Anal. Bioanal. Chem. (Original Paper)

HPLC determination of methylphenidate and its metabolite, ritalinic acid, by high-performance liquid chromatography with peroxyoxalate chemiluminescence detection

Mitsuhiro Wada[a], Keisuke Abe[a], Rie Ikeda[a], Ruri Kikura-Hanajiri[b], Naotaka Kuroda[a] Kenichiro Nakashima[a*]

[a] Graduate School of Biomedical Sciences, Nagasaki University, 1-14 Bunkyo-machi, Nagasaki 852-8521, Japan

[b] National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya, Tokyo 158-8501, Japan

*Corresponding author.

Tel.: +81-95-819-2450; Fax: +81-95-819-2450.

E-mail address: naka-ken@nagasaki-u.ac.jp (K. Nakashima)

Abstract

An HPLC-peroxyoxalate chemiluminescence (PO-CL) method for simultaneous determination of methylphenidate (MPH) and ritalinic acid (RA) was developed. Furthermore, the proposed method was applied to monitor MPH and RA after an administration of MPH to rat. The deproteinized plasma spiked with 1-(3-trifluoromethylphenyl)piperazine (IS) was dried up and labeled with 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F). The labeled sample was cleaned-up with two kinds of solid-phase extraction cartridges, and the DBD-labels were separated on an ODS column with gradient elution using a mixture of CH_3CN and imidazole-HNO_3 buffer. The separation of MPH and RA can be achieved within 33 min. The LODs of MPH and RA at signal-to-noise ratio of 3 were 2.2 and 0.4 ng/mL, respectively. Moreover, monitoring of MPH and RA after MPH administration (10 mg/kg) to rat could be performed. The concentration of RA at 480 min after administration was 8 times higher than that of MPH.

The proposed HPLC-PO-CL method was useful for determination of MPH and RA in rat plasma and was successfully applied to monitor these substances after MPH administration. (172 words)

Keywords: methylphenidate, ritalinic acid, HPLC, peroxyoxalate chemiluminescence, 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3- benzoxadiazole

Introduction

Methylphenidate (MPH), a phenethylamine derivative, is a well-known medicament for attention-deficit hyperactivity disorder and narcolepsy [1]. It is known that there is marked individual variability in the dose-response relationship for MPH, and therefore dosage must be titrated for optimal effect and avoidance of toxicity in each patient [2,3]. However, it is unclear whether this variability is predominantly pharmacokinetic or pharmacodynamic. MPH is reported to be absorbed rapidly and completely from the intestine after oral administration and hydrolyzed at the methyl ester moiety to its main metabolite, ritalinic acid (RA) [4]. And in human carboxylesterase contributes to hydrolyze MPH stereo-selectively to RA [5]. In order to fully assess the processes associated with metabolic clearance, simultaneous determination of MPH and RA might be needed, though RA is a major inactive metabolite.

Due to its similar pharmacological effect of amphetamine, MPH has been illicitly abused [2,6]. Recent fatalities due to the abuse of MPH have been reported [6]. To distinguish MPH abuse from external contamination of sample by MPH, identification of MPH and its metabolite in the sample was simultaneously required. Therefore, a simple and reliable analytical method to determine MPH and RA is in requisite, due to their clinical and forensic interests described above. However, only a few methods for simultaneous determination of MPH and RA in biological samples have been reported [7-9]. Chromatographic behavior between MPH and RA is different, because polarity of RA is higher than that of MPH. This is one of reasons for difficulty on simultaneous determination of MPH and RA.

Several analytical methods have been reported for the determination of MPH and/or RA in biological matrices. Gas chromatography-mass spectrometry [10,11], HPLC-UV

[12-14], -fluorescence (-FL) detections [15], liquid chromatography (LC-MS) [5,7-9,16,17] and LC-tandem mass spectrometry (LC-MS/MS) [2,18,19] methods were widely used. Quite recently, MS detection for very low-level quantification of MPH in biological samples has been frequently used. However, the use of MS detector, which is large and expensive equipment, is restricted. Simultaneous determination of MPH and RA by FL detection with nano-g level of sensitivity has never been achieved previously. On the other hand, a peroxyoxalate chemiluminescence (PO-CL) method, which is a powerful tool to determine fluorophore, has been used to determine trace amounts of biologically active compounds such as drugs in biological samples due to its high sensitivity and selectivity [20]. Thus sensitive determination of MPH and RA might be achieved by an HPLC-PO-CL method combined with FL labeling technique, although no previous achievement has been ever reported. In our previous study, we already developed a sensitive HPLC-PO-CL method for determination of MDMA-related compounds [21].

