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Validation of a radioimmunoassay for the determination of human plasma concentrations of lamotrigine*

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Abstract: A precise and sensitive radioimmunoassay to determine human plasma lamotrigine (430C78, Lamictal) is described. The method is a direct double antibody procedure employing a rabbit polyclonal first antibody raised to a BSA conjugate of lamotrigine, and an iodinated tyrosine methyl ester of lamotrigine as the tracer. Both reagents are added simultaneously to samples containing lamotrigine prior to an overnight incubation at 4°C. The method has a sensitivity of 20 ng ml^{-1}, when plasma samples are initially diluted 1:20 with phosphate buffer and sample volumes of 20 μl are used. The intra-assay precision at 40, 80 and 160 ng ml^{-1} was 6.2, 2.1 and 4.8%, respectively, and the inter-assay precision at 500, 2000, 5000 and 10,000 ng ml^{-1} was 4.6, 5.7, 4.6 and 5.9%, respectively. The method was specific and showed reasonable correlation with an HPLC method [1].

Keywords: *Validation; radioimmunoassay; lamotrigine; Lamictal; human plasma; ^{125}I-labelled tracer.*

Introduction

Lamotrigine [Lamictal, 430C78, 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine] a novel compound synthesized by The Wellcome Foundation, is an antiepileptic drug for use in the treatment of partial seizures and generalized tonic clonic seizures.

It was proposed to develop a high throughput radioimmunoassay to support the Phase III clinical trials of this compound. Antisera were therefore raised against a BSA conjugate of lamotrigine in rabbits. A radioiodinated tracer was selected because of the high throughput requirement, hence a tyrosine methyl ester of lamotrigine was synthesized. The radioimmunoassay and its validation is described here.

Experimental

Materials

Potassium dihydrogen phosphate, disodium hydrogen phosphate and sodium dihydrogen phosphate were purchased from BDH Chemicals Ltd (Poole, UK). Chloramine T, sodium metabisulphite, BSA (fraction V, RIA grade), diazepam, phenytoin, clonazepam, phenobarbital, sodium valproate and carbamazepine were brought from the Sigma Chemical Co. (Poole, UK). The structural analogues of lamotrigine, 563C78, 583C80, 261C78, 123W79, 288U51, 122W79, 125C78 and 1162C77 were obtained from the Department of Medicinal Chemistry, Wellcome Research Laboratories, (WRL, Beckenham). Lamotrigine was obtained from The Wellcome Foundation (Dartford). The free carboxyl intermediate (74W86) of lamotrigine was produced by Mr D.A. Hill of the Chemical Development Laboratory (CDL) at The Wellcome Foundation (Dartford). Donkey anti-rabbit Sac-Cel was purchased from IDS (Washington, UK). The iodine-125 (IMS 30) was supplied by Amersham International PLC (UK). The methanol and ethanol were purchased from Rathburns (Walkerburn, UK). The blank human plasma was obtained from the Occupational Health Centre, Wellcome (Beckenham).

Equipment

The C8 Bond Elut cartridges (1 ml) were purchased from Analytichem International

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(Harbor City, USA). The disposable plastic tubes (12 × 75 mm) and 1.5-ml micro vials were obtained from Sarstedt Ltd (UK). The Tecan robotic sample processor RSP 5052 was obtained from Tecan UK Ltd (Hemel Hempstead, UK). The 1277 Gammamaster and 1224 Multicalc laboratory data management program were purchased from Pharmacia (Milton Keynes, UK). All pipettes were purchased from Gilson Medical Electronics (France).

Methods

Preparation of immunogens. Two different carrier proteins, bovine serum albumin (BSA) and keyhole limpet haemocyanin (KLH) were conjugated to the free carboxyl intermediate of lamotrigine using the mixed anhydride method [2]. This work was carried out by Dr S. Wilkinson of the Department of Medicinal Chemistry at WRL (Beckenham).

Immunization procedure. Each of the conjugates was suspended in a 0.25% celacol–distilled water solution (w/v) to give a concentration of 2 mg ml^{-1}. Each suspension was then emulsified with an equal volume of Complete Freund's Adjuvant to give a final concentration of 1 mg ml^{-1}. Two groups of six female New Zealand white rabbits were immunized, one group with the BSA conjugate and the other with the KLH conjugate. The animals were primed with 1 ml (1 mg) of the appropriate conjugate by multiple (15–20) subcutaneous injections. This procedure was repeated twice more at weekly intervals. The rabbits were subsequently boosted at 4–6 week intervals using the same procedure, with the exception that Incomplete Freund's Adjuvant was used. Seven to 10 days after every boost, 10 ml of blood was collected from each rabbit, allowed to clot overnight and then separated by centrifugation. The serum was then assessed for lamotrigine antibodies.

