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COMPARISON OF HOMOGENEOUS ENZYME IMMUNOASSAY (EMIT) AND GAS-LIQUID CHROMATOGRAPHIC MEASUREMENTS OF PLASMA PHENOBARBITAL, DIPHENYLHYDANTOIN AND PRIMIDONE CONCENTRATIONS IN EPILETIC PATIENTS

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Abstract

The plasma PB (N=47), DPH (N=39) and primidone (N=30) levels in epileptic patients were determined by using EMIT and GLC and then, the determined values obtained by each of the two methods were compared. The high correlation was observed between the two methods in all three drugs as follows: $\gamma=0.974$ for PB, $\gamma=0.981$ for DPH, and $\gamma=0.962$ for primidone. EMIT has a considerably high accuracy and well reproducibility in addition to the simplicity of its operation, therefore, it may be possible to be applied as a routine clinical laboratory examination in the near future.

INTRODUCTION

For the quantitative determination of antiepileptics, titrimetry, colorimetry, ultraviolet spectrophotometry, thin-layer chromatography and gas-liquid chromatography (GLC) have been performed, but the measurement by GLC is said to be most accurate among them at the present stage. Miyamoto had reviewed on the measurement methods of antiepileptics[1]. Each of methods mentioned above, however, has some inadequate points as a routine clinical examination, so that sufficient informations on the plasma concentration of the drugs are not now available to assist the drug therapy for epileptic patients.

On the other hand, the enzyme immunoassay measurement reagent kit (EMIT developed by Ullman et al.[2,3]) using its exclusive measurement apparatus is revealed to have advantages of its rapid and easy operation, in addition to the higher sensitivity in μg/ml unit which is said to be sufficient to measure plasma concentrations of antiepileptics.

Therefore, we determined plasma phenobarbital (PB), diphenylhydantoin (DPH) and primidone levels by using EMIT in parallel with GLC. Following the comparison of the two results obtained by these two measurements, we observed a high correlation about which we report here.

SUBJECTS AND METHOD

Subjects: We selected for this study 53 cases of in- and outpatients of epilepsy with clinical seizures of various types receiving PB, DPH or primidone. The blood sampling was performed via vein by using heparin instillation syringe at the time of attending outpatients and before the morning dose for inpatients, and these samples were kept at 4°C in a refrigerator until used. Besides, the patients receiving a single or more than 2 doses of the drugs mentioned above, patients receiving other antiepileptics were also included in this study.

Measurement: Enzyme immunoassy measurement using pipettor-dilutor; 50 μl of plasma was diluted with 250 μl of 0.055 M Tris HCl buffer solution (pH 7.9) in a 1.0 ml disposable beaker. This diluted sample was diluted with 250 μl of the same buffer again in the same manner. Then 50 μl of reagent A (containing antibody, substrate and co-enzyme) and reagent B (containing enzyme labelled drug) respectively were added to the diluted sample. Immediately upon addition of Reagent B the contents of the beaker was aspirated into the spectrophotometer. The absorbancy after 15 and 45 seconds (of NADH produced from NAD at 340 nm) was measured and the sample was assayed by utilizing the difference of the two absorbancy and the relation of drug concentration. The apparatus used in this measurement was consisted of Gilford -300 T type spectrometer with a 30°C thermally regulated flow-cell inside connected with SYVA Timer Printer enabling to set the time of measurement. In addition, the samplings of the test solution and reagents were performed by using SYVA pipettor-dilutor with the increased accuracy for EMIT use.

GLC; The method described by Miura et al.[7], combing with the methods by Chin et al.[4], Cooper et al.[5] and Estas[6], was used. Namely, after 1 ml of plasma having been acidified by HCl was extracted with ether and concentrated, cholestane (methanol solution) was applied as the internal standard substance to which the methanol solution of trimethylanilinium hydroxide was added. A liquote of the mixture was injected onto GLC column and measured the resulting peaks after having flash-heater methylation. The GLC apparatus used was Shimazu GC-

4CM with pyrex column, 3 mm×200 cm of inside diameter, and packed with OV-17 (3 %). The analysis was performed on the conditions: the column temperature at 180-270°C; the rate of increased temperature at 6°C/min; the flow rate of gas at 50 ml/min for N_2 and 40 ml/min for H_2; and finally the detection was done by using FID.