In this study, an HPLC- PO-CL method for simultaneous determination of MPH and RA labeled with 4-(*N*,*N*-dimethylaminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (DBD-F) was developed. To clean-up labeled sample, two types of solid-phase extraction (SPE) cartridges were used. Furthermore, the proposed method was applied to monitor MPH and RA after an administration of MPH to rat.

Materials and methods

Chemicals

MPH was purchased from Sigma Chemical Co. (MO, USA). RA and 1-(3-trifluoromethylphenyl)piperazine (TFMPP), used as an internal standard (IS), were gifted from National Institute of Health Sciences (Tokyo, Japan). DBD-F and imidazole were obtained from Tokyo Kasei Kogyo Co. (Tokyo). Bis(2,4,5-trichloro-6-carbopentoxyphenyl)oxalate (CPPO) was kindly gifted from Lumica Co. (Fukuoka, Japan). Other reagents used were of analytical reagent grade. Water was purified by passing through a Pure Line WL21P system (Yamato Scientific Co., Tokyo, Japan). A stock standard solution (1 mg/mL) of MPH and RA were prepared by dissolving in MeOH and stored at -30°C until analysis.

Pretreatment of plasma sample

Fifty-μL of plasma spiked with 10 μL of IS in CH_3CN were deprotenized with 500 μL of CH_3CN. After centrifugation (3500*g*) at 20℃ for 10 min, the resulted supernatant was dried-up and the residue was applied to labeling reaction with DBD-F.

Each 50-μL of borate buffer (100 mM, pH 8.5) and 120 mM DBD-F in CH_3CN were spiked to the residue, and heated at 80℃ for 30 min in the dark (Fig. 1). The reaction mixture was cooled in ice-water to stop the reaction.

The reaction mixture with DBD-F was applied to SPE cartridges to eliminate the excess reagent and interfering materials for detection of MPH- and RA-DBD. Cleaned-up with 2 kinds of SPE cartridges, which are Varian Bond Elut® C18 for MPH and IS, and Waters Oasis® MAX for RA were used. Both cartridges were conditioned with H_2O and CH_3CN before use. The labeled sample was applied to Varian Bond Elut®

C18 and washed with 3 mL of CH_3CN/H_2O (=40:60, v/v). Then the MPH-DBD was obtained by eluting with 500 μL of MeOH. The washing solution obtained from Varian Bond Elut® C18 was applied to Waters Oasis® MAX. The Waters Oasis® MAX was washed with 2 mL of MeOH and eluted with 500 μL of 2% acetic acid in MeOH for RA-DBD. Then both eluates were combined and dried-up under N_2 gas. Finally, residue was reconstituted with 100 μL of CH_3CN and applied to HPLC analysis.

Fig. 1

HPLC conditions

The HPLC system for separation of MPH- and RA-DBD labels is shown in Fig. 2. It was consisted of three chromatographic pumps to flow mobile phase (2×LC-20AD, Shimadzu, Kyoto, Japan) and CL reagent (LC-10AD$_{VP}$, Shimadzu), a system controller (SCL-10 AD$_{VP}$, Shimadzu), a 7125 injector with a 20-μL of sample loop (Rheodyne, CA, USA), a Daisopak-SP-ODS-BP column (250×2.0 mm, i.d., 5 μm, Daiso, Osaka, Japan), a UNI-1 noise cleaner (Union, Gunma, Japan) which smoothes the baseline, a CL2027 Plus chemiluminescence detector (Jasco, Tokyo) and a recorder (FBR-2, Tosoh, Tokyo). The solutions, CH_3CN (MP 1) and 10 mM imidazole-HNO_3 buffer (pH 7.0, MP 2) were used as mobile phases and total flow rate was set at 0.3 mL/min. The gradient program to separate DBD labels was set as follows; the ratio of MP 1 was initialized at 10% (0 to 10.0 min), linearly ramped to 60% (10.1 to 14.0 min), preserved 60% (14.1 to 35.0 min), changed to 90% (35.1 to 45.0 min) for washing and then 10% (45.1 to 55.0 min) for equilibration of the analytical column. A mixture of 4 mM CPPO and 37.5 mM H_2O_2 in CH_3CN was used as a post column CL reagent and pumped at 0.45 mL/min of flow rate.

Fig. 2

Validation of the method

The standard of MPH and RA solutions spiked plasma were independently prepared adequate times in validation study. For determination of MPH and RA, calibration curves using rat plasma spiked with known concentration of standards prepared independently three times were prepared by the internal standard method. The IS solution containing standards were spiked to prepare the calibration curves. The calibration equations and correlation coefficients using the model of least squares were calculated by the software JMP® 8.0.2 (SAS Institute Japan, Tokyo). Plasma samples spiked with MPH ranging from 10 to 1000 ng/mL plasma and RA ranging from 100 to 10000 ng/mL plasma were prepared. The limit of detection (LOD) and quantification (LOQ) were defined as a concentration given signal-to-noise (S/N) ratio of 3 and 10. Recovery of the proposed method was expressed as the peak height ratio between standards spiked in plasma with SPE step and standards without clean-up with SPE. Accuracy and precision for inter-day and intra-day assay were evaluated by using plasma samples spiked with 10, 50 and 500 ng/mL of MPH, and 100, 500 and 5000 ng/mL of RA, respectively. Data was expressed as mean±SD (n=6).

