

Chapter 10

The Application of Analytical Bioluminescence to the Enzyme Multiplied Immunoassay Technique for the Assay of Anticonvulsant Drugs in Plasma

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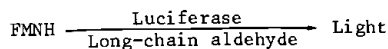
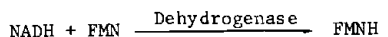
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INTRODUCTION

Analytical bioluminescence has been used to measure ATP (adenosine triphosphate) and NADH (reduced nicotinamide adenine dinucleotide) at levels below those accessible by conventional means, such as spectrophotometry, and typically the enhancement in sensitivity is around one hundred-fold. The procedure depends on the production of light when the enzyme or enzyme complex, a specific luciferase, acts on one of the above substrates. The photons emitted from the reaction mixture can be measured in the liquid scintillation counter (if the photomultipliers are switched out of coincidence) and thus gives a direct measure of the amount of either ATP or NADH.

Recently a technique for measuring anticonvulsant drugs in blood plasma has become available in which NADH is the indicator species determined. This is the so-called enzyme-multiplied immunoassay technique, EMIT[®]. In the conventional assay procedure a spectrophotometer is set at 340 nm to appraise the rate of NADH production over the reaction period of thirty seconds. In this presentation it will be shown that the NADH can also be measured using the luciferase from *Photobacterium* by counting the photons using a liquid scintillation spectrometer and applied to the estimation of the anticonvulsant drug, phenytoin.

The luciferase or more correctly the dehydrogenase luciferase complex catalyses the following reactions:



FMN(H) = (reduced) flavin mononucleotide

The number of photons produced is thus proportional to the concentration of NADH, providing FMN and aldehyde are present in saturating amounts.

A number of recent publications concerning enzyme immunoassay are to be found in a special issue of *Clinical Chemistry*¹ and the present author has reviewed analytical bioluminescence in a previous volume of this series² and also last year³ and has described the bioluminescence assay of NADH⁴. A slightly modified version of the latter is used herein.

EQUIPMENT

A Searle Isocap 300, Model 6970 was used and its detector chamber was set to operate at 20 °C. The instrument was fitted with bialkali EMI 9805/A photomultipliers which

have a frosted face of borosilicate glass. The photomultipliers were operated out-of-coincidence and counts were appraised from the photomultiplier tube with the lower background. This was approximately 15 K cpm for an integral window i.e. 0-2000 KeV.

For some of this work the pulses from the channel were also passed to a Searle Model 8954 ratemeter and the analogue signal so produced was plotted on a recorder. The time constant of the ratemeter was 0.5 s for the 10^6 cpm range and was too small to influence the results significantly.

All work with the instrument was carried out under tungsten light since fluorescent lighting is known to cause spurious effects (phosphorescence of vials) in this type of work^{2,3}.

COUNTING VIALS

Packard Tru-Lite glass vials were used in this work although they were not the containers of the reagents. Instead they served as a convenient carrier for a large (2 ml) polystyrene autoanalyser cup which was the reagent vessel.

REAGENTS

Enzyme-multiplied immunoassay technique, EMIT[®], reagents were obtained through Varian (Australia) Pty. Ltd., from the Syva Corporation, Palo Alto, California, U.S.A. The reagents were reconstituted according to the makers' instructions. For the assay, 0.1 M phosphate, pH 7.0 was used instead of the tris buffer supplied. A pipettor-dilutor (Cavro Scientific Instruments Inc., Los Altos, California, U.S.A.) was used for dispensing reagents A and B as well as the phosphate buffer.

Decanal was obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A. and was prepared freshly each day as a 1% solution in ethanol.

A phosphate buffer, 0.1 M, pH 7.0 was also prepared from sodium dihydrogen phosphate and potassium hydrogen phosphate and it too was filtered prior to use.

Flavin mononucleotide, FMN, was prepared as a 2 μ M solution in the phosphate buffer and stored for up to a week at 4°C and filtered before use on each occasion.

