Magnetizable Solid-Phase Fluoroimmunoassay of Phenytoin in Disposable Test Tubes

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We developed a fluoroimmunoassay for phenytoin in serum or plasma, based on the magnetic separation technique. The method involves sheep anti-phenytoin serum coupled to magnetizable cellulose/iron oxide particles, with a fluorescein-labeled phenytoin analog as tracer. After magnetic sedimentation of the solid phase from assay mixtures, the free fraction of the tracer is aspirated, removing endogenous fluorophores and other interfering components of the sample. The antibody-bound tracer is then eluted from the solid phase into a methanolic buffer medium and quantitated fluorometrically. The entire procedure, including fluorometry, is performed within disposable polystyrene test tubes. The assay involves only simple reagents and equipment, and correlates closely with established radioimmunoassay (r = 0.97) and gas-liquid chromatographic (r = 0.98) techniques.

Additional Keyphrases: flurometry • therapeutic drug monitoring • drug assay • anticonvulsant drugs

Non-isotopic immunoassays have been shown to be sufficiently reliable and sensitive for routine monitoring of circulating phenytoin concentrations during therapy with antiepileptic drugs. The homogeneous enzyme immunoassay method (1, 2), which involves no separation procedure, has been widely and successfully adopted. Fluoroimmunoassay (FIA) methods for phenytoin have also been developed. Non-separation FIA may be performed with fluorescence polarization (3) or the elegant substrate-labeling (4) technique; however, these methods require either specialized instrumentation or reagents. We developed an FIA for phenytoin based on separation with sodium sulfate and involving only simple reagents and equipment (5). Separation FIA has the advantage that appropriate assay design enables the removal of endogenous fluorophores and other interfering components of serum or plasma samples before the endpoint determination is made. Recently introduced magnetizable solid-phase reagents (6–9) enable rapid separation in immunoassays by the application of a magnetic field, and obviate the need for centrifugation. We describe an FIA for phenytoin that involves anti-phenytoin magnetizable solid phase and a fluorescein-labeled phenytoin analog as tracer. The solid phase, carrying the antibody-bound fraction of the tracer, is separated from the immunoassay mixture; then the tracer is eluted from the solid phase into a methanolic buffer medium and its fluorescence determined. The entire assay procedure, including the fluorometric endpoint measurement, is carried out in disposable polystyrene test tubes.

Materials and Methods

Materials

Phenytoin (5,5-diphenylhydantoin) was obtained from Parke-Davis, Pontypool, Gwent, U.K.; bovine albumin type A4503 from Sigma London Chemical Co., Poole, Dorset, U.K.; and methanol (AR grade) from BDH, Poole, Dorset, U.K.

Fluoresceinthiocarbamyl/α,α-diphenylglycine (FTC-DPG) was prepared as described previously (3).

Cellulose/iron oxide magnetizable particles, prepared according to published procedures (10), were provided by Technia Diagnostics, London EC1, U.K.

Phenytoin immunogen. Phenytoin-3-ω-valeric acid was prepared according to a published method (11), except that sonication was omitted. The final product was recrystallized three times from ethyl acetate. A mass spectrum showed the expected molecular ion at m/e 352.

The phenytoin derivative was coupled to bovine albumin by carbodiimide condensation (12). The insoluble product obtained was used for immunization.

Anti-phenytoin sera. Three sheep were each immunized with 2.5 mg of immunogen in 4 mL of an emulsion of physiological saline/complete Freund’s adjuvant (1/3, by vol). The injected immunogen was divided among several sites, both intramuscular and subcutaneous. Six weeks later, booster immunizations with 1.25 mg of immunogen were administered in the same way, but with incomplete Freund’s adjuvant. The booster injections were repeated at two-week intervals, and the sheep were bled 10 days after every other booster injection. Serum from one bleeding taken after the third booster injection of one animal was used in the studies described below.

Anti-phenytoin magnetizable solid phase. We activated 1 g of cellulose/iron oxide particles by the cyanogen bromide method (13), then coupled them to 2 mL of whole anti-phenytoin serum, following the procedures of Wide (14). The antibody solid-phase product was suspended in 20 mL of diluent buffer containing 2.5 mL of Tween 20 detergent per liter, and stored at 4 °C.