Preparation of tracer. A tyrosine methyl ester of lamotrigine (739C87) was prepared by Dr S. Wilkinson of the Department of Medicinal Chemistry (WRL, Beckenham). The iodination procedure, a modification of the method of Greenwood *et al.* [3], was carried out in a 1.5-ml micro vial to which 20 μl of sodium phosphate buffer (0.05 M, pH 7.5), 10 μl of a solution of 739C87 in ethanol (0.25 mg ml^{-1}), and 5 μl (0.5 m Ci) of carrier free sodium [^{125}I]iodide were added using a M25 Microman pipette. The reaction was initiated by the addition of 10 μl chloramine T in sodium phosphate buffer (500 μg ml^{-1}), and was allowed to proceed for 30 s, during which time the contents of the vial were continuously mixed. The reaction was terminated by the addition of 100 μl of sodium metabisulphite in sodium phosphate buffer (120 μg ml^{-1}). The reactants were further diluted by the addition of 0.5 ml of sodium phosphate buffer. The entire volume was then loaded onto a C8 Bond Elut cartridge which had been prewashed with 3 ml of methanol followed by 3 ml of distilled water. The column was initially washed with 2 ml of sodium phosphate buffer, followed by 2 ml of distilled water. The iodinated lamotrigine was eluted by washing with methanol (6 × 1 ml). The peak fraction was further diluted 1:10 with methanol, stored at −20°C, and used in the radioimmunoassay for about 4–6 weeks.

Preparation of buffer. 1/15 M phosphate buffer (pH 7.0) was prepared by the addition of 608 ml of 1/15 M disodium hydrogen phosphate to 392 ml of 1/15 M potassium dihydrogen phosphate. Assay buffer was prepared by the addition of 0.4% BSA (w/v) to 1/15 M phosphate buffer.

Preparation of standards and quality control samples. Lamotrigine was dissolved in methanol to give a stock standard solution (1 mg ml^{-1}). This was appropriately diluted in assay buffer to produce the assay standard (50 ng ml^{-1}). Quality control samples with concentrations of 100, 500, 2000, 5000 and 10,000 ng ml^{-1} were produced by spiking volumes of pooled blank human plasma with the appropriate dilutions of the stock standard.

Assay procedure. A range of assay standards was produced by a series of six double dilutions of the 50 ng ml^{-1} standard. All samples and quality control samples (QC) were initially diluted 1:20 with 1/15 M phosphate buffer, subsequent dilutions were with assay buffer. The assay was set up in duplicate, 20 μl of standard/QC/sample was diluted with 780 μl of buffer. A 900-μl aliquot of assay buffer was added to two tubes to determine non-specific binding (NSB).

Tracer (100 μl diluted in assay buffer to give approximately 20,000 cpm/100 μl) was added to all the tubes, including two tubes designated totals, followed by the addition of a 100 μl of

binder (diluted 1:1000 with assay buffer) to all the tubes except NSBs and totals. The tubes were then vortexed and incubated overnight (16–24 h) at 4°C.

The free and bound portions were separated using a solid phase second antibody. Sac-Cel donkey anti-rabbit coated cellulose (100 μl) was added to each tube, except the totals. The tubes were then vortexed and incubated at room temperature for 30 min. 1 ml of distilled water was added to each tube prior to centrifugation (1200*g*, 5 min).

The assay was then decanted and counted using an LKB 1277 gamma counter. The results were calculated using the 1224 Multicalc Laboratory Data Management Program. The standard curve was fitted using a five parameter logistic equation.

This method has been installed on a dual tipped Tecan RSP 5052, programmed using Version 7 of Immuno ami. Both arms handle samples and reagents, the buffer is dispensed through the system. Samples are initially screened at a 1:500 dilution and are then assayed at a number of serial dilutions in either a low or high dilution assay.

Results and Discussion

Antiserum (reference 9592/F)

Assessment of the sera on the basis of titre and displacement curves demonstrated that serum from one of the rabbits immunized with the BSA conjugate of lamotrigine was the most suitable antiserum for this assay.

Precision

The intra-assay precision of the method was determined by the analysis of 12 duplicate tubes at three different concentrations 40, 80 and 160 ng ml^{-1}. The relative standard deviations were 6.2, 2.1 and 4.8%, respectively. The inter-assay precision of the method was determined by analysis of the quality control samples, at 500, 2000, 5000 and 10,000 ng ml^{-1}. The relative standard deviations were 4.6, 5.7, 4.6 and 5.9%, respectively (see Table 1).

