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Complete two-dimensional separation for analysis of acidic compounds in plasma using column-switching reversed-phase liquid chromatography

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Abstract

A complete two-dimensional separation technique for the determination of acidic compounds in plasma was developed by using column-switching reversed-phase liquid chromatography. This technique was based on solute peak enrichment at the top of the second column during heart-cutting and an ion-pair chromatographic separation in the second column using tetrabutylammonium ion, where different separation modes in the first and second columns and solute peak enrichment at the top of the second column during heart-cutting were achieved coincidentally. Retention behaviors of two solutes, zidovudine-β-D-glucuronide (AZT-β-D-Gluc) and probenecid, in the first and second column and solute peak enrichment at the top of the second column were investigated for establishment of the system. Different retention behaviors of the solutes in the first and second column, which were evaluated by changes in capacity factor versus acetonitrile concentration in the mobile phases, and peak enrichment could be accomplished by using ion-pair chromatography in the second column. System suitability was confirmed by assessing the number of theoretical plates (N) of the second column for the solutes after heart-cutting. The N values in the second column after column switching were almost same as those in the case that the solutes were directly injected onto the second column. These results indicate that complete two-dimensional separation should be achieved by using this system. Furthermore, this technique was applied to method development for the determination of AZT-β-D-Gluc and probenecid in rat plasma. The peaks of each analyte in the plasma extract obtained by deproteinization were well separated from those of endogenous substances, and easy determination of the analytes could be accomplished at the ng/ml level only by changing the acetonitrile concentration in the mobile phases. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Two-dimensional separation; Acidic compounds; Zidovudine-β-D-glucuronide; Probenecid

1. Introduction

In order to elucidate the mechanism of drug metabolism in vivo, determination of metabolites as well as the parent drug in biological fluids is required in pharmacokinetic studies. After a drug is administered, it is generally oxidized to a hydrophilic form in the body, in which the parent drug is frequently metabolized to the carboxylic acid. Laborious sample pre-treatment procedures have to be carried out for

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sensitive and selective liquid chromatographic determination of acidic metabolites, since there are many hydrophilic endogenous organic acids in biological specimens.

Liquid chromatographic determination based on column-switching techniques is already in widespread use, and several analytical modes have been applied to achieve the selective or sensitive analysis of drugs in biological fluids, e.g., on-line solid-phase extraction (SPE) [1–3], different types of columns for the first and second column [4–8], mobile phases with different pH values [9] and, ion-pair chromatography in only the first column [10–12]. These methods are generally based on faster elution of the solute from the second column than the first column [13], by which the peak of the analyte could be compressed in the second column and the influence of diffusion of the peak in the first column could be minimized (Fig. 1a). Therefore, it is difficult to select the eluting conditions in the second column independently against those in the first column. The selectivity of these methods is insufficient in the case of analysis of a compound in a more complex matrix, like a hydrophilic acidic metabolite of a drug in plasma. A more selective analytical mode is required where the eluting condition in the second column is independent of that in the first column. Such an analytical mode should accomplish a complete two-dimensional analysis (Fig. 1b). For the direct injection analysis of acidic compounds, we have developed an on-line SPE technique, in which the influence of solute peak broadening in the first column is minimized by peak enrichment at the top of the second column [14,15]. Therefore, it is expected that the independent analysis in the second column could be realized against the conditions in the first column by using the peak enrichment technique.

In the present study, a complete two-dimensional system was established using model compounds, zidovudine-β-D-glucuronide (AZT-β-D-Gluc) and probenecid (Fig. 2), and applied to method development for the determination of these compounds in rat plasma.

2. Experimental

2.1. Materials

AZT-β-D-Gluc sodium salt was purchased from Sigma (St. Louis, MO, USA). Probenecid (biochemical grade), methanol and acetonitrile (HPLC grade) were purchased from Wako (Osaka, Japan). Tetra-*n*-butylammonium bromide (TBAB, ion-pair grade) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Deionized water was purified by using a Milli-Q laboratory water purification system (Nihon

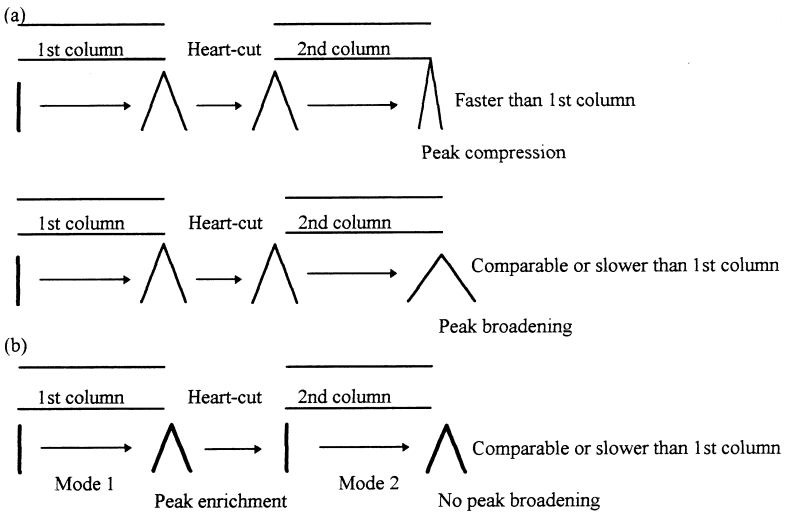

Fig. 1. Influence of the analytical mode on the peak shape after column switching.

