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A new generic column switching system for quantitation in cassette dosing using LC/MS/MS

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Abstract

Cassette dosing is a method in which multiple drugs are administered to a single animal at the same time, and the plasma concentrations of the individual compounds are simultaneously determined. This method enables high-throughput rapid screening for pharmacokinetic assessment of new drug candidates. An available gradient method was modified for cassette dosing analysis to attain the advantages of high sensitivity and applicability to a wide range of compounds. However, two problems arose; (1) the time-consuming optimization of mobile phases for each compound group, which limited applicability and (2) the remarkable suppression of ionization by polyethyleneglycol, which is commonly used in intravenous administration. To resolve these problems, a new column switching method was established to attain wider applicability and avoid the ionization suppression. This column switching system is very simple because the trap column and the analytical column are specified and the mobile phase is selected from only two species. Method optimization requires only the selection of the mobile phase and takes only a few hours. About 200 compounds, which were administered as about 50 cassettes, were analyzed using this column switching system. Assay validation of one cassette was carried out, and good accuracy and precision were obtained. About 90% of the compounds could be determined within 20% bias. These results showed that this new column switching system for cassette dosing is accurate enough for the screening of drug candidates and offers wide applicability for various compounds. This system was shown to be very useful for the determination of cassette dosing samples, containing multiple compounds.

Keywords: Cassette dosing; Generic column switching LC/MS/MS; Effect of vehicle

1. Introduction

In recent years, the number of synthesized compounds has remarkably increased with the advance of synthesis methods such as combinatorial chemistry. Active compounds are identified in

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vitro, and this serves as the first screening process. However, in vivo evaluation of the efficacy and medicinal actions of all synthesized compounds, which are most important in new drug development, is too time-consuming to be feasible. Thus, active compounds need to be screened and classified before evaluation for efficacy in vivo.

Compounds can be characterized by the physical aspects of solubility and permeability evaluated using Caco-2 cells, the pharmacokinetic parameters of total clearance and bioavailability, and cytochrome P-450 (CYP) enzyme inhibition. Rapid evaluation systems are needed but are difficult to establish for pharmacokinetic characterization, which can only be conducted in vivo.

To speed up pharmacokinetic evaluation in vivo, Berman et al. proposed the cassette dosing method [1]. In this method, a drug cocktail containing multiple drugs is administered to a single animal for simultaneous pharmacokinetic evaluation of multiple compounds. The greatest advantage of this method is acceleration of the pharmacokinetic evaluation in proportion to the number of compounds simultaneously administered. Also, individual differences, which have been a great problem in pharmacokinetic screening, are negligible, and the number of animals used can be greatly reduced [1–5].

One disadvantage of cassette dosing is drug–drug interaction. However, this can be minimized in three ways: (1) by keeping the concentration of drugs in plasma in the nM range using lower dosages, (2) by excluding compounds with strong inhibition ability against the main CYPs and (3) by reducing the number of drugs in a cassette [6].

Even if drug–drug interaction occurs when using cassette dosing, it is likely that the concentrations of drugs in plasma will be higher than that for the case of no interactions and thus the results give an underestimation of total clearance or an overestimate of the area under curve. However, these 'false positive' results offer an overestimation of pharmacokinetic characteristics and are not actually a disadvantage in the early stage of pharmacokinetics screening.

In cassette dosing screening, the most difficult and time-consuming step is establishing the analytical method. Since the drugs administered to a single animal should be determined simultaneously to maximize the evaluation speed, a generic method applicable to various compounds is urgently needed. High sensitivity for lower dosage and short analysis time for high-throughput is also required. However, no generic method for cassette dosing satisfying these requirements has been reported.

The objective of this study is to establish an analytical method for cassette dosing which:

1) Possesses wide applicability to minimize the time for optimization.
2) Avoids matrix effects from the administration vehicles and/or biofluids.
3) Is very robust offering constant sensitivity to eliminate the need for internal standards.
4) Is very sensitive for low dosages.
5) Involves simple preparation of samples such as precipitation by organic solvent.