Pharmacokinetic study of MPH and RA

Wistar male rats (n=3, 280-300 g, Otsubo experimental animals, Nagasaki, Japan) were used. Rats were anesthetized with ethyl carbamate (1.5 g/kg) before cannulation. From cannulated femoral artery, blood samples were collected into the test tube containing EDTA-2Na for preparation of plasma. Blood sampling was performed at 0, 15, 30, 45, 60, 90, 120, 180, 240, 300, 360, 420 and 480 min after administration of MPH (10 mg/kg, *i.p.*). After centrifugation (1000*g*) at 4˚C for 10 min, plasma was kept at -30˚C

until analysis. The pharmacokinetic parameters such as elimination rate constant (K), elimination half-life ($T_{1/2}$), distribution volume (V) and clearance (CLR) of MPH were manually calculated with one-compartment model analysis. The area under the curve (AUC) of MPH and RA was manually calculated by trapezoidal method. The data was presented as mean±SD (n=3).

This experiment was performed with an approval of Nagasaki University Animal Care and Use Committee.

Results and discussion

Optimization of pretreatment conditions of sample

Labeling conditions such as DBD-F and borate buffer (pH 8.5) concentrations and reaction time were examined. 1.0 μg/mL of MPH standard solution was used and its chemiluminescence intensity (CLI) and decomposition ratio to RA were evaluated. At first, the effects of DBD-F concentration (20-320 mM) on the CLI were examined. DIB-MPH gave maximum CLI with 120 mM and decomposition ratio of MPH decreased with increase of DBD-F concentration, thus the following experiments were done using 120 mM DBD-F. Next, concentration of borate buffer ranging from 25 to 150 mM was examined, and maximum constant CLI was achieved using more than 75 mM of borate buffer with low decomposition ratio of MPH, thus 100 mM was selected. Finally, reaction time at room temperature was studied in the range of 10-60 min. Maximum CLI was given by 30 min of reaction time, and longer reaction time than 30 min brought decomposition of MPH. The following experiments were done at room temperature for 30 min. In our preliminary study, acceleration of decomposition of MPH by heating was found (data is not shown), so labeling reaction at room temperature was demonstrated.

Deprotenization of plasma sample with CH_3CN was performed before labeling. It improved the recovery of MPH and FA.

To remove excess DBD-F, clean-up with SPE cartridge was performed. Varian Bond Elut® C18 could retain MPH- and IS-DBD labels, although RA-DBD was eluted with washing solution. On the other hand, the Waters Oasis® MAX, a mixed-mode sorbent consisting of a strong anion exchanger and a non-polar *N*-vinylpyrrolidone/divinylbenzene polymer, well-retained RA-DBD, however, the

recoveries of MPH- and IS-DBD labels were not satisfactory. So the reaction mixture with DBD-F was applied to Varian Bond Elut® C18 at first to recover the MPH- and IS-DBD labels. Then the washing solution of the Varian Bond Elut® C18 containing RA-DBD was applied to the Waters Oasis® MAX to recover the RA-DBD. Then both eluates were collected and reconstituted with CH_3CN after drying-up under N_2 gas. The recoveries of MPH- and RA-DBD were 50.0% and 43.8%, respectively. The recoveries of MPH and RA in previous reports demonstrated simultaneous determination these compounds were not mentioned clearly [7, 9]. And moderate recoveries (75% for MPH and 60% for RA) were obtained by LC-MS with SPE (Oasis HLB cartridge, Waters) [8]. To improve recovery, further examination with other SPE cartridges should be needed.

HPLC and CL detection conditions

At first, the isocratic separation of MPH- and RA-DBD was attempted. Since chromatographic behavior of both labels on reversed phase column (Daisopak-SP-ODS-BP column) was remarkably different, their satisfactory separation from interfering peaks with acceptable analysis time could not be obtained. Therefore gradient elution with CH_3CN (MP 1) and 10 mM imidazole-HNO_3 buffer (pH 7.0, MP 2) was performed. The retention times of MPH-, RA and IS-DBD labels were 29.5, 13.0 and 33.0 min, respectively. Total run time including washing and equilibration steps after eluting of IS-DBD label was 55 min.

