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Measurement of Plasma Phenytoin by EMIT in the Monarch Centrifugal Analyzer: Studies on the Elimination of Within-Rotor Drift

Philip R. Wenham and Heather M. Barbour

We investigated the measurement of phenytoin in plasma by the EMIT® method, in the Monarch centrifugal analyzer. When we used the standard protocol supplied by Instrumentation Laboratory, significant drift was observed across a full rotor loaded with 31 replicates of a single specimen. Although the cause remains obscure, the drift was eliminated by using a load–spin–reload–spin procedure. This makes it possible to analyze large batches of samples without excessive use of standards and reagents.

Measurement of therapeutic drugs is now provided as a service by many clinical chemistry departments. For this reason it is desirable to use assays that not only are both accurate and precise, but also are inexpensive and relatively easy to perform. Several techniques have been used for measuring therapeutic drugs, including ultraviolet spectroscopy (*1, 2*) and gas–liquid (*3*) and high-performance liquid chromatography (*4, 5*). Such techniques are not ideally suited to the service laboratory that has a large workload, but immunoassay techniques such as fluorescence-polarization (*6*) and enzyme-multiplied immunoassays (*7*) lend themselves to automation and are therefore potentially more useful in the busy routine department.

We had been using the EMIT (enzyme-multiplied immunoassay technique; Syva Co., Palo Alto, CA 94304) system as supplied by the company. To provide a more cost-effective assay with increased throughput, the EMIT methods were adapted for use in the Monarch centrifugal analyzer (Instrumentation Laboratory Ltd (IL), Warrington, Cheshire, U.K.). However, preliminary experiments revealed unacceptable precision when more than 10 samples were run as a batch. Here we describe how we established a protocol that resulted in acceptable precision for batches of as many as 31 samples.

Materials and Methods

Materials

EMIT reagents. EMIT reagents for the measurement of phenytoin were purchased from Syva. The standards, antibody-substrate (reagent A), and enzyme-bound drug (reagent B) were reconstituted according to the manufacturer's instructions.

Centrifugal analyzer. A Monarch 760 was used with the standard Rev. 4.2 version of the software provided by the manufacturer. A nonlinear interpolation model was used for all estimations, based on a six-point standard curve.

Department of Clinical Chemistry, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, U.K.

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Procedures

Monarch phenytoin method supplied by IL. The Monarch-phenytoin method is outlined in Table 1. Noncritical items (e.g., test name) that are included in the Monarch settings list are omitted, for brevity. The original IL protocol recommended a temperature of 30 °C. However, all of the other assays on the Monarch were performed at 37 °C, so we decided to use this temperature. This approach was necessary because considerable time could be wasted while the analyzer equilibrated from one temperature to another. Reagents A and B from the Syva kit (lot no. 6B 009UL) were reconstituted and left for a minimum of 8 h at room temperature. Working EMIT buffer was prepared by diluting the entire contents of the EMIT buffer-concentrate bottle to 200 mL with distilled water. Working reagents A (1 mL reagent A + 2 mL of working EMIT buffer) and B (1 mL reagent B + 7 mL of working EMIT buffer) were prepared and used immediately or within two weeks.

Statistical analysis. We used nonparametric analysis to confirm the presence of within-rotor drift [Mann–Whitney U test (*8*)]. We used parametric methods to confirm improvement in precision and for correlation analysis.

Results

Observation of Within-Rotor Drift

Thirty-one analyses of a patient's plasma with a phenytoin concentration of 72 μmol/L, in a single batch, gave a within-rotor CV of 7.2% (mean 82, SD 5.9 μmol/L). When the data were plotted (Figure 1), drift across the whole rotor was demonstrable. Comparison of the first and last group of 10 samples showed a significant difference ($P < 0.001$), confirming that drift had occurred.

