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Determination of antiepileptic drug concentrations in animal blood by the SLFIA method

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Summary

This study was designed to investigate whether antiepileptic drug concentrations in the blood of small animals could be determined by Ames TDA kits based on substrate-labeled fluorescent immunoassay (SLFIA) method. Six other drugs have been evaluated by this method using only a 6-μl serum sample. The concentrations of phenobarbital (PB), sodium valproate (VAP) and primidone (PRM) in the blood were determined by the SLFIA method in mice and rats, and were compared with those determined by gas-liquid chromatography (GLC) method. The drug concentrations in the blood collected from the tail wounded slightly in the upper part of tail vein were compared with those in the blood obtained by decapitation of mice and from the inferior vena cava of rats. A good correlation between the GLC and the SLFIA method was observed in the blood concentrations of VAP and PB in mice and rats. When determined by the SLFIA method using the blood obtained from the tail vein, inferior vena cava or decapitation, there was good agreement between the drug concentrations in blood obtained by each sampling technique. Using this assay and the blood sampling technique from the tail vein, the pharmacokinetic parameters for PRM and derived PB after an oral administration of PRM in rats were obtained following one-compartment open model. These results indicated that the SLFIA method could be applied to evaluate the blood antiepileptic drug concentrations in mice and rats.

Introduction

When pharmacological activities of a drug are evaluated, the drug concentration in the blood is rarely considered. This is especially true when small animals (e.g., mouse or rat) are used because of small drug dosage, in spite of the fact that these kinds of animals are frequently used in pharmacological experiments. Although blood concentrations can be estimated in small samples, conventional methods usually require at least 1–5 ml of blood. This means that, in the case of small animals, one animal must be sacrificed for blood collection. If pharmacokinetics in the blood is studied at the same time, a larger number of animals must be sacrificed.

Recently, determination of drug concentrations in various humors (such as blood, saliva and milk) has become popular in drug therapy, and there has been an increase in interest in therapeutic

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drug monitoring (TDM). As a result, enzyme immunoassay (EIA), which involves no chemical procedures, including pretreatment, is being used more frequently than conventional methods such as gas–liquid chromatography (GLC) and high-performance liquid chromatography (HPLC), which were previously the most popular methods for blood concentration analysis.

In our previous studies (Fukuda et al., 1983, Furuno et al., 1985, Sugino et al., 1985), the blood concentrations of phenytoin, phenobarbital, primidone, carbamazepine, ethosuximide and valproate were measured in patients suffering from epilepsy using the Fluorostat System, which is an instrument designed for use with Ames TDA kits based on the substrate-labeled fluorescent immunoassay (SLFIA) method, an EIA method. In these studies, we found that the 6 drugs could be evaluated using only a 6-μl serum sample, which was diluted to 1580 times with buffer in the kits.

The present study was undertaken to determine whether antiepileptic drug concentrations in the blood of small animals could also be measured accurately by the SLFIA method.

Materials and Methods

Animals

Male Sprague–Dawley (JCL–SD) rats, weighing 250–280 g, and male ICR mice, weighing 25–30 g, were used in this study. Food and water were available ad libitum before and during the experiments.

Drugs

Phenobarbital (PB; Fujinaga Pharmaceutical Co., Japan), primidone (PRM; Dainihon Pharmaceutical Co., Japan) and sodium valproate (VAP, Kyowa Hakko Kogyo Co., Japan) were suspended in 0.5% carboxymethylcellulose sodium solution. The test drugs were administered orally by a gastric tube at a volume of 2ml/kg b.wt.

Blood collection

Blood was collected 2 h after oral administration of each drug. Following conventional methods, rats were laparotomized under ether anesthesia in order to collect the blood from the inferior vena cava, while mice were decapitated for blood collection.

As a new blood collection method, the upper part of the tail of all animals was wounded slightly with a surgical knife, and the blood which bled from the tail vein was collected into a capillary made for plasma separation (60 μl; Miles-Sankyo Co., Japan). Fifty μl and 30 μl of blood were collected from rats and mice, respectively. Serum separation was performed using a hematocrite centrifuge (COMPUR M 1100, Miles-Sankyo Co.).