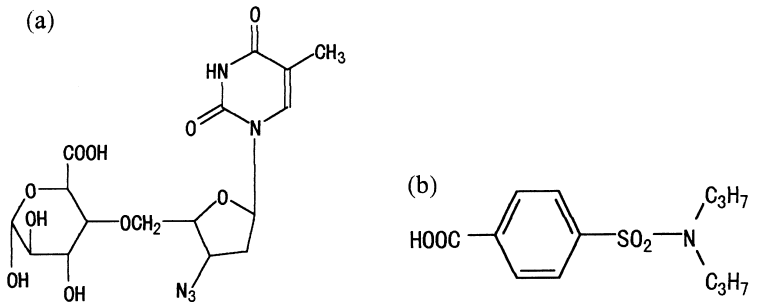

Fig. 2. Chemical structures of AZT-β-D-Gluc and probenecid.

Millipore, Tokyo, Japan) . All other chemicals were of reagent grade and used without purification.

2.2. Instrumentation and liquid chromatographic (LC) conditions

2.2.1. Instrumentation

The LC system consisted of three LC-10AD pumps, two SPD-10A ultraviolet (UV) detectors, FCV-2AH six-way switching valve, a CTO10AC column oven and an SIL-10A autosampler with a sample cooler, all of which were controlled by an SCL-10A controller (all from Shimadzu, Kyoto, Japan). UV detection was carried out at 268 nm for AZT-β-D-Gluc and at 249 nm for probenecid. Peak height of the analytes was measured using a Waters 805 data station (Nihon Waters, Osaka, Japan) or a VStation chromatography Data System (GL Science, Japan). Samples injected into the LC system were kept at 10°C in an SIL-10A autosampler until just before analysis.

2.2.2. LC conditions

A schematic diagram of the LC system is shown in Fig. 3 The columns (first and second column) were Inertsil ODS-3 columns (particle size, 3 μm; 100×4 mm I.D.; GL Science) for AZT-β-D-Gluc and Inertsil ODS-2 columns (particle size, 5 μm; 150×4.6 mm I.D.) for probenecid. The column temperature was 40°C. The flow-rate during re-equilibration of the first column for the determination of probenecid was 1.5 ml/min, and the flow-rate in all other cases was 1 ml/min. The mobile phases (MP1, MP2 and MP3) and other conditions are described in the following sections.

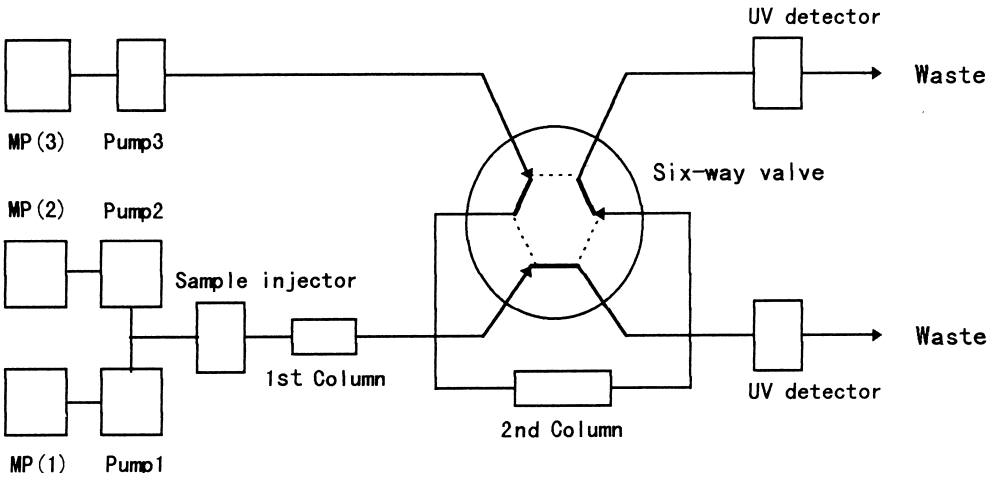

Fig. 3. Schematic diagram of the LC system.

2.2.2.1. Investigation of retention behavior in the second column. Samples were directly injected into the second column. Mixtures of 0.02 *M* phosphate buffer (pH 7) and acetonitrile with or without 10 m*M* TBAB were used as the mobile phases (MP3). The acetonitrile content was changed from 1 to 25% for AZT-β-D-Gluc and from 15 to 40% for probenecid. When the effect of tetra-*n*-butylammonium (TBA) ion on the stationary phase was investigated, the second column was pre-equilibrated with a mixture of 0.02 *M* phosphate buffer (pH 7) and acetonitrile containing 10 m*M* TBAB, and the mobile phase was switched to the same mixture not containing TBAB immediately after injection of the analytes.

2.2.2.2. Investigation of peak enrichment at the top of the second column. For AZT-β-D-Gluc, MP1 (for the first column) was a mixture of 0.02 *M* phosphate buffer (pH 7)–acetonitrile (96:4, v/v) and MP3 (for the second column) was the same as MP1 or mixtures of 0.02 *M* phosphate buffer (pH 7)–acetonitrile (90:10, v/v) containing 5, 7.5, 10 or 15 m*M* TBAB. For probenecid, a mixture of 0.02 *M* phosphate buffer (pH 7)–acetonitrile (758:242, v/v) was used for MP1 and mixtures of 0.02 *M* phosphate buffer (pH 7)–acetonitrile (758:242, v/v) not containing TBAB or (65:35, v/v) containing 5, 7.5, 10 or 15 m*M* TBAB were used for MP3. MP2 was not used in these cases.

