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# Reduction of carry-over in column-switching HPLC/MS system with automated system washing procedure for highly sensitive direct analysis of donepezil in dog plasma

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## Abstract

To solve the problem of carry-over – a persistent chromatographic challenge for bioanalytical assays with highly sensitive detector such as a mass spectrometry (MS) – a new on-line sample pretreatment HPLC/MS system using a column-switching technique was established. This system was designed to reduce carry-over based on a hydrophobic interaction mechanism using a washing function (multi-mobile phase flow system), as well as to remove impurities on-line in a mobile phase for the pretreatment. As a result, a washing function in this system was enabled to reduce carry-over and to remove impurities in a mobile phase by automatic operation. Therefore, concentration levels of donepezil (DH) in dog plasma as low as 10 pg/mL could be determined using this on-line sample pretreatment HPLC/MS system. In addition, method validation results of specificity, linearity, accuracy, precision, lower limit of quantitation (LLOQ), and carry-over demonstrated that this on-line sample pretreatment HPLC/MS system was robust and valid as a practical assay of drugs and metabolites in the biological samples.
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*Keywords:* Carry-over; Pharmacokinetics; RAM; Column-switching; Donepezil; On-line sample pretreatment

## 1. Introduction

In the pharmaceutical field, HPLC/MS with high sensitivity and selectivity is a powerful tool for drug and metabolite analysis in biological fluids. Recently, several restricted access media (RAM) columns, capable of direct injection of biological fluids, were developed, e.g. internal surface reversed phase silica [1–4], mixed functional phase silica [5], semipermeable surface silica [6], and diol silica [7,8]. Approaches to the acceleration of method development and reduction analysis times have involved a column-switching HPLC technique [9–12], in which the pretreatment RAM column eliminates macromolecules such as proteins from plasma samples, and is followed by an analytical column that is used for analytical separations. In particular, a methylcellulose-immobilized reversed-phase pretreatment column was developed [13] and applications to analysis of drugs in plasma with UV detection [13,16] and MS detection [15] were reported about high recovery, durability, and reproducibility. Moreover, a methylcellulose-immobilized strong cation-exchange pretreatment column for hydrophilic polar compounds was developed [14] and applied to pharmacokinetic studies of atenolol and sulpiride in rat plasma with UV detection [14] and determination of tricyclic antidepressants in rat plasma with MS detection [18]. In addition, this cation-exchange RAM column was successfully applied to determination of basic compounds and their metabolites in rat bile using tandem pretreatment columns [17].

However, impurity peaks originated from the mobile phase and/or the carry-over are significant problems for the HPLC/MS assays with highly sensitive detectors. In particular, carry-over is a commonly encountered chromatographic problem that can compromise the precision and accuracy of HPLC/MS assays, in which carry-over can reduce the reliability of analytical data, reproducibility, lower limit of quantitation (LLOQ), and dynamic linear range. One of major sources of carry-over is existed in the autosampler [19–21], steps to correct carry-over

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problems [22] and a case study in the elimination of autosampler carry-over in a bioanalytical HPLC/MS/MS method is reported [23]. Moreover, an on-line sample pretreatment HPLC system (column-switching HPLC system) increases the risk of carry-over owing to switching valves and complex lines. The existing literature provides only a limited reports of reduction carry-over in on-line sample pretreatment HPLC.

In this study, a new direct injection HPLC/MS system for the biological samples was established, capable of reducing carry-over and automating of the HPLC/MS bioanalytical assays coupling with RAM column and column-switching techniques.

This report describes a case study in reducing carry-over using an on-line sample pretreatment (column-switching) HPLC/MS system with an automated system washing procedure that was employed for the direct assay of donepezil in dog plasma. To evaluate the effect of automated system washing function in this system, a highly sensitive direct assay method for donepezil in dog plasma was developed using a column-switching HPLC/MS system as a model.

## 2. Experimental

### 2.1. Chemicals, reagents

Acetonitrile (HPLC/MS grade and analytical grade), acetic acid (analytical grade), and ammonium acetate (analytical grade) were purchased from Wako Pure Chemicals (Osaka, Japan). Trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, USA). All other reagents were analytical grade and used without further purification. Water was deionized and purified by a Milli-Q® purification system obtained from Millipore (Bedford, MA, USA).

Donepezil hydrochloride (DH) and donepezil-$d_7$ hydrochloride (IS) were obtained from Eisai Co., Ltd., Japan. The chemical structures are shown in Fig. 1.

### 2.2. Blank plasma

Blank dog plasma for the preparation of calibration curves and spiked samples were obtained from BMR division of Sunplanet Co., Ltd. (Gifu, Japan).

