In Vivo Microdialysis for Pharmacokinetic Investigations: A Plasma Protein Binding Study of Valproate in Rabbits

Mikiro Nakashima, Naoko Takeuchi, Motoko Hamada, Kenji Matsuyama, Masataka Ichikawa, and Shigeru Goto

Department of Hospital Pharmacy, School of Medicine, Nagasaki University, 1–7–1 Sakamoto, Nagasaki 852, Japan and Faculty of Pharmaceutical Sciences, Kyushu University, 3–1–1 Maidashi, Higashi-ku, Fukuoka 812, Japan.

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The use of microdialysis to study the binding of valproate (VPA) to plasma proteins was evaluated in rabbits. Prior to an in vivo microdialysis, in vitro relative recovery of VPA respectively from Ringer's solution, 5% (w/v) of albumin solution and plasma sample via a microdialysis probe was examined. The in vitro relative recovery was defined as a ratio of the VPA concentration determined in the dialysate to the free VPA concentration in the sample solution surrounding the membrane of the microdialysis probe. When the sample solution was well stirred at 700 rpm and maintained at 37℃, the in vitro relative recovery of VPA was significantly different among them. It increased in the order of Ringer's solution (34.3 ± 2.6%) > 5% (w/v) of albumin solution (25.7 ± 4.6%) > rabbit plasma sample (15.8 ± 1.2%).

Thereafter, pharmacokinetics of VPA was determined using both microdialysis sampling via the rabbit femoral vein and collection of whole blood via the rabbit ear vein after intravenous administration of VPA at a dose of 43 mg/kg. Free concentrations of VPA in plasma were determined by ultrafiltration method as opposed to microdialysis method. There was no difference in the elimination half-life of VPA determined by microdialysis, 1.09 ± 0.22 h, or ultrafiltration, 1.22 ± 0.21 h. The AUC of VPA in dialysate was 15 ± 4 µg/ml/h, which corresponded to 15% of that in ultrafiltrate (103 ± 17 µg/ml). The value was in good agreement with the in vitro relative recovery of VPA from plasma sample (15.8 ± 1.2%). On the basis of the obtained recovery of 15.8 ± 1.2%, free VPA in dialysate was corrected to calculated free VPA in plasma, followed by determination of the extent of plasma protein binding of VPA. The extent of plasma protein binding of VPA determined by microdialysis was found to be 69.2 ± 6.9%, which was the almost same as that determined by ultrafiltration (66.6 ± 2.7%).

In vivo determination of plasma protein binding of VPA was successfully performed by microdialysis when the in vitro relative recovery of VPA was calculated using plasma sample of the tested animal.

Keywords microdialysis; valproate; plasma protein binding; pharmacokinetics; in vitro relative recovery; rabbit

Most drugs are bound to plasma proteins, mainly to a serum albumin. Only a free drug is considered to be responsible for therapeutic effects and adverse reactions. Binding of drugs to a serum albumin is an important factor in determining their pharmacokinetic and/or pharmacodynamic behavior. Plasma protein binding studies of drugs have typically been performed by either equilibrium dialysis or ultrafiltration.1–5 However, neither method is suitable for in vivo determination of the free drug concentrations in blood.

Recently, microdialysis has been applied to the in vivo determination of free drug concentrations in plasma6–8 and the extent of drug binding to plasma proteins.6 In these papers, the in vivo free drug concentrations in plasma were corrected by the in vitro relative recovery of the drug via a microdialysis probe obtained from Ringer's solution6–8 or an aqueous solution.7

We selected valproate (VPA) as a model drug and here describe the most suitable method for determining its in vitro relative recovery via the microdialysis probe, using rabbit plasma, different concentrations of albumin solutions and Ringer's solution. Thereafter, in vivo microdialysis sampling was carried out through the rabbit femoral vein by intravenous administration of VPA to the rabbit, followed by in vivo determination of plasma protein binding of VPA. The result obtained from the microdialysis was compared with that from ultrafiltration method.

MATERIALS AND METHODS

Materials Sodium valproate was supplied by Kyowa Hakko Industry Co., Ltd. (Tokyo, Japan). Serum albumin (fraction V) of rabbit was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The TDX assay reagent set for fluorescence polarization immunoassay (FPIA) of VPA was purchased from Abbott Laboratories (Abbott Park, IL, U.S.A.). All other chemicals were of special reagent grade.