2. Experimental

2.1. Chemicals and reagents

All compounds analyzed in this paper were synthesized at Discovery Research Laboratories. Distilled water for HPLC, acetic acid (AcOH), ammonium bicarbonate (NH_4HCO_3), polyethyleneglycol 400 (PEG) and *N*,*N*-dimethylacetamide (DMAA) were purchased from Nacalai Tesque (Kyoto, Japan). Dimethylsulfoxide (DMSO) and polypropyleneglycol (PG) were purchased from Wako Pure Chemicals (Osaka, Japan) and methanol from Sigma-Aldrich Japan (Tokyo, Japan). Acetonitrile (CH_3CN) was purchased form Kanto Chemical Co., Inc. (Tokyo, Japan). All purchased reagents were of analytical grade.

2.2. Animals

Male Jcl:SD rats or female Crj:BALB/cA mice were used. All cassettes were administered intravenously at 0.5 mg/1.2 ml/kg, and orally at 1 mg/5 ml/kg. The intravenous administration vehicle was DMAA/PEG = 1/4, DMAA/PG = 1/1 or DMAA/

PG = 1/2. The oral administration vehicle was 0.5% methylcellulose suspension.

2.3. Equipment and analytical conditions

The HPLC system used in the modified gradient method was a 2690 separation module (Waters, USA). The HPLC system used in the column switching method consisted of the Agilent 1100 series HPLC system (binary pump, autosampler, on-line degasser and column compartment) and an additional isocratic pump (Agilent Technologies, USA). The column switching valve was put in the column compartment and the entire HPLC system was controlled by Agilent Chemistation software.

An Oasis® HLB Extraction Column (1.0 mm i.d. × 50 mm, Waters) was used in the modified gradient method. For the column switching system, the trap column used was XTerra™ MS C18 (2.1 mm i.d. × 10 mm, Waters) and the analytical column used was CAPCELL PAK C18 MG (2.0 mm i.d. × 35 mm, Shiseido, Japan). Some of the compounds were analyzed only with the trap column because of the poor shape of the peaks with the analytical column. The mobile phase was selected from two types in both methods, an acidic type of 0.1% AcOH/CH_3CN and a weak basic one of 10 mM NH_4HCO_3/CH_3CN. Elution from the trap column was conducted with 0.1% AcOH/CH_3CN = 20/80 (v/v) or 10 mM NH_4HCO_3/CH_3CN (v/v) = 20/80, which was selected according to the mobile phase employed above, at a flow rate of 0.2 ml/min using an isocratic pump.

A TSQ7000 (API2) mass spectrometer (ThermoQuest, USA) was used to detect analytes, being operated in the selected reaction monitoring (SRM) mode. Electrospray ionization was employed and the heated capillary temperature was set at 350 °C for all compounds. The sheath gas pressure and the auxiliary gas flow were set at 80 psi and 10 arbitrary units.

Monitored ions for compound A–E are listed below as 'precursor ion–production @collision energy'. Monitored ions of compound C were used to obtain a lower baseline in each method. The scan time was set at 90 ms for each channel.

Compound A: m/z 475–m/z 312 @30 eV
Compound B: m/z 402–m/z 235 @30 eV
Compound C: m/z 523–m/z 360 @25 eV (modified gradient method), m/z 523–m/z 312 @30 eV (column switching method)
Compound D: m/z 433–m/z 270 @40 eV
Compound E: m/z 499–m/z 288 @40 eV

2.4. Preparation of stock and standard solutions

All analytes were dissolved in DMSO at the concentration of 1 mg/ml (stock solution). Standard solutions were prepared by dilution of the stock solution with methanol.

2.5. Preparation of standard samples and QC samples

Standard samples were prepared by spiking 20 μl of standard solution at each concentration into 20 μl of control plasma, followed by precipitation with 180 μl of methanol. Precipitation was conducted under 14 000 g for 1 min, and the supernatant was subjected to analysis.