RESULTS

1. The calibration curve of EMIT, DPH phenobarbital and primidone.

The calibration curve was obtained from the determined values in the following series of concentrations by using EMIT AED calibration on the market: 2.5, 5, 10, 20, 30 μg/ml for DPH, 5, 10, 20, 40, 80 μg/ml for PB and 2.5, 5, 10, 15, 20 μg/ml for primidone. The linear relation of PB, DPH and primidone was observed on the specific graph paper in each case. The DPH correlation curve is shown in Fig. 1 for an example.

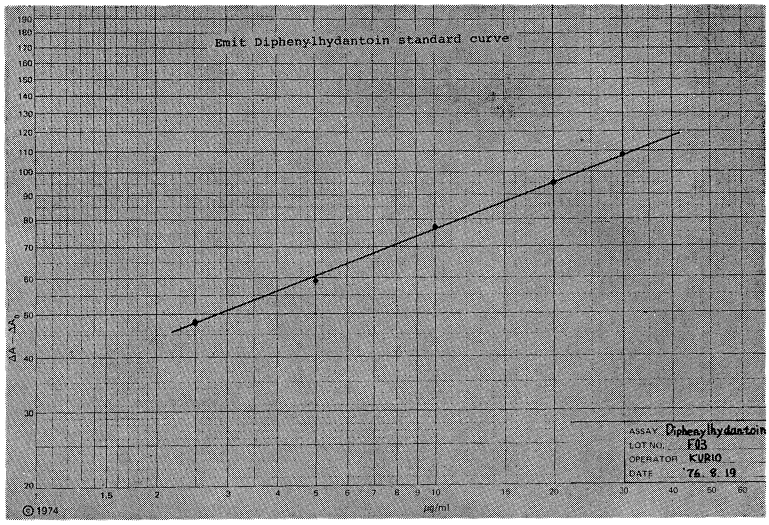

Fig. 1. EMIT Diphenylhydantoin Standard Curve

2. The plasma concentration of DPH, PB and primidone in 51 epileptic patients treated with the drugs.

The plasma samples obtained from 51 epileptic patients under the treatment with PB, DPH or primidone, and/or further with plural drugs were determined by GLC and EMIT. The average level of PB in all patients was 31.11 ± 16.80 μg/ml (±SD) by EMIT and 30.96 ± 16.00 by

GLC, therefore, no significant difference was observed between the two. Two average blood DPH levels obtained by using EMIT and GLC were 4.85 ± 4.99 μg/ml and 4.57 ± 4.59 μg/ml and those of primidone were 8.10 ± 4.46 μg/ml and 7.98g ± 4.55 μg/ml respectively, so that there also no significant difference between the two measurements.

Comparison of EMIT and GLC Measurements of Plasma PB, DPH and Primidone in Epileptic Patients

		EMIT	GLC	statistic differences
phenobarbital	Mean plasma concentration (μg/ml)±SD	31.11±16.80	30.96±16.00	t=0.044 N.S.
	Number of patients	47	47	
diphenyl-hydantoin	Mean plasma concentration (μg/ml)±SD	4.85±4.99	4.57±4.59	t=0.285 N.S.
	Number of patients	39	39	
primidone	Mean plasma concentration (μg/ml)±SD	8.10±4.46	7.98±4.55	t=0.111 N.S.
	Number of patients	30	30	