Nature of the Within-Rotor Drift

We undertook to ascertain whether the drift was the result of (*a*) a gradual change, throughout the rotor, in the sensitivity of the reagent system to the drug being mea-

Table 1. Critical Monarch Settings

Response algorithm	Final–initial
Result algorithm	Nonlinear interpolation
Loading type	Load, analyze
Sample volume	3 μL
1st reagent (B)	120 μL
2nd reagent (A)	45 μL
Temperature	37 °C
Delay time	30 s
Interval time	120 s
No. of data points	2
No. of calibrators	6

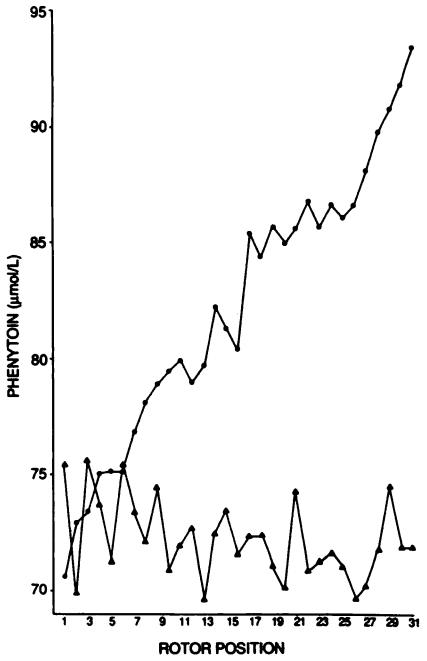

Fig. 1. Results of analysis of full rotor of plasma samples with nominal phenytoin concentration of 72 μmol/L
●—●, original loading procedure; ▲—▲, modified loading procedure

sured, (*b*) side reactions between sample and reagent A, or (*c*) a shift in the reagent baseline. Attempting to identify the nature of the drift, we designed experiments involving analysis of full sample rings of water, zero standard, and plasma (with and without phenytoin) to test each of the above options. Firstly, experiments involving plasma-containing drug were designed to demonstrate any change in reagent sensitivity. Secondly, studies with plasma (with and without drug) were designed to demonstrate the presence of any drift caused by side reactions occurring as reagent A was incubating with plasma. Thirdly, the additional experiments involving water and zero standard as well as plasma (with and without drug) were designed to demonstrate any shift in reagent baseline. The results (Table 2) showed that the same amount of drift in delta absorbance was obtained for all of the fluids studied. These findings suggest that the drift was predominantly ascribable to an increase in the reagent baseline, because the presence of neither plasma nor phenytoin was essential for its demonstration.

On running a full rotor with either reagent A or reagent B replaced by water, no delta absorbance values were obtained. Both reagents were required for any reaction to proceed.

Attempts at Eliminating Drift

In the above protocol (Table 1), sample and reagent A (antibody and substrates) were loaded simultaneously into the inner (sample) chamber of the rotor while reagent B (enzyme-labeled drug) was loaded into the outer reagent well. The contents of the inner well were then simultaneously transferred by centrifugal force to the outer well, initiating the reaction with reagent B. Antibody and free drug are therefore in contact up to about 4 min longer in the earlier-loaded samples than in those loaded later. We thought that part or all of the drift could be related to this phenomenon, so we attempted to decrease this time difference.

Increasing pre-incubation time. The Monarch has a facility whereby preloaded sample and reagents may be incubated for intervals specified by the operator before the mixing step is initiated. Pre-incubation for periods up to 5 min resulted in drift undulating consistently across the whole rotor (Figure 2). However, no improvement in precision was obtained with the two plasma samples studied (sample 1; mean 92, SD 6.0 μmol/L, CV 6.5%; sample 2; mean 85, SD 6.2 μmol/L, CV 7.3%).

Altering the delay time. Neither decreasing the delay time—the time between the start of the reaction (i.e., mix) and the first data point—to 5 s nor increasing it to 900 s had any effect upon the observed drift.

Switching reagents. Reagent B was loaded into the inside sample well and reagent A was loaded into the outer reagent well according to a loading protocol that maintained the same ratio of enzyme to antibody. This ensured that all samples were in contact with reagent A for an equal time. However, although the drift disappeared, there was a 10% loss in sensitivity, and analysis of a plasma sample gave a within-rotor CV of 4.3% (mean 63, SD 2.7 μmol/L). Moreover, the measured phenytoin concentrations ranged from 59 to 70 μmol/L. These latter two findings were considered unsatisfactory.

