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DETERMINATION OF PLASMA ANTIEPILEPTIC DRUGS BY A NEW KIT (MARKIT) BASED ON A COMPETITIVE ENZYME-IMMUNOASSAY

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Abstract

Three kinds of antiepileptic drugs, phenytoin (DPH), phenobarbital (PB) and primidone (PM), in plasma samples from patients with epilepsy were measured by a new technique "MARKIT" based on a competitive binding enzyme-immunoassay, and the obtained values were compared with these by the other method-EMIT based on homogeneous enzyme-immunoassay.

Correlative coefficient of the values between MARKIT (x) and EMIT (y) was r=0.980, n=85 for DPH ; r=0.978, n=117, for PB ; and r=0.978, n=66, for PM. The equation of a regression line of the obtained values was close to y=x, with all three drugs.

MARKIT has a merit to be applicable to routine apparatuses used in the ordinary clinical laboratories. It requires about two hours for one sample assay, but only 3-5 minutes per one sample when it comes to simultaneous assays for 50-100 samples.

INTRODUCTION

In the drug therapy of patients with epilepsy, administration of minimum effective dosage of antiepileptic drugs by monitoring their blood levels has been recognized to be important not only for enhancing the effective treatment, but also for avoiding undesirable toxic side effects.[1,2] Therefore, development of a simple and precise method for the determination of blood levels of drugs is in great demand.

We have already reported a good correlation between the plasma levels of antiepileptic drugs measured by a gas liquid chromatographic technique (GLC) and EMIT (Syva Co. U. S. A.) based on a homogeneous enzyme-immunoassay technique.[3] Recently, another new method (MARKIT) based on a competitive

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enzyme-immunoassay technique has been developed by Kurooka et al.[4] This report is to describe the performance of MARKIT as compared to EMIT and GLC for the determination of plasma phenytoin (DPH), phenobarbital (PB), and primidone (PM) levels.

MATERIALS AND METHODS

Assay samples ..... Blood samples used were from both in-patients and out-patients on several antiepileptic drugs, including DPH, PB and/or PM. The blood samples were taken from their arm veins by a heparinized syringe. Sampling was done when the patients visited our hospital or before the in-patients on drugs take breakfast. The blood samples were centrifuged and the obtained plasma samples were kept frozen at -20°C until they were used for the assay.

ASSAY METHODS

(1) MARKIT method.....MARKIT-phenytoin for DPH assay, MARKIT-phenobarbital for PB assay, and MARKIT- primidone for PM assay used in this study were supplied from Dainippon Pharmaceutical Co., Ltd., Osaka, Japan.

a) Principle of MARKIT ..... A constant amount of enzyme labelled drug (β-D-galactosidase-DPH, PB, or PM) and known amount of standard drug (DPH, PB, or PM) or samples with unknown amount of drugs are subjected to a competitive immuno-reaction against a constant amount of insolubilized antibody (anti-DPH, -PB, or-PM immunoglobulin bound to bacterial cell walls).

In this competitive immuno-reaction, enzyme labelled drug not bound to the antibody (=free enzyme labelled drug) increases as the amount of unlabelled drug in the samples increases, and *vise versa*.

The immuno-reaction mixture is centrifuged and the free enzyme labelled drug in the supernatant can be separated from the bound enzyme labelled drug in the precipitate.

The enzyme activity of the free enzyme labelled drug in the supernatant can be measured by addition of the enzyme substrate (o-nitrophenyl-β-D-galactopyranoside) at 410 nm (due to liberated o-nitrophenol anion). The absorption (=enzyme activity) reflects drug concentration in the plasma samples.

b) Assay method by MARKIT ..... The assay method is summarized in Table 1. Into series of glass test tubes (1.3×8.5 cm), add firstly 0.1 ml of blood plasma diluted to one tenth with water or 0.1 ml of standard drug solutions (6 levels), secondly 0.5 ml of enzyme labelled drug solution, and finally 0.2 ml of insolubilized antibody suspension. Mix well and incubate at 37°C for 60 min to complete the immunoreaction. After centrifugation (3,000

rpm, 10 min), transfer the supernatant into another corresponding test tubes, and add 0.1 ml of substrate solution.

TABLE 1

Assay procedure of plasma antiepileptic drugs using MARKIT

Diluted plasma or Standard solution	0.1 ml
Enzyme labelled antigen	0.5 ml
Insolubilized antibody suspension	0.2 ml

Incubation at 37°C for 60 min.

