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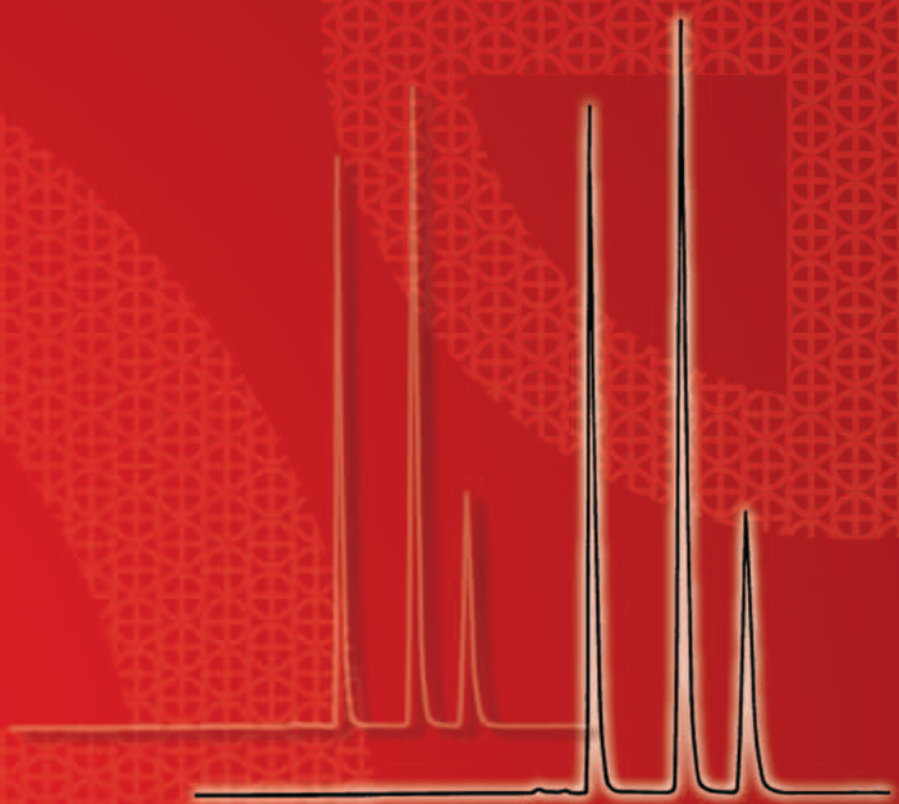
Direct Analysis of Drugs in Blood Plasma using 2D-HPLC for Bioanalysis "Co-Sense for BA"

Shimadzu
HPLC Application Report No. 23



HPLC APPLICATION REPORT NO. 23

Direct Analysis of Drugs in Blood Plasma using “Co-Sense for BA” Bio-Sample Pretreatment System

HPLC APPLICATION REPORT NO. 23

Direct Analysis of Drugs in Blood Plasma using "Co-Sense for BA"

Bio-Sample Pretreatment System

Introduction

Analysis of drugs using HPLC (High Performance Liquid Chromatography) in pharmacokinetics studies and clinical tests is an effective means of elucidating the behavior of drugs and their metabolites in living organisms.

Normally, when conducting HPLC analysis of drug-containing bio-samples, such as blood plasma and blood serum, etc., it is first necessary to eliminate proteins and other contaminants through pretreatment. This pretreatment generally consists of using an organic solvent, etc. to remove the proteins, and then performing centrifugal separation. However, not only are these pretreatment procedures cumbersome, analytical accuracy can be compromised due to the reliance on manually performed procedures. For this reason, it is becoming preferable to automate these types of procedures.

Recently, advances have been made in the development of pretreatment columns designed specifically for removing proteins and other contaminants[1~4], and there is growing expectation that with these columns in combination with a column switching system, the pretreatment operations can be automated to provide better analytical accuracy and enhanced specimen processing capability.

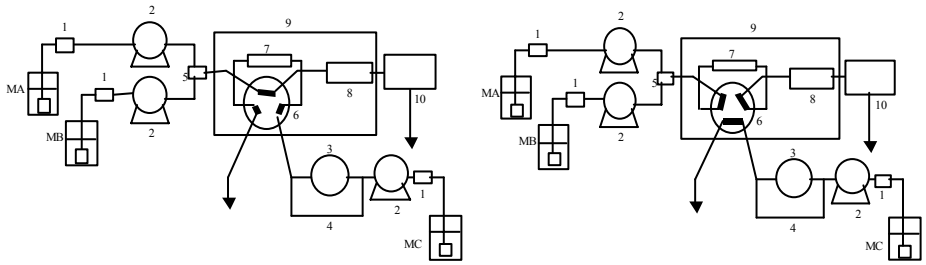
Here we report on the principles and features of a bio-sample analysis column switching HPLC system (Co-Sense for BA), equipped with the newly developed Shim-pack MAYI-ODS[5] bio-sample pretreatment column and unique on-line dilution channel, in addition to application examples utilizing direct injection of blood plasma to perform drug analysis.

2. What is Co-Sense for BA?

Co-Sense for BA is a column-switching HPLC system equipped with the newly developed Shim-pack MAYI-ODS pretreatment column and a unique on-line dilution bypass channel, providing an automated series of processes from deproteinization of the blood plasma bio-sample to analysis. This system, with its UV detector, serves as a front-end HPLC system for a mass spectrometer, enhancing the efficiency of analyzing drugs in bio-samples in pharmacokinetics studies and clinical tests.

The Shim-pack MAYI-ODS is an inner surface type reversed-phase pretreatment column providing high-rate deproteinization and excellent durability. In addition, the on-line dilution bypass channel enables automatic dilution of the injected sample with the ideal sample introduction (injection) mobile phase to achieve a high recovery of drugs, even exhibiting those a high rate of protein binding.

Figure 1 shows the flow diagram of the Co-Sense for BA system.



Apparatus; 1: Degasser, 2: Pump, 3: Auto sampler, 4: Dilution bypass line,
5: Mixer, 6: High-pressure flow selection valve, 7: Pretreatment column,
8: Analytical column, 9: Column oven, 10: Detector,
MA, MB : Mobile phase for sample separation,
MC: Mobile phase for sample injection

Figure 1 : Flow diagram of Co-Sense for BA

The process flow is described in sequential order.

① With the high-pressure flow line selection valve (6) in the state shown in the left-hand diagram of Fig. 1, a bio-sample such as blood plasma is injected from the injector (3).

② The injected sample is automatically diluted with sample injection mobile phase (MC) in the dilution bypass line (4) to separate the target components from proteins in the sample.

③ The sample is then introduced into the Shim-pack MAYI-ODS pretreatment column (7). Low molecular components like drugs are trapped, while proteins pass straight through and out of the column.

④ The high-pressure flow line selection valve (6) makes in 60 degree revolution, and then assumes the state shown in the right-hand diagram of Fig. 1, after

which the analysis mobile phase (MA, MB) transports the drugs that were trapped in the Shim-pack MAYI-ODS pretreatment column (7) onto the analysis column (8).

⑤ The drugs are separated and eluted from the analysis column (8) for detection (10).

3. Principle of Shim-pack MAYI-ODS

The Shim-pack MAYI-ODS pretreatment column effectively removes proteins from bio-samples like blood plasma, while trapping low molecular weight compounds like drugs. This is one of the important features of the Co-Sense for BA system.

3-1. Column Surface of the Shim-pack MAYI-ODS Pretreatment Column

Figure 2 illustrates the column surface of the Shim-pack MAYI-ODS pretreatment column.

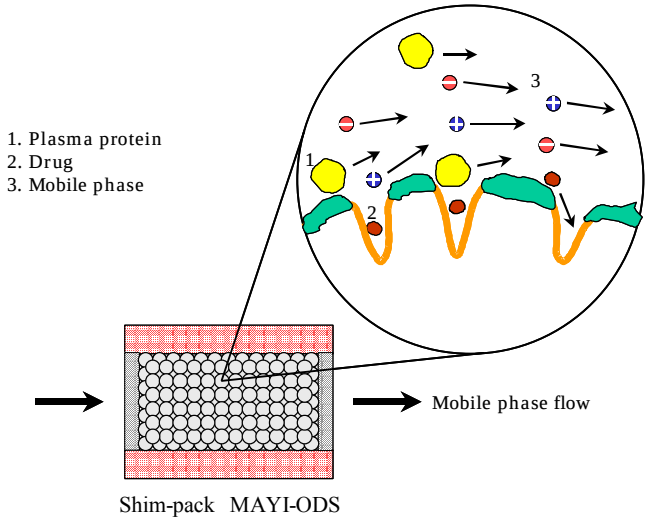


Figure 2 : Extraction of Drugs from Plasma Protein using the Shim-pack MAYI-ODS Column

The Shim-pack MAYI-ODS is an inner surface type reversed-phase column, in which the outer surface of silica gel (50μm) is coated with a water-soluble polymer, and only the pore interior is chemically modified with an octadecyl group.

As shown in Fig. 2, macromolecules such as proteins, which cannot enter the pore interior blocked by the water-soluble polymer on the outer surface, are easily eluted and not retained by the stationary phase. Other organic, low molecular weight compounds, however, permeate into the pore interior and are retained by the stationary phase of the inner surface.