Specificity

As a measure of the specificity of the antiserum various concomitant drugs, structural analogues and the primary metabolite of lamotrigine were tested for cross-reactivity according to the method of Abraham [4] in which execssive masses of potential cross-reactants are incubated under the predetermined immunoassay conditions (see Table 2). None of the levels of cross-reactivity shown should cause a problem when using this method to assay clinical trial samples.

Matrix

No matrix effects were observed. Samples diluted in parallel to the standard curve displayed no systematic bias. Similar recovery levels were obtained when a low sample was spiked with a low, medium or high concentrations of lamotrigine (see Table 3).

Sensitivity

Since there is an initial dilution of 1:20 the minimum concentration which this method is capable of distinguishing from zero with a confidence of 95% is 20 ng ml^{-1}.

Comparability

A comparison was made with an HPLC

Table 1
Intra-assay and inter-assay accuracy and precision of method

Concentration of lamotrigine (ng ml^{-1})	Accuracy (%)	Relative standard deviation (%)	Number of duplicate radioimmunoassay tubes analysed
Intra-assay			
40	92.8	6.2	12
80	100.9	2.1	12
160	95.4	4.8	12
			Number of assays in which duplicate RIA tubes were analysed
Inter-assay			
500	117.8	4.6	11
2000	114.5	5.7	11
5000	121.9	4.6	11
10,000	105.9	5.9	11

Table 2
Specificity of rabbit anit-lamotrigine 9592/F

Compound	% Cross-reactivity
(i) Concomitant medications	
Carbamazepine	<0.008
Clonazepam	<0.008
Diazepam	<0.008
Phenobarbital	<0.008
Phenytoin	<0.008
Sodium valproate	<0.012
(ii) Metabolite and analogues of lamotrigine	
Lamotrigine-N^2-glucuronide (M1)	0.52
563C78	6.00
123W79	1.85
122W79	1.64
583C80	0.12
125C78	4.20
1162C77	1.80
261C78	1.67
288US1	1.00

$$\% \text{ Cross reactivity} = \frac{\text{Amount of lamotrigine required to give 50\% inhibition}}{\text{Amount of potential cross-reactant required to give 50\% inhibition}} \times 100$$

Table 3
The per cent recovery obtained when spiking a low sample

Spike concentration (ng ml^{-1})	% Recovery
2	122.6
5	111.0
10	107.7

method which uses liquid–liquid extraction and normal-phase chromatography with UV detection at 306 nm to determine lamotrigine concentration [1]. Eighty-six clinical samples from various studies were analysed by both methods. The R^2 value was 0.96, the gradient 0.87 and the intercept 0.67 (see Fig. 1).

The major difficulty in developing this method has been the high concentrations of lamotrigine found in the clinical samples. Levels are normally in the range of 4–8 μg ml^{-1}, occasionally 16 μg ml^{-1}. This means most samples have to be diluted 1:500 to 1:2000 fold in order to stay within the range of the calibration curve. The use of the Tecan enables serial dilutions of each unknown to be easily assayed improving precision but reducing sample throughput.

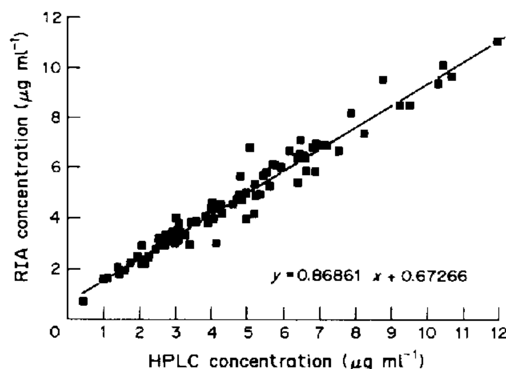

Figure 1
A comparison between RIA and HPLC methods for the determination of plasma concentrations of lamotrigine.

References

[1] A.F. Cohen, G.S. Land, D.D. Breimer, W.C. Yuen, C. Winton and A.W. Peck, *Clin. Pharmacol. Ther.* **42**, 535–541 (1987).
[2] B.F. Erlanger, *Pharmacol. Rev.* **25**, 271 (1973).
[3] F.C. Greenwood, W.M. Hunter and J.S. Glover, *Biochem. J.* **89**, 114–123 (1963).
[4] G.E. Abraham, *J. Clin. Endocr.* **29**, 866–870 (1969).

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