**Validation of a HPLC method for the measurement of erythrocyte encapsulated thymidine phosphorylase (EE-TP) activity**

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Abstract

A sensitive and simple reverse-phase high performance liquid chromatographic (HPLC) assay has been validated for the determination of thymine as a measure of thymidine phosphorylase activity encapsulated in erythrocytes (EE-TP), a formulation which is under clinical development as an enzyme replacement therapy for the treatment of mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). Diluted erythrocyte lysates were incubated in 100 mM sodium phosphate buffer and 10 mM thymidine at 37oC for 10 minutes and the reaction stopped with 40% trichloroacetic acid. Following centrifugation, the supernatant was washed with water saturated diethyl ether, and injected onto a Spherisorb C18 column (125mm x 4.6, 5 µm), with a mobile phase (40 mM ammonium acetate, 5 mM tetrabutyl ammonium hydrogen sulphate, pH 2.70) delivered at a flow rate of 1.0 ml/min and run time of 8 minutes. Ultraviolet detection (UV) was employed at 254 nm. The method was linear in the range of 5 to 500 nmol/ml (*r2*=0.992), specific with intra- and inter -day precisions of < 9.6 and accuracies within ± 20%. Limits of detection and quantification were 1.2 nmol/ml and 10 nmol/ml, respectively. The method was applied to quantify thymidine phosphorylase activity in samples of in-process controls and batches of EE-TP manufactured for clinical use.

Keywords: HPLC, Thymidine phosphorylase, Erythrocyte encapsulated thymidine phosphorylase, Validation, MNGIE

1. Introduction

Thymidine phosphorylase (EC 2.4.2.4) is part of the pyrimidine nucleoside salvage metabolic pathway and catalyses the reversible phosphorylation of the pyrimidine nucleosides, thymidine and deoxyuridine to 2-deoxyribose 1-phosphate and their respective bases, thymine and uracil. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) is a fatal autosomal recessive disorder caused by a deficiency in thymidine phosphorylase [1, 2]. Erythrocyte encapsulated thymidine phosphorylase (EE-TP) is under clinical development as an orphan designated enzyme replacement therapy for the treatment of MNGIE (EC register number: EU/3/11/856). In this approach thymidine phosphorylase is encapsulated within the patient’s own erythrocytes *in vitro* and the erythrocytes then returned to the patient to enable the elimination of the pathological plasma metabolites that accumulate in MNGIE [3-6].

Previously described methods for the measurement of thymidine phosphorylase activity include radiochemical [7], spectrophotometric [8] and reversed-phase high performance liquid chromatography (HPLC) [9]. Limitations of these methods include the use of radioactive isotopes, non-specific absorbance of interfering substances in the erythrocyte lysates, and inadequate separation of thymidine and thymine peaks.

The aim of this study was to validate a simple and sensitive isocratic HPLC method according to International Conference on Harmonization guidelines (ICH) [10] to facilitate the regulatory development of EE-TP. The proposed method was applied to the measurement of enzyme activity in EE-TP batches manufactured for a clinical evaluation study in two patients with MNGIE.

2. Experimental

2.1 Chemicals and reagents

Recombinan*t E. coli* thymidine phosphorylase (specific activity 178 to 211 IU/mg protein) was produced by Sigma-Aldrich, Israel. Ammonium acetate (BioXtra ≥ 98%), concentrated hydrochloric acid (HCl, ACS grade), methanol (HPLC grade, ≥99.9), sodium phosphate dibasic (ACS reagent, ≥99.0%), thymidine (HPLC grade, ≥ 99.0%), thymine (HPLC grade, ≥ 99.0%), trichloroacetic acid (TCA; ACS reagent ≥ 99.0%) and Tris (hydroxmethyl) aminomethane (ACS reagent, ≥99.8%) were all from Sigma-Aldrich, United Kingdom. Diethyl ether (AnalaR NORMAPUR) was from VWR. Purified deionised water (18.2 megohm/cm) obtained from an Arium water tower (Sartorius) water purification system was used for the preparation of all solutions.