3-2. Deproteinization Effect with Shim-pack MAYI-ODS

The deproteinization effect of the Shim-pack MAYI-ODS pretreatment column is shown in Fig. 3, and the analysis conditions are shown in Table 1. The evaluation was performed using isopropylantipyrine-spiked blood plasma, and as indicated by the lower chromatogram in Fig. 3, the proteins were clearly eluted within 3 minutes. After the proteins were expelled, the drugs and other low molecular weight compounds were selectively introduced into the analysis column by the switching action of the high-pressure flow line selection valve, providing the chromatogram shown in the upper level of Fig. 3.

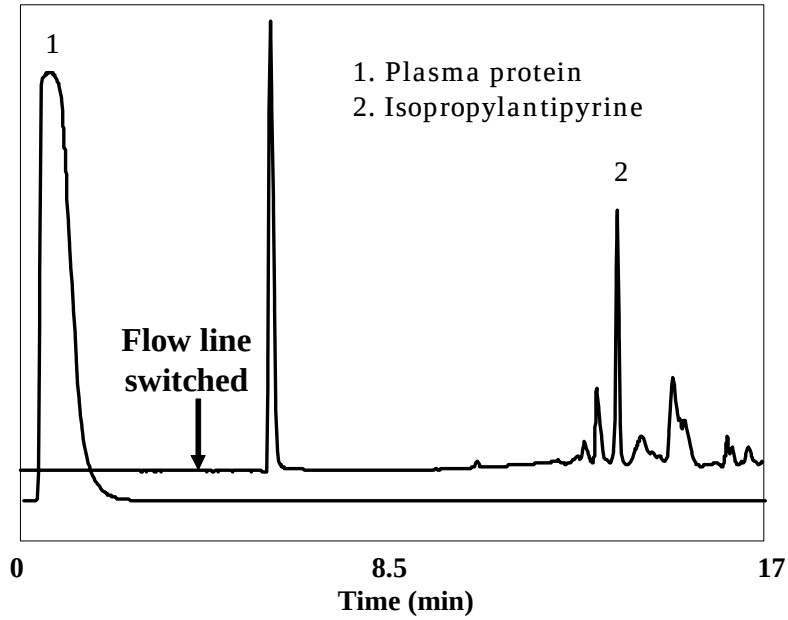


Figure 3 : Deproteinization Effect with Shim-pack MAYI-ODS Column
(1μg/mL spiked, 100μL injected)

Table 1 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	0.1% Phosphoric acid / Acetonitrile = 95 /5 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack VP-ODS (150mmL.×4.6mmI.D.)
Mobile phase	(A) : Water
	(B) : Acetonitrile
	Linear gradient (B) 5% to 95% (12min)
Flow rate	1.0mL/min
Temperature	40°C
Detection	275nm : Isopropylantipyrine
	280nm : Plasma protein
	(SPD-M10A*VP*)

4. On-line Dilution Bypass Effect

When analyzing drugs in blood plasma and other bio-samples, Co-Sense for BA adopts a unique on-line dilution bypass channel which effectively recovers target compounds from the sample.

4-1. Recovery of Drugs from Bio-Samples

In most cases, drugs in blood plasma and blood serum are bound to proteins, and by performing deproteinization, both proteins and drugs may be removed, thereby contributing to diminished drug recovery. Since proteins and drugs bind together due to ionic interaction or hydrophobic interaction, the protein-drug bond can be weakened by changing the pH or ionization strength of the sample environment by such means as adding organic solvent, etc.

The Co-Sense for BA system provides that the sample pass through a dilution bypass flow line directly after injection. Here, the sample environment is changed by performing an 8-fold dilution with an appropriate sample injection mobile phase to weaken the protein-drug bond before introduction into the deproteinization pretreatment column.

4-2 Dilution Bypass Effect

Figure 4 shows the effect of the dilution bypass flow line following a 500μL injection of blood plasma spiked with indometacin (1μg/mL), which displays a relatively high rate of binding with proteins. The analytical conditions that were used are shown in Table 2. The recovery rate was about 50% without using the dilution bypass, while a nearly 100% recovery rate was achieved when using the dilution bypass, clearly confirming the effect.

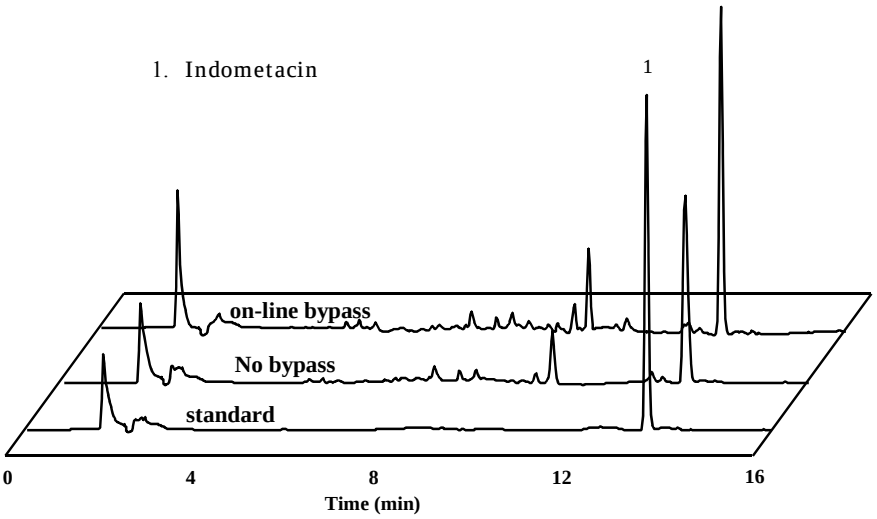


Figure 4 : Effect of On-line Dilution Bypass on the Recovery of Drug
(1μg/mL spiked, 500μL injected)

Table 2 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	0.1% Phosphoric acid / Acetonitrile = 95 /5 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack VP-ODS (150mmL.×4.6mmI.D.)
Mobile phase	(A) : 0.1% Trifluoroacetic acid
	(B) : Acetonitrile containing 0.1% trifluoroacetic acid
	Linear gradient (B) 5% to 95% (12min)
Flow rate	1.0mL/min
Temperature	40°C
Detection	315nm (SPD-M10A*VP*)

The dilution bypass effect on recovery of 6 other drugs was also evaluated, and the results are summarized in Table 3. Drug recovery rate values vary slightly depending on the composition of the sample injection mobile phase (pH, salt concentration, organic solvent content, etc.); however, for the most part, a 100mM acetate (Na) buffer solution (pH 4.7) was used here. The samples consisted of blood plasma spiked with drugs at a concentration of 2μg/mL.

Table 3 : Recovery of Drugs in Plasma

	Injection Volume(μL)	Recovery (%)		
		Ketoprofen[1)]	Naproxen[1)]	Warfarin[1)]
Bypass	50	98	100<	100<
	250	97	90	100<
	500	96	83	99
No Bypass	500	54	21	60

	Injection Volume(μL)	Recovery (%)		
		Chlorpropamide[1)]	Ibuprofen[1)]	Acetohexamide2[2)]
Bypass	50	94	99	100<
	250	-	-	99
	500	100<	100<	99
No Bypass	50			77
	250	-	-	56
	500	71	57	-

1) Mobile phase for sample injection : 100mM Acetate (Na) buffer (pH=4.7) / Acetonitrile = 9 / 1 (v/v)
2) Mobile phase for sample injection : 100mM Acetate (Na) buffer (pH=4.7)

With the dilution bypass, high recovery rates are possible even with 500μL injections. This demonstrates the high drug recovery rates achieved with Co-Sense for BA even with large-volume injections, making analysis of drugs in blood plasma with high sensitivity possible.

5. Linearity and Reproducibility

To evaluate the peak area linearity of drugs for spiking blood plasma, phenytoin and carbamazepine were added to plasma in concentrations of 0.2 μg/mL, 1μg/mL, 4μg/mL and 20μg/mL. The linearity results obtained after analysis using Co-Sense for BA are shown in Figure 5. The analytical conditions used and the chromatograms are shown in Chapter 8. Application Examples. It is clear from the figure that good linearity was obtained over this concentration range.

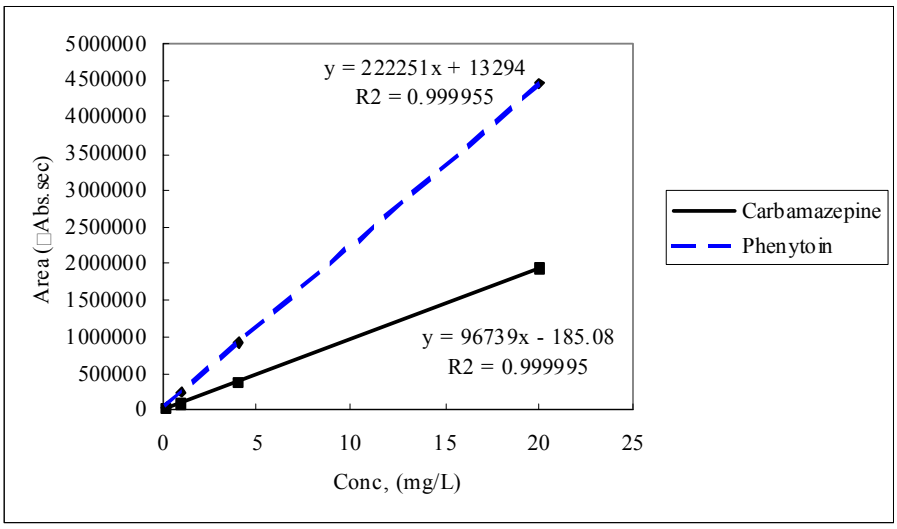


Figure 5 : Linearity of Peak Area of Phenytoin and Carbamazepine in Plasma

In addition, 1μg/mL of phenytoin and carbamazepine were added to plasma, and the reproducibility of area values obtained in five repeat analyses is shown in Table 4.

