Phenytoin Immunoassay Measurements in Serum Samples From Patients With Renal Insufficiency: Comparison With High-Performance Liquid Chromatography

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The debate continues regarding the possible interference of phenytoin metabolites in phenytoin immunoassays, and its clinical importance for patients with renal failure. The aim of this study was to compare the results obtained using the Abbott fluorescence polarization immunoassay (FPIA), Dade enzyme-multiplied immunoassay technique (EMIT), and high-performance liquid chromatography (HPLC) to establish the significance of the differences in conditions of renal failure. Thirty-six adult patients who had been treated with phenytoin and whose renal function ranged from normal to severely impaired were chosen for this study. In accordance with previously established validation criteria for analytical methods for the determination of drugs, a 15% bias from the HPLC phenytoin values was considered an acceptable limit. The mean

($\pm$ SEM) glomerular filtration rate (GFR) of the patients was 37.5 ± 4.6 mL/min (range = 10–102 mL/min). The mean values found using FPIA (10.8 ± 1.2 μ g/mL) and EMIT (10.8 ± 1.3 μ g/mL) presented acceptable deviations with respect to HPLC (10.5 ± 1.2 μ g/mL), and a high correlation was found among the results ($N = 36$) of the different methods ($r \geq 0.987$, $P < 0.001$). An FPIA deviation above the 15% bias limit with respect to HPLC was found only in two cases with very low serum phenytoin concentrations and low GFR values (< 20 mL/min), although it does not appear to be important in terms of adjusting drug dosage. According to our data, FPIA and EMIT gave accurate results for total phenytoin in serum samples from patients with renal failure. *J. Clin. Lab. Anal.* 21:119–123, 2007. © 2007 Wiley-Liss, Inc.

Key words: phenytoin immunoassays accuracy; renal insufficiency; phenytoin metabolites

INTRODUCTION

Phenytoin is mainly metabolized in the liver by the cytochrome P450 system to its pharmacologically inactive metabolite 5-(4'-hydroxyphenyl)-5-phenylhydantoin (HPPH), which is subsequently conjugated with glucuronic acid and then eliminated in urine (1,2). HPPH is present in plasma almost entirely as the glucuronic acid conjugate (HPPG), although it has been demonstrated that there is a large interindividual variability for HPPH glucuronidation in humans (3,4).

The serum concentrations of HPPG are increased 10-fold in renal failure, resulting in levels four to 10 times higher than phenytoin (5). In relation to HPPH, it has been reported that the serum concentrations of this metabolite in uremic and non-uremic individuals are similar (6) and do not exceed levels of 1.5 μ g/mL (7); however, concentrations of up to 4.0 μ g/mL have been

observed in patients with renal failure (8). In one study (5), a significant increase in the serum concentration of HPPH was found in patients with renal failure, but did not exceed 10% of the phenytoin concentration in any case. Little is known about the serum levels of other minor phenytoin metabolites, or to what extent they may accumulate in renal insufficiency.

High-performance liquid chromatography (HPLC) methods have the advantage of separating the parent drug from its metabolite HPPH. The cross-reactivity of

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different phenytoin immunoassays with the metabolites HPPH and HPPG has been explored in different studies, but conflicting results have been obtained regarding its clinical significance (9–17). Recent results obtained in a College of American Pathologists TDM Survey suggesting a significant HPPH interference in several phenytoin immunoassays, especially in the Abbott fluorescence polarization immunoassay (FPIA) (17), have reopened the debate regarding the possible interference of phenytoin metabolites in the phenytoin immunoassays in conditions of renal insufficiency (17–19).

In this study we compared the results of the FPIA and enzyme-multiplied immunoassay technique (EMIT) with those found via HPLC for phenytoin in serum samples from patients with various degrees of renal impairment.

