

Cost-Effective Assays for Use in Monitoring Carbamazepine, Phenobarbital, and Phenytoin in Serum

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Using Syva "EMIT" reagents and the Cobas Bio centrifugal analyzer, we have developed cost-effective assays for therapeutic drug monitoring of carbamazepine, phenobarbital, and phenytoin. With these optimized methods one can assay up to 2470 samples with a single 100-test EMIT kit with good precision and accuracy. Between-batch CVs, based on results of repeat analysis of routine patients' samples, were 4.6%, 7.0%, and 5.3%, respectively. Results correlated well with those by methods previously used [gas chromatography and fluorescence polarization (Abbott TDx)], but there was a negative bias (-10.9%) of our method for phenobarbital results in patients' samples, as compared with those obtained with the TDx.

Additional Keyphrases: *enzyme immunoassay · centrifugal analyzer · gas chromatography and fluorescence polarization compared · therapeutic drug monitoring*

Therapeutic drug monitoring is a valuable aid in management of epilepsy, but individuals vary widely in rates of absorption, metabolism, and excretion and in their response to anti-epileptic drugs, and the therapeutic efficacy of the drugs correlates better with concentrations in plasma than with the dose. In addition to providing for individualization of drug therapy, monitoring drug concentrations in plasma is useful for detecting and discouraging noncompliance and helps to prevent unnecessary use of different drugs (1).

Two analytical techniques that are widely used in therapeutic drug monitoring are chromatography and immunoassay. The chromatographic procedures, gas chromatography (GC) and "high-performance" liquid chromatography (HPLC), are excellent methods for separating drugs and metabolites. Using them, one can simultaneously analyze for several different anti-epileptic drugs and their metabolites in the same sample. However, these methods are labor-intensive and require skilled operators—although these disadvantages may be minimized by using a micro-computer to measure peak retention times and peak heights and to simplify identification of peaks—and they are not well suited for use as emergency assays outside of regular workshift hours. The initial equipment cost is high, but the cost of consumables is low.

Several homogeneous immunoassays are commercially available for therapeutic drug monitoring, including enzyme-multiplied immunoassays ("EMIT"; Syva Co., Palo Alto, CA), polarization fluoroimmunoassay (TDx; Abbott Laboratories Ltd., Wokingham, U.K.), and substrate-labeled fluorescent immunoassay (Optimate; Ames Division, Miles Laboratories Inc., Stoke Poges, Slough, U.K.). These assays are simple to use and rapid, and results correlate

well with those obtained by other procedures, including GC and HPLC (2-5). However, the cost of consumables is higher than in the chromatographic methods, and a different immunoassay method is required for the measurement of each drug.

Various procedures have been described (6-8) for modifying EMIT assays for use in the Cobas Bio centrifugal analyzer (Roche Products Ltd., Diagnostic Division, Welwyn Garden City, U.K.) to minimize reagent consumption and hence decrease the cost per sample. Here we describe the use of EMIT kits for carbamazepine, phenobarbital, and phenytoin modified for use with the Cobas Bio analyzer, along the lines of the cost-effective system devised by Sung and Neeley (8) for measurement of theophylline. We compared the results with those obtained by the methods we previously used in this laboratory (TDx for carbamazepine and phenobarbital; GC for phenytoin) and assessed the precision and reliability of the modified procedures. By using these cost-effective systems one can assay as many as 2470 samples with one 100-test EMIT kit.

Materials and Methods

TDx method. The TDx procedure was performed according to the manufacturer's instructions for carbamazepine and phenobarbital (1986 Assay Systems Manual).

GC method. Phenytoin was extracted from acidified serum or plasma into diethyl ether and measured by GC of the methylated derivative. Tolyphenylhydantoin was used as the internal standard. GC conditions were as follows: Sigma 3 instrument (Perkin-Elmer Ltd., Beaconsfield, U.K.) with nitrogen detector; column, 2% CDMS (cyclohexanedimethanol succinate) on Chromosorb W (1 m length); oven temperature, 220 °C; injector and detector temperature, 240 °C; and carrier gas flow, 60 mL/min.