Determination of drug concentration in serum

As for PB and VAP, their concentrations were determined by the SLFIA method employing the Fluorostat System (Miles-Sankyo Co.) (Thomas, 1984) and by the GLC method (Nishina et al., 1976), and the results were compared. Reagents used for the determination by the SLFIA method were contained in Ames TDA kits for phenobarbital, primidone and sodium valproate. A 5- or 10-μl serum sample obtained from the tail vein was used for the determination of each drug concentration. A gas chromatograph (Model 163, Hitachi, Japan) equipped with a flame ionization detector was used for analyses of PB and VAP. The column for PB, 0.3 cm in diameter and 200 cm in length, was packed with Chromosorb W (AW-DMCS, 100–200 mesh) coated with 1% OV-17 and its temperature was programmed to increase from 180°C to 270°C at the rate of 6°C/min. The column for VAP, 0.3 cm in diameter and 100 cm in length, was packed with Chromosorb W(AW-HMDS, 60–80 mesh) coated with 5% DECA and 1% H_3PO_4, and its temperature was 140°C. The internal standard solutions used were 200 μg/ml cholestanethanol solution for PB and 50 μg/ml diphenyl-ethyl acetate solution for VAP.

Pharmacokinetics of PRM in rats after oral administration

Six male rats were used in this experiment. A single dose of PRM (30 mg/kg) was given orally to each animal. At each specific time point, blood samples were collected 30 min, 1, 2, 4, 8, 12 and 24 h after the drug administration from the tail

vein. Concentrations of PRM and derived PB in serum were determined by the SLFIA method. These concentrations were plotted against time, and pharmacokinetic parameters were processed using a one-compartment open model. Parameters were assessed by conventional methods.

Results and Discussion

As for conventional drug concentration determinations in small animals, as much blood as possible is collected from the inferior vena cava after laparotomy in rats and by decapitation in mice, because the GLC method and the HPLC method require 1–5 ml blood samples. Needless to say, many animals have been sacrificed for blood collection. With the development of EIA methods, the amount of blood needed has been reduced, and blood concentrations of 6 antiepileptic drugs have been estimated by the SLFIA method using the same 6-μl serum sample for all 6 drugs.

When PB concentrations in blood collected from the inferior vena cava of rats after oral administration were determined by the GLC and the SLFIA method, there was a satisfactory agreement ($r = 0.990$, $P < 0.01$) between the values obtained by the two methods (Fig. 1). The mean for the SLFIA method was 30.4 μg/ml, and for the GLC method, 33.6 μg/ml. The statistical analysis on the slope of regression line gives a significant difference from unity. This could mean that by the SLFIA method lower values are measured than by the GLC method. When the PB concentration values were separated two ranges, in high PB concentration range (more than 40 μg/ml) the mean for the SLFIA method was significantly smaller ($P < 0.01$) than that for the GLC method, as shown in Table 1. On the other hand, in the low PB concentration range (less than 40 μg/ml) no significant difference was observed (Table 1). In the low PB concentration range, good correlation between the determinations by the SLFIA (abscissa, x-axis) and the GLC method (ordinate, y-axis) was obtained and the regression equation was $y = 1.01x - 0.5$ ($n = 14$, $r = 0.995$, $P < 0.001$) and in the high PB concentration range, $y = 1.84x - 31.3$ ($n = 7$, $r = 0.953$, $P < 0.01$). When the calibrators of 0, 10, 20, 40 and 60 μg/ml in the PB kit were determined by the SLFIA method using the Fluorostat System, the calibration curve should be drawn by connecting the points obtained with the fluorescence value of each calibrator (y-axis) and its concentration (x-axis), but not by drawing the best straight line for all of the points. Furthermore, the fluorescence value ranges among the calibrators narrow following the increase from 0 to 60 μg/ml of PB concentration. For instance, the fluorescence value ranges were 22 for 0–10 μg/ml, 11 for 10–20 μg/ml, 11 for 20–40 μg/ml and 5 for 40–60 μg/ml in which we demonstrated. These results are reasonably understood

