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Rapid, real-time sampling of R-84760 in blood by in vivo microdialysis with tandem mass spectrometry

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Abstract

A new technique involving rapid sampling of R-84760 in real-time was achieved using a combination of microdialysis (MD) and tandem mass spectrometry (MS/MS). After collecting the analyte in real-time by MD and separating it by MS/MS, the ion intensities are adapted to the data without any subsequent chromatographic separation or flow injection analysis. The R-84760 concentration was obtained from the plateau part of the ion intensities or the corrected values using an internal standard, after immersing the MD probe into the dialysis solution containing the drug for a definite time. Since contamination of the ion source was prevented by using an organic solvent for the perfused solution, it was possible to establish a stable analysis method. For an MD membrane of length 4 mm, the R-84760 concentration in saline was linear over the range 5–541.1 ng/ml ($r^2 = 0.9997$). Moreover, the R-84760 concentration in rat whole blood was linear over the range 24.9–1868.9 ng/ml ($r^2 = 0.9993$). As this method allowed the measurement of free drug concentration in rat blood, the analysis was also able to provide data needed for determining the protein-binding ratio. The protein-binding ratio obtained from the calibration curve in saline and rat whole blood was 87–90%, which was close to the result obtained by another analysis method. The concentration profile of R-84760 in blood as obtained by the MD-MS/MS method correlated well with the concentration profile in plasma, which was simultaneously monitored by LC-MS/MS.

Keywords: Microdialysis-tandem mass spectrometry; Real-time analysis; R-84760, in vivo protein binding; Rat whole blood

1. Introduction

Quantitative drug analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) has rapidly increased due to advances in ionization techniques. It is obvious that LC-MS/MS is more popular than other instrumental analyses, except for the high initial cost of the equipment, so it is often the first choice as an analytical technique for the measurement of drug concentrations in biological fluids.

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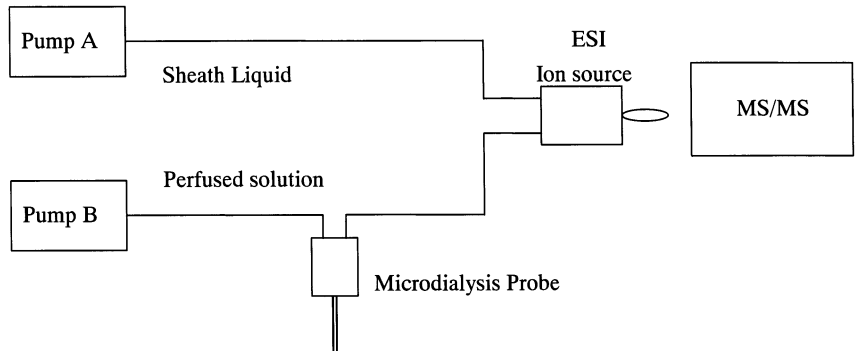

Fig. 1. Schematic manifold of MD-MS/MS system.

High selectivity is one of the features of LC-MS/MS. Impurities are hardly detectable following the separation of the second MS and only one peak derived from the analyte is often observed in the mass chromatogram. Chromatographic separation is not essential for LC-MS/MS. The role of the LC column becomes more important to avoid ion suppression. It is able to determine the analyte, following a suitable clean-up procedure, by flow injection analysis [1]. Therefore, we paid close attention to the selectivity of MS/MS. We attempted to develop a new analysis method by applying a combination of microdialysis (MD) and MS/MS without any chromatographic separation or flow injection analysis.

MD is useful for monitoring an analyte dynamically because endogenous and exogenous substances can be sampled directly and continuously from the target organ or matrix. MD is widely used in physiology and pharmacology, especially in the neurosciences. It has also been applied to pharmacokinetic and pharmacodynamic studies, e.g. protein binding [2–4]. A few online analyses by coupling MD and MS/MS have already been reported [5,6]. It has also been reported that the drug concentration in blood after its administration to experimental animals can be monitored in real-time [5,6]. However, these methods are the same as a conventional LC-MS/MS or flow injection analysis, after the sample collected by MD is stored in the loop of a high-pressure valve at a definite time. These systems are not efficient enough for continuous sampling, which is one of the features of MD. In this report, we describe the development of a real-time analysis method achieved by continuously and directly introducing the perfusate into the ion source without any chromatographic separation and then adapting the ion intensity to the data.