Bacterial luciferases derived from *Photobacterium* sp. were used in this work. Commercially available material also contains the dehydrogenase^{2,3} necessary for the reactions employed. A number of different batches from Sigma Chemical Co., St. Louis, Missouri, U.S.A. were used in this work. Luciferase₁ was prepared as a 250 μ g ml⁻¹ solution in phosphate buffer containing 10 mg ml⁻¹ bovine serum albumin and 1 mg ml⁻¹ dithiothreitol and then aged for one hour on ice before centrifuging at 2000 g or filtering through a 1.2 μ Millipore filter to remove any particulate matter. It was stored on ice at all times.

PRINCIPLE

A recent development in the assay of anticonvulsant drugs involves the Enzyme Multiplied Immunoassay Technique, EMIT[®].

It is a homogenous immunoassay technique which may be applied to the microanalysis of drugs, hormones etc. in biological fluids. In the case considered here, the anticonvulsant phenytoin, is labelled with an enzyme, a bacterial glucose-6-phosphate dehydrogenase. When this labelled drug is bound to the antibody the activity of the enzyme is reduced. The free drug present in the sample competes with the enzyme labelled drug for binding sites on the antibody and in this way the inactivation of enzyme is reduced. As a consequence the estimation of enzyme activity can be correlated with the drug present in the plasma sample. The principle is shown diagrammatically in Fig. 1.

Using the manufacturers nomenclature, reagent A containing the antibody for phenytoin and the substrates for the enzyme, nicotinamide adenine dinucleotide (NAD) plus glucose-6-phosphate is mixed with a small volume of plasma. Binding of the sample drug then occurs. Reagent B containing the enzyme labelled drug is then added and this last combines with any unfilled binding sites and the enzyme activity is thereby

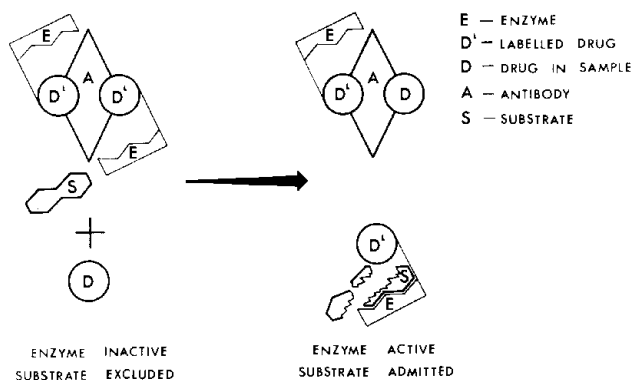


Fig. 1 A diagrammatic representation of the enzyme immunoassay process.

proportionately reduced. This equilibrium is set up rapidly and thus the assay may be performed immediately. The enzyme activity is measured by following the rate of production of reduced NAD, NADH, at 340 nm in a spectrophotometer for a period of thirty seconds.

ASSAY PROTOCOL

An autoanalyser cup was placed in a scintillation vial and 2 μl luciferase solution placed in it using an SMI micropipettor (Scientific Manufacturing Industries, Rosemere, Quebec, Canada). The micropipettors used were shown to have a coefficient of variation of less than 1%. Immediately following this, the immunoassay reaction was initiated.