Altering assay temperature to 30 °C. Altering the assay temperature to 30 °C had two effects. There was an approximate 25% loss in sensitivity of the reaction and the phenom-

Table 2. Delta Absorbance Values Obtained with Various Fluids

Fluid	Delta absorbance		Increase in delta absorbance
	Cup 1	Cup 31	Cup 1 to 31
Phenytoin-containing plasma	0.6362	0.6723	0.0361
Phenytoin-free plasma	0.4731	0.5081	0.035
Zero standard	0.4758	0.5092	0.0334
H_2O	0.4843	0.5149	0.0306

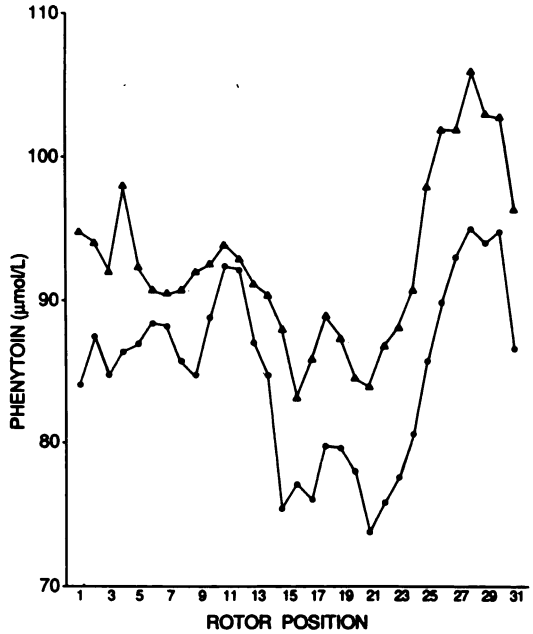

Fig. 2. Analysis of two plasma samples by the old loading procedure and 5-min pre-incubation stage
▲—▲, sample 1; ●—●, sample 2

enon of upward drift was replaced by one of downward drift across the whole rotor. This resulted in sample concentrations loaded at the end of the rotor measuring 9% less than those samples loaded at the beginning, an unacceptable observation.

Altering loading procedure. The Monarch analyzer has a loading option whereby sample and reagent A are loaded into the inner and outer well, respectively, and then mixed by centrifugal force in the outer well. Reagent B is then added to the inner well, transferred to the outer well by centrifugal force, and mixed with sample and reagent A. This procedure ensures that all samples are in contact with each reagent for the same time. However, the maximum volume of reagent allowed in the inner well is 89 μL. Therefore, it was necessary to decrease the volume of reagent B to 80 μL (and therefore that of reagent A to 30 μL) to utilize this loading facility. This resulted in a decrease in the reagent/antibody ratio, but there was no loss of sensitivity. A within-batch CV of 2.4% was obtained after running the original plasma sample (with a nominal phenytoin concentration of 72 μmol/L) 31 times in a single rotor (mean 72, SD 1.73 μmol/L) (Figure 1). This precision was significantly different from that obtained when using the original loading procedure ($P < 0.01$). Table 3 gives the critical loading variables of the modified procedure. We decided to use a longer run time to obtain increased delta absorbance values and thus obtain a steeper standard curve.

Correlation and Precision Studies

During one week we measured the phenytoin concentration in 37 patients' samples that had been assayed by gas–liquid chromatography (*3*). The results are shown in Figure 3; the regression equation was y (EMIT) = $1.06x$ (gas–liquid chromatography) − 5.03 (r = 0.982).

Between-batch precision over one month was assessed with a bi-level assayed anti-convulsant/anti-asthmatic control (Gilford Instrument Labs., Irvine, CA 92714). This gave a CV of 6.2% at a mean value of 28 μmol/L (SD 1.75 μmol/L) and 4.8% at a mean of 75 μmol/L (SD 3.6 μmol/L).