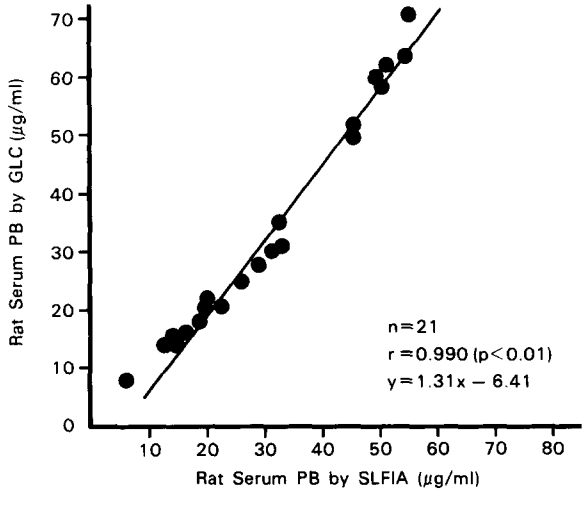

Fig. 1. Comparison of serum PB concentrations determined by the SLFIA method and the GLC method in rats administered PB (10–80 mg/kg, p.o.)

TABLE 1

Concentration dependency of rat serum PB determination obtained by GLC and SLFIA

PB concentration	n	GLC method (μg/ml)	SLFIA method (μg/ml)	Statistical comparisons
All	21	33.6±4.3	30.4±3.3	n.s.
Less than 40 μg/ml	14	20.8±2.1	21.1±2.0	n.s.
More than 40 μg/ml	7	59.2±2.8	49.2±1.5	$P < 0.01$

Values are the mean±S.E.M.

that the SLFIA method showed significantly lower values which may be calculated by the Fluorostat System in the high PB concentration range. Therefore, sera containing PB amounts more than 40 μg/ml may be diluted with the buffer before the analysis, since it was shown that dilution test by the SLFIA method indicates a good linearity over a wide range of drug concentrations (Furuno et al., 1985; Sugino et al., 1985; Tosoni et al., 1985).

Using blood collected by decapitation of mice and from the inferior vena cava of rats after oral administration of VAP, VAP concentrations in the serum were determined by the SLFIA and the GLC method. Both in mouse ($r = 0.996$, $P < 0.01$) and in rat ($r = 0.965$, $P < 0.01$) a good correlation between the SLFIA and GLC method was observed in the VAP concentrations (Figs. 2 and 3). The means in the mouse and rat for the SLFIA method were 29.7 and 11.1 μg/ml, and for the GLC method, 30.8 and 11.0 μg/ml, respectively. No significant differences between the two methods were observed in the mouse and rat. The calibration curve obtained with the VAP calibrators cannot be essentially drawn as a straight line such as with the PB determination. Moreover, Johno et al. (1986) reported that the values for the SLFIA method were significantly lower than those

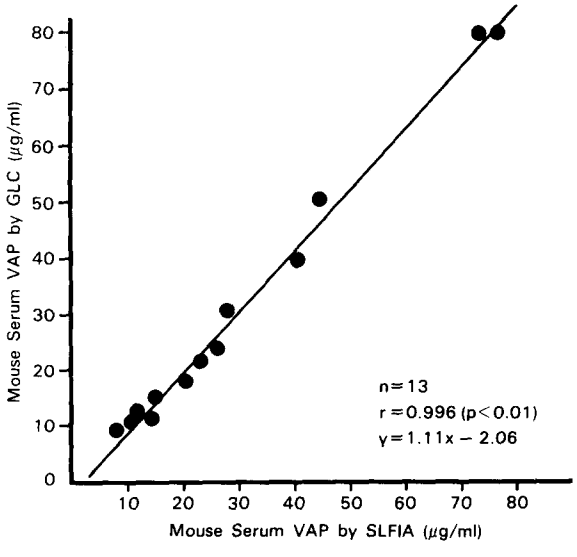

Fig. 2. Comparison of serum VAP concentrations determined by the SLFIA method and the GLC method in mice administered VAP (50–200 mg/kg, p.o.)