Optimization of PO-CL detection conditions such as concentrations of CPPO and H_2O_2 and flow rate of CL reagent was demonstrated. A mixture of CPPO and H_2O_2 in CH_3CN was used as a post column CL reagent. One μg/mL of MPH standard solution was used and its chemiluminescence intensity (CLI) was evaluated. CPPO ranging from

1 to 8 mM was used. More than 2 mM of CPPO gave maximum and constant CLI of MPH. Next, H_2O_2 concentration in the range of 25-100 mM (25.0, 37.5, 50.0, 75.0 and 100.0 mM) was examined, maximum CLI of MPH was obtained at 37.5 mM. Effect of flow rate of CL reagent on CLI of MPH was examined (at 0.30, 0.38, 0.45, 0.60 and 0.9 mL/min). More than 0.4 mL/min of flow rate gave the maximum and constant CLI of MPH. The following experiments were done by using CL reagent containing 4 mM of CPPO and 37.5 mM of H_2O_2 flowed at 0.45 mL/min.

Validation of the proposed method

Calibration curves of MPH and RA obtained with spiked plasma were linear with *r*-values greater than 0.998 in the ranges of 10-1000 ng/mL and 100-10000 ng/mL. Since concentration range of RA in plasma sample after administration might be 10 times higher than that of MPH, high concentrations for preparation of RA calibration curve were used. The LODs of MPH and RA at S/N ratio of 3 were 2.2 ng/mL (93.2 fmol on column) and 0.4 ng/mL (20.2 fmol on column), respectively, and the LOQs at S/N ratio of 10 were 7.3 ng/mL (MPH) and 1.3 ng/mL (RA).

Accuracy, intra- and inter-day precisions of the proposed method were evaluated by analyzing plasma spiked with known concentrations of MPH (10, 50 and 500 ng/mL) and RA (100, 500 and 5000 ng/mL) as shown in Table 1. Accuracy in the range of 91.6±8.9%-108.9±2.4% was obtained. The intra-day RSDs for MPH and RA were 9.7 (10 ng/mL), 2.2 (50 ng/mL) and 3.6 % (500 ng/mL), and 6.2 (100 ng/mL), 3.1 (500 ng/mL) and 2.7 % (5000 ng/mL), respectively. Inter-day precision (RSDs) of 9.3 (10 ng/mL), 5.0 (50 ng/mL) and 8.7 % (500 ng/mL) for MPH were obtained, while those for RA were 7.0 (100 ng/mL), 6.1 (500 ng/mL) and 6.7 % (5000 ng/mL). By our method,

decomposition of MPH to RA was not found. Because peak of RA did not appear when the plasma spiked with MPH was analyzed.

The sensitivity and plasma volume required for measurement of the proposed method and conventional methods were summarized in Table 2. Plasma volume required for measurement in our method was comparable to those of the conventional methods [7-9], though our method was less sensitive. The method reporting completed simultaneous determination of MPH and RA are rare. One of the reasons might be remarkable difference of chromatographic behavior of these compounds on reversed phase chromatography. To our knowledge, only LC-MS methods to determine MPH and RA simultaneously in plasma could be achieved [7-9]. The MS detection having excellent selectivity negotiates this difference on chromatographic behavior. In this study, the combination of fluorescence labeling of substances, clean-up step and gradient elution overcame it.

Table 1

Table 2

Pharmacokinetic study of MPH and RA

Furthermore, the proposed method was applied to monitor of MPH and RA concentration after MPH administration to rat. Chromatograms of rat plasma spiked with IS (A) and that obtained at 45 min after *i.p*. administration of MPH (10 mg/kg) (B) are shown in Fig. 2. An asterisk indicates that the detector sensitivity is set 5 times higher at that point of the analysis due to the higher concentration of RA compared with that of MPH. The concentrations of MPH and RA in the chromatogram corresponded to 482.5 and 238.8 ng/mL, respectively. And the concentration–time profiles of MPH and RA are shown in Fig. 3. The concentrations of MPH and RA in plasma were in the

range of 16.5-482.5 ng/mL and 219.4-745.9 ng/mL, respectively. The SD of RA concentrations were varied due to the variance of the time to reach plateau concentration of RA.

By using the proposed method MPH could be monitored after 480 min of administration. MPH was hydrolyzed to RA rapidly and this fact agrees with that in previous report [3]. The concentration of RA at 480 min after administration was kept high levels and 8 times higher than that of MPH. Pharmacokinetic parameters of MPH and RA was calculated and is summarized in Table 3. The K (0.0046±0.0027 min^{-1}) and CLR (0.025±0.009 L/min) of MPH obtained in this study were similar to those in previous report [3]. Kennerly *et al*., reported MPH is also metabolized to *p*-hydroxy methylphenidate in rat, but its concentration was lower than that of MPH [4]. In our study, no peak corresponding to *p*-hydroxy methylphenidate was found on the chromatogram. Applicability of the method could be clarified.