Table 4 : Reproducibility of Peak Area of Phenytoin and Carbamazepine (each 1μg/mL, 50μL injected)

	Phenytoin(μAbs·sec)	Carbamazepine(μAbs·sec)
NO.1	238911	98548
NO.2	240042	98367
NO.3	237208	98706
NO.4	238317	98481
NO.5	239678	98152
Average	238832	98451
Standard Deviation (s)	1127.9	207.3
Coefficient of Variation (%)	0.472	0.211

It is clear from the results that extremely high reproducibility was obtained for both drugs. Since variations due to deproteinization operations and human error are reduced through pretreatment automation, high reproducibility is obtained.

6. Suppression of Carryover

With respect to drugs present in blood, since there is a large difference in concentration between samples containing administered drugs and samples in which drugs have not been administered, it is extremely important to suppress carryover between samples to manage accuracy in analysis.

To suppress this carryover, we investigated materials that could be used in the autosampler flow lines. For the sampling needle, a special metallic coating was selected, while PEEK resin was selected for the injection port needle seal and switching valve seal. The results of the carryover suppression efficacy are shown in Table 5.

To prevent detection of proteins, less than 10% of the typical amount of organic solvent was used as the sample injection mobile phase in the Co-Sense for BA system. Higher carryover would be expected as compared with ordinary HPLC analysis; however, even with relatively strong hydrophobic drugs, adhesion was not evident due to the changes made to the materials.

This demonstrates the applicability for samples with a wide, low-to-high concentration range.

Table 5 : Carryover of Drug Standards

	Condition A[1)]	Condition B[2)]
Diazepam[3)]	0.09%	0.09%
Phenytoin[3)]	0.04%	0.02%
Carbamazepine[3)]	0.04%	0.05%
Phenylbutazone[3)]	0.28%	0.14%
Reserpine[3)]	0.08%	0.03%
Ketoprofen[4)]	0.05%	0.03%
Warfarin[4)]	0.03%	0.02%
Naproxen[4)]	0.19%	0.11%

1) mobile phase for sample injection : 100mM Acetate (Na) buffer（pH=4.7）
2) mobile phase for sample injection : 100mM Acetate (Na) buffer（pH=4.7）/ acetonitrile = 9 / 1 (v/v)
3) 200μg/mL standard solution
4) 10μg/mL standard solution

7. Analytical Conditions

The analytical conditions used when the UV / photodiode array detector is used are presented below. When the LC/MS is used, ammonium acetate, formic acid or acetic acid, etc. is used instead of phosphate buffer as the mobile phase. One example of this is presented in section 8.14.

7-1. Confirming Elution Behavior

Understanding the elution behavior of drugs using this system facilitates optimization of the analytical conditions. The basic analytical conditions are shown in Table 7.

The solutions used for the analysis mobile phase (mobile phase for separation) are shown in the column headings of Table 6. For the sample injection mobile phase, a 100mM phosphate (sodium) buffer solution (pH 2.1) was used for acidic drugs, while a 100mM phosphate (sodium) buffer solution (pH 6.9) was used for basic drugs. In addition, up to 10% organic solvent such as acetonitrile can be added; however, care must be taken in this regard because weakly hydrophobic drugs pass straight through the pretreatment column, preventing them from being trapped. As for acceptable sample injection buffer solutions, in addition to a phosphate buffer solution, an acetate buffer solution, etc. can also be used.

A drug retention time index based on these conditions is shown in Table 6. Only a few drugs are listed here, however, by performing analysis using the same conditions with the target drug, the elution behavior of that drug can be determined in the same way. Acidic drugs like ketoprofen and warfarin show longer retention times when using acidic buffer solutions as the analysis mobile phase, while basic drugs like verapamil and imipramine show a tendency toward longer retention times when using more neutral buffer solutions.

In this way, by understanding the elution behavior of drugs beforehand, it is possible to lessen the burden of finding the analytical conditions.

With respect to the target concentration range, if separation from the plasma matrix is achieved to some degree, those conditions can be applied for analysis of the drugs present in the plasma. If even better separation from the plasma matrix is desired, or if a shorter analysis time is desired, more optimal method conditions can be derived on the basis of this index.

Table 6 : Drug Retention Times Relative to Analysis Mobile Phase

	20mM Phosphate (Na) buffer (pH=2.5) 100mM Sodium perchlorate	20mM Phosphate (Na) buffer (pH=6.9)	100mM Acetate (Na) buffer (pH=4.7)
Cefloxime	12.6min	10.8min	11.6min
Caffeine	13.2min	14.2min	13.5min
Lidocaine	13.3min	21.5min	15.0 min
Aspirin	14.0min	8.2min	12.2min
Noscapine	14.7 min	21.6min	19.6 min
Salicylic acid	15.2min	9.5min	11.7min
Chlorpheniramine	15.6 min	21.2 min	16.6 min
Propranolol	16.9 min	20.7 min	16.8 min
Diphenhydramine	17.2 min	22.1 min	17.1 min
Phenytoin	17.2 min	17.0 min	17.1min
Isopropylantipyrine	17.7 min	17.7 min	17.8 min
Chlorpropamide	17.8 min	15.2 min	17.7 min
Verapamil	17.8 min	21.9 min	17.9 min
Carbamazepine	17.8 min	17.8 min	17.8min
Acetohexamide	18.3 min	15.7 min	19.2 min
Reserpine	18.6 min	22.7 min	
Nifedipine	18.7 min	19.0 min	18.9min
Imipramine	18.8 min	24.4 min	18.9 min
Alprazolam	-	18.9min	18.9min
Triazoram	-	18.7min	18.7min
Ketoprofen	19.5 min	16.7 min	18.7 min
Naproxen	20.1 min	16.4 min	
Diazepam	20.2 min	20.2 min	20.2min
Warfarin	20.5 min	15.3 min	19.3 min
Phenylbutazone	21.0 min	17.2 min	
Ibuprofen	22.2 min	19.2 min	21.3 min

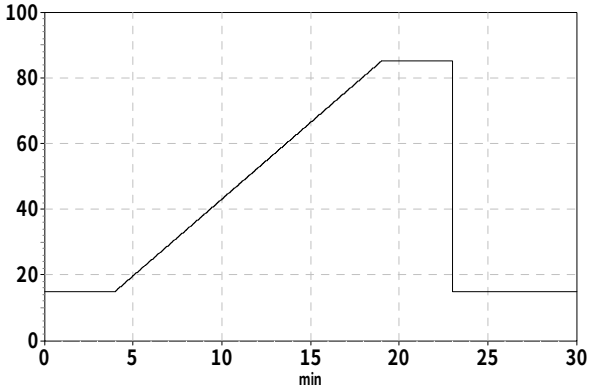
Table 7 : Analytical Conditions of Drugs in Plasma

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.× 4.6mmI.D.)
Mobile phase (C)	100mM Phosphate(Na) buffer (pH=2.1 or 6.9) / Acetonitorile = 100/0 to 90/10 (v/v) (Optimized for each drug)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack VP-ODS (150mmL.×4.6mmI.D.)
Mobile phase	(A) : Buffer solution (shown in Table 6) (B) : Methanol Linear gradient (B) 15% to 85% (4 to 19min.)
Flow rate	1.0mL/min
Temperature	40°C
Detection	205nm, 235nm, 250nm, 280nm (SPD-M10A*VP*)

Table 8 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
4.00	B.CONC (Mobile phase B%)	15
8.00	C.FLOW (Smpl. Inj.. Sol.C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol.C mL/min)	0.2
19.00	B.CONC (Mobile phase B%)	85
23.00	B.CONC (Mobile phase B%)	85
23.01	B.CONC (Mobile phase B%)	15
23.02	RV.A (Hi.Pr. Selection valve position)	0
23.95	C.FLOW (Smpl. Inj. Sol.C mL/min)	0.2
24.00	C.FLOW (Smpl. Inj. Sol.C mL/min)	2
30.00	STOP	



7-2 Establishing Analysis Conditions

The analysis conditions are established based on the elution behavior information of section 7-1.

① Mobile Phase for Analysis

Analysis is performed using a gradient of phosphate buffer solution and methanol or acetonitrile.

The elution conditions can be determined by modifying the conditions (Table 7) so that the plasma matrix and drug are separated within the target concentration range. The conditions in the elution index (Table 7) apply to a methanol concentration range from 15 to 85%; however, by modifying them so that the range is from 50 to 70%, separation can be improved. If the drug peaks can be separated from the inherent plasma peaks, this does not necessarily make these conditions acceptable as the final elution conditions.

With respect to the elution index of Table 7, for the mobile phase, selecting a buffer solution with a pH that weakens retention will improve peak shape and facilitate separation from contaminants. For example, according to Table 6, verapamil shows a retention time of 21.9 minutes when using a neutral phosphate buffer solution (pH 6.9), but with the acidic phosphate buffer solution (pH 2.5), retention time becomes 17.8 minutes, indicating that the acidic buffer solution (pH 2.5) should provide a more favorable separation.