### 2.3. Sample preparation

#### 2.3.1. Preparation of stock and working solutions

Stock solutions of donepezil hydrochloride (DH) and donepezil-$d_7$ hydrochloride (IS) were dissolved separately in a mixture of 0.1 M hydrochloric acid and methanol (1:1, v/v) having a concentration of 0.3 mg/mL and stored at 4 °C until use. Working solutions of DH, at concentrations ranging from 300 pg/mL to 600 ng/mL, were prepared by diluting the stock solution of DH with a mixture of 0.1 M hydrochloric acid and methanol (1:1). Similarly, working solutions of IS were prepared by diluting the stock solution of IS with a mixture of 0.1 M hydrochloric acid and methanol (1:1) to a concentration of 150 ng/mL.

#### 2.3.2. Preparation of calibration standards and standard samples

Calibration standards, format concentrations of 10 pg/mL to 20 ng/mL were prepared by addition of 20 μL of DH working solutions and 20 μL of IS working solutions to 600 μL of plasma, giving a final internal standard concentration of 5 ng/mL. Standard samples for recovery test were prepared by addition of DH and IS working solutions to 600 μL of a mixture of water and acetonitrile (7:3, v/v) in the same manner.

#### 2.3.3. Preparation of processed samples (injection samples)

Processed samples were prepared by diluting two times with 5 mM aqueous ammonium acetate solution of spiked samples and vortexed, then 400 μL each of processed samples was injected directly into the HPLC.

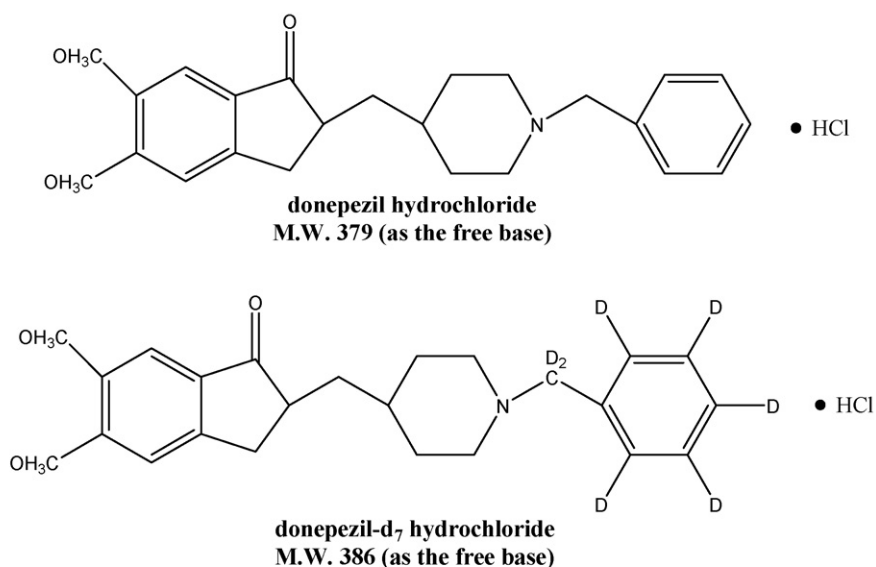


Fig. 1. Chemical structures of donepezil hydrochloride (DH) and donepezil-$d_7$ hydrochloride (IS).

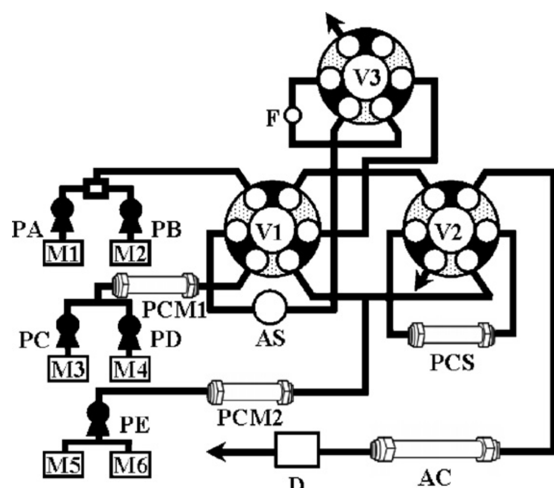


Fig. 2. Schematic diagram of co-sense® for BA/MS system. M1 and M2: mobile phase for analysis; M3 and M5: mobile phase for pretreatment; M4 and M6: mobile phase for washing; PA, PB, PC, PD, PE: pump; PCM1 and PCM2: column using the purification of mobile phase for pretreatment; AS: auto sampler; V1, V2, V3: six-port valve; F: line filter; PCS: column for pretreatment; AC: ODS column for separation; D: detector (mass spectrometer).

### 2.4. Chromatographic conditions and column-switching techniques

The basic instrumentation used in this study was composed of a biological sample analysis system, Prominence Co-Sense® for BA obtained from Shimadzu (Kyoto, Japan). A schematic diagram is shown in Fig. 2. This system was composed of three LC-20AD pumps (PC, PD and PE) for pretreatment, LC-20AB pump (PA and PB) for separation with gradient elution mode, DGU-20A$_5$ and DGU-20A$_3$ vacuum degassers, SIL-20AC auto sampler (AS), CTO-20AC column oven, CBM-20A system controller, LCMS2010 single stage quadrupole mass spectrometer (D), FCV-12AHi six-port valves (V1, V2), and FCV-12AH$_2$ six-port valve (V3). These components were used for the on-line pretreatment and analysis of biological samples. The pump (PE) was used for an on-line seven-fold dilution of biological samples with a mobile phase for pretreatment with the goal of recovering analytes from plasma [15]. The mobile phases M3 and M5 were used for the pretreatment, and M1 and M2 were used for the separation with a gradient elution, whereas M4 and M6 were used for system washing. The composition of each mobile phase is shown in Table 1.

