quantified.28)

Quantification of MDMA related compounds in abusers’ hair samples by a semi-micro column HPLC-FL method was performed. These drugs were extracted from hair into 5% trifluoroacetic acid in CH$_3$OH. The limits of detection at $S/N=3$ for MDMA, MDA, MP and AP were 0.25, 0.15, 0.25 and 0.19 ng/mg, respectively. The method was applied to determine 6 abusers’ hair samples.29)

A sensitive semi-micro column HPLC-CL method for determining MDMA-related compounds in hair was developed by employing fluorescent labeling with DBD-F followed by PO-CL detection. Washed hair samples were digested with 1 M NaOH at 80°C for 25 min and the analytes were extracted with n-heptane. Derivatized analytes with DBD-F were injected into the HPLC-column switching system. The mixture of bis(2,4,5-trichloro-6-carbopentoxyphenyl)oxalate (CPPO) and H$_2$O$_2$ in CH$_3$CN was used as a post-column CL reagent. The method allowed highly sensitive determination of stimulants and the limits of detection at $S/N=3$ for MDMA, MDA, MP and AP were 0.03, 0.16, 0.02 and 0.15 ng/mg, respectively. The proposed method was applied to determine MDMA and its related compounds in multi-drug abused patients’ hair which were treated in a Chemical Dependency Unit of Mississippi State Hospital. Figure 4 shows tentative chromatograms obtained from patients’ hair samples.30)

Pentazocin (PZ) has been used in the management of patients with post-operative pain or initial carcinogenic pain due to its short-acting narcotic-antagonist analgesic effect. Additionally, PZ has also been known as a drug of abuse. An HPLC-FL method for determination of PZ in rat hair was developed. PZ in 1 mg of hair washed with 0.1% sodium dodecyl sulfate (SDS) and H$_2$O was extracted in CH$_3$OH containing 5% HCl with sonication for 1 hr at room temperature. Following fluorescence labeling with DIB-Cl, the reaction mixture was cleaned-up by an solid phase extraction (SPE) cartridge. The limit of detection at $S/N=3$ was 0.18 ng/mg. The precision for intra- and inter assays and recovery were 5.7, 4.8 and 103%, respectively. By the proposed method, incorporation rates [calculated from PZ concentration in hair/area under the curve (AUC) of PZ in plasma] of PZ for white and black hairs obtained from Zucker rat were 0.053 and 0.007, respectively.31)

Figure 5 shows a reaction scheme of PZ with DIB-Cl. Typical chromatograms of DIB-PZ in normal and spiked hair samples are shown in Fig. 6.
Anorectics

An HPLC-FL detection for Fen and Norf labeled with DIB-Cl was developed and applied it to human hair as a biomarker of N-Fen ingestion. The limits of detection at \( S/N = 5 \) were 36 pg/mg (2.7 pg on column) for Fen and 16 pg/mg (1.2 pg on column) for Norf. The method was applied to the segmental determination of Fen and Norf in hair samples obtained hospitalized patients diagnosed with hepatotoxicity, who were suspected to ingest N-Fen. Both Fen and Norf could be detected in these 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 ingest N-Fen for a period not less than 5 months. This showed that Fen and Norf could be used as biomarkers for N-Fen ingestion. Moreover, Fen and Norf were suggested to be incorporated in white hair, which was well corresponded to the results of N-Fen administration experiment to rats.\(^{12}\)

**DRUG-DRUG INTERACTION**

Nowadays, multi-medicament use was increased in clinical practice, especially, in the treatment of elder patients. Therefore, the drug-drug interaction of medicaments, which are expected to be co-administered in clinical practice, seems to be significant. Accumulation of this kind of knowledge may be helpful for rational use of medicament in practice. In this mean an HPLC method is a powerful technique to study on drug-drug interaction.