2.2.2.3. Determination of AZT-β-D-Gluc and probenecid in rat plasma. For AZT-β-D-Gluc, MP1, MP2 and MP3 were as follows: MP1, a mixture of 0.02 *M* phosphate buffer (pH 7)–acetonitrile (96:4, v/v); MP2, a mixture of 0.02 *M* phosphate buffer (pH 7)–acetonitrile (40:60, v/v); and MP3, a mixture of 0.02 *M* phosphate buffer (pH 7)–acetonitrile (90:10, v/v) containing 10 m*M* TBAB. For probenecid, MP1, MP2 and MP3 were as follows: MP1, a mixture of 0.02 *M* phosphate buffer (pH 7)–acetonitrile (71:29, v/v); MP2, a mixture of 0.02 *M* phosphate buffer (pH 7)–acetonitrile (40:60, v/v); and MP3, a mixture of 0.02 *M* phosphate buffer (pH 7)–acetonitrile (60:40, v/v) containing 10 m*M* TBAB.

The time programs for the LC system are described in Table 1. Following the injection of the plasma extract into the first column, the solute was eluted with MP1. Immediately before the elution of the solute from the first column, the position of the six-way valve was switched from position 1 (solid

Table 1
Time programs for the column switching system

Compound	Time (min)	MP1 + MP2 (ml/min)	MP1 concentration (%)	MP2 concentration (%)	Six-way valve
AZT-β-D-glucuronide	0.0	1.0	100	0	Position 1
	6.0		100	0	
	6.1		0	100	
	6.3				Position 2
	7.3				Position 1
	10.0		0	100	
	10.1		100	0	
	20.0	1.0	100	0	Position 1
Probenecid	0.0	1.0	100	0	Position 1
	5.5				Position 2
	5.8		100	0	
	5.9		0	100	
	6.3				Position 1
	10.0	1.0	0	100	
	10.1	1.5	100	0	
	17.0	1.5			
	17.1	1.0			
	18.0	1.0	100	0	Position 1

line in Fig. 3) to position 2 (dotted line in Fig. 3) to connect the first and second column. After introduction of the solute into the second column (AZT-β-D-Gluc, 6.3–7.3 min; probenecid, 5.5–6.3 min), the six-way valve was switched back to the position 1. The solute introduced into the second column was eluted with MP3 and the first column was washed with MP2 (AZT-β-D-Gluc, 6.1–10.0 min; probenecid, 5.9–10.0 min) and re-equilibrated with MP1 for the next injection. The analysis time per sample was 20 min for AZT-β-D-Gluc and 18 min for probenecid.

2.3. Preparation of quality control (QC) samples

2.3.1. AZT-β-D-Gluc

AZT-β-D-Gluc (3 mg) was dissolved in 3 ml of water to obtain a 1 mg/ml stock solution which was stored at 4°C. Working solutions of AZT-β-D-Gluc (250, 50 and 10 μg/ml) for QC samples were prepared by diluting the stock solution with water. Then, 10-μl portions of the working solutions were added to 4990-μl portions of drug free plasma to obtain QC samples at the concentrations of 500, 100 and 20 ng/ml. The QC samples were stored at −20°C before use.

2.3.2. Probenecid

Probenecid (3 mg) was dissolved in 3 ml of methanol to obtain a 1 mg/ml stock solution which was stored at 4°C. Working solutions of probenecid (100, 25 and 5 μg/ml) for QC samples were prepared by diluting the stock solution with methanol. Then, 10-μl portions of the stock solution or the working solutions were added to 4990-μl portions of drug free plasma to obtain QC samples at the concentrations of 200, 50 and 10 ng/ml. The QC samples were stored at −20°C before use.

2.4. Preparation of calibration standards

2.4.1. AZT-β-D-Gluc

Working solutions of AZT-β-D-Gluc (2000, 500, 100, 20 and 10 ng/ml) for calibration standards were prepared by diluting the stock solution with water. Then, 150-μl portions of the working solutions were added to 150-μl portions of drug free plasma to obtain spiked plasma samples for the calibration standards which were equivalent to plasma samples at the concentrations of 2000, 500, 100, 20 and 10 ng/ml. The spiked plasma samples were prepared just before analysis.

2.4.2. Probenecid

Working solutions of probenecid (1000, 200, 50, 10 and 5 ng/ml) for calibration standards were prepared by diluting the stock solution with methanol. Then, 150-μl portions of the working solutions were evaporated under a stream of nitrogen and mixed well with 150 μl-portions of drug free plasma to obtain spiked plasma samples for the calibration standards at the concentrations of 1000, 200, 500, 10 and 5 ng/ml. The spiked plasma samples were prepared just before analysis.

2.5. Analysis of drug levels in rat plasma

2.5.1. AZT-β-D-Gluc

A 150-μl portion of the QC sample was mixed with 150 μl of water, and 1.5 ml of methanol was added to the mixture. After mixing for 30 s with a vortex mixer, the mixture was centrifuged at 1400 *g* for 3 min. The supernatant obtained was evaporated to dryness under a stream of nitrogen, and the residue was reconstituted with 150 μl of MP1. After filtration through a 0.45-μm membrane filter (UFC30HV00 membrane filter; pore size, 0.45 μm; Nihon Millipore), a 100-μl portion of the filtrate was injected into the LC apparatus.