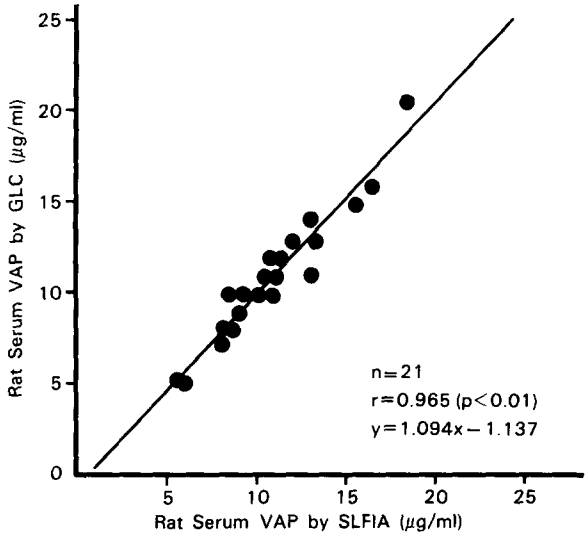

Fig. 3. Comparison of serum VAP concentrations determined by the SLFIA method and the GLC method in rats administered VAP (20 mg/kg, p.o.)

for the GLC method in rabbit sera containing VAP amounts of more than 150 μg/ml. Therefore, sera containing the VAP amounts considerably above the therapeutic concentration range or the toxic levels also can be diluted before the analysis.

These excellent correlations indicate that the SLFIA method could be applied to the determination of the blood PB and VAP concentrations in the mouse and rat such as in the human.

Since blood collection from individual subjects is usually performed several times in order to evaluate pharmacokinetics of a drug, the tail vein was thought to be a good candidate for a blood collection site without having to sacrifice the animals. Moreover, rodents have a relatively well-developed tail vein, which is routinely used for intravenous injections and shows good blood circulation. Therefore, it was thought that the drug concentration in the tail vein would be almost the same as that in larger vessels.

In rats, blood was first collected from the tail vein 2 h after oral administration of PB at 10–60 mg/kg, and then laparotomy was immediately performed in order to collect blood from the inferior vena cava. The determination of the PB con-

centration was performed using a 50-μl serum sample obtained from the inferior vena cava and a 5-μl serum sample from the tail vein according to standard procedures of the SLFIA method. As shown in Fig. 4, PB concentrations in sera obtained from the tail vein and the inferior vena cava showed an excellent correlation ($r = 0.987$, $P < 0.01$). The means were 31.8 for the tail vein and 30.4 μg/ml for the inferior vena cava and no significant difference was observed.

In mice, blood was collected from the tail vein 1 h after oral administration of 5–20 mg/kg of PRM, and then decapitation was immediately performed for blood collection. As shown in Fig. 5, PRM concentrations determined by the SLFIA method using a 5-μl serum sample from the tail vein and a 50-μl serum sample (a conventional amount) obtained by decapitation exhibited a very good correlation ($r = 0.988$, $P < 0.01$). The means were 5.2 for the tail vein and 5.3 μg/ml for decapitation and no significant difference was observed.

As a result of this experiment, it was confirmed that exactly the same drug concentrations in mice and rats were obtained by the SLFIA and GLC methods. Almost the same results were obtained by the SLFIA method using a small sample obtained from the tail vein as those obtained using blood collected by conventional procedures. Therefore, we investigated whether pharmacokinetics for PRM in rats after oral administration is evaluated by using this assay and the blood sampling technique from the tail vein.

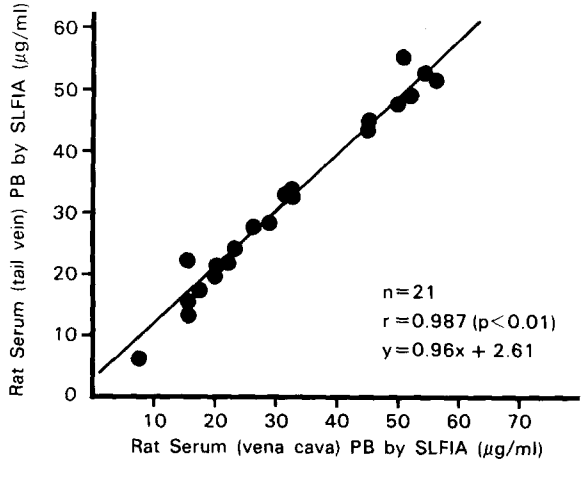

Fig. 4. Comparison of PB concentrations in the blood obtained from the tail vein and inferior vena cava in rats.