Analysis of VPA The concentrations of VPA were analyzed by the Abbott TDX automated fluorescence polarization analyzer (Abbott Laboratories, Irving, TX, U.S.A.) based on FPIA technology.9

Ultrafiltration Ultrafiltration was performed with Ultrafree (C3-LGC, Nippon Millipore, Tokyo, Japan). Four-hundred µl aliquots of plasma samples and albumin solution were ultrafiltered by centrifugation at 3500 rpm for 30 min. The ultrafiltrates were analyzed to obtain the free VPA concentrations.

Microdialysis System The microdialysis system consisted of a CMA/100 microinjection pump (Carnegie Medicin, Stockholm, Sweden) and CMA/10 microdialysis probes with a dialyzing membrane length of 10 mm and an outer diameter of 0.5 mm (Carnegie Medicin). The microdialysis probe was connected to the microinjection pump and perfused continuously with Ringer's solution at a rate of 4.0 µl/min during the experimental period.
Recovery Experiments In order to determine *in vivo* the concentration of VPA eliciting a detectable concentration in the perfusion medium, it is necessary to know the relative recovery *via* the microdialysis probe. *In vitro* relative recovery was defined as a ratio of the analyte concentration determined in the dialysate to the actual concentration of the analyte in the sample solution surrounding the membrane of the microdialysis probe. The *in vitro* relative recoveries of VPA were determined in fresh rabbit plasma, different concentrations of albumin solutions (1, 3, 4 and 5% w/v, pH 7.4) and Ringer’s solution (pH 7.4) at a concentration of 250 μg/ml, respectively. Fifteen ml aliquots of each sample solution was brought to 37 °C in a water bath set at a 700 rpm stirring condition. Furthermore, the effects of VPA concentration (250, 125 and 50 μg/ml) and stirring speed (700, 300 and 0 rpm) on the *in vitro* relative recovery of VPA were also examined both in plasma sample and Ringer’s solution. Dialysate samples were collected every 20 min in triplicate.

The *in vitro* relative recovery in Ringer’s solution was calculated by the following equation:

\[
\text{relative recovery (\%) = } \frac{\text{VPA concentration in dialysate}}{\text{VPA concentration in Ringer’s solution}} \times 100
\]

In the cases of *in vitro* relative recovery in plasma sample or each concentration of albumin solution, the following equation was used:

\[
\text{relative recovery (\%) = } \frac{\text{VPA concentration in dialysate}}{\text{free VPA concentration in plasma or albumin solution}} \times 100
\]

The free VPA concentration in plasma sample and albumin solutions was determined by ultrafiltration method as described above.

Free VPA fraction in plasma sample and albumin solutions was defined as a ratio of the free VPA concentration to the total VPA concentration in each sample solution.

Animal Experiments Male Japanese Albino rabbits, weighing 3.0 to 3.2 kg, were anesthetized with urethane (1.5 g/kg as 300 mg/ml, i.p.), and anesthesia was continued throughout the experimental period.

Prior to administration of a drug, the microdialysis probe was inserted into the rabbit femoral vein, using a guide cannula (Carnegie Medicin). One h after the probe insertion, VPA was administered via the ear vein to the rabbit at a dose of 43 mg (0.3 mmol)/kg. The dialysate samples were collected every 20 min for 2 h, and then hourly for 4 h. Collection of whole blood was performed at the midpoint of each microdialysis sampling period. The whole blood sample was immediately centrifuged for 10 min at 3000 rpm to give a plasma sample, and the plasma sample was divided into two parts, followed by analysis of the total and free concentrations of VPA.

The free VPA concentration in the plasma sample was determined by ultrafiltration method as described above.

In rabbit plasma, the concentration of albumin was measured by the bromocresol green method using a Clalyzer RX-40 (JEOL Co., Tokyo, Japan).