↓

Centrifugation at 3,000 r. p. m. for 10 min.

↓

Decantation

↓

Supernatant

↓ —Substrate solution 0.1 ml.

Incubation at 37°C for 30 min.

↓ —Alkaline solution 2.5 ml.

Measurement with spectrophotometer at 410 nm.

Incubate at 37°C for 30 min and stop the enzyme reaction by adding 2.5 ml of alkaline solution. Measure the absorbance at 410 nm in a cuvette of 1 cm light path against water. Make a calibration curve on a semi-log graphic paper by plotting the absorbance against standard drug concentrations (log scale), and read the drug concentrations of unknown plasma samples.

(2) EMIT method . . . . . The assay was carried out using the commercial EMIT kits (Syva Co., Ltd., U. S. A.) as described in the previous reports.

(3) Gas liquid chromatography (GLC) . . . . . The assay was carried out as described by Hammer et al.

EXPERIMENTAL RESULTS

1) *A calibration curve for the assay of DPH, PB, and PM concentrations MARKIT.* Typical standard calibration curves for the assay of plasma DPH, PB, and PM levels obtained by using MARKIT-phenytoin, -phenobarbital,

and -primidone are presented in Figure 1.

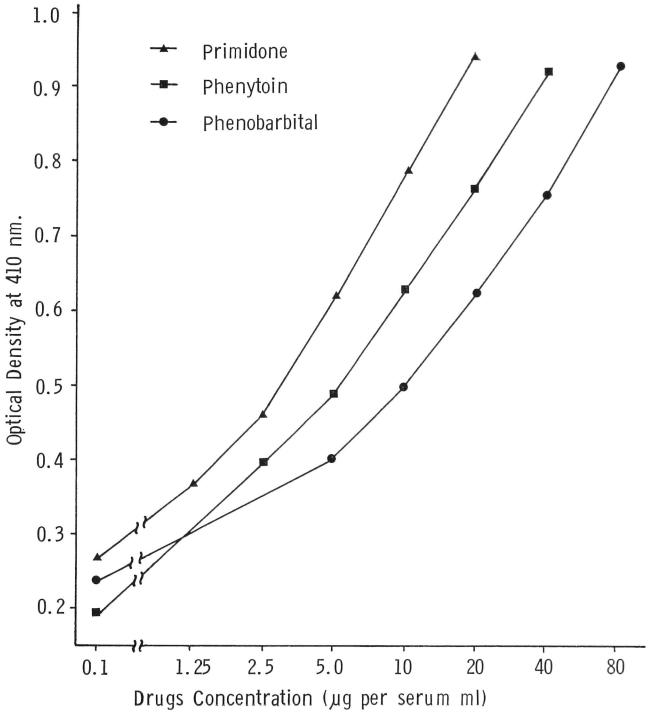

Fig. 1. Standard calibration curves for the determination of antiepileptic Drugs

2) *Comparison of DPH concentrations obtained by using MARKIT-phenytoin and EMIT kit.* Eighty five different plasma samples from epileptic patients treated with various antiepileptic drugs including DPH were measured in parallel by the two kits. The mean plasma DPH concentration for the whole samples was 9.4 μg/ml with MARKIT (x) and 9.5 μg/ml with EMIT (y), showing no statistically significant difference between the two methods ($p<0.001$). The equation of the regression line between the MARKIT (x) and EMIT (y) was $y=1.05\ x - 0.41$. The correlation coefficient (r) between the DPH values measured by the two methods was 0.989. It may be said that MARKIT and EMIT correlated well, indicating the good reliability of the two methods (Figure 2).

3) *Comparison of PB concentrations measured by MARKIT and EMIT* PB levels of 117 different plasma samples from patients treated with

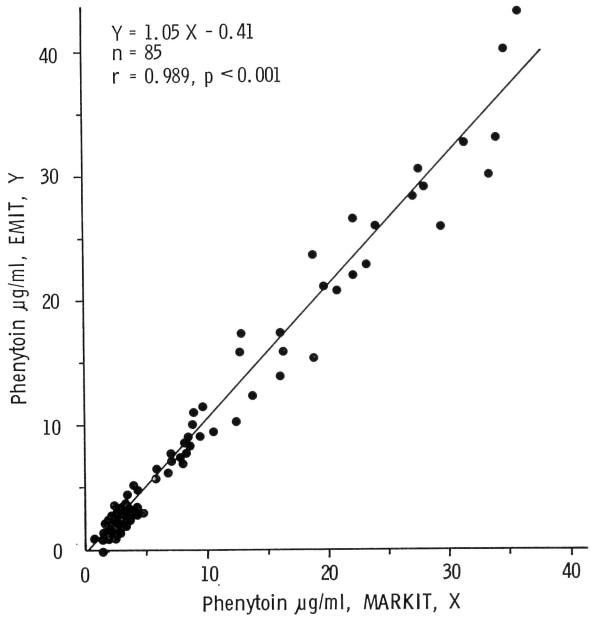

Fig. 2. Relationship of plasma phenytoin levels measured by MARKIT and EMIT method