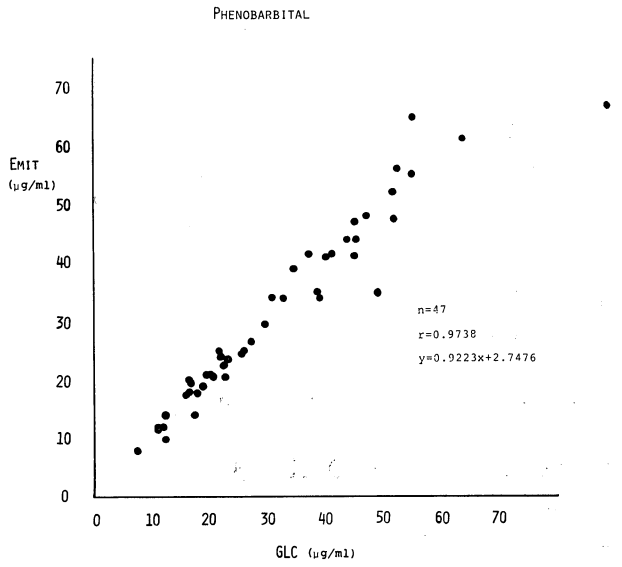

Fig. 2. Comparison of phenobarbital plasma levels (μg/ml) as measured by gas-liquid chromatography and homogeneous enzyme immunoassay in the same patients (N=47)

3. The correlation of the determined value by EMIT with that of GLC.

The comparison of the determined values obtained from the two measurements was performed in the same plasma sample taken from the patient and the coefficient of correlation γ was calculated as follows; γ EMIT/GLC=0.974 for PB (Fig. 2), γ EMIT/GLC=0.981 for DPH (Fig. 3) and γ EMIT/GLC=0.962 for primidone (Fig. 4). The positive correlation was observed in all three drugs by $P < 0.001$.

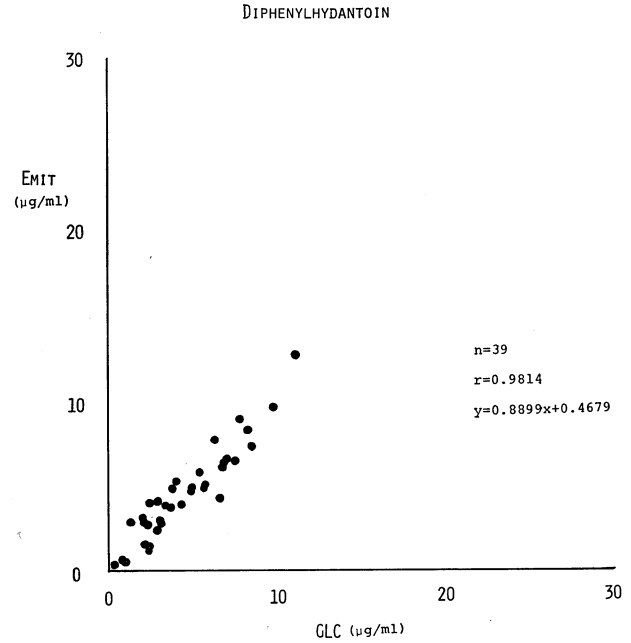

Fig. 3. Comparison of diphenylhydantoin plasma levels (μg/ml) as measured by gas-liquid chromatography and homogeneous enzyme immunoassay in the same patients (N=39)