Assay standards. Phenytoin, dissolved in 100 mmol/L aqueous sodium hydroxide, was added to pooled normal human serum.

Patients’ specimens. Serum or plasma specimens from well-monitored patients receiving phenytoin, either alone or in combination with other drugs, were obtained from the Department of Clinical Pharmacology, St. Bartholomew’s Hospital, London EC1, U.K., where gas-liquid chromatographic assays (15) were performed, and from the Chalfont Centre for Epilepsy, Chalfont St. Peter, Bucks., U.K., where determinations were made with phenytoin radioimmunoassay kits (The Radiochemical Centre, Amersham, Bucks., U.K.). We assayed these specimens by sodium sulfate separation FIA as described previously (5).

Fluorometry

A ratio-recording fluorometer (Model 1000; Perkin-Elmer

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Ltd., Beaconsfield, Bucks., U.K.) was used as described previously (5), except that wide-band interference filters types FITC (440 to 490 nm transmission) and DB2 (510 to 600 nm transmission) from Barr and Stroud, Anniesland, Glasgow, U.K., were fitted in the excitation and emission light paths, respectively.

Fluorescence was measured in disposable test tubes. To position the tubes in the fluorometer cuvette holder unit, an adapter was constructed from a 45-mm length of 0.5-in. (12.7 mm) square-section aluminum rod, drilled out to 10-mm diameter down most of its long axis. Vertical slits 5 mm wide were cut in the sides to allow passage of exciting and emitted light beams. The adapter was given a black anodized finish. It was permanently placed in the fluorometer cuvette holder, where it filled the space ordinarily occupied by a conventional cuvette. To accommodate the extra height of the test tubes, the normal cuvette compartment lid of the fluorometer was replaced with a standard Perkin-Elmer accessory unit (Ittrich sample cover).

Fluoroimmunoassay Procedure

Assay tubes. Disposable polystyrene test tubes, 75 x 10 mm (no. 55.480; Walter Sarstedt, Leicester, Leics., U.K.).

Diluent buffer. Sodium phosphate buffer, 100 mmol/L, pH 7.5, containing 1 g of bovine albumin and 1 g of sodium azide per liter.

Elution reagent. Methanol/sodium bicarbonate buffer (50 mmol/L, pH 9.0), 9/1 by vol.

Assay protocol. Carry out the entire procedure at room temperature and in duplicate. To 50 μL of serum or plasma sample, add 1 mL of diluent buffer. Transfer 50 μL of the diluted sample to a second test tube and add 100 μL of FTC-DPG (180 nmol/L in diluent buffer), followed by 50 μL of anti-phenytoin solid phase (16 g/L in diluent buffer). Incubate for 30 min, vortex-mixing every 10 min to prevent excessive gravitational settling of the particles. Add 1 mL of diluent buffer and sediment the solid phase on a multipolar ferrite magnet (Magnet Applications, City Road, London EC1, U.K.). Aspirate the supernate, add 1.5 mL of elution reagent, and vortex-mix. Again sediment the solid phase on the magnet, then place the assay tube in the fluorometer to measure the fluorescence of the supernate containing the eluted FTC-DPG. Construct the standard curve by plotting total fluorescence signal (arbitrary units) vs phenytoin serum standard concentration.

Results

Anti-Phenytoin Sera

Antiserum of similar titer, suitable for use in the FIA, was obtained from all three sheep.

Optimization of Assay Conditions

Binding of FTC-DPG to the walls of the plastic test tubes was prevented by including bovine albumin in the diluent buffer.

The fluorometric endpoint measurement could be made directly on the supernate after elution of FTC-DPG because the sedimented solid phase on the bottom of the test tubes was well removed from the fluorometer light paths. Other than FTC-DPG fluorescence, the only significant contribution to the final signal was a constant background from the elution reagent, about 4 units on the arbitrary scale chosen. Experiments performed in the absence of FTC-DPG confirmed that the endogenous fluorophores of serum or plasma samples were efficiently removed in the aspiration step. Accordingly, total fluorescence signals were routinely recorded and no blank or background corrections were necessary.