A 150-μl portion of drug free plasma was mixed with 150 μl of AZT-β-D-Gluc working standard solution instead of water for the spiked plasma (see Section 2.4).

2.5.2. Probenecid

To a 150-μl portion of the QC sample or the calibration standard, 1 ml of acetonitrile was added and then mixed for 30 s by using a vortex mixer. After centrifugation at 1400 *g* for 3 min, the supernatant obtained was evaporated to dryness under a stream of nitrogen, and the residue was reconstituted with 150 μl of MP1. After filtration through the 0.45-μm membrane filter, a 100-μl portion of the filtrate was injected into the LC apparatus.

2.6. Calculation

The number of theoretical plates (N) was calculated using the following equation.

$$N = 5.55 \cdot (W_r / W_h)^2 \quad (1)$$

where W_r and W_h are retention time of the solute and half the width of the solute peak, respectively. W_r for the second column after column switching was obtained by subtracting the retention time for the first column from the total retention time (1st column + 2nd column).

External standards were used to calculate the plasma concentrations of analytes. It is difficult to use internal standards because the heart-cutting technique is applied to the analytical system. A calibration curve was obtained as a weighted linear regression curve ($1/c$) which was calculated by using the peak heights of the analytes of calibration standards.

3. Results

3.1. Effect of TBA ion on the retention behaviors of AZT-β-D-Gluc and probenecid

Plots of capacity factor (k) of the analytes vs. acetonitrile concentration in the mobile phases in the absence (first column) and presence (second column) of TBA are shown in Fig. 4 Different slopes in the absence and presence of TBA were observed for both compounds, indicating that different retention behavior could be obtained by ion-pair chromatography using TBA.

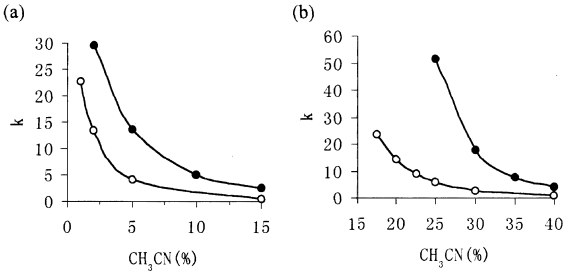

Fig. 4. Retention behavior of (a) AZT-β-D-Gluc and (b) probenecid: open symbol, mobile phase containing no TBA; closed symbol, mobile phase containing 10 m*M* TBA.

3.2. Effect of TBA on peak enrichment for AZT-β-D-Gluc and probenecid

Fig. 5 shows the ratio of the peak heights of the analytes obtained by column switching to that obtained by direct injection into the second column. The acetonitrile contents in the mobile phase for the second column were adjusted as the retention times of the analytes in the first and second columns were almost equivalent in the presence and absence of TBA. The peak heights of analytes were comparable under the conditions of column switching and of direct injection, when TBA concentrations were more than 5 and 10 m*M* for AZT-β-D-Gluc and probenecid, respectively.

3.3. Chromatograms and validation data

Chromatograms of the drug free plasma obtained using the first column are shown in Figs. 6 and 7. Large peaks caused by endogenous substances were observed at the retention times of AZT-β-D-Gluc and probenecid, especially in the case of AZT-β-D-Gluc where separation from these peaks would be impossible. Typical chromatograms of the drug free plasma and the plasma spiked with AZT-β-D-Gluc or probenecid obtained using the column switching technique are also shown in Figs. 6 and 7. Furthermore, chromatograms of plasma sample obtained from rats administered zidovudine or probenecid

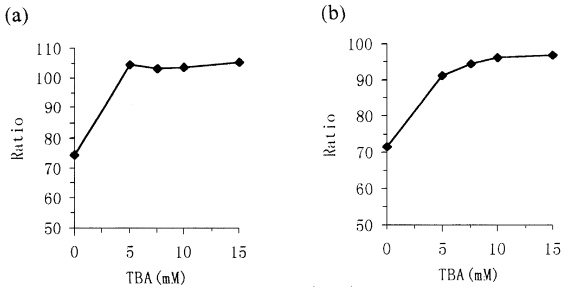

Fig. 5. Effect of TBA concentrations on the relative peak height of analytes: (a) AZT-β-D-Gluc, (b) probenecid. The peak heights were taken as 100% when the analytes were directly injected into the second column.