In addition, for basic drugs, which show distorted or tailing peak shapes, adding 100mM of sodium perchlorate can improve the effectiveness of the mobile phase.

② Sample Introduction (Injection) Solution

In general, solutions such as phosphoric acid, acetic acid or formic acid to which 5 - 10% acetonitrile has been added are used. Buffer solutions with a pH of 2 - 7.5 can be selected, but to prevent clogging of the flow lines with insoluble proteins in the plasma, a neutral buffer solution is recommended. However, acidic buffer solutions can be useful in cases of low recovery rate and low-volume injections.

Attention should be paid to whether the drugs are trapped with certainty and whether the peak shapes are distorted. Then, it is necessary to verify that recovery of the drugs from the plasma is adequate.

To increase the recovery rate of drugs from the plasma, set the salt concentration on the high side for ionic drugs. In the case of hydrophobic drugs, increase the amount of acetonitrile added to the sample injection solution according to the strength of retention. However, to prevent clogging of the flow lines with insoluble proteins in the plasma, if possible, keep the amount of acetonitrile to less than 10%.

・Attention with Acidic Drugs

When using a neutral sample injection solution, ionic interaction causes considerable binding to proteins. To prevent a reduction in drug recovery, set a higher salt concentration for the sample injection solution. For weakly retained drugs, it is effective to use an acidic pH value.

It is necessary to note that if the pH is near the drug pKa value, distortion of the peak shape may occur.

・Attention with Basic Drugs

Neutral sample injection solutions are suitable because they enable strong drug retention. Depending on the strength of retention, increase the amount of acetonitrile added to the sample injection solution. In addition, similarly as with the mobile phase, it is effective to add sodium perchlorate to improve peak shape and to slightly strengthen retention.

③ Analytical Column

Normally, a reversed-phase ODS column is used for drug analysis. For example, the Shim-pack VP-ODS and Shim-pack FC-ODS (for high-speed analysis) are used.

④ Detection

Generally, the wavelength with the greatest absorbance by the target component is selected as the detection wavelength. In cases where separation from the plasma matrix is inadequate, it is also recommended to select the wavelength at which the co-eluting compounds least affect the detection. The photodiode array detector is particularly useful and effective in this respect.

8. Application Examples

8-2 Considerations in Setting Conditions

In the following application examples, the analysis conditions were set based on “7. Analytical Conditions”. The considerations involved in setting the analysis conditions are summarized below.

Note that all samples consisted of control human plasma filtered beforehand through a 0.45μm membrane filter, and to which drugs were added.

① Mobile Phase Conditions

The elution index for the acidic and neutral mobile phases was used to select the mobile phase with the pH would provide the easiest separation from the inherent peaks of the blood plasma. In addition, the gradient conditions were set in consideration of the separation from the plasma-inherent peaks as well as the analysis time. At this time, column cleaning is also involved, so the gradient’s final methanol concentration was raised to 85% whenever possible. Depending on the sample, tailing peaks do occur, so in these cases, sodium perchlorate was added with the intention of improving the peak shape.

② Sample Introduction (Injection) Solution

Primarily, a solution containing up to 10% acetate buffer solution or acetonitrile was used. In the case of highly hydrophobic drugs, up to 15% acetonitrile was added.

③ Column for Analysis

To shorten the analysis time, the Shim-pack FC-ODS high-throughput, high separation column (Shimadzu Corp.) was used whenever possible. In addition, the Shim-pack VP-ODS general-use ODS column was also used.

④ Detection

A photodiode array detector was used, and wavelengths were selected so that the peaks of contaminants in the blood plasma did not overlap with those of the target drugs.

8-2 Analysis of Phenytoin

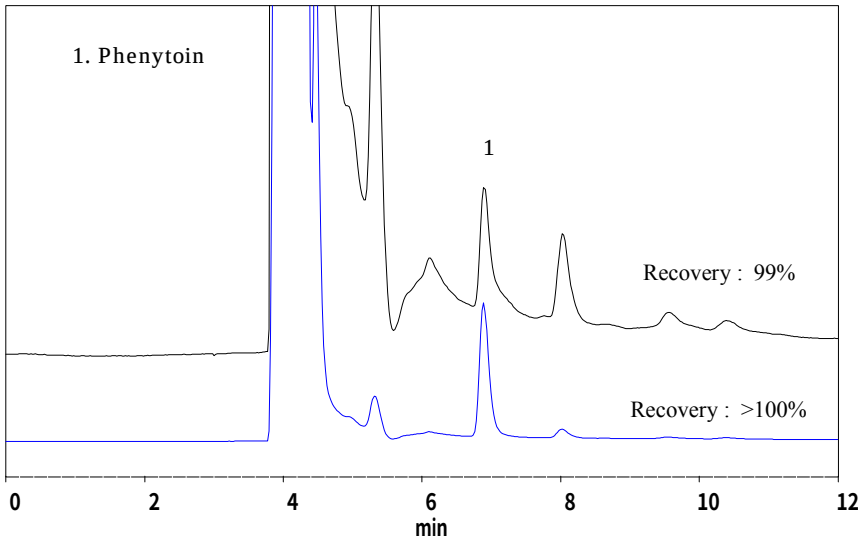


Figure 6 : Chromatogram of Phenytoin in Plasma
Upper: 0.2µg/mL spiked, 50µL injected
Lower: 2µg/mL spiked, 50µL injected

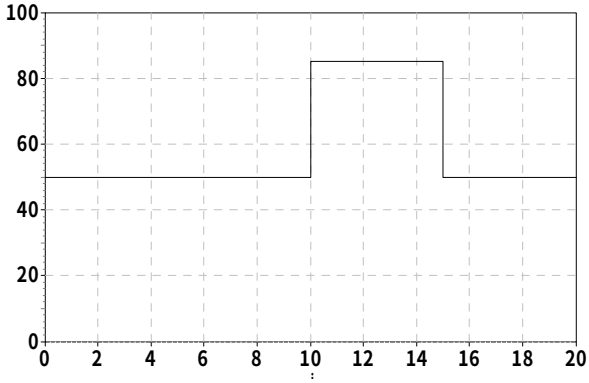
Table 9 Analytical Conditions

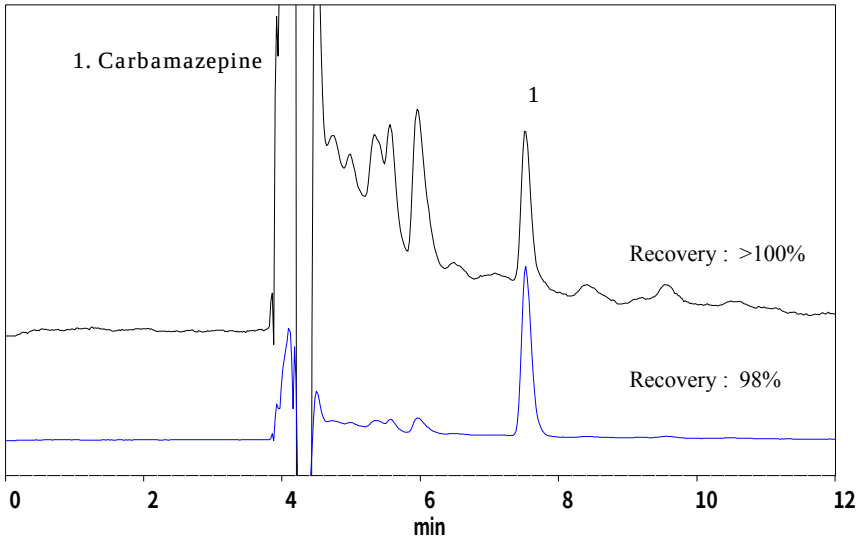
For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	100mM Acetate (Na) buffer (pH=4.7) / Acetonitrile= 95 /5 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack FC-ODS (75mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=2.5) (B) : Methanol (A) / (B) = 50 / 50 (v/v)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 210nm

Table 10 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
10.00	B.CONC (Mobile phase B%)	50
10.01	B.CONC (Mobile phase B%)	85
15.00	B.CONC (Mobile phase B%)	85
15.01	B.CONC (Mobile phase B%)	50
15.02	RV.A (Hi.Pr. Selection valve position)	0
15.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
16.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
20.00	STOP	





8-3 Analysis of Carbamazepine

Figure 7 : Chromatogram of Carbamazepine in Plasma
Upper: 0.1μg/mL spiked, 50μL injected
Lower: 1μg/mL spiked, 50μL injected

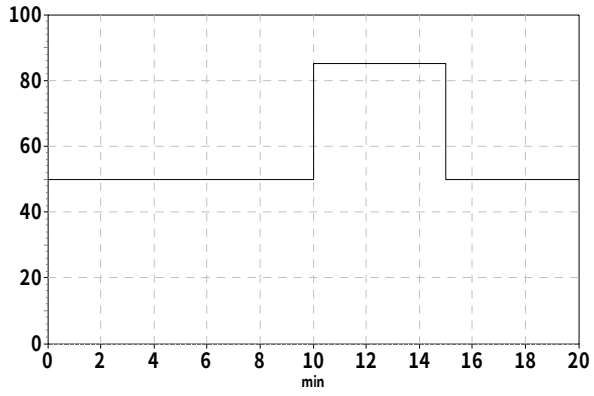
Table 11 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	100mM Acetate (Na) buffer (pH=4.7) / Acetonitrile= 95 /5 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack FC-ODS (75mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=2.5) (B) : Methanol (A) / (B) = 50 / 50 (v/v)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 300nm