Table 1
Composition of the mobile phase for pretreatment, separation, and system washing

| Mobile phase | Composition |
|---|---|
| M1, for separation | $H_2O:CH_3CN:TFA = 990:10:1$ (v/v/v) |
| M2, for separation | $CH_3CN:TFA = 1000:1$ (v/v) |
| M3, for pretreatment | $H_2O:CH_3CN:CH_3COONH_4 = 950:50:0.385$ (v/v/w) |
| M4, for system washing | $CH_3CN$ |
| M5, for pretreatment | $H_2O:CH_3CN:CH_3COONH_4 = 950:50:0.385$ (v/v/w) |
| M6, for system washing | $CH_3CN$ |

The analysis was performed using a methylcellulose-immobilized reversed-phase column for pretreatment, Shim-pack MAYI-ODS column (PCS) (30 mm × 4.6 mm i.d.; 50 μm, Shimadzu) [13] and Symmetry C18 column (AC) (100 mm × 2.1 mm i.d.; 3.5 μm, waters) for separation. Furthermore, two Shim-pack MAYI-ODS columns (PCM1, PCM2) were used for the purification of mobile phases M3 and M5.

Biological samples can be injected directly into this system which protects the lines and column from clogging with insolubles in biological samples using an in-line filter (F).

This system allows conduction of several column-switching techniques using six-port valves. The column-switching procedures are illustrated in Fig. 3. This column-switching system, composed of dual gradient elution (PA and PB for separation, PC and PD for pretreatment), was controlled by the time program as shown in Fig. 4.

First, the plasma samples including a target drug were injected by AS, and were transferred to the PCS using a M3 at a flow rate of 0.5 mL/min by PC and a M5 at a flow rate of 3.5 mL/min by a diluting pump (PE). Following removal of plasma proteins and other matrix components, target drugs were enriched on a PCS (Fig. 3a) via a solid-phase extraction mechanism. Target drugs enriched on a PCS were transferred in the backflush to the AC by switching V2 valves under the gradient elution mode of M1 and M2 at a flow rate 200 μL/min and separated at 35 °C then detected by MS (D) (Fig. 3b). During the separation and detection of the target drug, AS and valves (V1, V3) were washed by M3. Also, F was washed by M3 with backflushing (Fig. 3c). AS, PCM1, and valves (V1, V2) were washed by M4, and PCM2 and V2 valve were simultaneously washed by M6 (Fig. 3d).

### 2.5. Mass spectrometry

Analyses were conducted on a Shimadzu LCMS-2010 EV single-stage quadrupole mass spectrometer equipped with an electrospray ionization interface. Selected ion monitoring (SIM) was used for donepezil (DH; $m/z$ 380) and donepezil-$d_7$ (IS; $m/z$ 387). The prove voltage was set to 4.5 kV. The nebulizer gas flow was set to 1.5 L/min. Data processing was conducted using LCMS Solutions software.

## 3. Results and discussion

### 3.1. Elimination of impurities caused by the mobile phase for pretreatment

Reversed-phase (ODS) columns (e.g. Empore disk, 3 M) for removing impurities in mobile phase are commonly used for highly sensitive analysis. However, the off-line purification is considered a time-consuming step in the analytical process. On-line purification, such as ODS columns fitted directly on HPLC lines from pumps that provide the ability to trap impurities in the mobile phase, depends on column capacity. Therefore, on-line purification is inadequate for continual analysis. In this study, on-line elimination of impurities existing in mobile phase using HPLC equipment with a self-washing function was investigated.