MATERIALS AND METHODS

Serum concentrations of phenytoin were determined in 36 samples obtained for routine therapeutic drug monitoring and taken immediately before the next dose from 36 patients (17 males and 19 females, mean ($\pm$ SEM) age = 62.2 ± 3.9 years, range = 25–90 years) with normal to severely impaired renal function. The immunoassays used to measure phenytoin were FPIA on the TDx analyzer (Abbott Laboratories, Abbott Park, IL) and Emit 2000 on a V-Twin analyzer (Dade Behring, Marburg, Germany). Both immunoassays were calibrated and used in accordance with the recommendations of the manufacturers. The determination of serum phenytoin concentration by HPLC was made using an Agilent 1100 Series chromatographic system, and reagents and procedure from Chromsystems Instruments and Chemicals GmbH (Munich, Germany). This assay is carried out using an isocratic system with UV detection. HPPH appears at 2–3 min, and the parent drug appears at approximately 17.6 min in the high-resolution chromatogram (Fig. 1). Using the Chromsystems antiepileptic drugs serum controls, the between-run coefficient of variation ($N = 25$) was 0.82% for a mean ($\pm$ SEM) of 4.89 ± 0.01 $\mu\text{g/mL}$ (assigned value = 4.83 $\mu\text{g/mL}$), and 1.01% for 18.72 ± 0.04 $\mu\text{g/mL}$ (assigned value = 18.50 $\mu\text{g/mL}$). Serum cystatin C was measured using a particle-enhanced immunoassay in a BN ProSpec nephelometer (Dade Behring, Marburg, Germany). The predicted values for the glomerular filtration rate (GFR) derived from the serum cystatin C concentrations were calculated according to the equation proposed by Larsson et al. (20): $\text{GFR (mL/min)} = 77.24 \times (\text{serum cystatin C})^{-1.2623}$.

Statistical analysis of the data was performed with the Microsoft-Excel package (v.5.0), and the results were expressed as the mean $\pm$ SEM. Linear regression analysis was performed with the Passing-Bablok method, and

Fig. 1. High-resolution chromatogram of serum from an epileptic patient treated with phenytoin, phenobarbital, and carbamazepine.

the results were also compared using the Eksborg (21) difference plots. In accordance with previously published validation criteria of analytical methods for the quantitative determination of drugs and their metabolites in biological samples (22), the acceptable level for accuracy was a deviation of no more than $\pm 15\%$ from the considered nominal concentrations (HPLC values).

RESULTS

The 36 serum samples had a mean concentration of 2.5 ± 0.3 mg/dL (range = 0.4–7.9 mg/dL) for creatinine and 2.6 ± 0.2 mg/L (range = 0.8–4.9 mg/L) for cystatin C, with a highly significant correlation between both variables ($r = 0.705$, $P < 0.001$). The GFR value predicted by the cystatin C concentration for the group of patients was 37.5 ± 4.6 mL/min (range = 10–102 mL/min).

The mean levels found for the serum concentration of phenytoin using FPIA (10.8 ± 1.2 $\mu\text{g/mL}$) and EMIT (10.8 ± 1.3 $\mu\text{g/mL}$) presented acceptable deviations according to the criterion of accuracy used (22) with respect to HPLC (10.5 ± 1.2 $\mu\text{g/mL}$). Figure 2 shows the correlation and regression (A, C, and E) and the difference plots (B, D, and F) for the results obtained using FPIA, EMIT, and HPLC. Figure 3 shows the relationship of the relative differences between the phenytoin measurements with the GFR.

For the two immunoassays used, unacceptable deviations from the accuracy criterion used (22), both between each other and with respect to HPLC, were found only in two cases that simultaneously presented very low GFR values (< 20 mL/min) and phenytoin levels (see Figs. 1 and 2).

Fig. 2. Correlation, regression, and Eksborg difference plots for the FPIA, EMIT, and HPLC phenytoin results for serum samples from patients with GFR values lower (●) and higher (○) than 20 mL/min. The dotted lines correspond to the limits of the acceptance criterion for accuracy.

DISCUSSION

The serum concentration of cystatin C is currently considered to be a more suitable marker for estimating GFR than serum creatinine (23,24), and the present results indicate that the 36 patients had normal to severely impaired renal function (see Fig. 3).