Pharmacokinetic Analysis Pharmacokinetic parameters were calculated by MULTI.10 The VPA concentration data were estimated by fitting the following equation using the least-squares fit:

\[
C_t = C_0 e^{-K_e t}
\]

where \(C_t\) is the drug concentration at time \(t\), \(C_0\) is the drug concentration at time 0, and \(K_e\) is the elimination rate constant. The elimination half-life \((T_{1/2})\) was calculated by the following equation:

\[
T_{1/2} = \frac{\ln 2}{K_e}
\]

The area under the concentration–time curve (AUC) for blood sampling was calculated by the linear trapezoidal rule and extrapolated to time infinity by the addition of \(C_\infty /K_e\), where \(C_\infty\) is the concentration of the last measurement. For the microdialysis data, the concentration of VPA measured in dialysate is a time-averaged concentration. Accordingly, the AUC was obtained as the sum of the products of the measured concentrations and the collection time interval with the addition of \(C_\infty /K_e\) by the following equation:

\[
AUC = \sum_{i=1}^{n} \frac{C_i dt + C_\infty}{K_e}
\]

The total body clearance (CL) was calculated by the following equation:

\[
CL = \frac{\text{dose}}{AUC}
\]

where \(AUC\) is the AUC for total VPA.

Noncompartmental analysis was performed to obtain the mean residence time (MRT) and the volume of distribution at a steady state (\(V_{ss}\)). The MRT and \(V_{ss}\) were calculated by the following equation:

\[
MRT = \frac{AUMC}{AUC}
\]

\[
V_{ss} = \frac{\text{dose} \times MRT}{AUC}
\]

where \(AUMC\) is the area under the first moment curve.

The extent of VPA binding to rabbit plasma proteins was calculated by the following equation:

\[
\text{plasma protein binding (\%) = } \frac{AUC_t - AUC_i}{AUC_t} \times 100
\]

where \(AUC_t\) is the AUC for free VPA.

Statistical analysis was performed using the Student’s t-test.

RESULTS AND DISCUSSION

*In Vitro Relative Recovery of VPA via Microdialysis Probe* Prior to an application of microdialysis to *in vivo* plasma protein binding study of VPA, *in vitro* relative recovery of VPA via a microdialysis probe was determined from fresh rabbit plasma, 1, 3, 4 and 5% (w/v) of albumin solutions and Ringer’s solution at a concentration of 250 μg/ml, respectively. In all experiments, the test solutions were stirred at 700 rpm by a star-headed stirrer and maintained at 37 °C, so that the *in vitro* experimental system provided the hydrodynamic system to model flowing blood. The *in vitro* relative recovery of VPA and...
free VPA fraction obtained from each test solution are shown in Table I.

The in vitro relative recovery of VPA was increased in the order of Ringer's solution > albumin solutions > plasma sample. The Ringer's solution gave much higher recovery of 34.3 ± 2.6%, while the plasma sample gave the lowest recovery of 15.8 ± 1.2%. A significant difference was observed between them (p < 0.001). The in vitro relative recovery of VPA was found to be influenced by the existence of gelatinous substances, i.e., a serum albumin and so on. Regarding albumin solutions, 1% (w/v) of albumin solution exhibited a significantly higher recovery than the other albumin solutions. However, albumin solution of more than 3% (w/v) showed almost the same recovery ranging from 25 to 27%, although the free VPA fraction decreased with increasing albumin concentration. This result suggests that the in vitro relative recovery of VPA is independent of changes in the in vitro binding of VPA in the albumin solutions. In addition, the difference in the free VPA fraction was also observed between the plasma sample and the albumin solutions. This result almost corresponded to the previous report that the free VPA fraction in human plasma differed from that in 4% (w/v) of human serum albumin when determined by equilibrium dialysis method. Considering the fact that rabbit plasma used in the present experiment contained 5% (w/v) albumin, it was of special interest that the in vitro relative recovery of VPA from plasma sample was significantly lower than that from 5% (w/v) of albumin solution (p < 0.01). The significantly low recovery from plasma sample might be attributable to the existence of other biological colloidal substances.