Discussion

Centrifugal analyzers have an extremely good reputation for precision, and in the insert supplied with each kit Syva quotes typical within-batch CVs of 2.9 and 4.0%. Thus our finding of a within-batch CV as high as 7.2% when we used Syva reagents in the Monarch analyzer was quite unexpected. Syva protocols for instrumentation such as the "Advance" system involve the identical, sequential handling of both samples and reagents. In contrast, the original IL Monarch protocol involved loading sample together with reagent A into the same compartment (Table 1). This meant that sample and antibody were in contact with each other for decreasing periods of time from rotor position 1 to 31, and therefore interaction effects might occur. In contrast, elimination of the sequential addition of samples to reagent A with the load–spin–reload–(crit) incubate–analyze facility, was associated with both excellent within- and between-batch precision.

Table 3. Monarch Settings for Modified EMIT Drug Assay

Loading type	Load, spin, reload, (crit) incubate, analyze
Sample volume	3 μL
1st reagent (A)	30 μL
4th reagent (B)	80 μL
Delay time	5 s
Interval	200 s
No. of data points	2
Incubation time	0

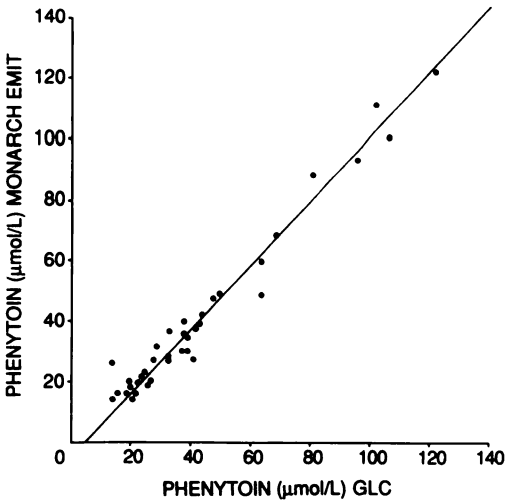

Fig. 3. Correlation of 37 patients' samples as measured by EMIT and gas–liquid chromatography

Within-rotor drift in the measurement of drugs by EMIT in centrifugal analyzers was observed previously (*9*). To eliminate it, Massey and Barta (9) modified the loading of reagents so that reagent B was loaded with sample and reagent A was loaded separately. However, we found this procedure unsuitable for two reasons: there was an apparent 10% loss of sensitivity in the standard curve and the within-rotor imprecision was about twice that obtained with the double-loading protocol. Moreover, their study (*9*) only improved the within-batch CV from 31.3% to 7.6%, a value we considered unsatisfactory. Neither increasing the delay time nor running the assay at 30 °C solved the drift problem. In the former case, undulating drift was obtained; in the latter, the drift appeared to be downward. Only by adopting the double-loading protocol could we eliminate drift altogether. Thereafter, the assay performed satisfactorily, as shown by the precision and correlation studies.

On analyzing full rotors of a single specimen, three types of drift were observed in this study: upward drift, downwards drift, and drift undulating up and down across the series of samples in a rotor. The chemical reactions causing each of them appear to be complex. The undulating drift obtained after increasing the delay time (Figure 2) may be due to "hot spots" on the analyzer table. However, this hypothesis was not supported by results obtained from the measurement of complete rotors of a single sample analyzed for various routine enzymes. Therefore, the cause of this undulating drift remains unknown. The downward drift observed after running the original protocol at 30 °C is also difficult to explain. It may be that at this lower temperature later-loaded samples do not reach equilibrium with antibody before the phenytoin–enzyme conjugate is added. This would lead to more conjugate binding to antibody in these later-loaded samples, resulting in lower enzyme activity and therefore a lower apparent concentration of drug. However, other unknown factors might also contribute to this particular drift phenomenon. The upward drift obtained after running the old protocol at 37 °C may, from the data presented in Table 2, be explained by the addition of water or of interaction with the plastic material of the rotor.