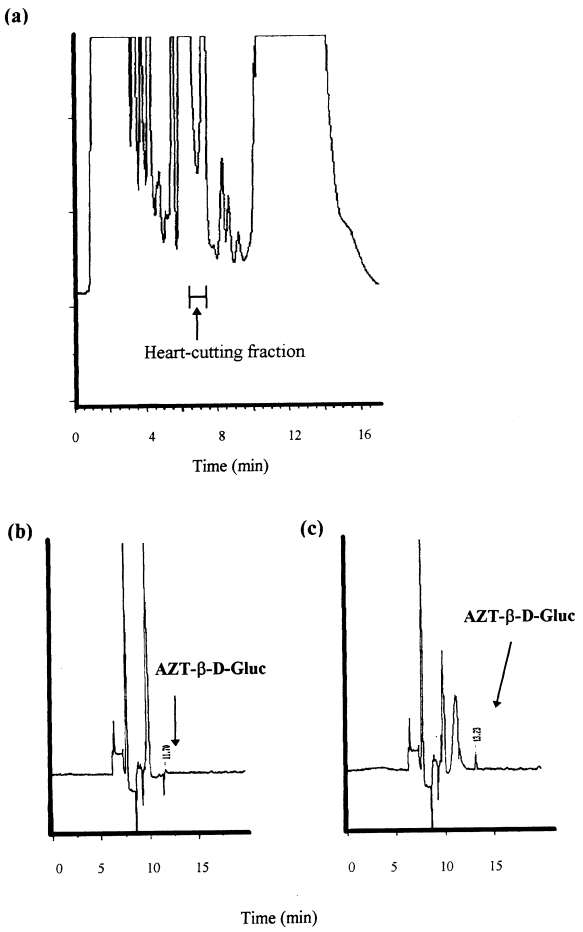

Fig. 6. Typical chromatograms of drug free plasma (a, b) and plasma spiked with AZT-β-D-Gluc (c, 20 ng/ml): (a) first column only; (b) and (c) column switching.

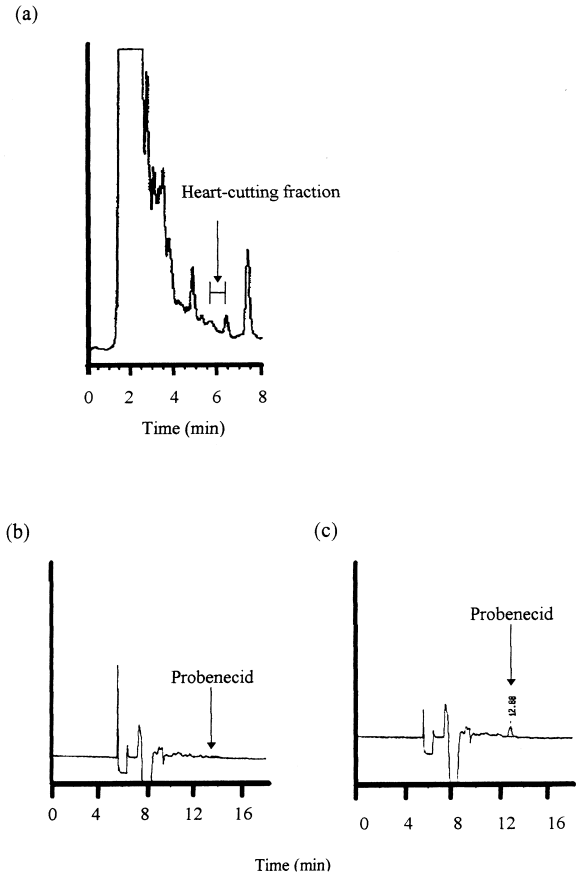

Fig. 7. Typical chromatograms of drug free plasma (a, b) and plasma spiked with probenecid (c, 10 ng/ml): (a) first column only; (b) and (c) column switching.

orally are shown in Fig. 8. No interfering peaks were observed at the retention time of AZT-β-D-Gluc or probenecid.

The linearity, accuracy, precision and reproducibility were satisfactory from 10 to 2000 ng/ml for AZT-β-D-Gluc and from 5 to 1000 ng/ml for probenecid (Tables 2–4). The linearity of the calibration curve was demonstrated by the correlation coefficient which was greater than 0.999 (Table 2). The lower limit of quantitation, which was defined as the lowest concentration when the relative error (R.E.) and coefficient of variation (CV) were not more than 20% [16], was 10 ng/ml for AZT-β-D-Gluc and 5 ng/ml for probenecid (Table 3). The CV values and relative errors for the QC samples for AZT-β-D-Gluc were not more than 1.6% and 4.0%, respectively, and those for probenecid were not more than 3.5% and 9.0%, respectively (Table 4).

4. Discussion

4.1. Two-dimensional analytical system

In order to accomplish complete two-dimensional analysis by column switching LC, the following conditions have to be fixed and combined. (1) Different separation modes in the first and second columns and (2) solute peak enrichment at the top of the second column during heart-cutting.