Fig. 2

Fig. 3

Table 3

Conclusion

The proposed HPLC-PO-CL method was useful for simultaneous determination of MPH and RA in rat plasma. The reason of difficulties of simultaneous determination of MPH and RA might be the remarkable difference of chromatographic behavior of both compounds. Using fluorescence labeling, two types of SPE cartridges for clean-up step, gradient elution, and PO-CL detection, sensitive and selective determination of these compounds was achieved. Validation parameters of MPH and RA in plasma obtained were acceptable. Furthermore, this method could be successfully applied to monitor these substances after MPH administration to rat. Due to these results, the proposed method was apparently simple and reliable, and suitable to apply over preclinical and forensic regions of research.

References

[1] Volkow ND, Fowler JS, Wang GJ, Ding YS, Gatley SJ (2002) Role of dopamine in the therapeutic and reinforcing effect of methylphenidate in humans: results from imaging studies. Eur Neuropsychopharmcol 12: 557-566.

[2] Patrick KS, Straughn AB, Minhinnett RR, Yeatts SD, Herrin AE, DeVane CL, Malcolm R, Janis GC, Markowitz JS (2007) Influence of ethanol and gender on methylphenidate pharmacokinetics and pharmacodynamics. Clin Pharmacol Ther 81: 346-353.

[3] Wargin W, Patrick K, Kilts C, Gualtieri CT, Ellington K, Mueller RA, Kraemer G, Breese GR (1983) Pharmacokinetics of methylphenidate in man, rat and monkey. J Pharmacol Exp Ther 226: 382-386.

[4] Kimko HC, Cross JT, Abernethy DR. (1999) Pharmacokinetics and clinical effectiveness of methylphenidate. Clin Pharmacokinet **37**: 457-470.

[5] Sun Z, Daryl J, Sanghani SP, Davis WI, Kedishvili NY, Zou Q, Hurley TD, Bosron WF. (2004) Methylphenidate is stereoselectively hydrolyzed by human carboxylesterase CES1A1. J Pharmacol Exp Ther **310:** 469-476.

[6] Masselo W, Capenter DA (1999) A fatality due to the intransal abuse of methylphenidate (Ritalin). J Forensic Sci 44: 220-221.

[7] Marchei E, Farrè M, Pellegrini M, Rossi S, Algar OG, Vall O, Pichini S. (2009) Liquid chromatography-electrospray ionization mass spectrometry determination oh methylphenidate and ritalinic acid in conventional and non-conventional biological matrices. J Pharm Biomed Anal 49: 434-439.

[8] Doerge DR, Fogle CM, Paule MG, Mccullagh M, Bajic S. (2000) Analysis of methylphenidate and its metabolite ritalinic acid in monkey plasma by liquid chromatography/electrospray ionization mass spectrometry. Rapid Commun Mass Spectrom 14: 619-623.

[9] Barbarin N, Mawhinney DB, Black R, Henion J. (2003) High-throughput selected reaction monitoring liquid chromatography-mass spectrometry determination of methylphenidate and its major metabolite, ritalinic acid, in rat plasma employing monolithic columns. J Chromatogr B 783: 73-83.

[10] Sticht G, Sevecke K, Käferstein H, Döpfner M, Rothschild MA. (2007) Detection of methylphenidate in the hair of children treated with ritalin. J Anal Toxicol 31: 588-591.

[11] Chan YM, Soldin SJ, Swanson JM, Deber CM, Thiessen JJ, Macledo S. (1980) Gas chromatographic/mass spectrometric analysis of methylphenidate (Ritalin) in serum.

Clin Biochem 13: 266-272.

[12] Zhang J, Deng Y, Fang J, Mckay G. (2003) Enantioselective analysis of ritalinic acid in biological samples by using a protein-based chiral stationary phase. Pharm Res **20**: 1881-1884.

[13] Soldin SJ, Chan YM, Hill BM, Swanson JM. (1979) Liquid-chromatographic analysis for methylphenidate (ritalin) in serum. Clin Chem 25: 401-404.

[14] Soldin SJ, Hill BM, Chan YM, Swanson JM, Hill JG. (1979) A liquid-chromatographic analysis for ritalinic acid [α-phenyl-α-(2-piperidyl)acetic acid] in serum. Clin Chem 25: 51-54.

[15] Zhu HJ, Wang JS, Patrick KS, Donovan JL, Devane CL, Markowitz JS. (2007) A novel HPLC fluorescence method for the quantification of methylphenidate in human plasma. J Chromatogr B 858: 91-95.