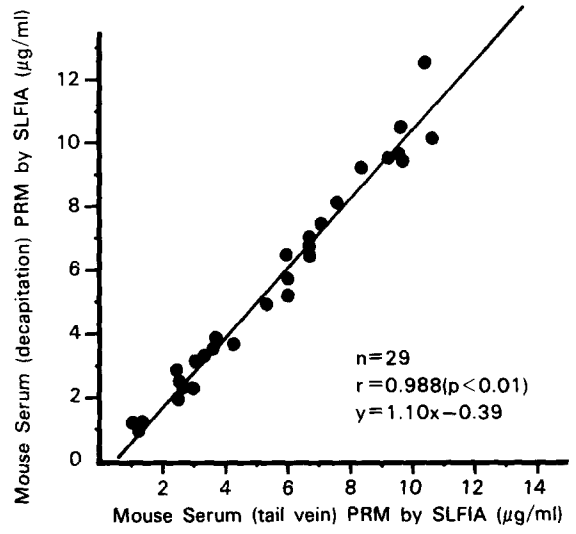

Fig. 5. Comparison of PRM concentrations in the blood obtained from the tail vein and by decapitation in mice.

The serum levels of PRM and derived PB after a oral administration of PRM (30 mg/kg) in rats are shown in Fig. 6. Pharmacokinetic parameters calculated from the serum concentrations of PRM

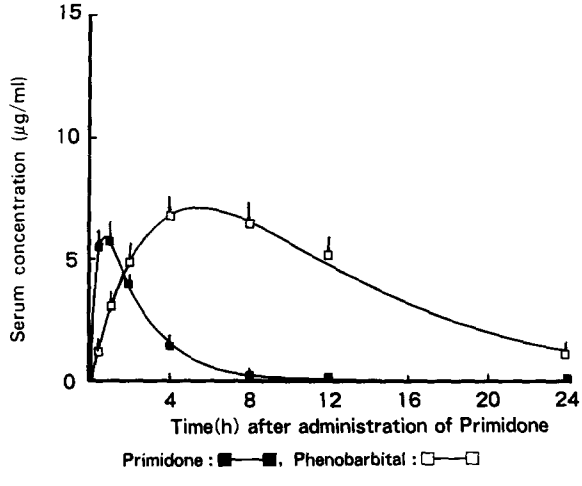

Fig. 6. Serum levels of primidone and derived phenobarbital after oral administration of primidone (30 mg/kg) in rats. Each point represents the mean of 6 rats and vertical lines represent the S.E.M.

TABLE 2

Pharmacokinetic parameters of PRM and derived PB after acute administration of PRM (30 mg/kg, p.o.)

Parameters	PRM	Derived PB
C max (μg/ml)	5.89±1.37	7.15± 2.30
t max (h)	0.78±0.34	5.48± 1.21
AUC (μg/ml·h)	17.88±1.70	109.80±42.32
$t_{1/2}$ (h)	1.45±0.21	5.15± 1.33
K_e (h^{-1})	0.48±0.18	0.13± 0.04
K_a (h^{-1})	2.70±1.05	

Values are the means ± S.E.M. of 6 rats.

and PB are shown in Table 2. Thus, the evaluation of pharmacokinetics of antiepileptic drugs for small animals can be performed by the SLFIA method.

In conclusion, the SLFIA method was easy to use for the determination of antiepileptic drugs in the blood of small animals, and performed precisely and accurately in the human therapeutic range of the drugs investigated, and in the high levels above its range or the toxic levels after sample dilution. This assay performed well in comparison with the GLC method, and showed no cross reactions in mice and rats.

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