QC samples were prepared in the three ways described below. QC-A was prepared by the same method as the standard samples. QC-A is measured for the guarantee of the constant sensitivity of the mass spectrometer during the analysis. QC-B samples were prepared by spiking 5 μl of standard solution into 120 μl of control plasma, followed by vortex mixing for about 10 s. A 20-μl portion of this was transferred into another tube and precipitated with 200 μl of methanol. The precipitation procedures were the same as for the QC-A samples. The ratio of QC-B to QC-A gives the recovery for the precipitation step. QC-C samples, in almost the same manner as the QC-B samples, were prepared using control plasma containing 4% vehicle of intravenous administration instead of intact control plasma. The ratio of QC-C to QC-B gives the effect of vehicles on the ionization of analytes.

The methanol volume for precipitation was changed to 980 and 1000 μl for the standard and QC (QC-B and QC-C) samples, respectively, for the analysis of high concentration samples.

2.6. Preparation of plasma samples obtained from animals

A 20-μl plasma sample was precipitated with 200 μl of methanol. Precipitation procedures were the same as for the standard and QC samples. The methanol volume for precipitation was increased to 1000 μl in the analysis of high concentration compounds for dilution.

3. Results

3.1. Development of a high-speed gradient method and the issues involved

A rapid gradient method was developed using Oasis® HLB Extraction Column. This column removes proteins and macromolecular substances included in plasma according to molecular size, while retaining small molecular substances such as drugs. A rapid analytical method for a drug using the combination of this column together with LC/MS/MS was described by Ayrton et al. [7]. However, two problems were found when this method was applied to cassette dosing. The first was the high flow rate of 4 ml/min employed to reduce the run time. Only 1/10 of the mobile phase could be introduced into the ESI interface by the splitter because of the flow rate limitation of the interface, which causes low sensitivity of the system in some cases. The second problem was the use of internal standard. In the assay of cassette dosing, it is quite difficult to find an internal standard suitable for all compounds in a cassette. To solve these problems, the rapid gradient method of Ayrton was modified for cassette dosing analysis.

The equipment overview and the profile of the modified gradient elution are shown in Fig. 1. The flow rate for the elution step is reduced to introduce the entire mobile phase into the ESI interface without splitting. Hundreds of compounds were evaluated for their pharmacokinetics using cassette dosing coupled with this modified rapid gradient method. Here we describe in detail the administration of five compounds in one cassette, which allowed identification of the one of the weak points of this analytical method. Typical chromatograms of these five compounds, A–E, are shown in Fig. 2. All compounds gave well-shaped peaks and wide range linearity was obtained for each compound without internal standards, as shown in Table 1.

However, as shown in Fig. 3, the peak area of compound A was significantly reduced when compared the peak areas obtained from rat plasma containing 4% of PEG with those from intact rat plasma. Sample preparation was the same in both cases. To specify the cause of the peak area reduction, the LC/MS spectrum was obtained at the retention time of compound A, resulting in the detection of PEG peaks (Fig. 4). Thus, the cause of the peak area reduction is considered to be the suppression of ionization by PEG eluting together with compound A. Variations of the gradient program of this method were tried to avoid the effect of PEG, but without success. A separate experiment showed that the ionization suppression of compound A was not due to co-elution with compounds B through E in the chromatography.

On the other hand, this method was found to require optimization of mobile phases for each compound group, therefore, the applicability was limited within each group.

In sum, advantages of this method were high sensitivity and a simple system while its disadvantages were its limited applicability and the effect of ionization suppression by PEG.

3.2. Development of a new column switching method

Plasma samples obtained by cassette dosing contain multiple drugs, generally 5–10, with all being targets of determination. Therefore, the analytical method for cassette dosing must have wide-range applicability to various compounds without time-consuming method optimization. This is the most important feature of the method of cassette dosing.