[16] Lindsay DC, Markowitz JS, Carson SW, Boulton DW, Gill HS, Ziah N, Craig RS. (2000) Single-dose pharmacokinetics of methylphenidate in CYP2D6 extensive and poor metabolizers. J Clin Psycopharmacol 20: 347-349.

[17] Marchei E, Muñoz JA, Alger ÓG, Pellegrini M, Vall O, Zuccaro P, Pichini S. (2008) Development and validation of a liquid chromatography-mass spectrometry assay for hair analysis of methylphenidate. Forensic Sci Int 176: 42-46.

[18] Fernandez MMR, Laloup M, Wood M, Boeck GD, Rivadulla ML, Waĺlemacq P, Samyn N. (2007) Liquid chromatography-tandem mass spectrometry method for the simultaneous analysis of multiple hallucinogens, chlorpheniramine, ketamine, ritalinic acid, and metabolite, in urine. J Anal Toxicol 31: 497-504.

[19] Eichhorst J, Etter M, Lepage J, Lehotay DC. (2004) Urinary screening for methylphenidate (Ritalin) abuse: a comparison of liquid chromatography-tandem mass spectrometry, gas chromatography-mass spectrometry, and immunoassay methods. Clin Biochem 37: 175-183.

[20] Nakashima K, Ikeda R, Wada M. (2009) Analytical studies on the development of high-performance liquid chromatographic methods with fluorescence or chemiluminescence detections and their practical applications. Anal Sci 25: 21-32.

[21] Nakamura S, Wada M, Crabtree BL, Reeves PM, Montgomery JH, Byrd HJ, Harada S, Kuroda N, Nakashima K. (2007) A sensitive semi-micro column HPLC method with peroxyoxalate chemiluminescence detection and column switching for determination of MDMA-related compounds in hair. Anal Bioanal Chem 387: 1893-1990.

Figure Captions

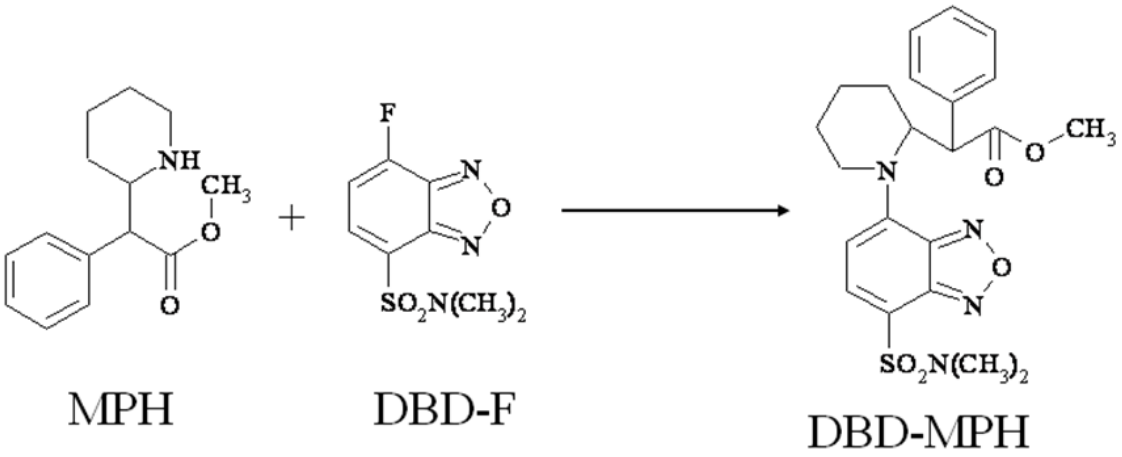

Fig. 1 Reaction scheme of MPH with DBD-F.