Table II shows the effect of VPA concentration range on the in vitro relative recovery of VPA via the microdialysis probe in both Ringer's solution and plasma sample. The in vitro relative recoveries of VPA from the two matrices were independent of changes in VPA concentration in the solutions. Similar to albumin solutions, it was also observed that the in vitro relative recovery of VPA from plasma sample was independent of changes in the free VPA fraction. On the other hand, stirring condition of the solutions affected the in vitro relative recoveries of VPA in both Ringer's solution and plasma sample (Table III). Both in vitro relative recoveries of VPA decreased as stirring speed slowed down from 700 to 0 rpm, with that from Ringer's solution being affected more markedly than that from plasma sample. When stirring speed was changed from 700 to 0 rpm, the in vitro relative recovery of VPA from Ringer's solution was reduced significantly from 34.3 ± 2.6 to 15.5 ± 2.6% (reduction of 50%), whereas that from plasma sample was reduced from 15.8 ± 1.2 to 13.0 ± 0.5% (reduction of 20%). Colloidal substances in plasma seemed to prevent the influx of VPA molecule into the membrane of the microdialysis probe, resulting in less effect of stirring speed on the in vitro relative recovery of VPA from plasma sample than from Ringer's solution. Teltig-Diaz et al. performed in vitro recovery experiments for theophylline using Ringer's solution, plasma sample and whole blood in a thermostated shaker bath (shaking speed of 50 rpm) to both maintain the temperature at 37°C and provide the hydrodynamic system to model flowing blood. In contrast to our present results, the in vitro relative recovery of theophylline was independent of the different matrices. The difference in their findings and ours might be attributable to a difference in the mixing conditions of test solutions in the in vitro recovery experimental methods.

Pharmacokinetic Studies for VPA Microdialysis was validated by simultaneous collection of whole blood samples through rabbit ear vein. The concentration–time profiles of total VPA in plasma, free VPA in ultrafiltrate and free VPA in dialysate after intravenous administration of VPA at a dose of 43 mg/kg to rabbits are shown in Fig. 1. Pharmacokinetic parameters obtained from the time profile data are also shown in Table IV. All of the pharmacokinetic curves were described as a one-compartment open model. There was no statistical difference
in the elimination half-life between free VPA in ultrafiltrate (1.22 ±0.21 h) and that in dialysate (1.09 ±0.22 h). There was also no difference in the MRT between free VPA in ultrafiltrate (1.82 ±0.30 h) and that in dialysate (1.64 ±0.31 h). On the other hand, C₀ and the AUC were significantly different between them. The value of C₀ for free VPA in dialysate was 10 ±3 µg/ml, which corresponded to 16% of that in ultrafiltrate (61 ±3 µg/ml). The value of AUC for free VPA in dialysate was 15 ±4 µg·h/ml, which corresponded to 15% of that in ultrafiltrate (103 ±17 µg·h/ml). Interestingly, these values were almost equal to the in vitro relative recovery of VPA obtained from plasma sample (15.8 ±1.2%).

On the basis of various in vitro relative recoveries of VPA obtained from Ringer’s solution, 5% (w/v) of albumin solution and plasma sample, we made a correction in the free VPA concentration in dialysate, as shown in Fig. 2. It was demonstrated that the calculated free VPA concentration–time curve in dialysate adjusted by the in vitro relative recovery from plasma sample (open squares) was in close agreement with the free VPA concentration–time curve obtained from ultrafiltration (closed squares). However, the calculated free VPA concentration–time curves in dialysate adjusted by that from Ringer’s solution (circles) and 5% (w/v) of albumin solution (triangles) were lower than the curve obtained from ultrafiltration.

On the basis of the AUC values, in vivo extent of VPA binding to rabbit plasma proteins was calculated. Binding percentage of VPA determined by ultrafiltration method was found to be 66.6 ±2.7. When the in vitro relative recovery of VPA from plasma sample (15.8 ±1.2%) was applied to the correction for free VPA concentration in dialysate, binding percentage of VPA calculated by the microdialysis method was 69.2 ±6.9, which was almost the same as the real binding percentage determined by the ultrafiltration method.

Microdialysis offers several advantages in that no blood need be drawn, and a large number of samples can be collected from a single experimental animal without a loss of fluid volume. Simultaneous sampling can be achieved using multiple microdialysis probes as reported in previous studies. 11-13)

When we apply microdialysis to the quantitative determination of drugs in plasma, the in vitro relative recovery of the drugs from a sample solution is important. Judging from our present results, we conclude that microdialysis offers good potential for use in the in vivo determination of plasma protein binding of a drug when the in vitro relative recovery of the drug is calculated using a plasma sample of the tested animal.

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