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Short Communication

Substrate-labelled fluorescence immunoassay of phenytoin

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Introduction

Substrate-labelled immunoassay systems are based upon competition between labelled analyte and unlabelled analyte for a limited number of anti-analyte antibody binding sites. An enzyme present in the reaction mixture reacts with antibody-free labelled analyte and is sterically inhibited from reacting with the antibody-bound fraction. The reaction product is fluorescent at longer wavelengths than the unreacted substrate, and the fluorescence at this long wavelength maximum is monitored. The rate of the enzyme-catalysed reaction, and hence the extent of the reaction after a fixed time interval, is directly related to the concentration of unlabelled analyte. Quantitation of the analyte is accomplished by the construction of a standard curve.

Previous substrate-labelled fluorescence immunoassays have employed β-galactosidase [1–7] or phosphodiesterase [7] and derivatives of 7-hydroxycoumarin (umbelliferone) as the fluorogenic substrate. β-Galactosyl or phosphate ester bonds involving the coumarin hydroxy group are cleaved, yielding the free coumarinolate anion at the pH employed for the reaction.

A different enzyme substrate system which exhibits similar chemistry and shows potential usefulness for the immunoanalytical quantitation of analytes has been utilized. Porcine esterase (carboxylic-ester hydrolase, EC 3.1.1.1) and a 2-acetoxy-8-naphthalene-sulfonamido-phenytoin derivative have been employed in an assay for phenytoin. Antibody-free substrate–phenytoin conjugate is available for reaction with the esterase to yield as product, the hydroxyaromatic form of the fluorophor at the assay pH. Relatively short wavelength excitation of the conjugate acid form of the probe, followed by excited state prototropic dissociation, results in long wavelength emission associated with the excited conjugate base. The emission of this fluorophor is at longer wavelength than that of the coumarin derivative, where there is background serum fluorescence interference. Serum fluorescence decreases in general at longer emission wavelengths.

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Phenytoin is a drug which is quite widely prescribed for the treatment of epilepsy. It is quite important to measure serum phenytoin concentrations because the drug is clinically effective over only a narrow concentration range. Overdosing associated with concentrations greater than 21 mg l^{-1} can result in a host of serious side-effects including convulsions. These are similar to symptoms of underdosing, when serum concentrations are less than 9 mg l^{-1} [8, 9]. Traditional methods of phenytoin quantitation include gas chromatography [10], high-performance liquid chromatography [11], radioimmunoassay [12] and an absorptiometric homogeneous enzyme immunoassay [13]. Fluorescence immunoassays for phenytoin have also been developed recently including a double-antibody technique [14], a magnetizable solid-phase method [15], a fluorescence polarization immunoassay [16] and the reactant-labelled assay [2].

Experimental

Esterase from porcine liver (EC 3.1.1.1) was obtained from Sigma Chemical Co. (St Louis, MO). 5, 5-Diphenylhydantoin was obtained in 99% purity from Aldrich Chemical Co. (Milwaukee, WI). Rabbit anti-phenytoin-3-ω-valeryl-BSA (anti-P) was obtained from Miles-Yeda Ltd (Rehovot, Israel). Antisera stock solutions were prepared by diluting the antisera 1:10 into a pH 7.5, $\mu = 0.10$ phosphate buffer containing 0.1% sodium azide. 3-[(2″-acetoxynaphthyl-8″-sulfonyl)-2′-amino]ethyl-5,5-diphenylhydantoin (2-8-DPH) was synthesized by a procedure previously reported [17].

Fluorescence measurements were obtained using a Perkin–Elmer MPF-2A fluorimeter. All measurements and incubations were carried out at 30°C.

Assay procedure

An aliquot of 2.0 ml containing phosphate buffer (pH 7.5, $\mu = 0.10$), 0.1% sodium azide, 22 μg esterase and 75 μl antibody stock solution was added to a cuvette. To this was added 50 μl of a serum solution prepared as follows. To a reaction vial were transferred 100 μl serum and 100 μl 0.1 M NaOH. This mixture was then sonicated for 5 min to destroy all endogenous esterase activity and 50 μl of this mixture was added to the cuvette. A fluorescence measurement was then taken at the excitation and emission maxima of the enzyme-cleaved conjugate, 337 and 480 nm, respectively. Lastly, 50 μl 1.2×10^{-5} M 2-8-DPH was added, and well mixed. After precisely 15 min another fluorescence measurement was taken and the value corresponding to the background fluorescence was subtracted from this value to yield the corrected relative intensity.

Typical standard curves are depicted in Fig. 1 for various incubation time intervals. Fifteen minutes was chosen for the analytical procedure for reasons of convenience.