Table 12 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
10.00	B.CONC (Mobile phase B%)	50
10.01	B.CONC (Mobile phase B%)	85
15.00	B.CONC (Mobile phase B%)	85
15.01	B.CONC (Mobile phase B%)	50
15.02	RV.A (Hi.Pr. Selection valve position)	0
15.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
16.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
20.00	STOP	



8-4 Analysis of Diazepam

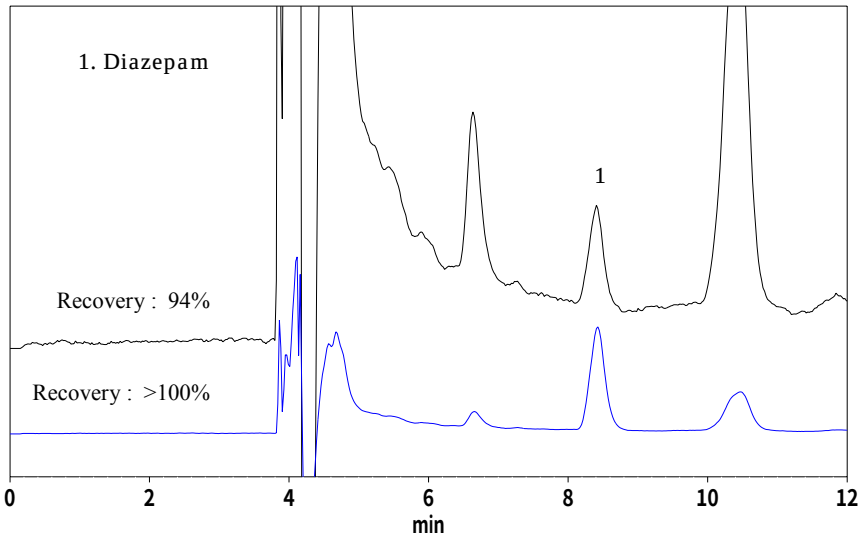


Figure 8 : Chromatogram of Diazepam in Plasma
Upper: 0.2µg/mL spiked, 50µL injected
Lower: 2µg/mL spiked, 50µL injected

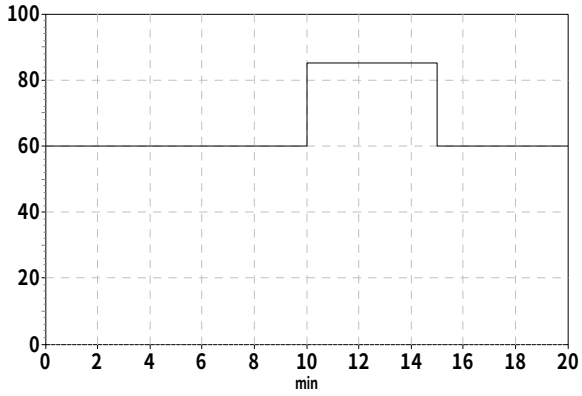
Table 13 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	100mM Acetate (Na) buffer (pH=4.7) / Acetonitrile = 95 /5 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack FC-ODS (75mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=2.5) (B) : Methanol (A) / (B) = 40 / 60 (v/v)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 312nm

Table 14 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
10.00	B.CONC (Mobile phase B%)	60
10.01	B.CONC (Mobile phase B%)	85
15.00	B.CONC (Mobile phase B%)	85
15.01	B.CONC (Mobile phase B%)	60
15.02	RV.A (Hi.Pr. Selection valve position)	0
15.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
16.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
20.00	STOP	



8-5 Analysis of Reserpine

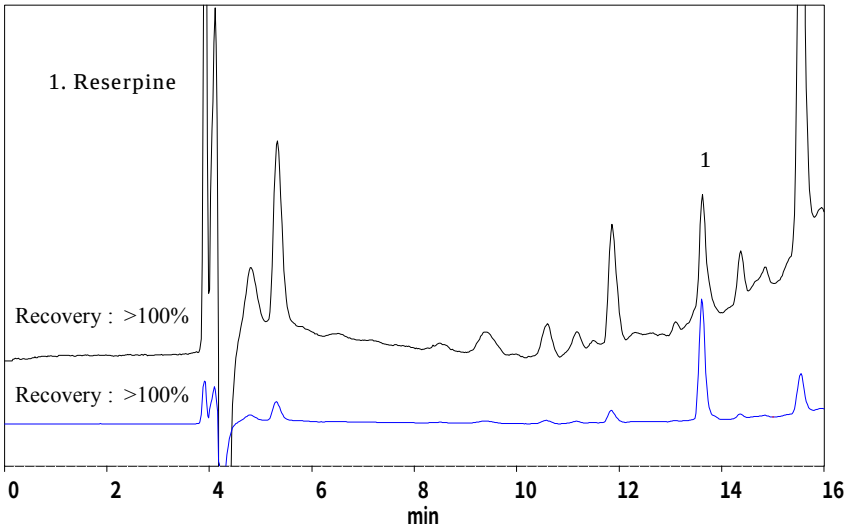


Figure 9 : Chromatogram of Reserpine in Plasma
Upper: 0.1μg/mL spiked, 50μL injected
Lower: 1μg/mL spiked, 50μL injected

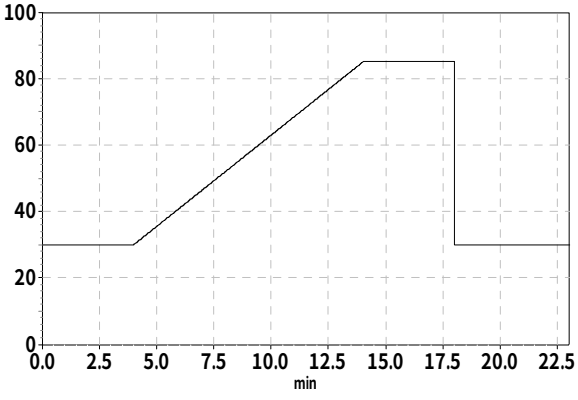
Table 15 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	100mM Acetate (Na) buffer (pH=4.7) / Acetonitrile = 95 /5 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack FC-ODS (75mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=2.5) 100mM Sodium perchlorate (B) : Methanol Linear gradient (B) 30% to 85% (4 to 14min.)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 300nm

Table 16 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
4.00	B.CONC (Mobile phase B%)	30
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
14.00	B.CONC (Mobile phase B%)	85
18.00	B.CONC (Mobile phase B%)	85
18.01	B.CONC (Mobile phase B%)	30
18.02	RV.A (Hi.Pr. Selection valve position)	0
18.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
19.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
23.00	STOP	



8-6 Analysis of Phenylbutazone

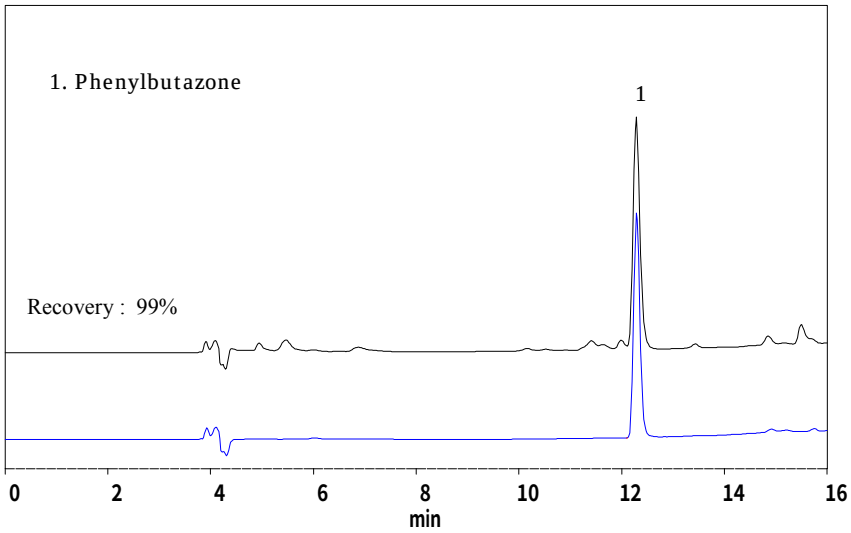


Figure 10 : Chromatogram of Phenylbutazone in Plasma
Upper: 4μg/mL spiked, 50μL injected
Lower: 4μg/mL of the standard drug, 50μL injected

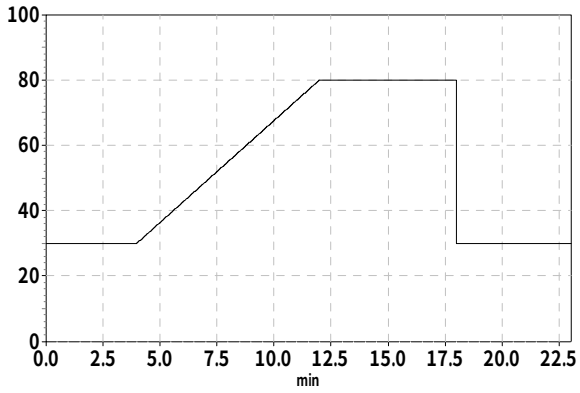
Table 17 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	100mM Acetate (Na) buffer (pH=4.7) / Acetonitrile = 95 /5 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack FC-ODS (75mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=6.9) 100mM Sodium perchlorate (B) : Methanol Linear gradient (B) 30% to 80% (4 to 12min.)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 265nm