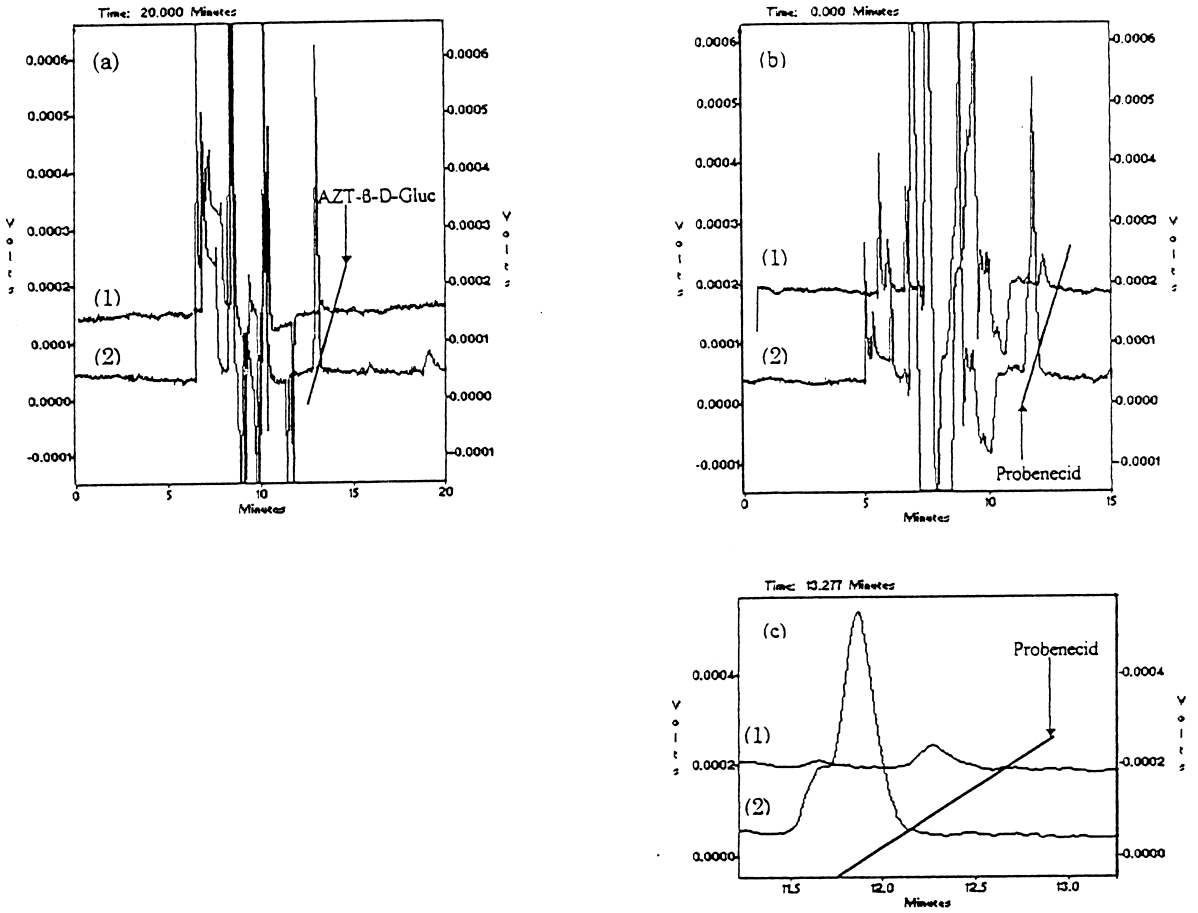

Fig. 8. Chromatograms of plasma samples obtained from dosed rats (a, zidovudine, 5 mg/kg p.o.; b and c, probenecid, 10 mg/kg p.o.): (a, b, c-1), before administration; (a-2), 1 h after administration, AZT-β-D-Gluc=66 ng/ml ; (b and c-2) 2 h after administration, probenecid=73 ng/ml . Solid lines in the figures indicate the retention time of elutes. (c) Magnification of (b) around the retention time of probenecid.

The different separation modes in the first and second columns would be generated by using ion-pair chromatography with TBA in the second column. The *k* values of model compounds for acidic drugs, AZT-β-D-Gluc and probenecid, vs. acetonitrile concentrations in the mobile phases were plotted to determine whether the solutes exhibit different retention behavior. The different slopes of the plots

Table 2
Regression parameters for the validation of analytes in plasma[a]

Compound	Concentration[b] (ng/ml)	Slope (CV, %)	Intercept	r^c (CV, %)
AZT-β-D-glucuronide	10-2000	5.3 (1.3)	4.1	0.999 (0.01)
Probenecid	5-1000	8.9 (1.1)	18.5	0.999 (0.04)

[a] Weighted linear regression: $1/c$. Regression parameters were obtained as the mean values of five standard curves.
[b] See Table 3.
[c] Correlation coefficient.

Table 3
Intra-day precision of the standard curves of the methods for plasma[a]

Compound	Nominal concentration (ng/ml)	Observed concentration (ng/ml)	CV (%)	R.E.[b] (%)
AZT-β-D-glucuronide	10	9.9	4.0	-1.0
	20	20.3	2.5	1.5
	100	99.6	1.8	-0.4
	500	$5.03 \cdot 10^2$	0.6	0.6
	2000	$20.0 \cdot 10^2$	1.6	0.0
Probenecid	5	5.63	10.8	12.6
	10	8.84	5.7	11.6
	50	48.62	1.1	2.8
	200	$2.02 \cdot 10^2$	1.1	1.1
	1000	$9.97 \cdot 10^2$	1.1	-0.3

[a] Back-calculated from calibration curves ($n=5$).
[b] Relative error.

indicate that different separation modes could be obtained under the normal and ion-pair conditions (Fig. 4). The log–log plot of the k values of probenecid vs. acetonitrile concentrations in the mobile phases shows a linear relationship with different slopes (Fig. 9). Therefore, the change in the retention behavior of probenecid under the normal and ion-pair conditions can be explained by enhancement of the hydrophobic interaction of probenecid with the stationary phase (ODS column) through ion-pair formation of probenecid and TBA in the ODS column [17]. On the other hand, the log–log plot for AZT-β-D-Gluc, which is a hydrophilic compound, exhibits parallel curves under the normal and ion-pair conditions, indicating the change in the retention behaviors of AZT-β-D-Gluc cannot be explained by enhancement of the hydrophobic interaction (Fig. 9). An extrapolated k, lipophilic index,

Table 4
Intra-day and inter-day precision and accuracy of the methods for plasma[a]