Acceptable differences according to the criteria of accuracy used (22) among the mean phenytoin values determined by FPIA, EMIT, and HPLC, and high correlation coefficients among them ($r \geq 0.987$, $P < 0.001$) were found (Fig. 2). In most of the patients, the deviations were lower than 16% independently of renal function. In two cases with GFR < 20 mL/min and

Fig. 3. Relationship of the relative differences among FPIA, EMIT, and HPLC phenytoin results with the GFR for serum samples with phenytoin levels lower (●) and higher (○) than 10 µg/mL. The dotted lines correspond to the limits of the acceptance criterion for accuracy.

very low phenytoin levels, the FPIA provided higher results than HPLC or EMIT, with deviations above the acceptable limit (Figs. 2 and 3). In these two cases, since a higher analytical imprecision is expected, the phenytoin determinations were carried out in duplicate, and the means were 0.9 µg/mL and 0.9 µg/mL for HPLC, 1.5 µg/mL and 1.8 µg/mL for FPIA, and 1.0 µg/mL and 1.1 µg/mL for EMIT. These levels were above the

detection limit of the different techniques used (approximately 0.5 µg/mL); however, they were below the concentration of low FPIA and EMIT calibrators (2.5 µg/mL), which should hamper interpretation of the results.

The FPIA and EMIT phenytoin assays do not present a significant cross-reactivity with HPPG (13,18), although this metabolite appears to produce a slight negative interference in the EMIT (13). The phenytoin-N-glucuronide shows a significant cross-reactivity with FPIA and other phenytoin immunoassays (15); however, this is a minor phenytoin metabolite. Soldin and coworkers (17) suggested that HPPH presented a 19% cross-reactivity in the FPIA, which was the more affected of the most widely used immunoassays, and no doubt accounted for a significant HPPH interference in some phenytoin immunoassays (19). However, other authors found a lower HPPH cross-reactivity (5–15%) for FPIA (13–16). In some studies the EMIT showed an even lower cross-reactivity with HPPH (12,13,17), and did not exhibit any significant interference in specimens from renally impaired patients (12,13).

The importance in clinical practice of HPPH interference in phenytoin immunoassays should be connected with the relative proportion of the metabolite concentration with respect to the parent drug concentration. The serum HPPH/phenytoin ratio may be modulated by the rate of HPPH formation, the rate of HPPH glucuronidation, and impairment of renal function. In general, the ratio of the serum concentration of a metabolite to the unchanged drug depends on the metabolic capacity of the liver. This capacity for metabolizing phenytoin is limited, reaching the saturation of the liver's oxidative enzyme system for drug serum concentrations even within the therapeutic range (1,25). A large interindividual variability for the glucuronidation of HPPH in phenytoin-treated patients has been described (3,4). However, this variability was not related to genetic polymorphisms of UDP-glucuronosyltransferases, and may be affected by other factors, such as diet, environmental conditions, and disease state (4). Consequently, only in some cases with very low phenytoin concentrations and GFR values, the FPIA deviation with respect to HPLC and even to EMIT would be greater than the 15% considered as the acceptable limit (22). Although a bias limit of 10% has also been recommended for maximum phenytoin inaccuracy (26), the possible metabolite interference does not appear to be of practical importance in terms of drug dosage adjustment.

In accordance with the findings of Rainey et al. (13), our study suggests that both FPIA and EMIT provide accurate results for total serum phenytoin in specimens from patients with renal insufficiency. However, since

HPPH and HPPG are not strongly protein bound, and the ratios of the free metabolites to the free phenytoin concentrations will be increased in relation to the ratios of total concentrations (13,17), the possible interference of HPPH in free phenytoin measurements using FPIA would be more relevant.