A previous report described the adaptation of the ion intensity to data for a qualitative analy-

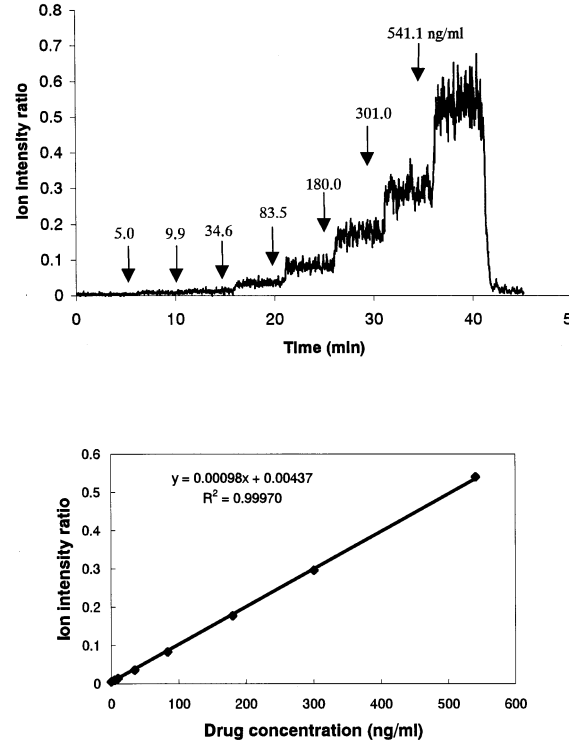

Fig. 2. MD-MS/MS chart and calibration curve of R-8476 in saline.

Table 1
Validation data obtained from calibration curve of R-84760 in saline[a]

Concentration (ng/ml)	Mean ($n = 100$)	S.D.	Accuracy (%)	CV (%)		
				a	b	c
Ion intensity ratio (R-84760/I.S.)						
0.0	0.0044	0.0018	–	41.9	21.9	17.9
5.0	0.0086	0.0026	12.5	29.7	16.5	12.9
9.9	0.0129	0.0043	11.8	33.3	18.0	11.6
34.6	0.0352	0.0069	8.9	19.5	12.1	8.9
83.5	0.0829	0.0121	4.0	14.6	9.8	6.7
180.0	0.1764	0.0197	2.5	11.2	7.2	5.3
301.0	0.2956	0.0297	1.3	10.0	7.4	6.2
541.1	0.5392	0.0493	−0.9	9.1	5.7	4.3
	$r^2 = 0.9997$					
Ion intensity						
0.0	10134	4245	–	41.9	22.1	18.2
5.0	20005	6202	6.4	31.0	17.1	12.6
9.9	30141	10055	4.7	33.4	17.7	11.5
34.6	81304	15026	2.9	18.5	11.5	8.7
83.5	188949	26695	−1.0	14.1	10.2	7.1
180.0	398112	38697	−1.7	9.7	5.7	4.5
301.0	650460	60712	−0.4	9.3	6.3	6.1
541.1	1153592	88010	0.3	7.6	5.5	4.8
	$r^2 = 0.9999$					

[a] The CV was calculated from raw data (a, $n = 100$), averaging sequential data at 3 (b, $n = 33$), or 5 (c, $n = 20$) points.

sis[7], but until now it has not been adapted for quantitative calculations. Through the latter approach, we were able to develop a method to rapidly sample R-84760 in real time.

R-84760, a novel central analgesic agent [8,9], was selected as the drug to be measured. R-84760 acts at very low concentrations and its disposition has already been reported following quantitative analysis using a highly sensitive and specific LC-MS/MS method [10]. This report describes the results of an evaluation of the assay and the determination of the drug concentration profile in blood after intravenous administration of R-84760 to rats using a new MD-MS/MS method.