Compound	QC sample (ng/ml)		Intra-day assay			Inter-day assay
			Day 1	Day 2	Day 3	
AZT-β-D-glucuronide	20	CV (%)	1.6	0.5	1.0	3.6
		R.E. (%)[b]	−4.0	−2.5	−1.0	−2.5
	100	CV (%)	0.6	0.6	0.6	2.5
		R.E. (%)	−1.0	−2.3	−3.1	−2.1
	500	CV (%)	1.0	1.3	0.1	1.3
		R.E. (%)	−0.8	−1.7	−2.0	−1.5
Probenecid	10	CV (%)	3.5	2.6	1.8	2.0
		R.E. (%)	−7.3	−9.0	−8.4	−8.3
	50	CV (%)	1.5	0.2	3.3	1.1
		R.E. (%)	−3.6	−3.6	−4.4	−3.9
	200	CV (%)	0.1	2.6	1.6	1.7
		R.E. (%)	1.0	−0.4	0.0	0.2

[a] $n=5$ for intra-day assay and $n=3$ for inter-day assay.
[b] Relative error.

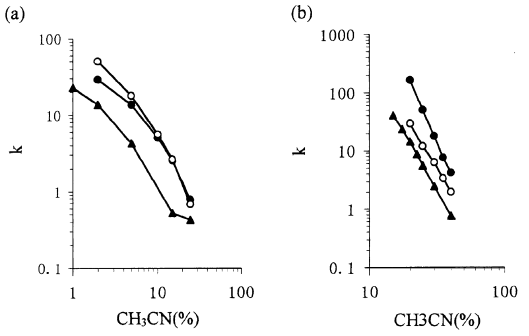

Fig. 9. Hydrophobic interaction of (a) AZT-β-D-Gluc and (b) probenecid with the ODS stationary phase: closed triangle, column without TBA; closed circle, column with TBA; open circle, column pre-equilibrated with TBA.

which is the intercept of vertical axis obtained from the semi-log plot of the *k* value vs. the solvent concentration in the mobile phase, is frequently used for evaluation of the lipophilicity of a solute [18,19]. The lipophilic index of AZT-β-D-Gluc was comparable with and without TBA (1.64 with TBA and 1.52 without TBA). Therefore, it was suggested that adding TBA to the mobile phase would have no effect on the hydrophobic interaction between the stationary phase and AZT-β-D-Gluc. The effect of TBA on the stationary phase was investigated in order to elucidate the retention behavior of AZT-β-D-Gluc under the ion-pair conditions. The retention behavior of AZT-β-D-Gluc in the column pre-equilibrated with TBA was examined by eluting with the mobile phase not containing TBA. The log–log plot of the *k* value vs. acetonitrile concentration under these conditions was superimposed over that obtained using the mobile phase for the ion-pair condition which contained 10 m*M* TBA (Fig. 9). Since TBA is known to be strongly adsorbed onto an ODS stationary phase and not to be washed out easily, the column pre-equilibrated with TBA could act as an anion-exchange column. Therefore, the retention behavior of AZT-β-D-Gluc under the ion-pair conditions would be explained by both anion-exchange and hydrophobic interaction. In the case of probenecid, the log–log plot of the *k* value vs. acetonitrile concentration for the column pre-equilibrated with TBA was halfway between the plots obtained from the normal and ion-pair mode (Fig. 9), and the slope for the pre-equilibrated mode was parallel to that for the normal mode. Although an anion-exchange interaction exists, the retention behavior would be dominated by the ion-pair interaction. Thus, a different separation mode could be obtained using the ion-pair chromatography with TBA, although it was shown that the retention mechanism in the ion-pair mode might differ with the hydrophilicity of the solute.

As discussed above, since TBA is strongly adsorbed onto the ODS stationary phase and is not washed out easily by the small volume of mobile phase not containing TBA which is introduced into the second column during heart-cutting, acidic compounds would be retained on the column by ion-pair formation or an anion-exchange interaction [14,15]. Thus the peak enrichment for acidic compounds would be anticipated at the top of the second column. The peak enrichment for AZT-β-D-Gluc and probenecid during heart-cutting was evaluated by using the ratios of solute peak heights in the second column obtained from column switching and direct injection (single column) modes. As shown in Fig. 5, the peak height ratios increased with TBA concentration, and comparable peak heights were obtained with and without column switching at a TBAB concentration of more than 10 m*M* for both compounds. This is explained by the peak enrichment for both compounds at the top of the second column. In these experiments, the acetonitrile concentration in the mobile phase used for the elution of solutes from the second column is higher than that used for the introduction of solutes from the first column to the second column (during heart-cutting), therefore, peak compression might be induced by the gradient effect caused by the difference in the acetonitrile concentration. In order to rule out this possibility, the acetonitrile content in the mobile phase for the second column was adjusted to give almost the same retention time as that in the first column, by which the retention time in the second column with and without TBA became the same.

System suitability was confirmed by assessing the *N* values of the second column for the solutes under column switching and single column modes. The N values for AZT-β-D-Gluc and probenecid in the second column obtained after column switching were comparable to those obtained with direct injection of the analytes into the second column when the TBA

Table 5
N values of the second column with and without column switching[a]

Compound	TBA (m*M*)	*N*		
		1st + 2nd[b]	2nd only[c]	Ratio
AZT-D-glucuronide	0	3963	6856	0.58
	5	10 502	8473	1.24
	7.5	10 608	8559	1.24
	10	10 372	8214	1.26
	15	9180	8226	1.12
Probenecid	0	8435	10 286	0.82
	5	9467	10 898	0.87
	7.5	10 407	11 732	0.89
	10	10 485	11 822	0.89
	15	10 412	11 880	0.88

[a] *N*: Number of theoretical plates.
[b] With column switching.
[c] Without column switching.

concentration in the mobile phase for the second column was more than 10 m*M* (Table 5). This means that the different separation modes and the peak enrichment would be combined well and complete two-dimensional separation should be achieved using the present system.