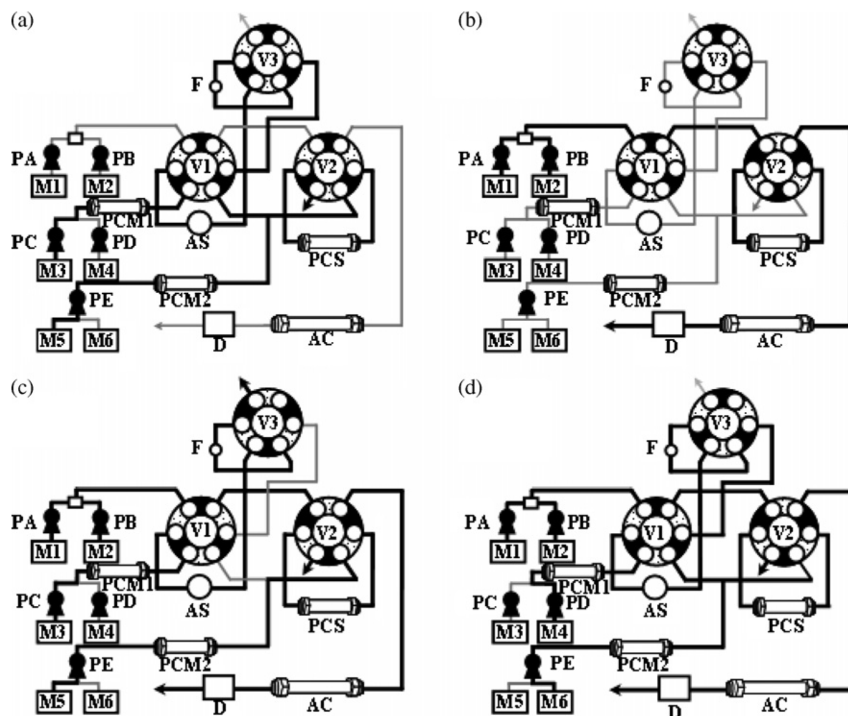


Fig. 3. Column-switching procedures of co-sense® for BA/MS system. (a) Trapping the analytes on the pretreatment column (PCS). (b) Transferring the analytes trapped to the analytical ODS column (AC) from PCS and separation with gradient elution mode through MS analysis. (c) Washing the system with water-rich solutions during analysis. (d) Washing the system with organic solvent-rich solutions during analysis.

The effect of ODS columns (PCM1 and PCM2 in Fig. 2) used for the purification of mobile phases for pretreatment on the elimination of the impurities in mobile phase was evaluated.

SIM chromatograms obtained from the HPLC/MS with and without ODS columns (PCM1 and PCM2 in Fig. 2) are shown in Fig. 5.

Without PCM1 and PCM2 columns, impurity peaks were observed at 17 and 25–30 min. Since the impurity peak in mobile phase for pretreatment eluted at 17 min and had similar eluting behavior to DH, it was challenging to separate each by changing the columns, mobile phases, and gradient conditions. Moreover, the increase in peak response was observed in proportion to the length of pretreatment time, namely the increase in total volume of the mobile phase for pretreatment through PCS. This suggests that these impurity peaks were caused by the mobile phase for pretreatment. Impurities in the mobile phase for pretreatment were enriched in PCS. To the contrary, no impurity peaks were observed in HPLC equipped with PCM1 and PCM2 columns, the system peak at 17 min was not detected, and peak responses around 25–30 min were found to be remarkably reduced. PCM1 and PCM2 columns are thought to retain impurities in the mobile phase for pretreatment, and reduce impurities that exist on-line as a result. Therefore, M4 and M6 for washing PCM1 and PCM2 were built into this system to conduct continual analyses. PCM1 and PCM2 were washed by the M4 and M6 each time, and impurities eluted from PCM1 and PCM2 were automatically drained off each time using the column-switching technique for evaluation of accurate carry-over and highly sensitive assay.

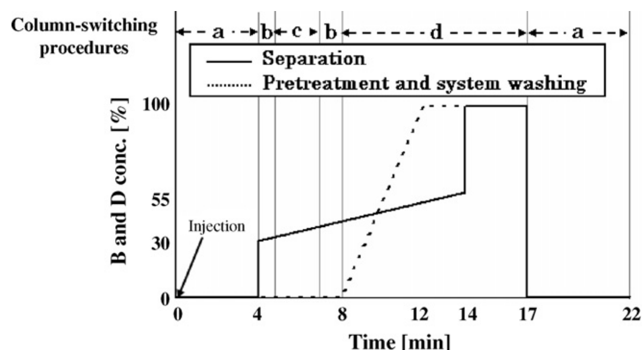


Fig. 4. Time programs of the gradient curve for separation, pretreatment, and system washing.

### 3.2. *Reduction of carry-over by on-line sample pretreatment HPLC*

Carry-over is a commonly encountered and persistent chromatographic problem that can compromise the precision and accuracy of HPLC/MS assays and, therefore, the reliability of analytical data. The risk of carry-over in the on-line sample pretreatment HPLC composed of column-switching valves and complex lines is commonly larger than that in conventional HPLC. The main difference between on-line sample pretreatment HPLC and conventional HPLC is the solvents (the mobile phase) used to transfer the sample from the sample loop in AS to the column. In conventional HPLC, the sample loop is flushed with a relatively good solvent (organic solvent rich), whereas in on-line sample pretreatment HPLC, the sample loop is flushed with a relatively poor solvent (organic solvent poor) in order to