**Stimulants and Narcotics**

Simultaneous determination of MDMA and MDA in rat blood and brain microdialysates by HPLC-FL was developed. Microdialysates were directly subjected to labeling with DIB-Cl. The limits of detection for MDA and MDMA were 1.2 and 4.2 ng/ml for blood and 1.3 and 4.8 ng/ml for brain, respectively. The proposed method was successfully applied for the monitoring of MDMA and its metabolite MDA in rat blood and brain microdialysates. The pharmacokinetic parameters of MDMA and MDA in the microdialysates after administration of MDMA (5 mg/kg, i.p.) with or without caffeine...
(20 mg/kg, i.p.) were evaluated. MDMA could easily pass through the blood-brain barrier due to its hydrophobic nature and small molecular size. The parameters suggested that caffeine may interfere with renal clearance of MDMA. On the other hand, caffeine inhibited the transportation of MDMA and MDA to brain via the blood-brain barrier, although the exclusions of MDMA and MDA were not influenced by caffeine.\(^{10}\)

Morphine (Mor), one of the most famous narcotics, is a very important medicament for the pain treatment, and has been for sometimes used illegally. A sensitive HPLC-FL method for determination of Mor in rat brain and blood microdialysates was developed using DIB-Cl as a label. The limits of detection of Mor in brain and blood microdialysates were 9.4 and 0.6 ng/ml at \(S/N = 3\), respectively. The proposed method was applied to the preliminary study of potential pharmacokinetic interaction between Mor and diclofenac (Dic). After administration of Mor (10 mg/kg, i.p.) with/without Dic (5 mg/kg, i.p.) to rats, pharmacokinetic parameters were determined and evaluated. No significant difference could be found between the two groups in brain and blood for any parameters. However these results may help the clinical treatment of Mor with Dic.\(^{32}\)

**Anorectics**

Pharmacokinetic study by using an HPLC-FL was examined for Phen and Fen in rat brain and blood microdialysates after i.p. administration. The limits of detection were less than 23 fmol (\(S/N = 3\)) on column in both brain and blood microdialysates. The method was applied for a pharmacokinetic drug-drug interaction study of Phen and Fen following individual and combined i.p. administration to rats. 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.\(^{33}\)

The mechanism involved in the serious adverse effects associated with PPA has not yet been clarified, and PPA in usual cases is not being ingested without other drugs. Therefore we studied to characterize the possibility of pharmacokinetic interactions between PPA and most often combined drugs, e.g., caffeine and CPA, existing in the same dosage. PPA in rat brain and blood microdialysates was determined by HPLC-FL method using DIB-Cl as a label. As a result, the single i.p. administration of caffeine (5 mg/kg) with PPA (2.5 mg/kg) caused 1.6-fold increase in the AUC of PPA compared to the single administration of PPA, and was comparable to the 1.5-fold increase caused by CPA (0.4 mg/kg). The multiple combinations caused an increase in the AUC by 1.9-fold, which is comparable to the increase in the AUC of PPA obtained from the OTC product (2.2-fold). The adverse effect with PPA use could be related to the increase in its level in the brain.\(^{34}\)

**Other Drugs**

A semi-micro column HPLC method for TZ in rat plasma and brain microdialysates was developed for drug-drug interaction study of TZ with itraconazole (ITZ). As a result, ITZ seriously interfered with pharmacokinetic parameters of TZ when single simultaneous administration of TZ with ITZ and single administration of TZ after daily pretreatment with ITZ were performed.\(^{35}\)

The possibility of interaction of aspirin (Asp) or clopidogrel (CG) on DP in rats by HPLC-FL was investigated. DP levels in rat plasma with a single administration of DP (5 mg/kg, i.p., group I) and those with a co-administration of Asp [200 mg/kg, per os (p.o.), group II or 200 mg/kg, i.p., group III] or CG (5 mg/kg, p.o., group IV) were monitored. The DP concentrations determined in rat plasma ranged from 25.0 to 336.1 ng/ml. The pharmacokinetic parameters for these groups were calculated and compared with one another. No significant difference was observed on the comparison of group I with other groups except for mean resident time of group IV (\(p = 0.012\)). These basic findings may help clinical inference when DP is co-administered with Asp and CG to human.\(^{36}\)

**CONCLUSION**

Analysis of biologically active compounds is very important to obtain clinically valuable data for human health. Especially, analysis of drugs of abuse can predict of and prevent from the risks of human health, and is growing issue in toxicological and forensic sciences. The recent rapid development of electronic technology has provided highly sensitive and selective determination methods such as GC-MS and LC-MS. Also, detection techniques utilizing FL and CL gave highly sensitive HPLC methods, which should be improved in sensitivity and selectivity, and down-sized in their systems for more practical use.
We believe that our research results will be helpful for analytical researchers, and would like to continue to make progress from now on.

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