2. Experimental

2.1. Materials

R-84760 and d_8-R-84760 ((3R)-3-(1-d_8-pyrrolidinylmethyl)-4-[(1,S)-5,6-dichloro-1-indancalbonyl]-tetrahydro-1,4-thiazine hydrochloride; internal standard, I.S.) were synthesized in the Medicinal Chemistry Research Laboratories, Sankyo (Tokyo, Japan). Other reagents and solvents were of analytical grade and were used without further purification.

2.2. System manifold and instrumentation

The manifold and operating conditions of the MD-MS/MS system are shown in Fig. 1. A commercially available microdialysis probe was used (BAS, West Lafayette, IN, USA). The membrane of the MD probe was made from cuprophan which is resistant to organic solvents, and was 4 or 10 mm in length. Perfusate, which was composed of ethanol:water (1:1, v/v), was supplied to the experimental system at a flow rate of 10 μl/min by syringe pump (CMA/100, BAS). The sheath liquid consisted of acetic acid:methanol:

water (0.1:50:50, v/v/v) containing 10 ng/ml I.S. and the flow rate was maintained at 50 μl/min by syringe pump (Model 22, Harvard Apparatus, South Natick, MA, USA). All data was acquired on a triple-stage mass spectrometer (TSQ-700, Thermoquest, San Jose, CA, USA) equipped with an ESI ion source (Thermoquest). The mass spectrometer conditions were basically the same as in a previous report [10]. Modifications for the MD-MS/MS were as follows: the capillary temperature was 250°C; no auxiliary gas was used and the sheath gas pressure was 70 psi. R-84760 was monitored at m/z 328.2 from 399.2 and the I.S. was monitored at m/z 328.2 from 407.2 by the selected reaction monitoring (SRM) method. The scan ranges switched over at 1 s intervals.

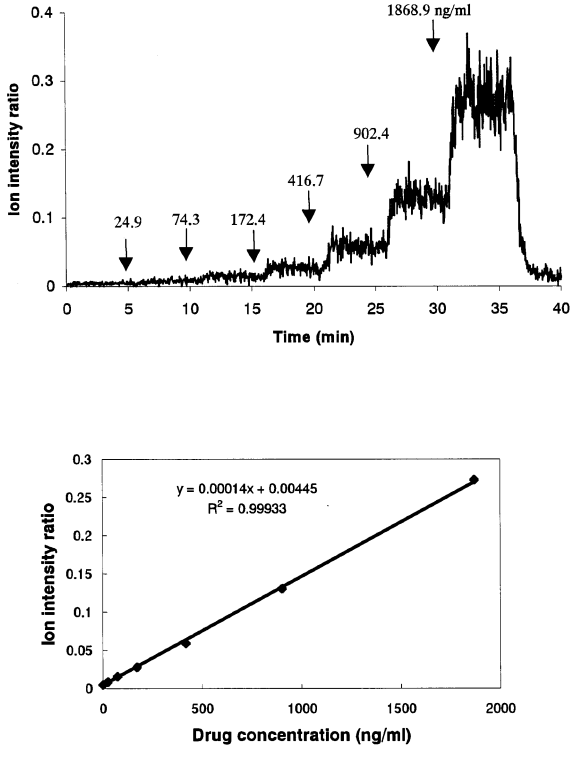

Fig. 3. MD-MS/MS chart and calibration curve of R-84760 in rat whole blood.

2.3. Animals

Male Sprague–Dawley (SD) rats (age: 8 weeks, body weight: 270 g) were used for the in vivo study or to obtain blood samples. Whole blood was collected by heparinized syringe and used immediately.

2.4. Calibration curve

The in vitro study of drug concentration measurement was performed by the titration method or the tube-exchange method. The calibration curve was constructed using the titration method. Saline or whole blood was stirred in a beaker and the probe was immersed. A suitable amount of R-84760 solution was introduced every 5 min while stirring the dialysate.

2.5. The drug profile in blood after intravenous administration of R-84760 to the rat

R-84760 (2 mg/ml) in 10% dimethylacetamide (DMA) solution was prepared for administration.