EMIT reagents. EMIT kits containing reagents and calibrators were from Syva Co. Calibrators, controls, and stock "antibody/substrate" and "enzyme-labeled antigen" reagents were reconstituted and stored as recommended in the manufacturer's package insert. Stock "G6P/NAD coenzyme" and "substrate booster" were prepared by adding 1.24 g of glucose 6-phosphate (G6P) monosodium salt (cat. no. G-7879) and 2.91 g of NAD⁺ (cat. no. N-7004; both from Sigma Chemical Co., Poole, Dorset, U.K.), to 10 mL of distilled water. This yielded 12 mL of a solution containing, per liter, 366 mmol of G6P and 366 mmol of NAD⁺, which we divided into five aliquots and stored at -20 °C. The working buffer was prepared by diluting the stock buffer according to the manufacturer's instructions. Working antibody/substrate reagent was prepared by mixing stock reagent (1.5 mL), working buffer (103.5 mL), and stock G6P/NAD⁺ (2.1 mL). Working enzyme reagent was prepared by adding stock reagent (1.5 mL) to working buffer (9.0 mL). The manufacturer states that reconstituted kit reagents are stable for three months at 4 °C.

Cobas Bio procedures. Reagents, standards, tests, and controls were removed from the refrigerator and allowed to reach ambient temperature. Table 1 lists the Cobas Bio test

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Table 1. Settings for Cobas Bio Centrifugal Analyzer for Assay of Carbamazepine

	$\mu\text{mol/L}$
1. Units	1000
2. Calculation factor	1000
3. Standard 1 concentration	0.00
2 concentration	8.47 ^a
3 concentration	16.95
4 concentration	33.90
5 concentration	50.85
6 concentration	84.75
6. Limit	0.0
7. Temperature ($^{\circ}\text{C}$)	37.0
8. Type of analysis	7.3
9. Wavelength (nm)	340
10. Sample volume (μL)	3
11. Diluent volume (μL)	70
12. Reagent volume (μL)	170
13. Incubation time (s)	20
14. Start reagent volume (μL)	17
15. Time of first reading (s)	5.0
16. Time interval (s)	10
17. No. of readings	19
18. Blanking mode	0
19. Printout mode	1

^a The corresponding standard concentrations 2–6 for phenobarbital are 21.55, 43.10, 86.21, 172.4, and 344.8 $\mu\text{mol/L}$; for phenytoin, 9.92, 19.84, 39.68, 79.37, and 119.1 $\mu\text{mol/L}$.

settings for carbamazepine. Except for the concentrations of the standards, the same settings were used for phenobarbital and phenytoin. Concentrations of the EMIT-a.e.d. (anti-epileptic drug) controls can be expected to be within 10% of the value specified by Syva. The system was calibrated before each analytical run (current cost of calibration, about £1.20, \$2.03).

Controls. We used "Seronorm" (Nyegaard & Co., Oslo, Norway) control serum and EMIT-a.e.d. controls in assessing precision.

Results

Carbamazepine. To assess the extent to which the calibration curve slope varies between analytical runs (with use of the same batch of working reagents), we examined curves for 22 runs. To negate the effect of between-assay "shifts," we subtracted the reaction rate for the zero calibrator from the reaction rate values for the standards. The results (Table 2) show that the slope of the curve is very constant. However, the variation we observed was not entirely random: two of the lowest range values come from the same curve and all five of the highest from another one.

The precision of replicate measurements of carbamazepine was assessed by using Seronorm controls for within-batch precision, and EMIT-a.e.d. controls, Seronorm controls, and repeat analysis of patients' samples for between-batch precision (Table 3). Precision was good, the mean CVs being $\leq 4.5\%$. We assessed the accuracy of the modified method by comparing the results obtained for 77 patients' samples with the results obtained by the TDx method. The correlation between the two methods is shown in Figure 1a ($y = 0.97x + 1.59 \mu\text{mol/L}$, where $x = \text{TDx method}$ and $y = \text{Cobas Bio method}$). The correlation coefficient was 0.97.