Table 18 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
4.00	B.CONC (Mobile phase B%)	30
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
12.00	B.CONC (Mobile phase B%)	80
18.00	B.CONC (Mobile phase B%)	80
18.01	B.CONC (Mobile phase B%)	30
18.02	RV.A (Hi.Pr. Selection valve position)	0
18.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
19.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
23.00	STOP	



8-7 Analysis of Warfarin

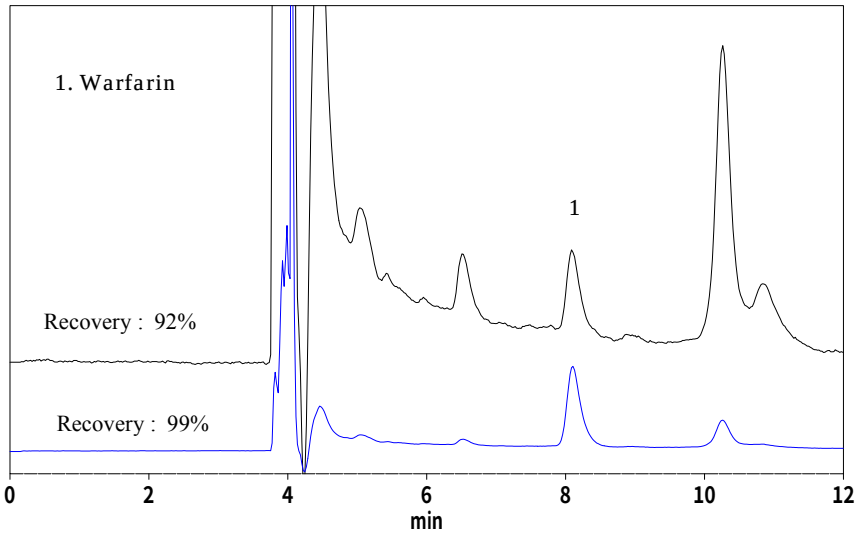


Figure 11 : Chromatogram of Warfarin in Plasma
Upper: 0.1μg/mL spiked, 50μL injected
Lower: 1μg/mL spiked, 50μL injected

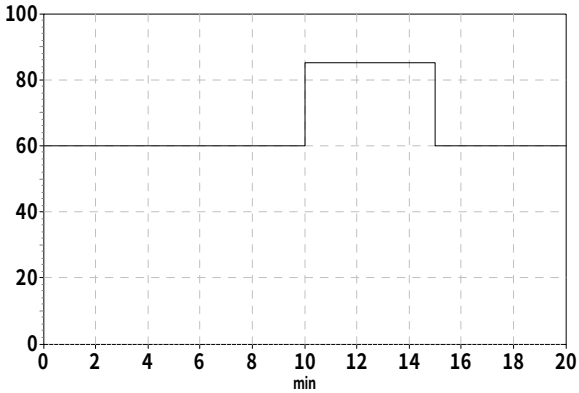
Table 19 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	100mM Acetate (Na) buffer (pH=4.7) / Acetonitrile = 95 /5 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack FC-ODS (75mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=2.5) (B) : Methanol (A) / (B) = 40 / 60 (v/v)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 315nm

Table 20 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
10.00	B.CONC (Mobile phase B%)	60
10.01	B.CONC (Mobile phase B%)	85
15.00	B.CONC (Mobile phase B%)	85
15.01	B.CONC (Mobile phase B%)	60
15.02	RV.A (Hi.Pr. Selection valve position)	0
15.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
16.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
20.00	STOP	



8-8 Analysis of Naproxen

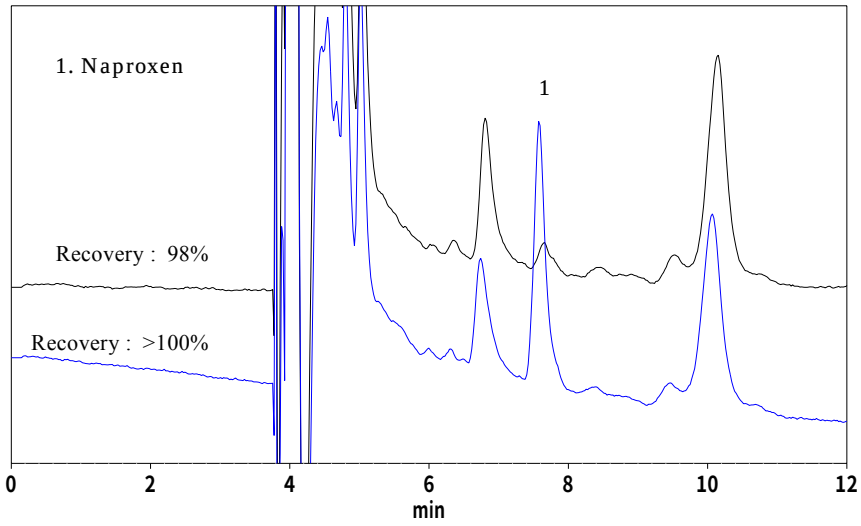


Figure 12 : Chromatogram of Naproxen in Plasma
Upper: 0.1µg/mL spiked, 50µL injected
Lower: 1µg/mL spiked, 50µL injected

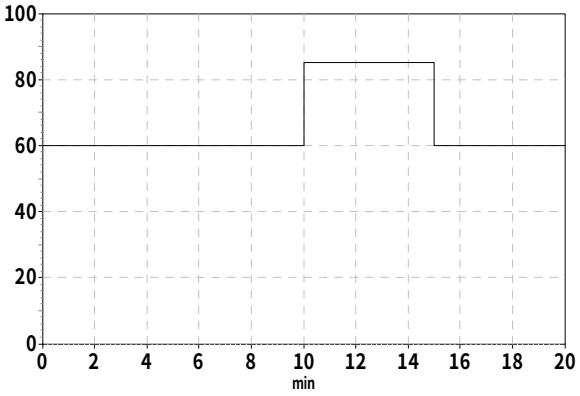
Table 21 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	0.1% Phosphoric acid / Acetonitrile = 95 /5 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack FC-ODS (75mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=2.5) 100mM Sodium perchlorate (B) : Methanol (A) / (B) = 40 / 60 (v/v)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 330nm

Table 22 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
10.00	B.CONC (Mobile phase B%)	60
10.01	B.CONC (Mobile phase B%)	85
15.00	B.CONC (Mobile phase B%)	85
15.01	B.CONC (Mobile phase B%)	60
15.02	RV.A (Hi.Pr. Selection valve position)	0
15.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
16.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
20.00	STOP	



8-9 Analysis of Phenobarbitol, Phenytoin and Carbamazepine

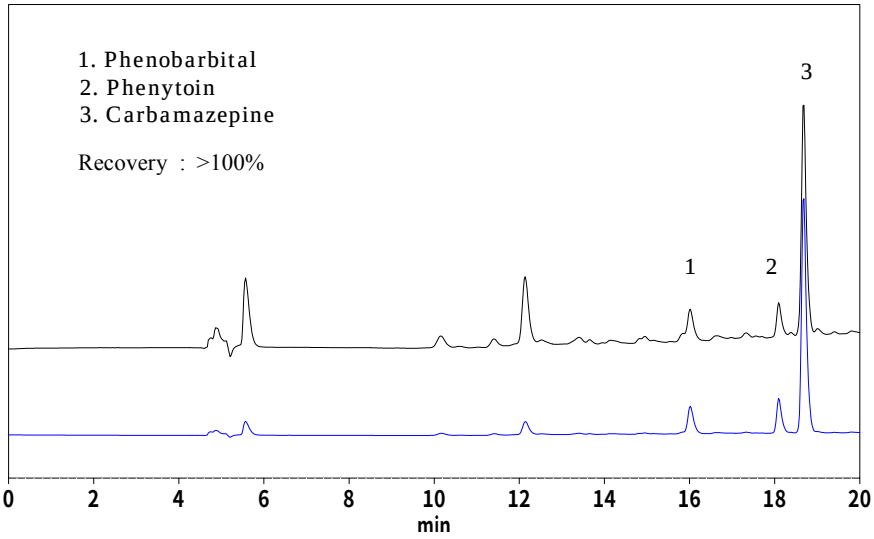


Figure 13 : Chromatogram of Phenobarbital, Phenytoin and Carbamazepine in Plasma
Upper: Spiked with 2μg/mL of each drug, 50μL injected
Lower: Spiked with 10μg/mL of each drug, 50μL injected