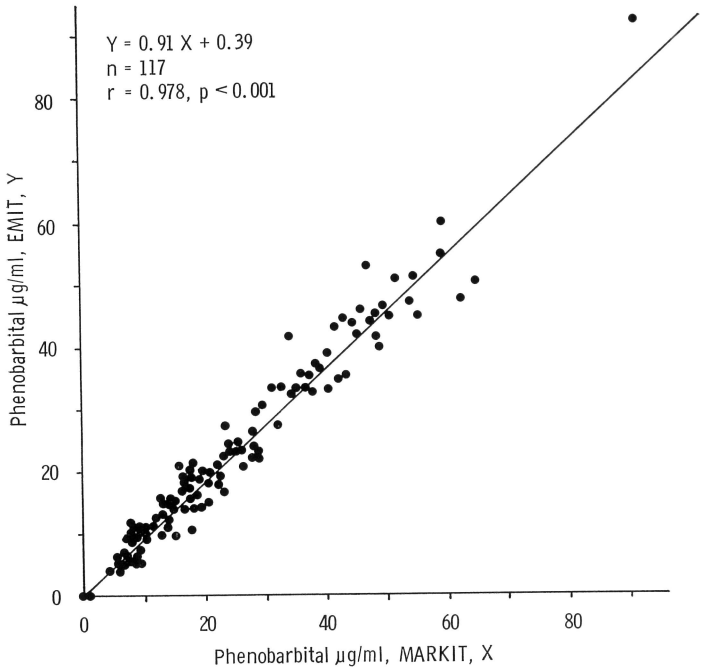

Fig. 3. Relationship of plasma phenobarbital levels measured by MARKIT and EMIT method

various antiepileptic drugs including PB were measured by the two methods. The mean plasma PB concentration for the whole samples was 25.6 μg/ml with MARKIT (x), and 23.6 μg/ml with EMIT (y), showing no statistically significant difference between the two methods ($p<0.001$). The equation of the regression line being $y=0.91\ x + 0.39$. The correlation coefficient (r) between the PB values measured by the two methods was 0.978. Both methods seems to be correlated well.

4) *Comparison of plasma PM levels measured by MARKIT and EMIT* PM levels in 66 different plasma samples from epileptic patients treated with various antiepileptic drugs including PM were measured by the two methods. The mean plasma PM concentration for the whole samples was 8.0 μg/ml with MARKIT (x) and 7.8 μg/ml with EMIT, indicating no statistically significant difference between them ($p<0.001$). The equation of the regression line being $y=0.97\ x \times 0.03$, and correlative coefficient (r) between the PM levels measured by the two methods was 0.978. Both methods were found to be correlated well.

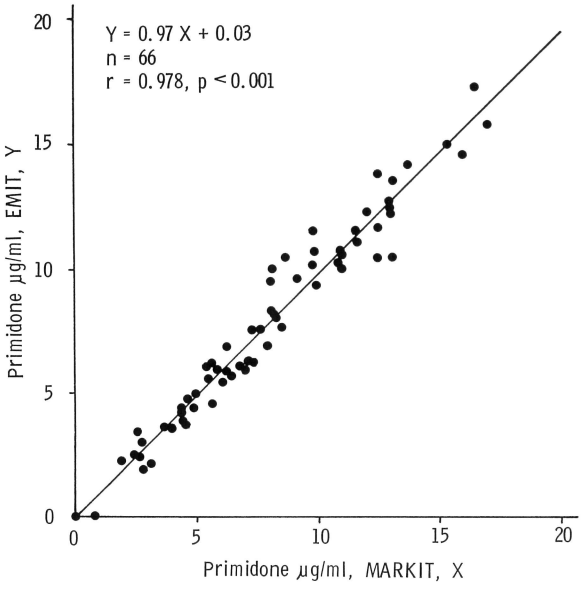

Fig. 4. Relationship of plasma primidone levels measured by MARKIT and EMIT method