A rat was fixed to the anatomy board under ether anesthesia and the jugular veins exposed. The probe (membrane length: 10 mm) was inserted into the left jugular via a guide cannula, which was an improvement on the guide made by BAS. After the ion intensity of the I.S. reached steady-state, 2 mg/kg R-84760 was administered to the rat tail vein under anesthesia.

Rat whole blood spiked R-84760 was measured by the tube-exchange method in order to correct the concentration obtained by the MD-MS/MS method in the in vivo study. R-84760 (0.1 and 1 μg/ml) in rat whole blood was prepared in Eppendorf® tubes and determined by immersing the probe.

Intravenous blood was collected from the right jugular simultaneously to compare the MD-MS/MS and the conventional method. Two hundred microliters of rat blood was collected with a heparinized syringe at 2, 5, 10, 20, 30, and 60 min. The collected blood was centrifuged at 12 000 $\times g$ for 3 min and 50 μl plasma was deproteinized by adding 200 μl CH_3OH including 10 ng/ml I.S. (d_8-R-84760). After shaking and centrifuging, the supernatant was measured by LC-MS/MS.

Table 2
Validation data obtained from calibration curve of R-84760 rat whole blood[a]

Concentration (ng/ml)	Mean ($n = 100$)	S.D.	Accuracy (%)	CV (%)		
				a	b	c
Ion intensity ratio (R-84760/I.S.)						
0.0	0.0044	0.0016	–	36.5	17.0	11.7
24.9	0.0081	0.0028	−2.2	35.4	22.1	16.6
74.3	0.0151	0.0045	−1.1	29.8	13.4	11.8
172.4	0.0274	0.0065	6.2	23.5	11.5	10.9
416.7	0.0587	0.0110	8.3	18.7	11.3	6.4
902.4	0.1302	0.0152	1.9	11.7	6.9	5.7
1868.9	0.2724	0.0329	−0.9	12.1	7.3	5.7
	$r^2 = 0.9993$					
Ion intensity						
0.0	10404	3794	–	36.5	17.0	11.7
24.9	17138	6213	−22.8	36.3	22.1	16.6
74.3	27052	7974	−1.7	29.5	13.4	11.8
172.4	50515	11671	−5.5	23.1	11.5	10.9
416.7	99785	17130	2.7	17.2	11.3	6.4
902.4	199425	21580	5.0	10.8	6.9	5.7
1868.9	442669	40645	−4.9	9.2	7.3	5.7
	$r^2 = 0.9977$					

[a] The CV was calculated from raw data (a, $n = 100$), averaging sequential data at 3 (b, $n = 33$), or 5 (c, $n = 20$) points.

2.6. LC-MS/MS analysis

The plasma sample, which had been collected in the in vivo experiment, underwent protein precipitation. Two hundred microliters of methanol was added to 50 μl plasma. After shaking for 2 min and centrifuging, 10 μl supernatant was measured by the LC-MS/MS method as reported previously [10].

2.7. Data processing

Each ion intensity of R-84760 and I.S. was transferred to a second terminal. The ion intensity ratio was calculated by dividing the ion intensity of R-84760 by that of I.S. which was detected next. The data observed were plotted against the corresponding time axis.

In the case of determination in the static state, e.g. the calibration curve, the ion intensity or the ion intensity ratio were used in the plateau phase. The data were calculated as means of the plateau phase.

3. Results

3.1. Calibration curve of R-84760 in saline and rat whole blood

The calibration curve of R-84760 and the chart showing saline with MD membrane of length 4 mm are shown in Fig. 2 and Table 1. The calibration curve of R-84760 and the chart showing rat whole blood are shown in Fig. 3 and Table 2. One hundred points of the ion intensity or its ratio

Table 3
Intra-day precision data for the analyses of R-84760 in saline[a]

Concentration (ng/ml)	CV (%)	
	Ion intensity	Ion intensity ratio
10.0	8.0	11.4
100.0	12.2	5.7
500.0	13.5	8.8

[a] The individual data was averaged ($n = 50$) and the analyses was repeated five times. ($n = 5$).