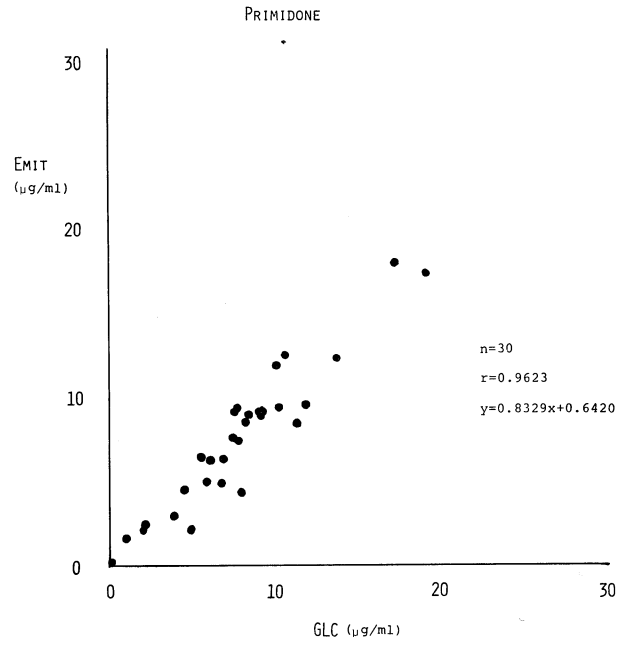

Fig. 4. Comparison of primidone plasma levels (μg/ml) as measured by gas-liquid chromatography and homogeneous enzyme immunoassay in the same patients (N=30)

DISCUSSION

By the explanatory statement on the package insert of EMIT, the minimum concentrations of DPH, primidone and PB which will be detectable from each calibration curve are said to be 2.5 μg/ml for DPH and primidone and 5 μg/ml for PB respectively, but it was sufficiently possible to determine DPH up to 0.4 μg/ml, PB up to 0.8 μg/ml and primidone up to 0.8 μg/ml by saving the first dilution of the samples even in the concentrations of less than the values mentioned above. As it has been already proved that the therapeutic concentrations of these drugs for epileptic patients in our country are lower than those reported in Europe and United States in either case[8,9,10], therefore, the determination in rather lower concentrations as we did may be considered useful in the clinical examination. Nonetheless we should bear in mind that the higher value produced by saving the first dilution need to be determined when using the sample containing a substance with high cross-reactivity.

No reports concerning the comparison of each plasma level of DPH,

PB and primidone determined by EMIT and GLC are available in Japan but there is a paper partly referring to the subject by Miyamoto[8]. As shown by the results of our study, EMIT was proved to have a high correlation with GLC in all cases of DPH, PB and primidone and to bring the identical result with that produced by GLC which is considered to be of the most accurate and precise measurement among conventional ones at the present stage. GLC has an advantage in that the serum concentrations of various antiepileptics can be determined simultaneously, which has not been applied to a routine clinical examination because the extraction of drugs from plasma samples by this method is so complicated, requiring much time. However, EMIT may enable us to determine the plasma levels even in a small amount of blood sample and its operation is so simple that the determination may take two minutes at most. Thus it is considered the method will be applicable to the routine clinical examination. As the method will require any equipment or machinery for the treatment of RI substance in comparison with radioimmunoassay, and the determination will be done on the enzyme activity directly without separation of the combined form (enzyme-drug complex-antibody) from the free-form (enzyme-drug complex), thus it will have an advantage of easy determination similar to other general enzyme activity measurements. As for the crossreactivity, however, we should keep in our mind that the phenomenon may appear at the ratio of PB to mephobarbital being 1:1 for PB, DPH to 5-(P-hydroxyphenyl)-5-phenylhydantoin being 1:4 for DPH and primidone to DPH, primidone to PB being 1:80 for primidone as stated in the explanatory statement on EMIT. Furthermore, there is a possibility to provoke a little cross-reactivity if a large amount of other anti-epileptics be present, but this point may give no trouble in clinical practice. As the average plasma PB, DPH and primidone levels in our patients were estimated in order to compare the determined values obtained by EMIT with those of GLC, we selected plasma samples to be distributed in a wide range of concentrations higher to lower in 116 patient determined by EMIT, and then GLC was performed. Therefore, these values do not represent the average concentration of PB or DPH in epileptic patients blood in Japan. As for the therapeutic concentration, we already reported about it in the previous paper[9,10].

It is also known that primidone will be transformed into the active metabolite such as phenobarbital or phenylethylmalonamide *in vivo*[11], thus the clinical implication of the plasma concentration of primidone

may be revealed only after the simultaneous determination of the blood concentration of these active metabolites. Further studies on this problem are being planned.

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