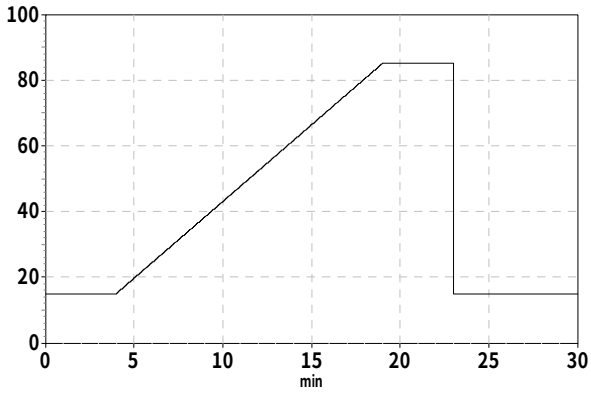
Table 23 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	100mM Acetate (Na) buffer (pH=4.7)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack VP-ODS (150mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=2.5)
	(B) : Methanol
	Linear gradient (B) 15% to 85% (4 to 19min.)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 250nm

Table 24 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
4.00	B.CONC (Mobile phase B%)	15
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
19.00	B.CONC (Mobile phase B%)	85
23.00	B.CONC (Mobile phase B%)	85
23.01	B.CONC (Mobile phase B%)	15
23.02	RV.A (Hi.Pr. Selection valve position)	0
23.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
24.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
30.00	STOP	



8-10 Analysis of 6 Acidic Drug Compounds

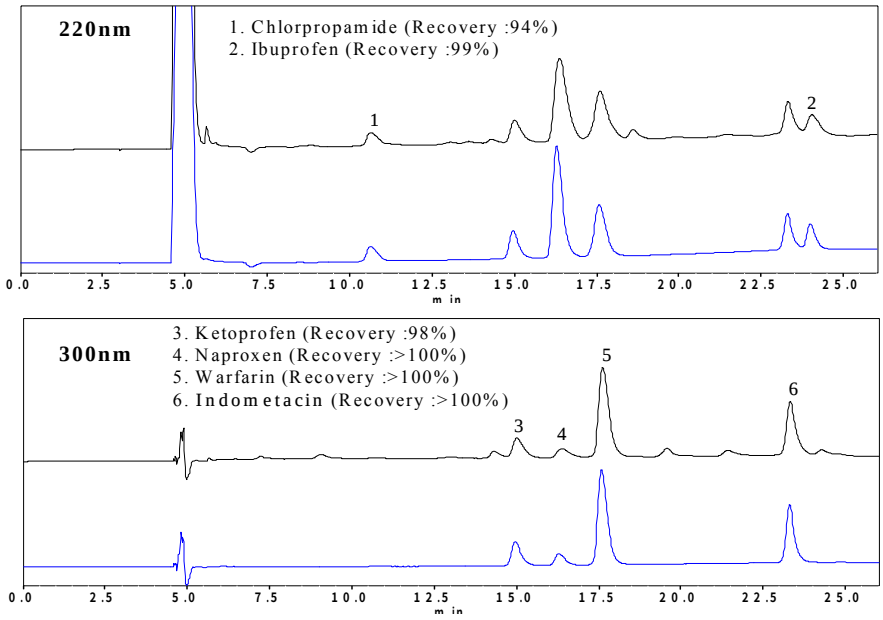


Figure 14 : Chromatogram of 6 Drugs in Plasma

Upper: Spiked with 2µg/mL of each drug, 100µL injected

Lower: 2µg/mL of each standard drug, 100µL injected

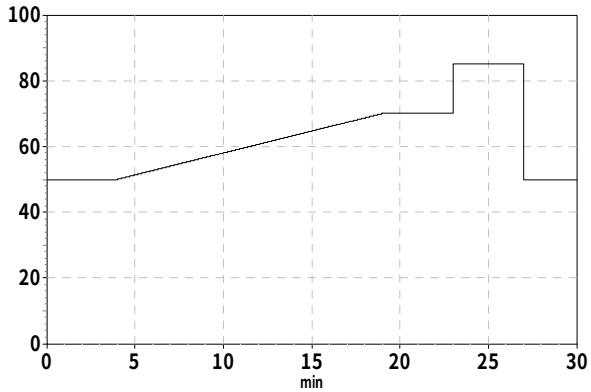
Table 25 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	100mM Acetate (Na) buffer (pH=4.7) / Acetonitrile = 90 / 10 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack VP-ODS (150mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=2.5)
	(B) : Methanol
	Linear gradient (B) 50% to 70% (4 to 19min.)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 220nm, 300nm

Table 26 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
4.00	B.CONC (Mobile phase B%)	50
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
19.00	B.CONC (Mobile phase B%)	70
23.00	B.CONC (Mobile phase B%)	70
23.01	B.CONC (Mobile phase B%)	85
27.00	B.CONC (Mobile phase B%)	85
27.01	B.CONC (Mobile phase B%)	50
27.02	RV.A (Hi.Pr. Selection valve position)	0
27.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
28.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
30.00	STOP	



8-11 Analysis of 8 Basic Drug Compounds

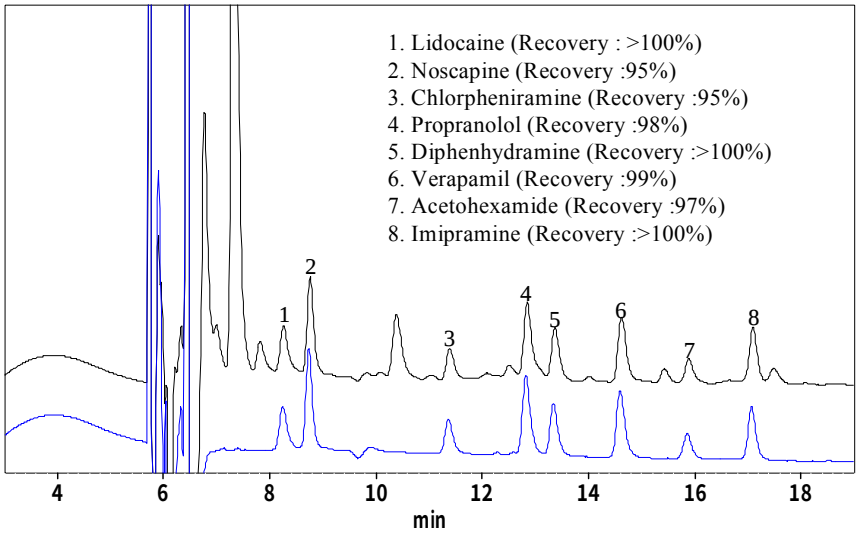


Figure 15 : Chromatogram of 8 Drugs in Plasma

Upper: Spiked with 0.5µg /mL of each drug, 50µL injected

Lower: 0.5µg /mL of each standard drug, 50µL injected

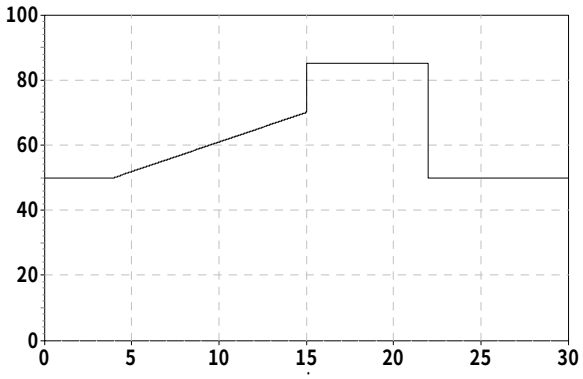
Table 27 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	100mM Acetate (Na) buffer (pH=4.7)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack VP-ODS (250mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=2.5) 100mM Sodium perchlorate (B) : Methanol Linear gradient (B) 50% to 70% (4 to 15min.)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 205nm

Table 28 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
4.00	B.CONC (Mobile phase B%)	50
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
15.00	B.CONC (Mobile phase B%)	70
15.01	B.CONC (Mobile phase B%)	85
22.00	B.CONC (Mobile phase B%)	85
22.01	B.CONC (Mobile phase B%)	50
22.02	RV.A (Hi.Pr. Selection valve position)	0
22.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
23.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
30.00	STOP	



8-12 Analysis of Ibuprofen

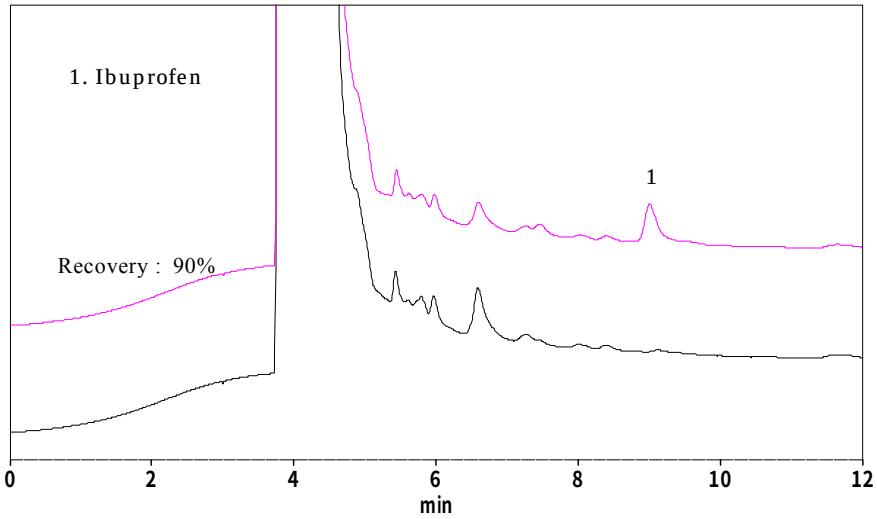


Figure 16 : Chromatogram of Ibuprofen in Plasma

Upper: 1μg/mL spiked, 50μL injected

Lower: Not spiked, 50μL injected

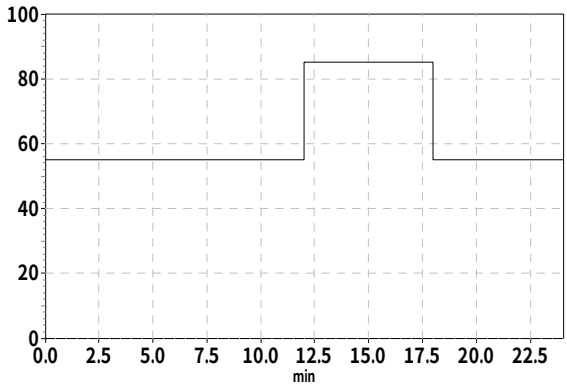
Table 28 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	100mM Acetate (Na) buffer (pH=4.7) / Acetonitrile = 90 / 10 (v/v)
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack FC-ODS (75mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=6.9) (B) : Methanol (A) / (B) = 45 / 55 (v/v)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 210nm