5) *Correlation of plasma antiepileptic drugs-concentrations between MARKIT and GLC* Levels of plasma DPH, PB, and PM in patients with

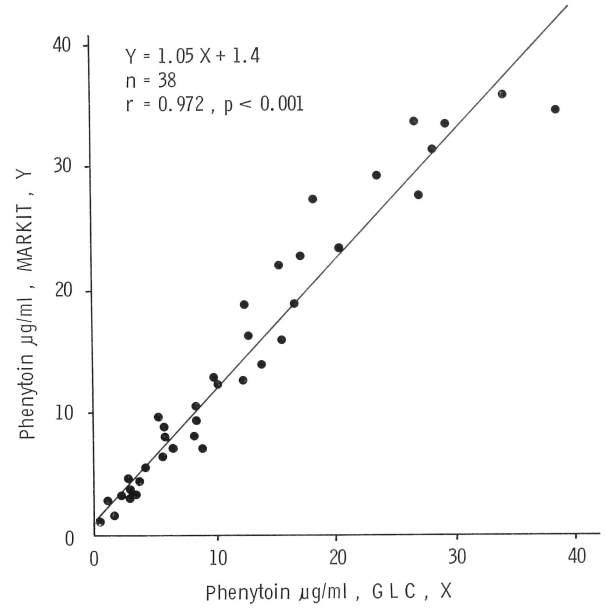

Fig. 5. Relationship of plasma phenytoin levels measured by MARKIT and GLC method

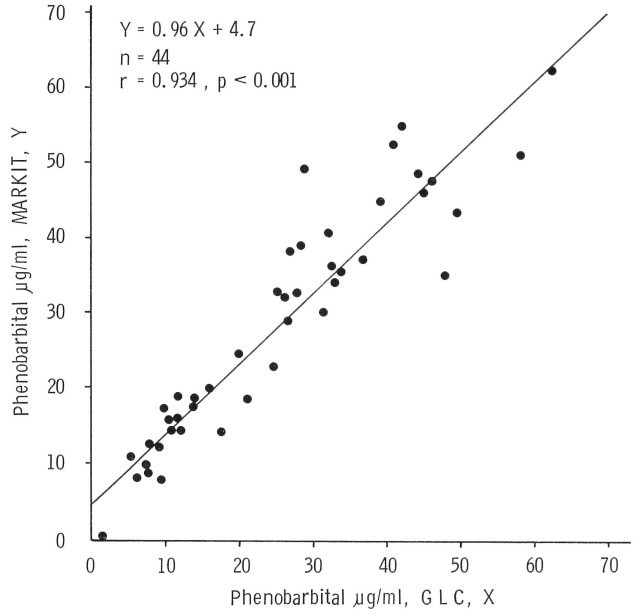

Fig. 6. Relationship of plasma phenobarbital levels measured by MARKIT and GLC method

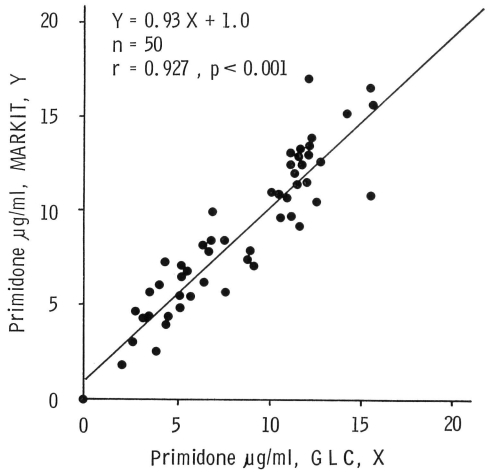

Fig. 7. Relationship of plasma primidone levels measured by MARKIT and GLC method

TABLE 2

Relationship of plasma antiepileptic drugs concentrations measured by MARKIT, EMIT and gas liquid chromatography (GLC) method.