To satisfy such requirements as wide applicability, avoidability of matrix effect, high robustness and high sensitivity, a new column switching system was established. The overview and time program of this system are shown in Fig. 5. The

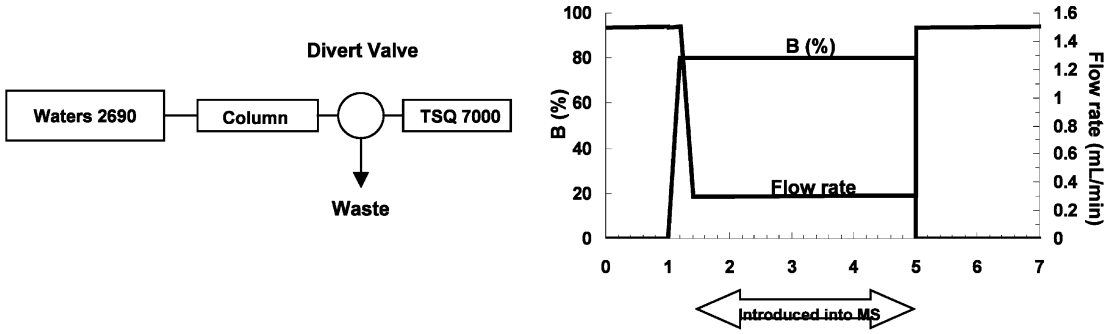

Fig. 1. Equipment overview and gradient time program of the modified rapid gradient method.

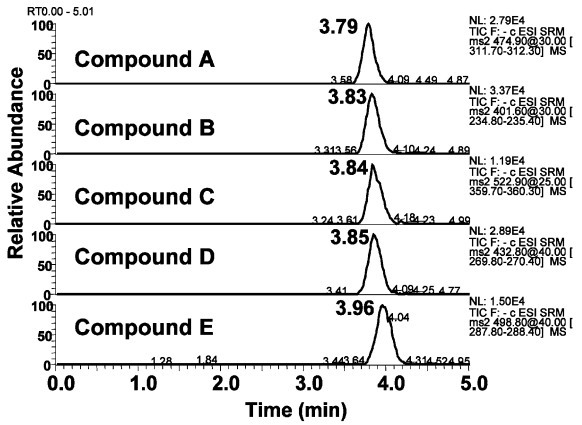

Fig. 2. Typical SRM chromatograms of five compounds obtained by the modified rapid gradient method.

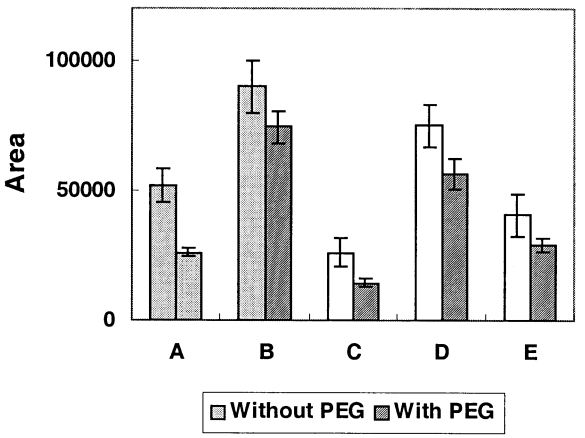

Fig. 3. Effect of vehicle on the peak areas of five compounds in using the modified rapid gradient method.

Table 1
Calibration parameters of A–E obtained by the modified gradient method

Compound	Equation	r^2
A	$Y = 9819.24 + 1309.00X$	0.9965
B	$Y = 2380.42 + 1859.48X$	0.9969
C	$Y = 1117.98 + 623.515X$	0.9959
D	$Y = 4223.62 + 1594.04X$	0.9974
E	$Y = 3977.55 + 971.825X$	0.9987

X: Concentration (ng/ml), Y: Peak area.

mobile phase is one of two types: an acidic type consisting of 0.1% AcOH in water and CH_3CN, or a weak basic type consisting of 10 mM NH_4HCO_3 and CH_3CN. Both the trap column and the analytical column are specified.

The mobile phase selection, which can be completed in few hours, is conducted by comparing the sensitivity and the peak shape obtained from the two types. Sample preparation is the same as that for the modified gradient method presented above.