Table 29 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
8.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
8.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
12.00	B.CONC (Mobile phase B%)	55
12.01	B.CONC (Mobile phase B%)	85
18.00	B.CONC (Mobile phase B%)	85
18.01	B.CONC (Mobile phase B%)	55
18.02	RV.A (Hi.Pr. Selection valve position)	0
18.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
19.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
24.00	STOP	



8-13 Analysis of Cefloxim

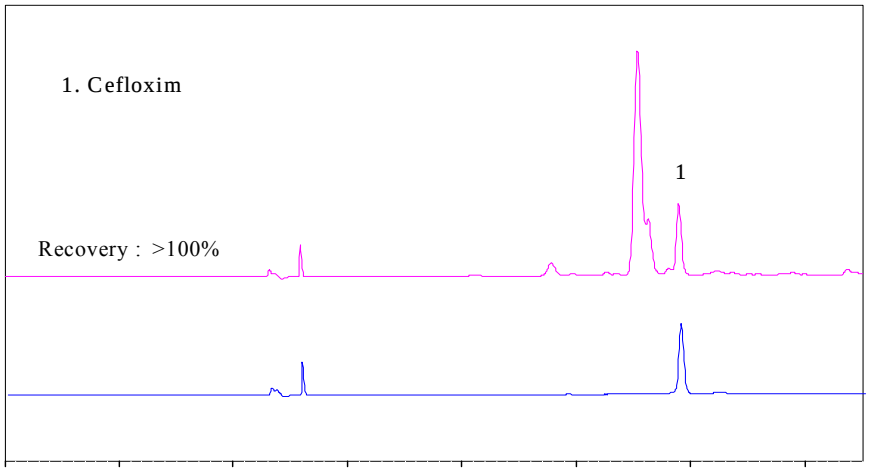


Figure 17 : Chromatogram of Cefloxime in Plasma
Upper: Spiked with 2µg/mL of each drug, 50µL injected
Lower: 2µg/mL of standard drug, 50µL injected

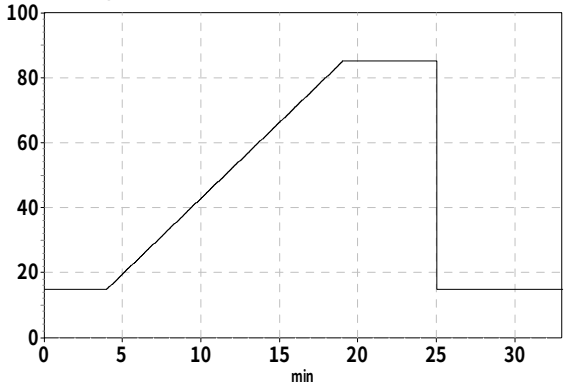
Table 30 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	20mM Phosphate (Na) buffer (pH=2.5) 100mM Sodium perchlorate
Flow rate	2.0mL/min
Dilution factor	8

For Separation	
Analytical column	Shim-pack VP-ODS (75mmL.×4.6mmI.D.)
Mobile phase	(A) : 20mM Phosphate (Na) buffer (pH=2.5) 100mM Sodium perchlorate (B) : Methanol Linear gradient (B) 15% to 85% (4 to 19min.)
Flow rate	1.0mL/min
Temperature	40°C
Detection	SPD-M10A*VP* at 270nm

Table 31 : Time Program

Time (min)	Function	Value
3.00	RV.A (Hi.Pr. Selection valve position)	1
4.00	B.CONC (Mobile phase B%)	15
6.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
6.10	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
19.00	B.CONC (Mobile phase B%)	85
25.00	B.CONC (Mobile phase B%)	85
25.01	B.CONC (Mobile phase B%)	15
25.02	RV.A (Hi.Pr. Selection valve position)	0
25.95	C.FLOW (Smpl. Inj. Sol. C mL/min)	0.2
26.00	C.FLOW (Smpl. Inj. Sol. C mL/min)	2
30.00	STOP	



8.14 Analysis of 4 Drug Compounds in Plasma using LCMS

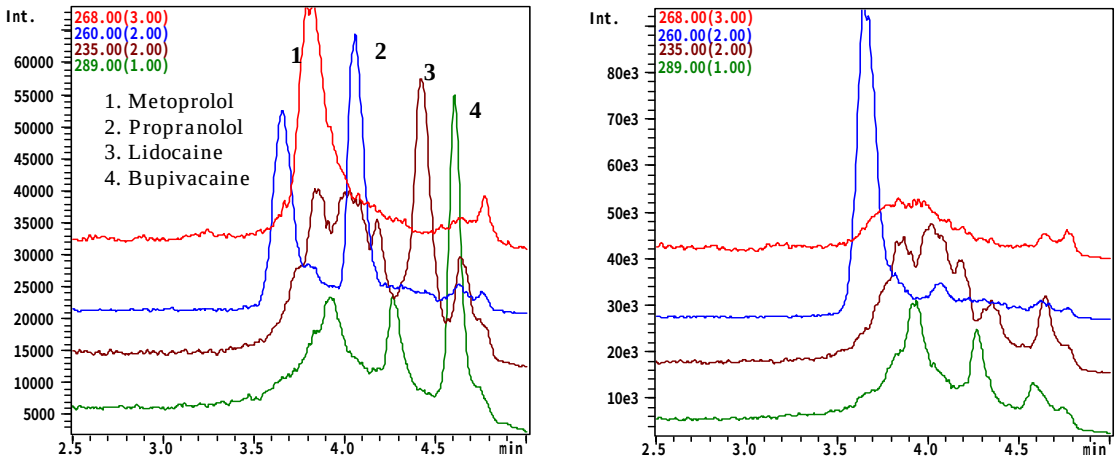


Figure 18 : SIM Chromatograms of 4 Drugs in Rat Plasma

Left: Spiked with 1ng/mL of each drug, 50µL injected, Right: Not spiked, 50µL injected

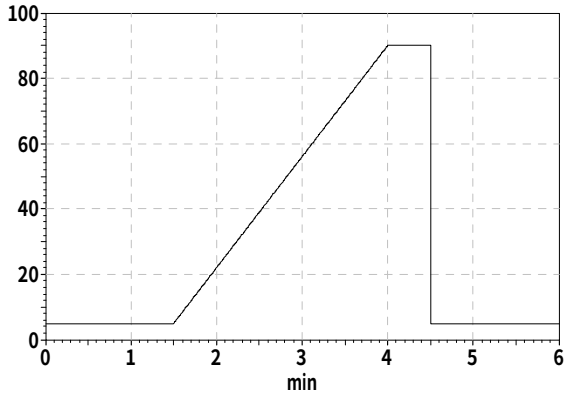
Table 32 : Analytical Conditions

For Sample Injection	
Pretreatment column	Shim-pack MAYI-ODS (10mmL.×4.6mmI.D.)
Mobile phase (C)	10mM Ammonium acetate / Acetonitrile = 95 / 5 (v/v)
Flow rate	3.0mL/min
Dilution factor	8

For Separation	
Analytical column	Phenomenex Mercury MS LUNA 5C18(2) (10mmL.×4.0mmI.D.)
Mobile phase	(A) : 10mM Ammonium acetate (B) : Acetonitrile Linear gradient (B) 5% to 90% (1.5 to 4min.)
Flow rate	0.5mL/min
Temperature	40°C
Detection	Ionization---Electrospray, positive Probe voltage---+4.5kV Nebulizing gas flow---1.5L/min

Table 33 : Time Program

Time (min)	Function	Value
1.50	B.CONC (Mobile phase B%)	5
2.00	RV.A (Hi.Pr. Selection valve position)	1
4.00	B.CONC (Mobile phase B%)	90
4.50	B.CONC (Mobile phase B%)	90
4.51	B.CONC (Mobile phase B%)	5
4.52	RV.A (Hi.Pr. Selection valve position)	0
6	STOP	



9. Acknowledgement

We would like to express our appreciation to Dr. Naoki Asakawa of Eisai Co., Ltd., co-developer of Co-Sense for BA, for his cooperation in the writing of this paper.

10. References

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Printed in Japan XXXX-XXXXX-00ATD