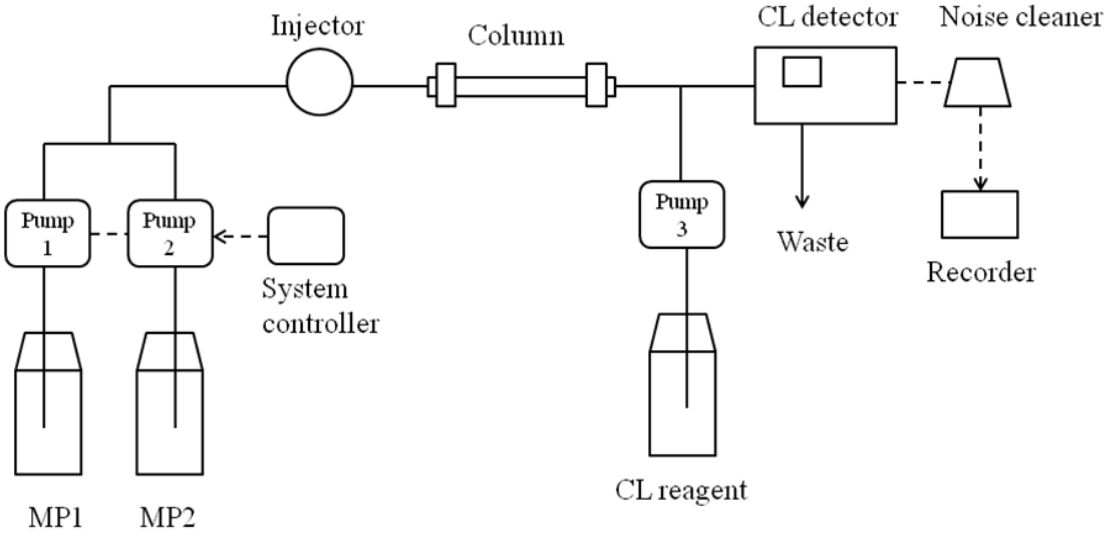

Fig. 2 Diagram of HPLC-PO-CL system for determination of MPH and RA.

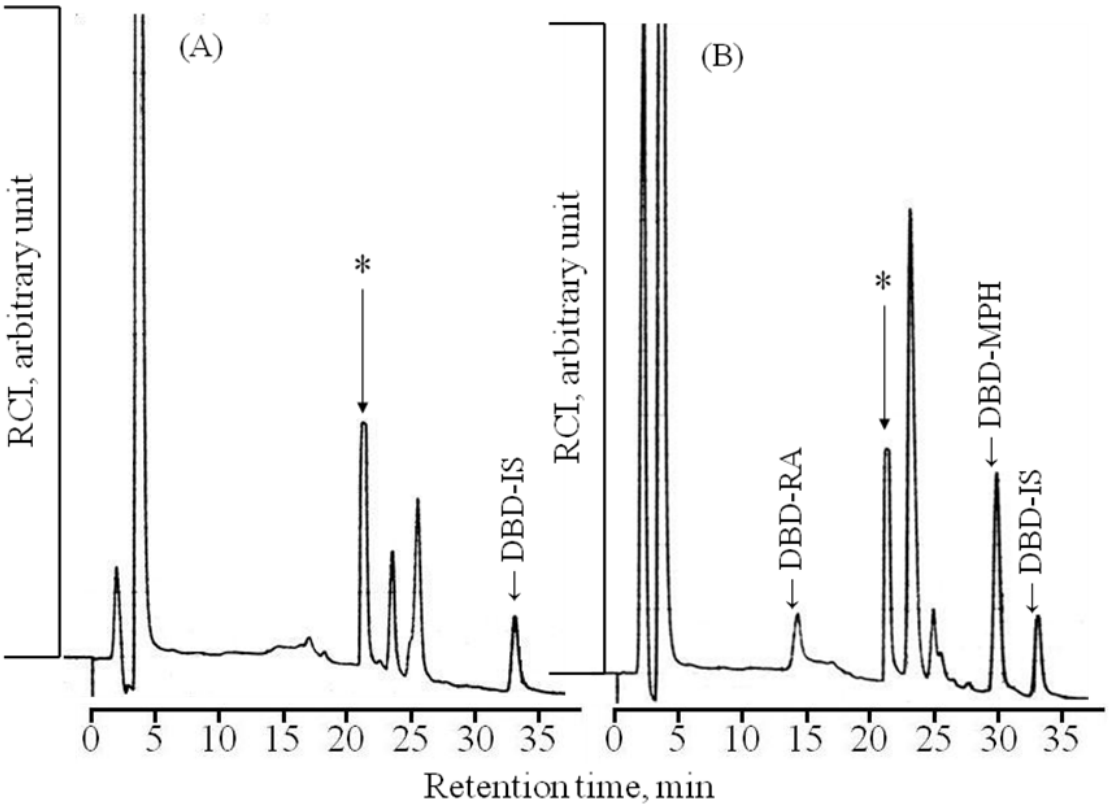

Fig. 3 Chromatograms of rat plasma spiked with IS (A) and that obtained at 45 min after *i.p*. administration of MPH (10 mg/kg) (B).

*An arrow indicates the changing point of sensitivity of detector to higher 5 times.