The effect of PEG on this method was estimated. The peak area of compound A is remarkably reduced by co-eluting PEG in the modified gradient method, but no effect of PEG is observed on detection of compound A in the column switching system, as indicated in Fig. 6. Close inspection of the mass spectrum at the retention time of compound A revealed no PEG peaks. These results demonstrated that the vehicle effect by PEG could be removed, which is a disadvantage of the modified gradient method.

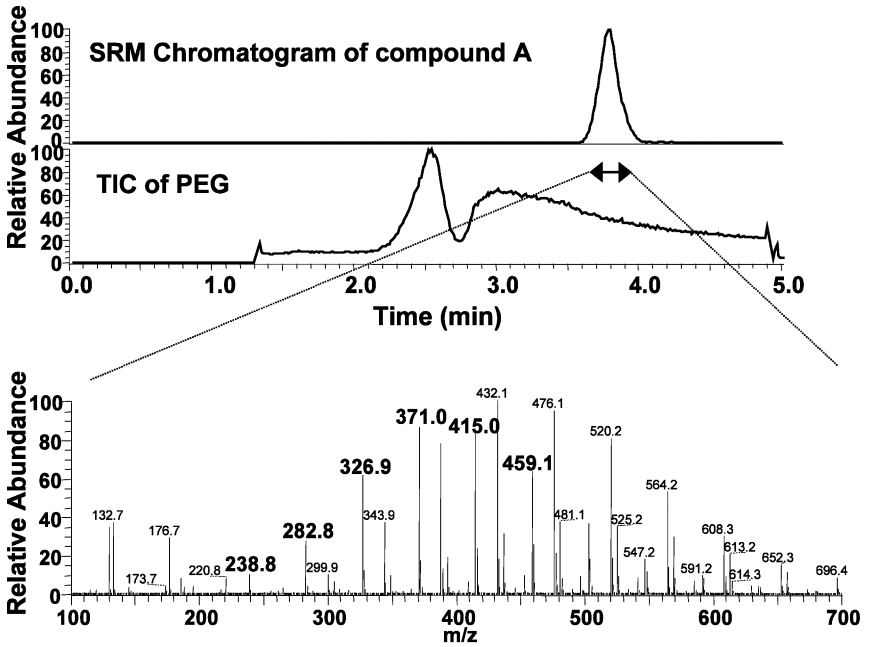

Fig. 4. Elution profile of PEG by the modified rapid gradient method. Top the total ion chromatogram of PEG (without compound A), and bottom, SRM chromatogram of compound A (without PEG). These chromatograms were obtained independently. The mass spectrum was obtained around the retention time of compound A.

Compounds A–E were examined using the column switching method. As all were acidic compounds, the acidic mobile phase was selected. Typical SRM chromatograms of compound A–E are shown in Fig. 7.

The eluted peaks were wide enough to conduct simultaneous determination of five compounds without remarkable decrease in sensitivity or sampling points.

The calibration curve parameters and intra-assay validation results are shown in Table 2 and Table 3, respectively. These results indicated good linearity, good reproducibility and validity of this method for these compounds.

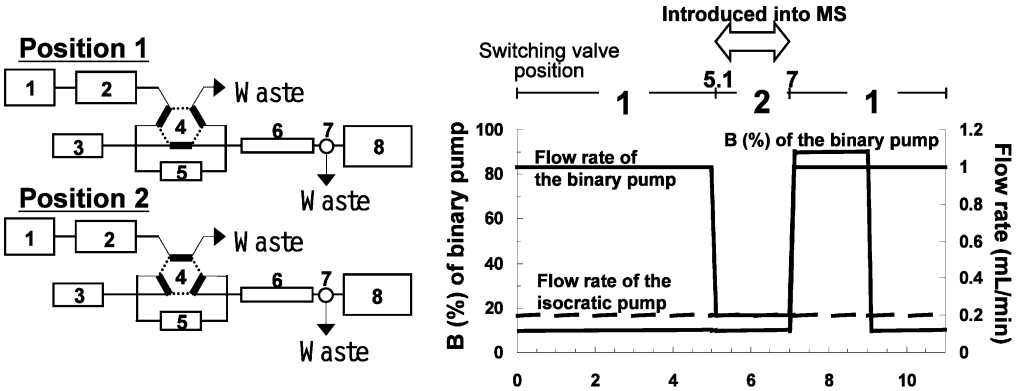

Fig. 5. Equipment overview and time programs of new column switching system. (1) Gradient pump, (2) autosampler, (3) isocratic pump, (4) column switching valve, (5) trap column, (6) analytical column, (7) divert valve, (8) mass spectrometer.