Table 4
Relationship between recovery from rat whole blood and probe membrane length[a]

Membrane length:	4 mm			10 mm		
Concentration (ng/ml)	Mean ion intensity	S.D.	CV(%)	Mean ion intensity	S.D.	CV (%)
0.0	10404	3794	36.5	14385	4588	31.9
24.9	17138	6213	36.3	63549	12684	20.0
74.3	27052	7974	29.5	153007	22846	14.9
172.4	50515	11671	23.1	313343	38709	12.4
416.7	99785	17130	17.2	720089	74419	10.3
902.4	199425	21580	10.8	1642373	140272	8.5
1868.9	442669	40645	9.2	3093040	245410	7.9
	$r^2 = 0.9977$			$r^2 = 0.9990$		

[a] Where $n = 100$.

Table 5
Protein binding ratio calculated by the in vitro MD-MS/MS method and ultracentrifuge method

	Concentration (ng/ml)	Calculated by	Protein binding ratio (%)
MD-MS/MS method	–	Ion intensity ratio	86.9
	–	Ion intensity	89.8
Ultra-centrifuge method	100	–	90.5*
	500	–	93.9*

* Where $n = 3$.

were extracted from the plateau phase and the mean calculated. A correlation coefficient of $r^2 = 0.9997$ was obtained over the range 5–541.1 ng/ml in saline by using the ion intensity ratio. The R-84760 concentration in rat whole blood was linear over the range 24.9–1868.9 ng/ml ($r^2 = 0.9993$). The CV was approximately 30% at low concentrations, but it was about 10% at higher concentrations. By averaging sequential data at three or five points, the CV was about twice as precise. The ion intensity of the I.S. did not fluctuate during determination of the calibration curve in both saline and rat whole blood (Tables 1 and 2). However, the precision was improved by I.S. correction as a result of the intra-day precision (Table 3).

The relation between the membrane length of the MD probe and the ion intensity in rat whole blood is shown in Table 4. The ion intensity with the MD membrane of length 10 mm was fivefold (or more) to that of a membrane of length 4 mm.

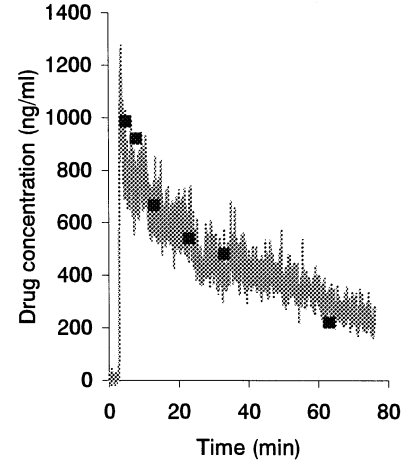

Fig. 4. Time-concentration profile of R-84760 in blood after intravenous administration to rat. Line: obtained by MD-MS/MS method. Closed square: obtained by LC-MS/MS method.

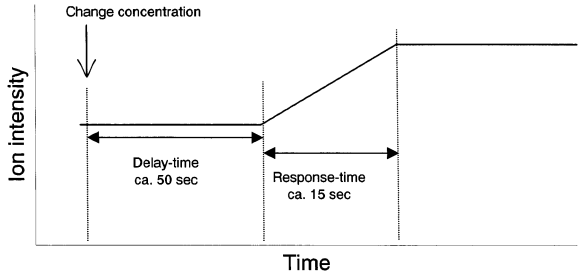

Fig. 5. Explanation of time parameter. The delay-time and response-time values were calculated from the calibration curve.

3.2. Protein binding ratio obtained from the calibration curve

The R-84760 concentration in saline and whole blood was assumed to represent the total and free drug concentration, respectively. The protein-binding ratio was calculated from the following expressions.

Protein binding ratio = {(Cout, saline-Cout, protein)/Cout, saline}*100.

Cout, protein: drug concentration in perfusate when rat whole blood is used for dialysate.

Cout, saline: drug concentration in perfusate when saline is used for dialysate.