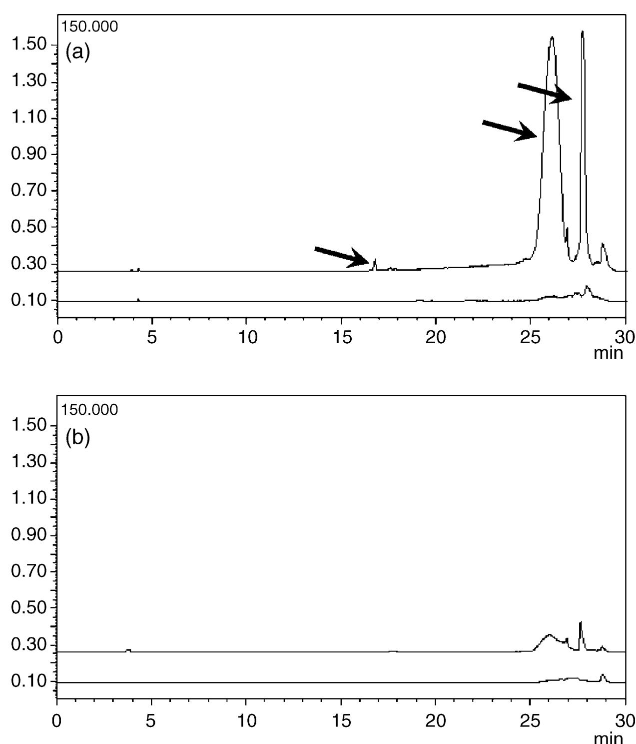


Fig. 5. Effect of removing impurities in mobile phase for pretreatment with MAYI-ODS columns (PCM1, PCM2) on the SIM chromatograms of mobile phase injection. (a) Without MAYI-ODS columns. (b) With MAYI-ODS columns. Upper: *m/z* 380 for donepezil hydrochloride (DH). Lower: *m/z* 387 for donepezil-$d_7$ hydrochloride (IS). Chromatographic conditions were same as Section 2.4 except for analytical column, Cadenza CD-C18 (AC) (75 mm × 2.0 mm i.d.; 3 μm, Imtakt), and the following gradient conditions: B. Conc. 0% (0–5 min); B. Conc. 30% (5.01 min); B. Conc. 60% (20 min); B. Conc. 100% (20.01 min); B. Conc. 100% (25 min); B. Conc. 0% (25.01–30 min).

retain the analytes on top of the pretreatment column. Therefore, in on-line sample pretreatment HPLC, a compromise must be made between carry-over and extraction recovery. If carry-over is not prevented, washing of sample loop and switching valves by the proper solvents is indispensable.

### 3.2.1. Initial assessment of carry-over

Significant carry-over was observed that corresponded to approximately 2.6% of the ULOQ in the absence of washing of sample loop and switching valves in on-line sample pretreatment HPLC using only the mobile phase for the pretreatment ($H_2O$:$CH_3CN$:$CH_3COONH_4$ = 950:50:0.385 (v/v/w)). A control plasma blank containing IS was injected immediately after the upper limit of quantitation plasma standard (ULOQ = 20 ng/mL). The calculated carry-over was obtained from the following equation: carry-over (%) = (peak area of plasma blank/peak area of previous, ULOQ sample) × 100.

### 3.2.2. Mechanism of carry-over

Wash solvent for sample loop and switching valves was investigated. Effect of acetonitrile (organic solvent) from 5% to 80% in wash solvent is shown in Fig. 6. Acetonitrile percent in wash solvent was found to affect the level of carry-over. This result showed that carry-over of DH in this HPLC system was based on a hydrophobic interaction mechanism. A higher percent organic solvent resulted in a better washing.

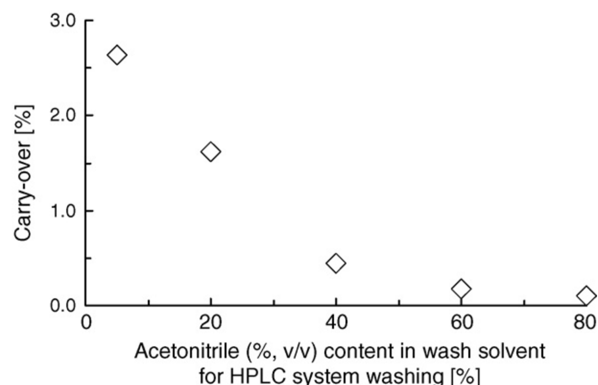


Fig. 6. Effect of acetonitrile in wash solvent on carry-over.

### 3.2.3. Effect of on-line sample pretreatment HPLC with automated system washing procedure on carry-over

Carry-over of DH in the on-line sample pretreatment HPLC system was based on a hydrophobic interaction mechanism. Therefore, acetonitrile was employed for wash solvent and the system was washed under the gradient mode of acetonitrile (M4) and a mobile phase for pretreatment (M3) using the gradient conditions as shown in Fig. 4.