By substituting anti-methotrexate particles, we determined the extent of nonspecific binding or entrapment of FTC-DPG by the solid phase to be about 4%.

Standard Curve

The dose–response curve of the magnetizable solid-phase FIA covered the therapeutic range of serum or plasma phenytoin (Figure 1).

Analytical Variables

Sensitivity. From the results of 20 replicate determinations, we calculated the standard deviation of the fluorescence response for zero dose and used it to estimate the sensitivity of the FIA according to a recommended method (16). The minimum detectable concentration of phenytoin in serum was 0.40 mg/L at the 95% confidence level. The minimum detectable dose, as defined by Rodbard (16), was 0.95 ng of phenytoin.

Precision. Three patients' serum specimens were used to assess within-assay precision (10 determinations). Mean assay results were 9.0, 15.8, and 20.5 mg/L, with CVs of 3.6, 2.8, and 5.4%, respectively.

Two specimens measured in 10 different assays gave mean results of 5.5 and 7.8 mg of phenytoin per liter, with between-assay CVs of 8.4 and 5.3%, respectively.

Accuracy. Phenytoin was added at a concentration of 10...
mg/L to nine individual normal human sera and nine individual lipemic sera (triglycerides between 1.7 and 4.0 mmol/L). Mean analytical recovery (and SD) by FIA was 95 (9.4)% for the normal sera (range 89 to 117%) and 102 (9.6)% for the lipemic sera (range 85 to 113%).

Specificity. Drugs that might be administered in combination with phenytoin were tested for their cross reactivity in the FIA system by the method of Abraham (17). Carbamazepine, ethosuximide, ethotoin, phenobarbital, phenusimide, primidone, and sodium valproate showed 1% or less cross reaction. The phenytoin metabolite, 5-(p-hydroxyphenyl)-5-phenylhydantoin, cross reacted by 17%, relative to phenytoin.

Correlation with Other Assay Methods

Patients' serum or plasma specimens were obtained from independent laboratories, where they had been assayed by gas–liquid chromatography or radioimmunoassay. In our laboratory they were assayed by the magnetizable solid-phase FIA and also by the previously developed sodium sulfate separation FIA (5), in which anti-phenytoin serum raised in rabbits was used. Figure 2 shows the correlation between results by solid-phase FIA and radioimmunoassay, and Figure 3 that between solid-phase FIA and gas–liquid chromatography. The parameters of the regression line relating the results for each pair of compared assays were calculated (Table 1), with the assumption in each case that both methods had equal precision characteristics (18).

Discussion

The advantage of magnetic separation in the simplification of immunoassay procedures has been described (6–9). We applied the technique to an FIA for phenytoin. The separation step was also used to remove potentially interfering endogenous fluorophores and other components present in serum or plasma samples. Quantitation of the antibody-bound fraction of the fluorescein-labeled tracer reagent required its elution from the separated anti-phenytoin solid phase. A methanol/buffer mixture was found suitable for this purpose.

The entire assay procedure was performed within disposable polystyrene test tubes, which also served as fluorometer cuvettes. Despite use of a standard type of test tube of nonoptical quality, good precision of the assay endpoint response was obtained with a simple adaptation of an inexpensive fluorometer.

The solid-phase FIA was shown to be sufficiently sensitive, precise, accurate, and specific for the routine monitoring of phenytoin therapy. To accommodate the therapeutic concentration range, we diluted each sample 21-fold. Reduction of the dilution factor would increase sensitivity, and probably allow application to pharmacokinetic studies.

The cross reactivity of the unconjugated hydroxylated metabolite was not great enough to lead to inaccuracy, because its circulating concentrations are much lower than those of the parent drug in most patients, including uremic patients (19). Glucuronide-conjugated metabolite concentrations are relatively higher, and can exceed the phenytoin concentration in uremic subjects (19). The conjugated material has apparently never been tested for cross reactivity in any immunoassay system, owing to its unavailability (20). However, in an immunoassay involving antisem with specificity directed towards the α,α-diphenyl structure of phenytoin, the conjugated metabolite would be expected to cross react to a markedly lesser extent than the unconjugated metabolite (20). The good correlation with gas–liquid chromatography (Table 1 and Figure 3) suggests that any interference in the FIA is insignificant in routine practice.

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