The initial steps of the standard enzyme immunoassay procedure were carried out at room temperature, 22°C, and only one fifth of the normal volumes were used, that is 10 μl plasma was mixed with 50 μl phosphate buffer. To this was added 10 μl reagent A (antibody plus enzyme substrates) together with 50 μl buffer, then 10 μl reagent B (enzyme labelled drug) was added with a further 50 μl buffer. One microlitre of this reaction mixture was immediately sampled with a SMI micropipettor and transferred to the autoanalyser cup and thoroughly mixed with the luciferase with 10 μl reagent mixture C delivered from a SMO micropipettor. Reagent mixture C was made up every two or three hours and kept at 20°C in the sample changer and consisted of phosphate buffer 0.1 M, pH 7.0, containing flavin mononucleotide 2 μM and decanal, 0.01% (prepared from a 1% solution in ethanol). Following the mixing of the three components on a vortex mixer the autoanalyser cup was placed in the scintillation vial which was immediately loaded into the spectrometer. The elapsed time between adding reagent B and the commencement of counting was set at 8 s. Repeat counting sequences of 0.1 min were made for one minute. Each counting sequence consisted of 0.1 min counting and a printing cycle. The total counting sequence time was 14 s. NADH was produced by the EMIT® reagents and this was coupled directly to the luciferase. As a result the production rate of photons increased over the one minute period and this could be used as a measure of free enzyme-drug concentration and thus the concentration of drug in the plasma sample could be measured.

It was necessary to set up a standard curve covering the commonly encountered range. In this work with the anticonvulsant drug, phenytoin, standards were set up for 0, 10, 20, 40, 80, 120 $\mu\text{mol l}^{-1}$.

RESULTS AND DISCUSSION

Figure 2 shows the increase in counting rate for each of the six standards.

Figure 3 shows a calibration curve. It is a plot of the counts accumulated during the fifth counting sequence (approximately 1 min after initiating the immunoassay) vs $\mu\text{mol l}^{-1}$ phenytoin for the range of interest. Note that the blank is not indicated since this is a quasilinear plot. Instead the blank value has been subtracted

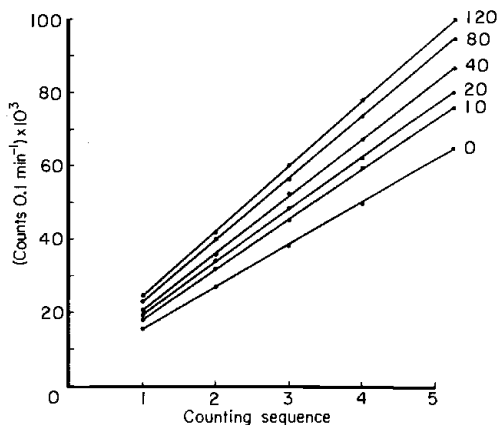


Fig. 2 The photon output for the standards. The numbers beside each line are $\mu\text{mol l}^{-1}$ phenytoin. The time for each counting sequence was 14 s.

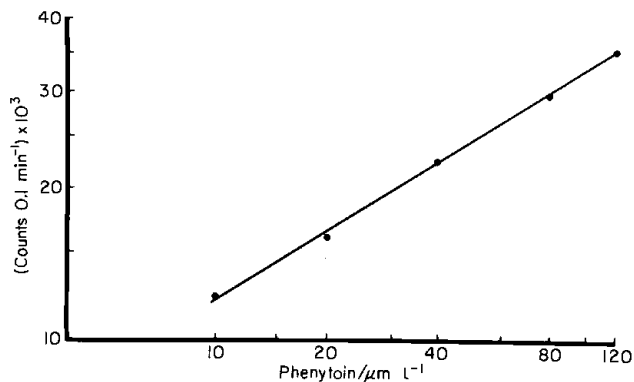


Fig. 3 A calibration curve for phenytoin vs counts per 0.1 min for the fifth counting sequence (see Fig. 2). The blank has been subtracted from each of the five points and the scales for the x and y axes are those supplied on the calibration graphs supplied by the manufacturer of the assay kits.

from each standard. The form of scales on the graph is that given by the manufacturers for use in the spectrophotometric procedure.

Fifteen plasma samples drawn from patients who had been prescribed phenytoin, were analysed by the standard spectrophotometric procedure and the one described here. The values covered the range zero to $100 \mu\text{mol l}^{-1}$. A regression line for the standard procedure on the bioluminescence method yielded a slope of 1.062 and an intercept of $-5.1 \mu\text{mol l}^{-1}$. The correlation coefficient was found to be 0.914 and thus highly significant.