Effect of the on-line sample pretreatment HPLC equipped with automated system washing procedure (i.e. the flow system of multi-mobile phases) on carry-over was evaluated in three cases (A–C) of experiment in preparing calibration curves ranging from 10 pg/mL (LLOQ) to 20 ng/mL (ULOQ) of DH in dog plasma. In experiment A, each calibration standard (0.01, 0.02, 0.2, 2, 10, and 20 ng/mL) was run sequentially from low to high concentrations with washing procedure (Fig. 3c and 3d) according to previous chromatographic conditions described in Section 2.4, assuming this case to have no influence of carry-over. In experiment B, the same series of standards were run in low samples following ULOQ (20 ng/mL) standard without washing procedure. In experiment C, the calibration standards were run in low samples following ULOQ standard with washing procedure (Fig. 3c and d). Effect of washing procedure on carry-over in on-line sample pretreatment HPLC system is shown in Table 2.

In experiment A, the correlation coefficient ($r$) was 0.9996 for a low-to-high sequence. The equation of the calibration plot was $y = 0.2233x + 0.0018$. In experiment B, there was poor correlation ($y = 0.2397x + 0.1269$, $r = 0.9680$). In particular, the $y$-intercept value was larger than the experiment A value obtained by a low-to-high sequence, and bias from experiment A results was at an unacceptable level of carry-over at a low concentration. To the contrary, carry-over was reduced by washing procedure in experiment C, and parameters of calibration curve ($y = 0.2262x + 0.0025$, $r = 0.9995$) were equivalent to results in experiment A. Moreover, bias from experiment A results obtained by a low-to-high sequence decreased to within ±15% at every concentration except for the lower limit of quantitation (LLOQ), where bias was within ±20%. The extent of

Table 2
Effect of washing on carry-over in three cases of experiments

| Concentration (ng/mL) | Experiment A | Experiment B | | Experiment C | |
|---|---|---|---|---|---|
| | $Ratio_A$ | $Ratio_B$ | Bias (%) | $Ratio_C$ | Bias (%) |
| 0.01 | 0.00401 | 0.12873 | 3110.2 | 0.00480 | 19.7 |
| 0.02 | 0.00633 | 0.13376 | 2013.1 | 0.00703 | 11.1 |
| 0.2 | 0.04810 | 0.17788 | 269.8 | 0.04973 | 3.4 |
| 2 | 0.43255 | 0.57363 | 32.6 | 0.46254 | 6.9 |
| 10 | 2.19106 | 2.29057 | 4.5 | 2.16716 | −1.1 |
| 20 | 4.51956 | 4.36529 | −3.4 | 4.47758 | −0.9 |
| Slope (*a*) | 0.2233 | 0.2397 | | 0.2262 | |
| *y*-Intercept (*b*) | 0.0018 | 0.1269 | | 0.0025 | |
| Correlation coefficient (*r*) | 0.9996 | 0.9680 | | 0.9995 | |

Bias (%) = ($ratio_{B\ or\ C}$ − $ratio_A$)/$ratio_A$ × 100. Experiment A: injection of calibration standards in a low-to-high sequence with washing procedure. Experiment B: injection of calibration standards after the upper limit of quantitation plasma standard (ULOQ = 20 ng/mL) without washing procedure. Experiment C: injection of calibration standards after the upper limit of quantitation plasma standard (ULOQ = 20 ng/mL) without washing procedure.

carry-over was below the level generally considered acceptable for bioanalytical assays. Hence, the on-line sample pretreatment HPLC/MS system could be developed to reduce carry-over.

### 3.3. Direct analysis of donepezil in dog plasma and method validation

The highly sensitive direct analysis of donepezil in dog plasma was performed with automated system washing function (multi-mobile phase flow system) using Co-Sense® for BA/MS system (Fig. 2). Method validation was conducted to ensure the reliability of this system, specificity, linearity, accuracy, precision, lower limit of quantitation (LLOQ), and carry-over as validation characteristics.

#### 3.3.1. Specificity

Typical SIM chromatograms for blank plasma and plasma spiked with donepezil (DH) are shown in Fig. 7. No system peaks were observed at the retention times of DH and IS; both were clearly separated from the endogenous plasma components.

#### 3.3.2. Linearity

Calibration standards were run sequentially from low to high concentrations. Peak area ratios of DH to IS were linear over