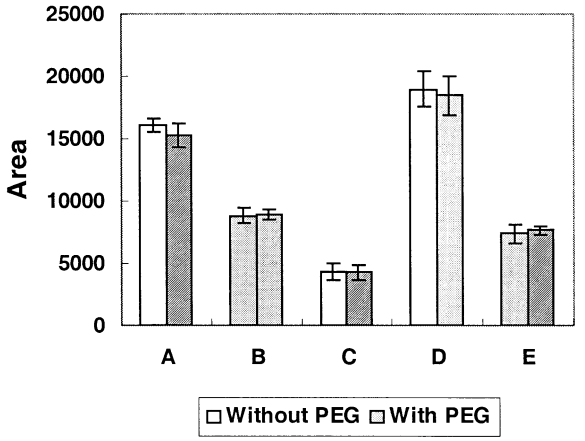

Fig. 6. Effect of vehicle on the peak areas of five compounds in using the column switching method.

Next, the applicability of this column switching system to various types of compounds was examined. More than 200 compounds, administered by cassette dosing in 52 cassettes, were determined using this system.

The compounds simultaneously administered were combined into groups having the same skeletal structure, but not with overlapping molecular weights. The number of compounds in one cassette was from 1 to 7. A separate examination

Table 2
Calibration parameters of A–E obtained by the column switching method

Compound	Equation	r^2
A	$Y = 996.665 + 110.341X$	0.9937
B	$Y = -197.263 + 379.041X$	0.9963
C	$Y = 350.868 + 69.895X$	0.9901
D	$Y = 232.138 + 178.857X$	0.9945
E	$Y = 175.523 + 123.476X$	0.9960

X: Concentration (ng/ml), Y: Peak area.

indicated that the ionization suppression ability of PG is weaker than that of PEG. Therefore, these compounds were all administered intravenously using PG/DMAA as a vehicle to verify the wide applicability of this method with minimization or avoidance of the vehicle effect.

The LOQ is lower than 10 ng/ml in plasma for most compounds, which is sensitive enough to evaluate the total clearance and bioavailability. As the validation study for each cassette could not be carried out, the reliability of each assay was assessed by the accuracy of the QC samples at one concentration level, which was determined together with plasma samples from the animals. The reliability of the assays was evaluated for more than 200 compounds, and the accuracy of

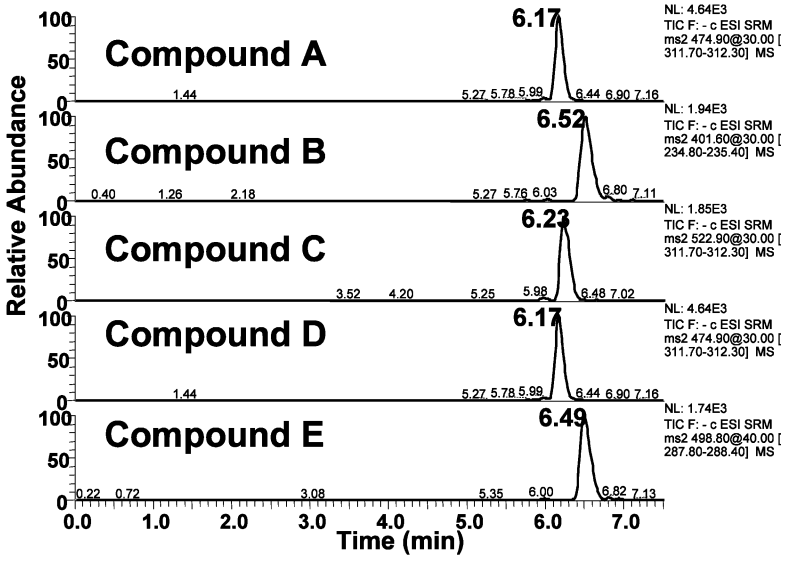

Fig. 7. Typical SRM chromatograms of five compounds obtained by the column switching method.