However, the latter effect would also be expected to occur with the revised protocol, which does not give rise to drift. Therefore plastic–reagent interaction is unlikely to be the cause of the observed drift, which is still unclear.

In conclusion, although we were unable to explain the physicochemical causes for all of the types of drift observed, we were able to eliminate them with the double-loading protocol. Moreover, our findings with the measurement of phenytoin may be applicable on a wider basis to the measurement of all therapeutic drugs in the Monarch.

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Changes in Magnesium, Zinc, Calcium, Potassium, Cholesterol, and Creatine Kinase Concentrations in Patients from Pre-Infarction Syndrome to Fatal Myocardial Infarction

Michelle Speich,[1] Simone Gelot,[2] Pierre Arnaud,[3] and Guy Nicolas[4]

We measured changes in concentrations of magnesium, zinc, calcium, potassium, cholesterol [total and high-density lipoproteins (HDL)], total creatine kinase (CK), and CK isoenzyme-MB in plasma (Pl) and/or erythrocytes (Erc) from apparently healthy subjects and from patients with either pre-infarction syndrome (PIS) or myocardial infarction (MI) with a favorable (MI_1) or fatal (MI_2) outcome, to assess the relationship of these changes to the increasing severity of ischemic disease. Significant sex-related differences led us to study men and women separately. In MI_1 and MI_2 patients, concentrations of Mg in Pl and Erc were increased as a function of time since the infarct, confirming that cardiac Mg leaves the heart and enters the circulatory compartment. Compared with concentrations in MI_1 patients, Zn concentrations in Pl were lower in MI_2 patients in the days before death. Significant negative correlations between Zn in Pl in MI_1 men or Zn in Erc in MI_2 men and CK or CK isoenzyme MB suggest that circulating Zn is taken up by non-necrotic myocardial tissue as part of the repair process. MI_2 patients had gradually decreasing Ca concentrations in Pl even more marked than those observed in PIS and MI_1 patients. We also noted a marked decrease in total and HDL cholesterol concentrations in both MI_2 men and MI_2 women shortly before death.

Laboratoires de [1] Biochimie Pharmaceutique, [2] Toxicologie et Hygiène Industrielle, and [3] Biophysique et Mathématiques, Faculté de Pharmacie, 1 rue Gaston Veil, 44035 Nantes Cedex, France.

[4] Clinique Cardiologique, Centre Hospitalier Régional et Universitaire de Nantes, B. P. 1005, 44035, Nantes Cedex, France.

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Additional Keyphrases: *sex-related differences · disease-related changes · erythrocytes · high-density lipoproteins · electrolytes*

The role of magnesium (Mg) in cardiac disease and energy metabolism and the study of its relationship with other cations have recently been thoroughly reviewed (*1–3*). In our earlier work (*4, 5*) we determined the concentrations of certain metals in cardiac ventricles after fatal myocardial infarctions (MI) but we did not study the concentrations of these cations in the blood before death.[5] Here we report the results of our investigations into this area in several groups of subjects with pre-infarction syndrome (PIS) or MI with favorable (MI_1) or fatal (MI_2) outcome. The variables investigated were plasma (Pl) and/or erythrocyte (Erc) concentrations of magnesium (Pl-Mg, Erc-Mg), zinc (Pl-Zn, Erc-Zn), calcium (Pl-Ca), potassium (Erc-K), cholesterol [total and high-density lipoproteins (HDL)], total creatine kinase (CK; EC 2.7.3.2), and isoenzyme CK-MB. We studied the changes in these variables as a function of the increasing severity of the disease and in view of our hypotheses about exchanges between the heart and the circulatory compartment (*6, 7*).

Subjects and Methods

Populations. Blood was sampled from 200 white subjects, all residents of the Nantes area in France, a region supplied with soft tap water. The 37 PIS patients included 26 men (ages 46–82 y) and 11 women (ages 53–78 y). All of these

[5] Nonstandard abbreviations: MI, myocardial infarction; PIS, pre-infarction syndrome; Pl, plasma; Erc, erythrocytes; HDL, high-density lipoproteins; and CK, creatine kinase.