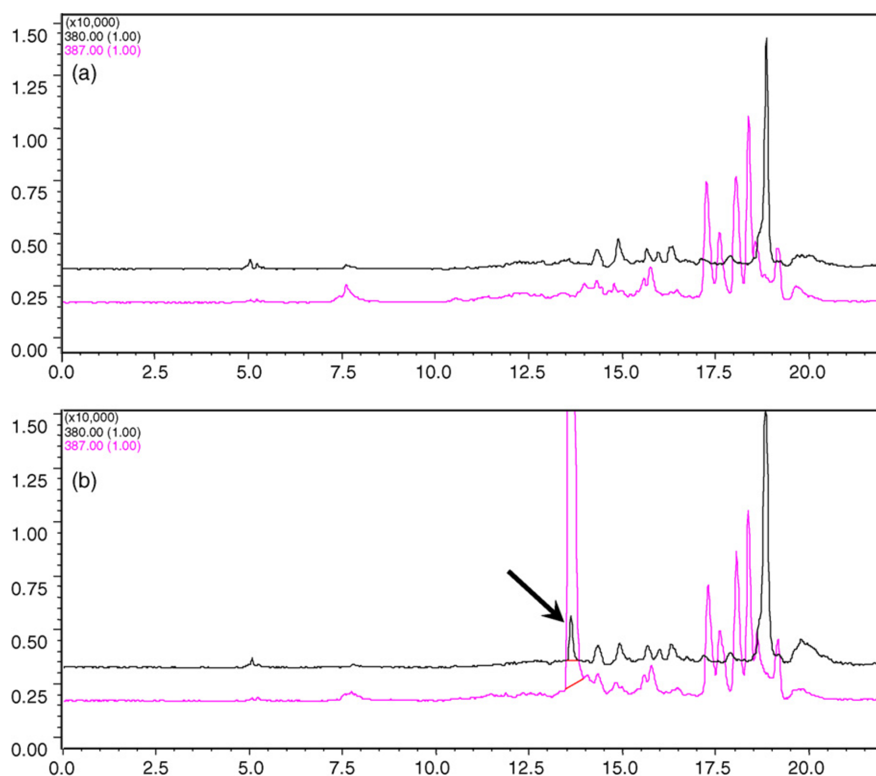


Fig. 7. Typical SIM chromatograms. (a) Blank plasma; (b) plasma spiked with DH (concentration: 10 pg/mL). Upper: *m/z* 380 for DH. Lower: *m/z* 387 for IS. Chromatographic conditions were the same as in Section 2.4.

Table 3
Absolute recoveries of DH from dog plasma

| Concentration added (ng/mL) | Recovery (%) | | | | | | Mean | S.D. |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | | |
| 0.02 | 100.5 | 101.8 | 101.7 | 98.0 | 103.2 | 100.0 | 100.9 | 1.8 |
| 1 | 103.4 | 94.9 | 99.1 | 102.4 | 103.4 | 90.4 | 98.9 | 5.3 |
| 20 | 92.0 | 96.5 | 98.0 | 96.9 | 100.3 | 98.1 | 97.0 | 2.8 |

Table 4
Intra-assay accuracy and precision of DH in dog plasma

| Concentration added (ng/mL) | Concentration found (ng/mL) | | | | | | Mean | S.D. | CV (%) | Accuracy (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | | | | |
| 0.01 | 0.0088 | 0.0100 | 0.0095 | 0.0108 | 0.0111 | 0.0099 | 0.0100 | 0.0008 | 8.4 | 0.2 |
| 0.02 | 0.0201 | 0.0205 | 0.0204 | 0.0193 | 0.0209 | 0.0199 | 0.0202 | 0.0006 | 2.7 | 0.9 |
| 1 | 1.0554 | 0.9675 | 1.0103 | 1.0452 | 1.0546 | 0.9209 | 1.0090 | 0.0548 | 5.4 | 0.9 |
| 20 | 18.9240 | 19.8460 | 20.1557 | 19.9413 | 20.6408 | 20.1813 | 19.9482 | 0.5721 | 2.9 | −0.3 |

Table 5
Inter-assay accuracy and precision of DH in dog plasma

| Concentration added (ng/mL) | Concentration found (ng/mL) | | | Mean | S.D. | CV (%) | Accuracy (%) |
|---|---|---|---|---|---|---|---|
| | First day | Second day | Third day | | | | |
| 0.01 | 0.0091 | 0.0097 | 0.0114 | 0.0101 | 0.0012 | 11.9 | 0.7 |
| 0.02 | 0.0198 | 0.0192 | 0.0193 | 0.0194 | 0.0003 | 1.7 | −2.8 |
| 1 | 0.9804 | 0.9918 | 1.0157 | 0.9960 | 0.0180 | 1.8 | −0.4 |
| 20 | 20.0425 | 19.8286 | 19.7448 | 19.8720 | 0.1535 | 0.8 | −0.6 |