Results

The assay could also have been carried out using an initial rate measurement but the single point measurement with background correction was deemed simplest. The within-day and between-day precisions obtained from this assay was quite good, as illustrated in Table 1.

These values compare well with those obtained in a commercial assay employing β-galactosidase and umbelliferone, demonstrating the effectiveness of the esterase/hydroxynaphthalene sulfonamide system. In the future the use of other hydroxyaryl-sulfonic acid derivatives could potentially result in more intense, more red-shifted

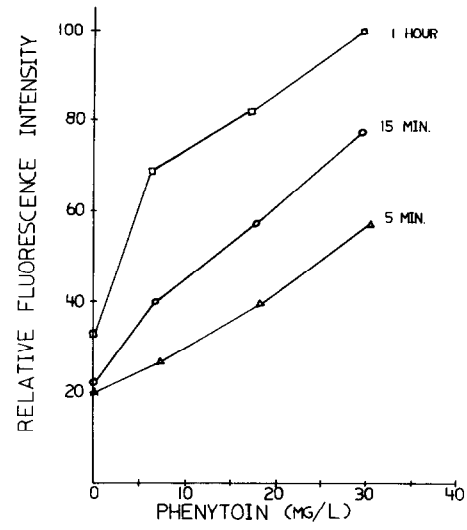

Figure 1
Substrate-labelled fluorescence immunoassay standard curves using 5 min, 15 min and 1 h incubation periods.

Table 1
Precision of substrate-labelled fluorescence immunoassay for phenytoin*

[Phenytoin] added (mg/l)	Within-day precision†	Between-day precision‡
5	6.25	7.81
10	5.47	6.72
15	4.45	6.55
20	4.51	7.34
25	5.02	8.12
30	5.57	9.21

*Percent relative standard deviation.
†Ten replications same day.
‡One measurement on five separate days.

fluorescence emissions. These include derivatives of 1-hydroxy-5-naphthalene sulfonic acid and pyrenol sulfonic acid.

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References

[1] J. F. Burd, R. C. Wong, J. E. Feeney, R. J. Carrico and R. C. Boguslaski, *Clin. Chem.* **23**, 1402–1408 (1977).
[2] R. C. Wong, J. F. Burd, R. J. Carrico, R. T. Buckler, J. Thoma and R. C. Boguslaski, *Clin. Chem.* **25**, 686–691 (1979).
[3] L. M. Kransz, J. B. Hitz, R. T. Buckler and J. F. Burd, *Therap. Drug. Monit.* **2**, 261–272 (1980).
[4] T. M. Li, J. L. Benovic, R. T. Buckler and J. F. Burd, *Clin. Chem.* **27**, 22–26 (1981).
[5] D. Worah, K. K. Yeung, F. E. Ward and R. J. Carrico, *Clin. Chem.* **27**, 673–677 (1981).
[6] T. M. Li, J. E. Miller, F. E. Ward and J. F. Burd, *Epilepsia* **23**, 391–398 (1982).
[7] K. J. Dean, S. G. Thompson, J. F. Burd and R. T. Buckler, *Clin. Chem.* **29**, 1051–1056 (1983).
[8] H. Kutt and F. McDowell, *J. Am. Med. Assoc.* **203**, 167–170 (1968).
[9] H. Kutt and J. K. Penry, *Arch. Neurol. (Chicago)* **31**, 283–288 (1974).

[10] A. Richens, in *Clinical Pharmacology of Anti-Epileptic Drugs* (H. Schneider, D. Janz, C. Gardner-Thorpe, H. Meinardi and A. L. Sherwin, Eds). Springer, Berlin (1975).
[11] P. M. Kabra, B. E. Stafford and C. J. Marton, *Clin. Chem.* **23**, 1284–1288 (1977).
[12] J. W. Paxton, F. J. Rowell and J. G. Ratcliffe, *J. Immunol. Methods* **10**, 317–327 (1976).
[13] H. E. Booker and B. A. Darcey, *Clin. Chem.* **21**, 1766–1768 (1975).
[14] M. J. Kurtz, M. Billings, T. Koh, G. Olander, T. Tyner, B. Weaver and L. Stone, *Clin. Chem.* **29**, 1015–1019 (1983).
[15] R. S. Kamel, J. Landon and D. S. Smith, *Clin. Chem.* **26**, 1281–1284 (1980).
[16] A. R. McGregor, J. O. Crookall-Greening, J. Landon and D. S. Smith, *Clin. Chim. Acta* **83**, 161–166 (1978).
[17] J. S. O'Neal and S. G. Schulman, *Anal. Chem.*, submitted for publication.

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