1 : Mean Values

Drugs	Number of Samples	Drugs Concentration (µg/ml)		
		MARKIT	GLC	EMIT
DPH	n=38	14.5	12.5	14.5
PB	n=44	28.7	25.0	27.7
PM	n=50	8.7	8.4	8.5

2 : Correlation

DPH (n=38)	MARKIT-EMIT	r=0.980	Y=0.91Z+1.2
	MARKIT-GLC	r=0.972	Y=1.05X+1.4
	EMIT-GLC	r=0.998	Z=1.13X+0.7
PB (n=44)	MARKIT-EMIT	r=0.973	Y=1.07Z−1.0
	MARKIT-GLC	r=0.934	Y=0.96X+4.7
	EMIT-GLC	r=0.934	Z=0.87X+5.9
PM (n=50)	MARKIT-EMIT	r=0.981	Y=0.99Z+0.3
	MARKIT-GLC	r=0.927	Y=0.93X+1.0
	EMIT-GLC	r=0.938	Z=0.93X+0.7

MARKIT (Y), EMIT (Z), GLC (X)

epilepsy treated with various antiepileptic drugs including the three drugs were measured by MARKIT and GLC. The correlationship of plasma levels of DPH, PB, and PM measured by the two methods are shown in Figure 5, 6 and 7, respectively. The values of the three drugs levels measured by the two methods were all well correlated with correlation coefficient (r) above 0.9. These drug levels in the samples were measured also by EMIT and the results are shown in Table 2. The average values obtained by the MARKIT were closely the same, whereas DPH and PB levels measured by MARKIT and EMIT were slightly higher than these by GLC.

DISCUSSION

Recently, in the field of drug therapy of epileptic patients, a remarkable progress had been made by using the index of monitored drug blood levels for deciding the optimal dosis or for preventing toxic side effect.[1,2] The progress owes greatly to the development of rapid and simple assay techniques for the blood drug levels. Especially, the enzyme-immunoassay developed by Ullman[5,6] gave a stimulus to apply the monitored blood drug levels for clinical treatment of patients with epilepsy. The EMIT has an advantage in the rapid assay of the blood drug levels, few minutes per sample, however the apparatus required for the assay is costly and its use is limited. On the other hand, the MARKIT developed by Kurooka et al.[4] can be applied to ordinary apparatus in the routine clinical laboratories. In the assay procedure, the MARKIT requires longer time in immuno reaction (60 min), centrifugation (10 min) for B/B separation, and enzyme reaction (30 min), however, when it comes to assay 50 to 100 samples simultaneously, it requires only 2 to 5 min per one sample. The supernatant containing the free enzyme labelled drug can be applied to the enzyme rate analyzer, enabling simplification of the assay procedure after B/F separation.

The present experimental data showed that plasma DPH, PB and PM levels measured by MARKIT and EMIT were well correlated, suggesting both methods can be applied for clinical test.

Although both MARKIT and EMIT are based on the enzyme-immunoassay, the enzyme and antibodies used are different each other. Therefore, different results were anticipated to be obtained before than evaluation, but there was no significant difference between them.

In the plasma DPH assay, MARKIT[4] showed a 1 % cross reaction with 5-(1-hydroxyphenyl)-5-phenyl hydantoin, whereas EMIT showed a 25 % cross reaction. In the plasma PB assay, MARKIT showed a 100 % cross reaction with p-OH PB and only 0.5 % cross reaction with N-methyl PB, whereas EMIT

showed a 100 % cross reaction with N-methyl PB which is often used in combination with PB for the treatment of epileptic patients.

In this sense, MARKIT has advantages over EMIT. p-OH PB which shows a 100 % cross reaction can be found in 1/100 of PB levels in the plasma and the assay result by MARKIT-phenobarbital reflect only PB in the plasma.

Both MARKIT and EMIT based on the enzyme-immunoassay technique were basically the same in precision. EMIT is interfered with plasma samples with hemolysis, lipidemia and bilirubinemia, whereas MARKIT is not interfered with plasma samples with hemoglobin (300 mg/dl), cholesterol (1,000 mg/dl), triglyceride (300 mg/dl), phospholipids (1,000 mg/dl), and bilirubin (20 mg/dl). Both methods can be used for the assay of plasma antiepileptic drug levels in the same concentration. In more details, the assay range of antiepileptic drugs covered with MARKIT are 0.5-40 μg/ml for DPH, 1-80 μg/ml for PB, and 0.5-20 μg/ml for PM, while EMIT covers 2.5-30 μg/ml for DPH, 5-80 μg/ml for PB, and 2.5-20 μg/ml for PM.

In comparison with European and American, Japanese patients are lower in their blood levels of effective antiepileptic drugs. For the plasma samples with lower drug levels, we had to increase the sensitivity of EMIT to 6 fold by elimination of a process to dilute the sample with buffer.

In this sense, MARKIT is useful for the clinical management of Japanese patients.

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