Table 3
Intra-assay validation results of determination of A–E by the column switching method

Compounds	40 ng/ml		800 ng/ml		8000 ng/ml	
	Bias (%)	RSD (%)	Bias (%)	RSD (%)	Bias (%)	RSD (%)
A	−5.2	7.8	13.6	8.4	3.2	8.5
B	7.4	5.4	8.5	6.3	1.6	7.3
C	11.0	11.2	10.2	7.8	−1.4	7.0
D	11.5	4.1	12.3	5.2	3.9	7.4
E	−0.4	8.7	5.5	4.8	1.0	3.4

over 90% of all compounds was within the range of −20 to +20%, suggesting that this method should be reliable enough for the screening at the early stage of drug discovery.

Recovery of the precipitation, which is used to prepare samples in this method, ranged from 80 to 120% for 99% of all compounds, indicating good recovery. The effect of vehicles for intravenous injection was also evaluated by the difference between the values obtained from QC samples with (QC-C) or without (QC-B) vehicles whose concentration spiked in plasma was 4% (v/v). Only few compounds deviated of the range from 80 to 120% of the spiked amount, indicating that no effects of vehicles were observed for almost all compounds.

The advantages and disadvantages of both methods are summarized in Table 4.

Table 4
Comparison of the characteristics of two methods

	Modified gradient method	Column switching method
Applicability	Limited	Wide
Matrix effect	Not avoidable	Avoidable
Robustness	High	High
Sensitivity	High	High
Sample preparation	Precipitation	Precipitation
Entire analysis time[a]	Long	Short

[a] Including the time for method development.

4. Discussion

4.1. Applicability to a wide range of c log P compounds

The distribution of *c* log *P* values of 202 compounds used to verify the reliability of this method, presented in Fig. 8, shows that these compounds possess a wide range of hydrophobicity. Thus, this column switching method should be a very useful generic method with applicability to diverse compounds possessing various levels of hydrophobicity. Compounds with *c* log *P* values smaller than two could be determined more accurately using formic acid rather than AcOH as the mobile phase, however, the results obtained using formic acid were accepted here. Attention must be paid to compounds with low *c* log *P* values, because they may require mobile phases other than those used in this method.

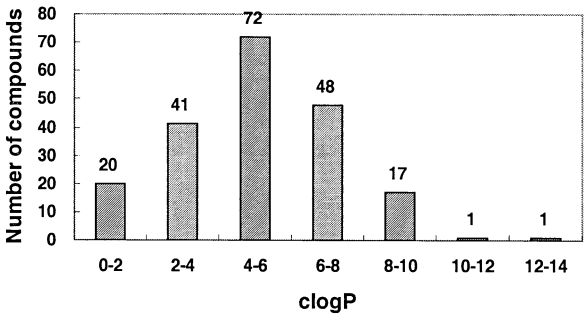

Fig. 8. Distribution of *c* log *P* values of compounds evaluated using the column switching system. Values of *c* log *P* were calculated by CS ChemDraw Pro™ (ver.6, Cambridge Soft Corporation, USA).

4.2. Avoiding matrix effects

As shown in Fig. 3, the response of some compounds may be reduced with ionization suppressed by vehicles such as PEG. However, the effect of PEG can be almost totally removed, because no effect of PG was observed on almost all of the 221 compounds and because no PEG or PG peaks were observed on the mass spectra acquired in the retention time range. Therefore, PEG400 or PG can be used as an administration vehicle, if necessary.

Also, no ionization suppression caused by matrices in plasma was observed with this method (data not shown), contributing to its robustness and good reproducibility without internal standard.

4.3. Possibility of reducing run time

As shown in Fig. 5, about 10 min are required for each sample using this method. To shorten the run time, a modified method with a run time of about 5 min was developed with the same reproducibility. However, as it is not unclear whether this modified method is applicable to other compounds, further investigation is needed.