The conditions for coupling the analytical bioluminescence procedure to the EMIT[®] technique, (that is the enzymic coupling) are quite narrow. A balance has been obtained between a number of conditions. The amount of EMIT[®] reagents in the final reaction mixture should not be so diluted as to prevent or severely retard the immunoassay reaction and the activity of the glucose-6-phosphate dehydrogenase. On the other hand too small a dilution gives rise to NADH levels (and thus photon production rates) which

are far too big to be handled by the counter. The dehydrogenase/luciferase level must be carefully manipulated. In the very dilute solution of the reaction mixture this enzyme complex tends to denature and give inconsistent results. Thus the enzyme complex is made up in a large excess of bovine serum albumin and dithiothreitol (to protect -SH groups) which act as protecting agents. Too much luciferase gives large photon production rates which again cannot be measured properly by the counter. The potential user should be alert to the range of activity and stability of commercial preparations of the luciferase complex. Thus the levels quoted herein may need to be changed according to the batch used.

The current method is not yet satisfactory for routine use, however, it does appear to have some potential. Firstly there is the obvious economic advantage. Only 1 μ l of the immunoassay reaction mixture is used instead of the 900 μ l in the standard assay. While the technology is not to hand for routinely carrying out the immunoassay at a one-nine-hundredth of the scale there would appear to be potential for a very considerable saving in the cost of EMIT[®] reagents. The cost of luciferase and other reagents is a good deal less than 1p per assay. Since this is a homogenous immunoassay presumably it could be adapted for use with an automatic enzyme analyser with a sensitive photomultiplier as the detector. Conventional EMIT[®] assays for anticonvulsant drugs have already been automated, see for example Ref. 5.

In the research area the procedure has the potential for considerably enhancing the sensitivity for phenytoin analysis in for example small tissue or fluid samples or where it is present at high dilution.

The disadvantages of the present method include the fact that it is not as convenient as the standard method and is not a rapid one. In addition technology is not, to the authors' knowledge, yet available to perform the immunoassay in 1 μ l and thus take full advantage of the potential of this very sensitive analytical technique. Further the reaction is quite temperature dependent and a constant temperature for the reactions is important. The presently described procedure is thus not ideal in this regard.

ACKNOWLEDGMENT

I thank Mrs Margot Peikert for very competent technical assistance and for preparing one of the diagrams.

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DISCUSSION

C. PALAIS: Is small sample volume (1 μ l) really essential in assays of this type?

P.E. STANLEY: It has been necessary to use this small volume since the NADH level must not be so high as to produce so much light that the counter cannot handle the pulse rate properly.

E.D. BRANSOME: I believe that a comment is appropriate to the papers of Drs Stanley and Redshaw and Professor Landon's lecture inasmuch as most of this audience are chemists and not clinicians. They may be confused by the need for specificity expressed by the last two speakers as opposed to the 'quick and dirty' approach referred to in an anecdotal fashion by Professor Landon. There are in fact two disparate needs. One is for rapid assays — ideally available at the bedside, which need not be precise but need only correlate in a semi-quantitative fashion with a drug level or the presence of a disease state. The other is for assays with rather precise stoichiometry and specificity. These are needed for pharmacokinetics, for clinical research, and to validate the less precise assays already mentioned. It may very well be that the problem of specificity may never be fully answered by an RIA technique but only striven for. Thus direct chemical measurement as with GC/MS is another burgeoning area of clinical analytical chemistry.

P.E. STANLEY: Some six months ago we changed our drug assay service for anticonvulsants from a once-a-day, three-hour turn-around for phenytoin to the conventional enzyme immunoassay which takes only ten minutes and can be done at any time of day. The quicker turn-around has provided the physician with almost instantaneous blood levels of the anticonvulsants and thus patient management has been substantially enhanced. This has been most noticeable in an outpatient clinic where blood levels on patients are measured just before they see the doctor. It takes only a few minutes and consequently it is not necessary for the patient to be seen at a later date. Patient convenience is thus improved.