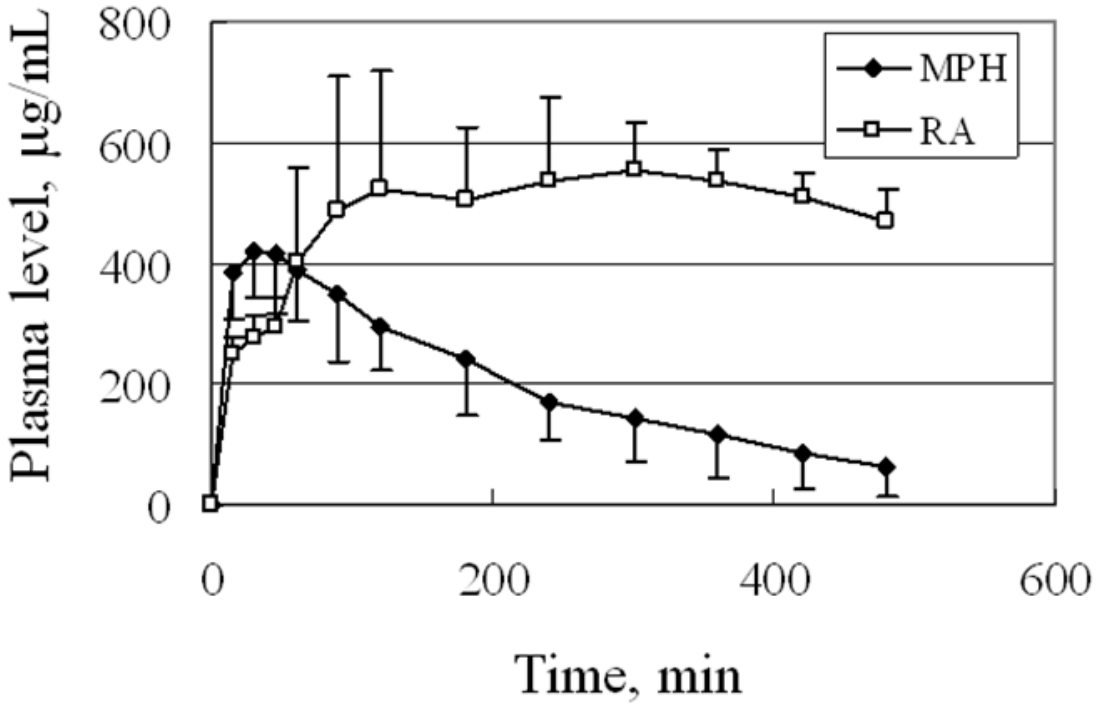

Fig. 4 Concentration-time profiles of MPH and RA in plasma after administration of MPH to rat.

Data was presented as mean±SD (n=3).

Table 1 Accuracies, intra- and inter-day assay precisions for MPH and RA.

Analyte	Spiked concentration, ng/mL	Accuracy[a] %	Precision % (RSD)	
			Intra-day	Inter-day
MPH	10	91.6±8.9	9.7	9.3
	50	108.9±2.4	2.2	5.0
	500	99.4±3.6	3.6	8.7
RA	100	93.6±5.8	6.2	7.0
	500	107.6±3.3	3.1	6.1
	5000	95.0±2.6	2.7	6.7

[a] Data was presented as mean±SD (n=6).

Table 2 The sensitivity and plasma volume required of the proposed method and conventional methods

Target compound	Method	LOD	LOQ	Plasma volume, μL	Ref.
MPH, RA	HPLC-PO-CL Labeled with DBD-F	MPH: 2.2 ng/mL RA: 0.4 ng/mL (S/N=3)	MPH: 7.3 ng/mL RA: 1.3 ng/mL (S/N=10)	50	-
MPH	HPLC-UV (λ=192 nm)	10 ng/mL (S/N=2)		300	13
MPH	HPLC-FL (λex=330, λem=460 nm) Labeled with DIB-Cl[1]		1 ng/mL (Accuracy within 80-120%, RSD<20%)	1000	15
MPH, RA	LC-MS	MPH and RA: 0.31 ng/mL (3 × SD of noise level)	MPH and RA: 1.0 ng/mL (10×SD of noise level RSD<20%)	100	7
MPH, RA	LC-MS	MPH and RA: 0.075 ng/mL (S/N=3)	MPH and RA: 0.25 ng/mL (S/N=10)	100	8
MPH, RA	LC-MS		MPH: 0.1 ng/mL RA: 0.5 ng/mL (RSD=20%)	25	9

[1] DIB-Cl: 4-(4,5-diphenyl-1*H*-imidazol-2-yl)benzoyl chloride.

Table 3 Pharmacokinetic parameters of MPH and RA.

Pharmacokinetic parameter	Mean±SD (n=3)
[MPH]	
K, min^{-1}	0.0046±0.0027
$T_{1/2}$, min	200±136
V, L	6.1±2.2
CLR, L/min	0.025±0.009
AUC, mg·min/L	98±22
[RA]	
AUC, mg·min/L	232±40

Data was presented as mean±SD (n=3).

Abbreviations: K, elimination rate constant; $T_{1/2}$, half-life time; V, distribution volume; CLR, clearance; AUC, area under the curve.