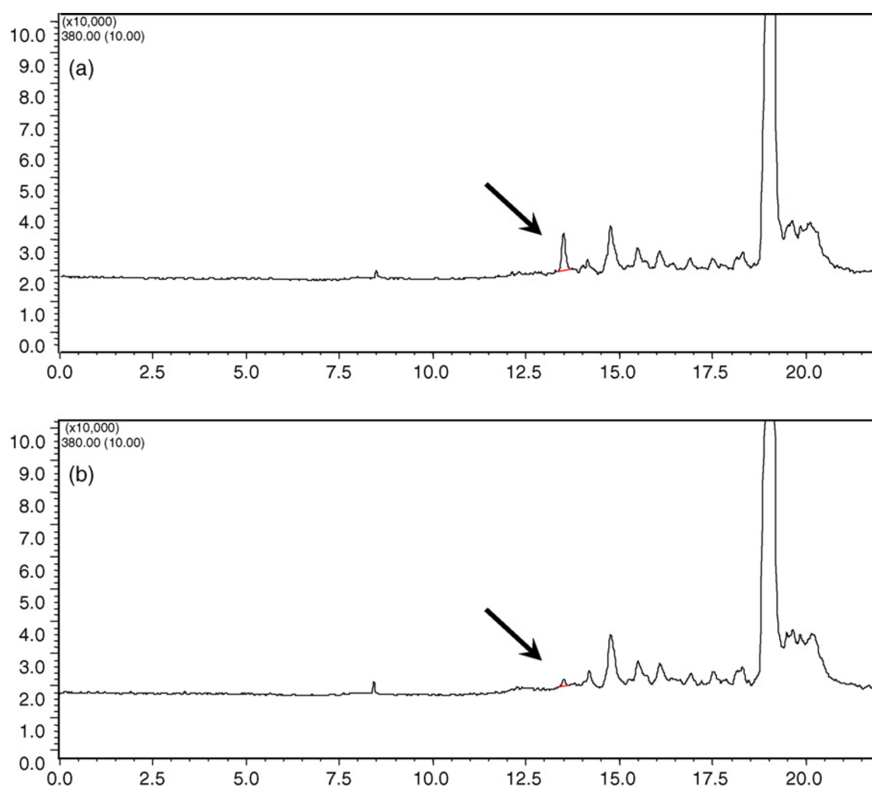


Fig. 8. SIM chromatograms ($m/z$ 380). (a) Plasma standard containing 10 pg/mL DH and (b) blank plasma injected immediately after a plasma standard containing 20 ng/mL DH (ULOQ). Chromatographic conditions were the same as Section 2.4.

the range of 10 pg/mL to 20 ng/mL. Correlation coefficients ($r$) were above 0.999 (experiment A, Table 2).

#### 3.3.3. Absolute recovery

The absolute recoveries of DH from plasma were evaluated at three concentration levels (20 pg/mL, 1 ng/mL, and 20 ng/mL) with their standard samples in six replicates. As shown in Table 3, the absolute recoveries were between 90.4% and 103.4%.

#### 3.3.4. Intra-assay accuracy and precision

The accuracy and precision of intra-assay variability were assayed at four concentration levels (10 pg/mL, 20 pg/mL, 1 ng/mL, and 20 ng/mL) in six replicates. As shown in Table 4, the accuracy and precision of intra-assay variation were −0.3% to 0.9% and 2.9%–8.4%, respectively.

#### 3.3.5. Inter-assay accuracy and precision

The accuracy and precision of inter-assay variability were assayed on three days at four concentration levels (10 pg/mL, 20 pg/mL, 1 ng/mL, and 20 ng/mL). As shown in Table 5, the accuracy and precision of inter-assay variation were −2.8% to 0.7% and 0.8% to 11.9%, respectively.

#### 3.3.6. Lower limit of quantitation (LLOQ) and limit of detection (LOD)

The sensitivity of the method was evaluated by determining the lower limit of quantitation (LLOQ) and limit of detection (LOD). LOD was defined as the concentration at a signal-to-noise ratio of 3, while LLOQ was defined as the concentration at a signal-to-noise ratio of 10. The chromatogram of LLOQ (10 pg/mL) is shown in Fig. 7b. The signal-to-noise ratio was approximately 10, while LOD was approximately 1 pg/mL at signal-to-noise ratio of 3.

#### 3.3.7. Carry-over

An acceptable level of carry-over (about 0.01%) was observed when blank plasma was injected immediately following ULOQ (20 ng/mL) plasma standard. This carry-over peak in the blank plasma was approximately below 20% the 10 pg/mL plasma LLOQ standard, as shown in Fig. 8.

These validation results indicate that the on-line sample pretreatment HPLC/MS system established in this study enabled reduction of carry-over and facilitated performance of a highly sensitive, accurate, and precise analysis.

## 4. Conclusions

A case study in reducing carry-over in an on-line sample pretreatment HPLC, equipped with a RAM column and column-switching HPLC/MS system, was described. The level of carry-over in an on-line sample pretreatment HPLC was unacceptable if it compromised the reliability data, reproducibility, LLOQ, and dynamic linear range. Therefore, a new direct injection HPLC/MS system with a washing function of an HPLC system was established by equipping it with the flow system of multi-mobile phase. This system allowed not only for reduction of carry-over based on a hydrophobic interaction mechanism by the flow system of multi-mobile phase, but also removal of impurities existing in mobile phase for pretreatment. As a result, this system could determine DH in plasma as low as 10 pg/mL. Evaluation results of specificity, linearity, accuracy, precision, limit of detection, limit of quantitation, and carry-over as analytical characteristics for method validation demonstrated that this system is useful and acceptable as a practical method that is highly sensitive, accurate, and precise. Therefore, it is expected that this on-line sample pretreatment HPLC/MS system will contribute to rapid, highly sensitive, and continual analysis of biological samples. For further application of this automatic washing HPLC system to the hydrophobic compounds, the use of highly hydrophobic solvents (e.g. 2-propanol, tetrahydrofuran) as a washing material is trying to reduce carry-over.

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