The ratio of the *N* values for probenecid was slightly less than 0.9 (Table 5). This would be explained by the incomplete peak enrichment during heart-cutting (enrichment ratio 0.95, Fig. 5). On the other hand, the *N* value ratio for AZT-β-D-Gluc was higher than 1. This result cannot be explained only by peak enrichment (enrichment ratio 1, Fig. 5). In the present study, the *k* values in the first and second columns were adjusted to become the same for the respective mobile phases. However, the retention capacity of the second column during heart-cutting would be much greater than that after the column switching, because AZT-β-D-Gluc was introduced into the second column by the mobile phase for the first column not containing TBA. Therefore, the peak compression phenomenon would occur in the second column through the gradient effect caused by the difference in retention capacity immediately after heart-cutting. These observations would indicate that a more hydrophilic compound was more advantageous in this system.

The total *N* value for this two-dimensional system (apparent *N*) is quite high (apparent $N > 40\,000$) and about two-fold higher than the total of the respective *N* values for the first and second columns, because the diffusion of solute in the first column is canceled out during heart-cutting. Therefore, it is expected that excellent resolution and selectivity should be obtained using the present complete two-dimensional system.

4.2. Method development for determination of AZT-β-D-Gluc and probenecid in rat plasma

The complete two-dimensional system was confirmed to be applicable to the development for determination of drugs in plasma under routine analytical conditions, establishing determination methods for AZT-β-D-Gluc and probenecid in rat plasma. We have already reported an on-line SPE system for the determination of acidic compounds in plasma which only requires the filtration of plasma for sample pre-treatment [14,15]. Although this system is simple and sensitive, some problems exist for clinical use, e.g., the biological hazard involved with the direct injection of clinical samples, difficulty of filtration for patient samples containing insoluble material and only moderate selectivity. The purpose of the present study is to develop a simple method for the determination of acidic compounds in plasma, in which only the deproteinization of plasma for sample pre-treatment and the changing of solvent

contents of the mobile phase under the LC conditions are required for application to various compounds. Using the complete two-dimensional system, the simple methods could be developed easily for determination of AZT-β-D-Gluc and probenecid in rat plasma. Thus the complete two-dimensional system should be applicable for the method development for simple analysis of acidic compounds in plasma taking into consideration for the conditions used in routine analysis.

5. Conclusion

A complete two-dimensional separation technique was developed for selective determination of acidic compounds. AZT-β-D-Gluc and probenecid in rat plasma could be easily determined using the present technique. This technique should be useful for the bioanalysis of drugs, especially hydrophilic acidic metabolites.

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References

[1] R. Wyss, F. Bucheli, B. Hess, J. Chromatogr. A 729 (1996) 315.
[2] I. Ono, K. Matsuda, S. Kanno, J. Chromatogr. B 678 (1996) 384.
[3] E.J. Woolf, B. Matuszewski, J. Chromatogr. A 729 (1996) 211.
[4] G. Zurcher, M.D. Prada, J. Chromatogr. 530 (1990) 253.
[5] B.M. Eriksson, B.A. Persson, M. Wikstrom, J. Chromatogr. 527 (1990) 11.
[6] V. Kircher, H. Parlar, J. Chromatogr. B 677 (1996) 245.
[7] M. Takahashi, H. Hoshino, K. Kushida, K. Kawana, T. Inoue, Clin. Chem. 42 (1996) 1439.
[8] M. Josefsson, B. Norlander, J. Pharm. Biomed. Anal. 15 (1996) 267.
[9] T. Miyabayashi, T. Okuda, M. Motohashi, K. Izawa, T. Yashiki, J. Chromatogr. B 677 (1996) 123.
[10] K. Yamashita, M. Motohashi, T. Yashiki, J. Chromatogr. 487 (1989) 357.
[11] T. Miyabayashi, K. Yamashita, I. Aoki, M. Motohashi, T. Yashiki, K. Yatani, J. Chromatogr. 494 (1989) 209.
[12] K. Yamashita, M. Motohashi, T. Yashiki, J. Chromatogr. 527 (1990) 196.
[13] H. Takahagi, K. Inoue, M. Horiguchi, J. Chromatogr. 352 (1986) 369.
[14] T. Okuda, M. Motohashi, I. Aoki, T. Yashiki, J. Chromatogr. B 662 (1994) 79.
[15] T. Okuda, K. Yamashita, M. Motohashi, J. Chromatogr. B 682 (1996) 343.
[16] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman, S. Spector, J. Pharm. Sci. 81 (1992) 309.
[17] A. Kaibara, C. Hohda, N. Hirata, M. Hirose, T. Nakagawa, Chromatographia 29 (1990) 275.
[18] T. Yamana, A. Tsuji, E. Miyamoto, O. Kubo, J. Pharm. Sci. 66 (1977) 747.
[19] T. Braumann, J. Chromatogr. 373 (1986) 191.