In routine use, based on repeat analysis of patients' samples, the mean CV for the assay was 4.6%; for the TDx

Table 2. Variation in Shape of Calibration Curves for Measurements of Carbamazepine by the Cobas Bio Method

Concn of standard, $\mu\text{mol/L}$	Reaction rate, $\Delta A/\text{min}^a$			
	Mean	Range	SD	CV, %
8.5	8.58	7.5–10.4	0.64	7.4
17.0	15.35	14.5–16.9	0.60	3.9
33.9	25.11	26.6–27.5	0.81	3.2
50.9	31.33	30.3–33.9	0.99	3.2
84.8	38.70	37.3–41.0	1.03	2.6

$n = 22$ each.

^a To negate the effect of between-assay "shifts," the reaction rate for the zero calibrator (mean 70.52, range 66.9–75.8, SD 1.99, CV 2.8%) was subtracted from the reaction-rate values for the standards.

method, the CV was 4.0%.

Phenobarbital. The within-batch precision was assessed by using Seronorm controls (target value 95 $\mu\text{mol/L}$), and the between-batch precision was evaluated by using calibration standards and Seronorm controls. The mean CV was 3.9%, significantly higher than that for the carbamazepine assay (2.1%). Evaluation of the between-batch precision gave a mean CV of 9.0% (Table 3) with individual values ranging from CV = 13.4% for a concentration of 20 $\mu\text{mol/L}$ to CV = 6.4% for 173 $\mu\text{mol/L}$.

We compared the results for 78 patients' samples obtained by the Cobas Bio method with those obtained by the TDx method. Regression analysis gave the following relation: $y = 0.89x + 0.19 \mu\text{mol/L}$, where $x = \text{TDx method}$ and $y = \text{Cobas Bio method}$ (Figure 1b). The correlation coefficient was 0.99. Values by the Cobas Bio method were consistently lower than by the TDx method. The mean values for the 78 patients' samples analyzed were 106.3 $\mu\text{mol/L}$ by the TDx method, 94.7 $\mu\text{mol/L}$ by the Cobas Bio method (concentration range of samples 8–308 $\mu\text{mol/L}$), giving a bias of -10.9% for the Cobas Bio method. Analysis of TDx standards and controls by the Cobas Bio method gave a bias of -6.5% to -17.4% (Table 4).

The CV for the assay in routine use, based on repeat analysis of patients' samples, was 7.0%, compared with a mean value of 2.2% for the TDx method.

Table 3. Precision of Cobas Bio Methods

	No. of samples	Concentration, $\mu\text{mol/L}$		Mean CV, %
		Mean	Range	
Carbamazepine				
Within-batch	95	30.9	28.8–33.7	2.1
Between-batch				
Controls (2)	40	—	23.2–32.9	4.5
RAC	20	34.5	10.6–52.4	2.3
Phenobarbital				
Within-batch	180	97.7	77.7–112.9	3.9
Between-batch				
Controls (5)	100	—	16.2–193.2	9.0
Phenytoin				
Within-batch	209	62.6	52.1–75.6	3.4
Between-batch				
Controls (6)	104	—	9.9–79.4	5.0
RAC	20	69.3	33.1–114.2	6.0

RAC, repeat analysis control: repeat analysis of patients' samples.