Each drug concentration was obtained from the slope of the calibration curve. Cout, saline was 2046.82 as calculated by ion intensity and 0.0009872 as calculated by the ion intensity ratio. Cout, protein was obtained from the slope of the calibration curve over the range 24.9–416.7 ng/ml using the corresponding range of the calibration curve in saline (Table 1). Cout, protein was 209.38, calculated by ion intensity and 0.0001295, calculated by ion intensity ratio. Thus, the protein-binding ratio was 89.8%, calculated by ion intensity and 86.9%, calculated by ion intensity ratio (Table 5).

3.3. The blood concentration of R-84760 after intravenous administration to rat

The concentration profile of R-84760 in rat blood by MD-MS/MS after i.v. administration is shown in Fig. 4. The drug concentration in rat whole blood, which had been measured by the tube-exchange method before the in vivo study, was extrapolated to the ordinate in Fig. 4. The ion intensity increased sharply 50 s after administration. Then, the profile showed a C_{max} after 30 s, followed by a gradual attenuation phase. This profile was appropriate for i.v. administration and was followed for 1 h or more.

The plasma concentration profile, which was simultaneously monitored, by LC-MS/MS is also shown in Fig. 4. The R-84760 concentration profile in blood by the MD-MS/MS method correlated well with that in plasma by the LC-MS/MS method following correction by the parameter on the time axis.

4. Discussion

4.1. Avoiding the effect of non-volatile salt

It is possible to find the extent of interference from non-volatile salt by monitoring the ion intensity of the I.S. or the current value of the ion source. Since non-volatile salt interferes with ionization, or contaminates the ion source, it is difficult to perform stable analysis by LC-MS. Physiological salt solutions like Ringer's, containing large amounts of non-volatile salts, are usually used for microdialysis perfusates. Moreover, the salt recovered from the dialyzed solution also interferes with the analysis. Therefore, the system was improved as follows.

At first, saline was used as the perfusate, but the problem of the effect of non-volatile salt occurred. Next, H_2O was used instead of saline, but a stable determination was impossible because of the effect of salt derived from the dialyzed solution. Then, organic solvent was used for the perfusate [11].

The solvent consisted of water:ethanol (1:1) in consideration of the damage to the animal during the in vivo study. As a result, the perfusate allowed stable determination with the sheath liquid as described in the next paragraph. It is assumed that the perfusate reduced the recovery of the salt in the dialyzate.

The lower the flow rate of the LC, the higher the sensitivity of the ESI analysis. The LC flow rate is inconsistent with the absolute sensitivity of the ESI analysis [12,13]. However, if the analyte is not well-cleaned up and is measured at a low flow rate, the sensitivity is markedly reduced. Moreover, the minute hole in the heated capillary where the ion is introduced becomes heavily contaminated. Therefore, sheath liquid was used in expectation of the effects associated with dilution and diffusion of the perfusate (Fig. 1). The influence of the matrix was suppressed as much as possible.

There is a negative correlation between the flow rate of the perfusate and the recovery of the analyte [14]. The flow rate of perfusate is usually set up at under 5 μl/min to achieve good recovery of the analyte. However, it has been reported that the absolute recovery is improved in parallel with the flow rate up to 10 μl/min [15]. Moreover, a high rate is to be expected in order to improve the response, shorten the delay time and limit diffusion. Therefore, the flow rate of the perfusate was set at 10 μl/min in this system.

4.2. System configuration (especially the problem of pressure associated with the probe)

At first, a post-addition system was developed, which involved adding make-up liquid directly to the perfusate from the output of the probe. However, since the flow rate of the make-up liquid was five to ten times higher than that of the perfusate, the pressure in the whole system increased and the membrane of the probe ruptured. Therefore, make-up liquid was added as a sheath liquid by the spray process in the ion source (Fig. 1). While the conventional online system, in which an injector is connected, needs a restricted tube length because of the load to the probe [16], it is unnecessary to limit the tube length in this present system.

4.3. Correction by I.S.

Since the ion intensity fluctuates widely and is not reproducible, internal standard correction is routine for quantitative analysis by LC-MS/MS. It was considered that correction by I.S. was necessary to obtain reliable data in this analysis method [17]. Correction by adding a fixed amount of I.S. in the sheath liquid was attempted.