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REFERENCES

1. Finn AL, Olanow CW. Phenytoin. Therapeutic use and serum concentration monitoring. In: Taylor WJ, Finn AL, editors. Individualizing drug monitoring. Practical applications of drug monitoring. Vol. 2. New York: Gross, Townsend, Frank, Inc.; 1981. p 63–85.
2. Maguire JH. Quantitative estimation of catechol/methylcatechol pathways in human phenytoin metabolism. *Epilepsia* 1988;29:753–759.
3. Nakajima M, Sakata N, Ohashi N, Kume T, Yokoi T. Involvement of multiple UDP-glucuronosyltransferase 1A isoforms in glucuronidation of 5-(4'-hydroxyphenyl)-5-phenylhydantoin in human liver microsomes. *Drug Metab Dispos* 2002;27:1165–1170.
4. Yamanaka H, Nakajima M, Hara Y, et al. Urinary excretion of phenytoin metabolites, 5-(4'-hydroxyphenyl)-5-phenylhydantoin and its O-glucuronide in humans and analysis of genetic polymorphisms of UDP-glucuronosyltransferases. *Drug Metab Pharmacokinet* 2005;20:135–143.
5. Borga O, Hoppel C, Odar-Cederlof I, Garle M. Plasma levels and renal excretion of phenytoin and its metabolites in patients with renal failure. *Clin Pharmacol Ther* 1979;26:306–314.
6. Hoppel C, Garle M, Rane A, Sjöqvist F. Plasma concentration of 5-(4'-hydroxyphenyl)-5-phenylhydantoin in phenytoin-treated patients. *Clin Pharmacol Ther* 1977;21:294–300.
7. Burgess ED, Friel PN, Blair Jr AD, Raisys VA. Serum phenytoin concentrations in uremia. *Ann Intern Med* 1981;94:59–60.
8. Eadie MJ, Tyrer JH, Bochner F, Hooper WD. The elimination of phenytoin in man. *Clin Exp Pharmacol Physiol* 1976;3:217–224.
9. Flachs H, Rasmussen JM. Renal disease may increase apparent phenytoin in serum as measured by enzyme-multiplied immunoassay. *Clin Chem* 1980;26:361.
10. Rubenstein K, Bastiani R. Renal disease may increase apparent phenytoin in serum as measured by enzyme-multiplied immunoassay [reply]. *Clin Chem* 1980;26:362.
11. Sawchuk RJ, Matzque GR. Contribution of 5-(4-hydroxyphenyl)-5-phenylhydantoin to the discrepancy between phenytoin analyses by EMIT and high-pressure liquid chromatography. *Ther Drug Monit* 1984;6:97–103.
12. Roberts WL, Rainey PM. Interference in immunoassay measurements of total and free phenytoin in uremic patients: a reappraisal. *Clin Chem* 1993;39:1872–1877.
13. Rainey PM, Rogers KE, Roberts WL. Metabolite and matrix interference in phenytoin immunoassays. *Clin Chem* 1996;42:1645–1653.
14. Datta P. Oxaprozin and 5-(p-hydroxyphenyl)-5-phenylhydantoin interference in phenytoin immunoassays. *Clin Chem* 1997;43:1468–1469.
15. Frank EL, Schwartz EL, Juenke J, Annesley TM, Roberts WL. Performance characteristics of four immunoassays for antiepileptic drugs on the IMMULITE 2000 automated analyzer. *Am J Clin Pathol* 2002;118:124–131.
16. Datta P, Scurlock D, Dasgupta A. Analytic performance evaluation of a new turbidimetric immunoassay for phenytoin on the ADVIA 1650® analyzer: effect of phenytoin metabolite and analogue. *Ther Drug Monit* 2005;27:305–308.
17. Soldin SJ, Wang E, Verjee Z, Elin RJ. Phenytoin overview–metabolite interference in some immunoassays could be clinically important. *Arch Pathol Lab Med* 2003;127:1623–1625.
18. Roberts WL, Rainey PM. Phenytoin overview–metabolite interference in some immunoassays could be clinically important. *Arch Pathol Lab Med* 2004;128:734.
19. Soldin SJ, Guo T, Wang E, Verjee Z, Elin RJ. Phenytoin overview–metabolite interference in some immunoassays could be clinically important. *Arch Pathol Lab Med* 2004;128:734–735.
20. Larsson A, Malm J, Grubb A, Hansson LO. Calculation of glomerular filtration rate expressed in mL/min from plasma cystatin C values in mg/L. *Scand J Clin Lab Invest* 2004;64:25–30.
21. Eksborg S. Evaluation of method–comparison data. *Clin Chem* 1981;27:1311–1312.
22. Shah VP, Midha KK, Dighe S, et al. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetics studies. *Eur J Drug Metab Pharmacokinet* 1991;16:249–255.
23. Dharnidharka VR, Kwon C, Stevens G. Serum cystatin C is superior to serum creatinine as a marker of kidney function: a meta-analysis. *Am J Kidney Dis* 2002;40:221–226.
24. Laterza OF, Price CP, Scott MG. Cystatin C: an improved estimator of glomerular filtration rate. *Clin Chem* 2002;48:699–707.
25. Perucca E, Richens A. Antiepileptic drugs: clinical aspects. In: Richens A, Marks V, editors. Therapeutic drug monitoring. Edinburgh: Churchill Livingstone; 1981. p 320–348.
26. Warner A, Privitera M, Bates D. Standards of laboratory practice: antiepileptic drug monitoring. *Clin Chem* 1998;44:1085–1095.