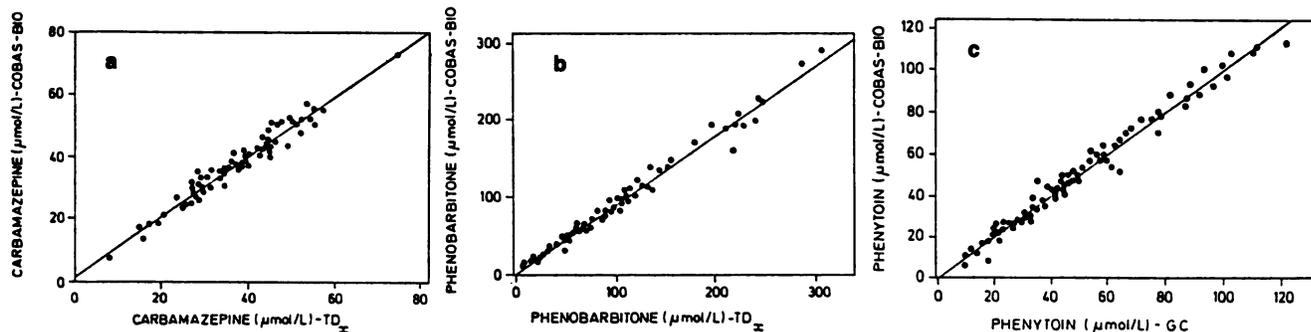


Fig. 1. Comparison of concentrations ($\mu\text{mol/L}$) measured by the TDx and Cobas Bio methods (a) Carbamazepine: $n = 77$; $r = 0.97$; slope = 0.97; and intercept = 1.59. (b) Phenobarbital (phenobarbitone): $n = 78$; $r = 0.99$; slope = 0.89; and intercept = 0.19. (c) Phenytoin: $n = 110$; $r = 0.99$; slope = 1.01; and intercept = -0.24

Phenytoin. Assessment of the within-batch precision based on 209 assays of a Seronorm control gave CVs in the range 1.7% to 5.7% with a mean of 3.4% (Table 3). To evaluate between-batch precision, we used EMIT phenytoin controls, Seronorm controls, calibration standards, and repeat analysis of patients' samples. Mean CVs of 6.0% or less were obtained (Table 3).

Regression analysis was based on a comparison of results for 110 patients' samples analyzed in 15 batches by the Cobas Bio and GC methods. The regression equation obtained was $y = 1.01x - 0.24 \mu\text{mol/L}$, where x is the GC method and y the Cobas Bio method; the correlation coefficient was 0.99 (Figure 1c).

The precision of the assay, based on the use of repeat analysis controls in routine operation, gave a CV of 5.3% as compared with 3.7% for the TDx method.

Discussion

Our modification of the EMIT system for use with the Cobas Bio analyzer has provided a method that is economical, rapid, and easy to perform, and is suitable for use outside of regular workshift hours. The between-batch CVs for the carbamazepine and phenytoin assays compared well with those for the TDx and GC methods we used previously. The precision of the Cobas Bio method for phenobarbital compares less well with the TDx method, and the Cobas Bio method shows a marked negative bias compared with the

TDx. Abbott Laboratories Ltd. reported good agreement between the TDx method and HPLC for phenobarbital (1986 Assay Systems Manual), whereas the EMIT method has been shown to compare less well with TDx (5) and with GC (2). However, because of wide interindividual variation in plasma concentrations required to achieve the desired anti-convulsant effect and the effects of tolerance on patients' responses to the drug, therapeutic drug monitoring for phenobarbital is of less value than for the other drugs studied, and the precision achieved with the Cobas Bio method for phenobarbital appears acceptable.

The costs per sample by the Cobas Bio methods (currently about 20 p, \$0.34) are considerably less than by the TDx method (about £3.10, \$5.24). The Cobas Bio method is not less expensive than the GC method for phenytoin, but it is much quicker and much less labor-intensive. The Cobas Bio methods described here have been in use for 10 months for phenytoin, eight months for carbamazepine, and two months for phenobarbital, and have been found to be very reliable in routine operation.

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Table 4. Analysis of TDx Phenobarbital Standards and Controls by the Cobas Bio Method

	Concentration, $\mu\text{mol/L}$		Bias, %
	TDx material, stated value	Cobas Bio, mean measured ($n = 6$)	
Standard B	21.6	20.2	-6.5
C	43.1	38.6	-10.4
D	86.2	75.4	-12.5
E	172.4	142.4	-17.4
Low control	64.7	56.4	-12.8
Medium control	129.3	110.0	-14.9
High control	215.5	189.0	-12.3