This correction did not cause a significant fluctuation in the ion intensity of the I.S. in the calibration curve (Tables 1 and 2), but it did lead to an improvement in the accuracy of the intraday precision (Table 3). Therefore, measurement of the ion intensity value of the I.S. is efficient for monitoring the fluctuations that are often observed during long-term analysis. Fluctuations in the analyte were synchronized to those of the I.S., so that correction was effective. Therefore, the I.S. was always determined simultaneously with the analyte. This correction is not efficient for fluctuations involving the microdialysis; e.g. recovery from the dialysis, but is for ionization and detection.

4.4. Microdialysis probe (especially material and inside diameter)

The polycarbonate used in microdialysis is generally not resistant to organic solvents. For this reason, cuprophan was used because it was resistant. The probe made from cuprophan has a small membrane dead volume, equal to 1 μl in a membrane of length 4 mm and this improved the response. It was suggested that there was correlation between the dead volume in the membrane and response to the concentration change of the analyte in the dialyzed solution.

The length of the probe membrane influences the recovery (Table 4). Probes with membrane lengths of 4 and 10 mm were examined. The longer membrane length allowed an increased recovery of both the analyte and the salt. Therefore, contamination in the ion source was accompanied by high recovery. The length of membrane depends on the aim of the analysis.

4.5. Time-axis parameter (response time when concentration changes; delay time; time resolution)

The delay-time described in this report indicates a lag-time in the detection for the dead volume in

the output part (Fig. 5). When the dead volume in the output part and the flow rate of perfusate are fixed, the delay time is reproducible. However, the flow line in the output part should be reduced as much as possible in order to suppress dispersion. The delay-time in this system was about 50 s, obtained as the time from changing the analyte concentration to the start of the change in ion intensity in the calibration curve (Figs. 1 and 2). The delay-time in the in vivo study was applied for this value.

The response-time described here is the time taken to attain the ion intensity corresponding to the concentration, when the concentration in the dialyzed solution is changed (Fig. 5). The response-time is the most important factor involved because real-time quantitative analysis needs a quick response. The response-time obtained from the calibration curve (Figs. 1 and 2) was approximately 15 s, calculated as the time required to shift the plateau value. It was considered that this response was good enough to monitor the blood drug concentrations.

The time-resolution depends on the scanning time of the analyte and I.S. One piece of data is obtained in 2 s since the scanning time required for the analyte and I.S. is 1 s for each. Therefore, the time-resolution is 2 s.

The response and delay times depend on the following factors: piping in the system, flow rate of the perfusate, stirring and composition of the dialyzed solution, probe dead volume and membrane length. To obtain a quick response or shorten the delay time, it is necessary to optimize these factors.

4.6. Evaluation of the MD-MS/MS method for quantitative analysis

One of the features of this method is that the ion intensity is used as a measurement parameter. Though the CV is approximately 10–30%, it is possible to obtain more accurate data by taking an average as shown in Tables 1 and 2. However, the data should be averaged in consideration of reducing the time-resolution.

Using ultracentrifugation, the protein-binding of R-84760 is 93–90% in rat serum (data was not shown), which is close to the value obtained from this analysis method.

When the in vivo study was terminated about 1 h after dosing, the amount of the salt adhering to the ion source did not have a major effect on the analysis. Moreover, it was the same pre- and post-calibration value in the in vivo study, so there was no probe effect on the recovery. It is anticipated that it will be possible to carry out determinations over a longer period.

It is assumed that the C_{max} value in the in vivo study was close to the C_o obtained from the extrapolation of the α phase in the intravenous administration study, taking into consideration the response to the concentration change. Thus, the MD-MS/MS method is very useful for studies that require a very quick response. It is no exaggeration to say that monitoring the analyte in tissues is very important during in vivo studies. When this MD-MS/MS method is applied to in vivo studies, it is not clear whether the organic solvent in the perfusate influences the recovery from body tissues.

It is very difficult to calculate pharmacokinetics parameters from numerical values supplied continuously by this analytical method using conventional software. It is possible to process data by conventional software by extracting some numerical values and calculating intermittently. However, the ideal would be to establish a technique that allows continuous analysis of all the data generated.

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