4.4. Comparison with published generic method for LC/MS/MS

The generic method demonstrated by Herman has the advantage of applicability to various compounds, but the disadvantage of requiring both a dedicated column and dedicated HPLC equipment for the turbulent flow [8]. The Prospekt® liquid chromatography system reported by Beaudry has the same disadvantage of dedicated cartridges and equipment being needed for automatic change of the cartridge [9]. For the new column switching system, only an isocratic pump needs to be added to ordinary LC/MS/MS. The trap column and analytical column are not unique and are readily available.

4.5. Robustness

This column switching method excels in robustness. The performance of the trap column is retained even after analysis of over 1500 plasma samples because the precipitation is done with at least 10-fold volume of methanol in the preparation step, and the trap column is washed with acetonitrile within every run. The performance of the analytical column is retained longer than the trap column because the sample introduced into the analytical column is cleaned in the trap column and isocratic elution is employed. No visible stains on the ESI interface can be observed after the analysis, and the sensitivity remains high for a long term without maintenance.

Various kinds of rapid analysis using LC/MS/MS have been reported recently. There are two important factors related to high throughput analysis, one is related to sample preparation and the other to the HPLC conditions.

4.6. Sample preparation

As reported by Jemal, direct analysis of plasma sample without any preparation is the fastest [10]. Jemal demonstrated a rapid and reliable analysis of Pravastatin by direct injection of plasma using the Oasis® Extraction Column, which is also used in our modified gradient method described above. Jemal's method is very useful for the analysis of Pravastatin, however, problems may arise in its application to other compounds because of difference in protein binding. In other words, the clean-up mechanism of the Oasis® Extraction Column used in this method operates by size exclusion, and therefore, the drug-protein interaction may affect the retention of compounds possessing high protein binding ratios yield poor chromatograms. For instance, compound F, which belongs to the same compound group as compound A–E, was eluted as two peaks on direct analysis of the serum without precipitation. Precipitated by organic solvent, compound F was eluted as a single peak and good results were obtained. Thus, while direct plasma analysis is effective for reducing the preparation time of some compounds, the plasma samples usually should be precipitated by organic

solvent. Precipitation is the simplest preparation method for plasma samples. Our method uses precipitation by organic solvent, which is considered to be the best for the analysis of cassette dosing samples.

4.7. Comparison with published high-speed gradient methods

In general, the high-speed gradient is used as an HPLC generic method that has wide applicability [11,12]. For analysis by high-speed gradient HPLC, an analyte is eluted as an extremely narrow peak, which requires an extremely short dwell time. In the analysis of cassette dosing, the dwell time for each compound is shorter than that in single compound detection because multiple analytes are included in plasma samples in cassette dosing.

The high-speed gradient method is considered to have lower sensitivity due to shortening of the dwell time or reduced reproducibility due to the few data points. Thus, careful study is needed before applying the high-speed gradient method to cassette dosing analysis. In contrast, the column switching system elutes analytes at a low flow rate, giving peak shapes that are wide enough to allow simultaneous detection of multiple compounds without a drastic decrease in sensitivity, indicating that the column switching system is more suitable for cassette dosing analysis than the high-speed gradient method.

4.8. Expansion to systems with higher resolution

Another advantage of this column switching system is the capability for expansion. With the cassette dosing analysis using LC/MS/MS, compounds do not necessarily have to be separated completely by liquid chromatography because compound discrimination is done by mass spectrometry based on molecular weight, thus, eliminating the need to separate compounds when using our column switching system.

If separation of the analytes is essential, this system can be easily modified for high resolution by optimization of the columns and the mobile phase for elution from the trap column.

5. Conclusion

The column switching system demonstrated in this paper is an excellent method with wide applicability to various types of compounds and the capability of expansion to a high-resolution system. This system allows reduction of the time for development of the analytical method and is appropriate for cassette dosing analysis. The combination of cassette dosing and this column switching method should enable construction of more rapid in vivo pharmacokinetics screening systems.

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