2.2 EE-TP formulation

Method validation was performed using EE-TP prepared from blood taken from healthy volunteers. The utility of the method was confirmed in EE-TP prepared from blood taken from two patients with MNGIE and manufactured for the purpose of treating these patients on compassionate grounds. Venous blood was collected into heparinised tubes, centrifuged at 1,500 *g* to separate the erythrocytes from the plasma and buffy coat. After three washes in phosphate-buffered saline (pH 7.4), the erythrocytes were subjected to a reversible hypo-osmotic dialysis technique as described by Bax *et al* [11-13]. Sham-loaded erythrocytes, i.e. erythrocytes subjected to the dialysis encapsulation process, but in the absence of thymidine phosphorylase were also prepared for the purpose of producing an enzyme-free erythrocyte matrix (see section 2.4) and for assessing assay specificity. EE-TP for therapeutic use was manufactured in accordance with the provisions of Schedule 1 of The Medicines for Human Use (Marketing Authorisations Etc.) Regulations SI 1994/3144, under a Manufacturer’s Specials Licence (MS) in pharmacy facilities. Clinical Research Ethics Committee approval and participant informed consent was obtained. Samples of EE-TP and sham-loaded erythrocytes were stored at -80oC until required for further processing and analysis.

2.3 Preparation of stock and standard solutions

Stock solutions of 100 mM sodium phosphate buffer, pH 6.5, 10 mM thymidine in deionised water, 125 mM Tris buffer, pH 7.4 and 40% TCA were prepared and stored at -20oC (stable for up to one year). Water saturated diethyl ether was prepared by adding 100 ml deionised water to 2.5 l ether and stored at room temperature (for up to one year). A primary stock solution of 3.5 mM thymine was prepared by dissolving thymine first in 0.1 mM sodium hydroxide, followed by dilution with acidified water (concentrated was HCl added drop-wise to purified deionised water to achieve pH 2.0). Thymine and thymidine scale factor standards were prepared by diluting both a 1 mM solution of thymine and 1mM solution of thymidine 1:31 with acidic water (pH 2.0).

2.4 Enzyme-free erythrocyte matrix pool

An erythrocyte matrix pool was created for the preparation of calibration standards and validation quality control (QC) samples. Sham-loaded erythrocytes prepared from six healthy volunteers were assayed to confirm the absence of thymidine phosphorylase and pooled using an equal volume from each preparation to provide a total volume of 30 ml. Sodium phosphate buffer (120 ml, 100 mM, pH 6.5) and 30 ml 40% TCA were added to the pool and mixed well. After centrifugation at 15,000 *g* for 6 minutes, the supernatant was washed twice with water-saturated diethyl ether with 2 minutes of vortexing to extract the TCA. To avoid ether interfering with HPLC separation, effective removal was achieved by exposing the matrix to the air for 5 minutes to allow evaporation of the ether. The matrix was then aliquoted and stored at -80oC until required.

2.5 Preparation of calibration standards and validation QC samples

The primary thymine stock solution was diluted with water to give working standard solutions of 0.07 and 0.7 mM. Known volumes of the thymine working solutions were added to the blank erythrocyte matrix and diluted with water to achieve calibration standards of 5 to 500 nmol/ml in a total volume of 175µl. Three QC samples at 10 nmol/ml (low thymine), 100 nmol/ml (medium thymine), and 400 nmol/ ml (high thymine) were prepared independently and were prepared on the day of analysis.

2.6 Preparation of samples for HPLC

Thymidine phosphorylase activity was determined in EE-TP samples by quantifying the rate of conversion of thymidine to thymine. Preliminary experiments were conducted to determine the linear metabolite formation kinetics with respect to time and enzyme dilution; the method was shown to be linear for up to 16 minutes, over a thymine phosphorylase range of 4.0 to 719 nmol/min/ml (corresponding to a sample dilution range of 10 to 9088. Lysates of pre-dialysis samples (thymidine phosphorylase mixed with erythrocytes), EE-TP and sham-loaded erythrocyte samples were prepared by diluting thawed samples 1:710 with 125 mM tris HCl, pH 7.4. Twenty five µl of the erythrocyte lysate were then added to 100 µl sodium phosphate buffer (100 mM, pH 6.5) and 25 µl thymidine standard (10 mM), mixed and incubated at 37°C for 10 minutes. The reaction was terminated with 25 µl 40% TCA. Assay blanks were prepared by adding TCA to the sodium phosphate buffer/thymidine incubation mixture prior to adding the erythrocyte lysate. Samples were centrifuged at 13,400 *g* for 2 minutes, and the supernatant washed twice with water-saturated di-ethyl ether with 2 minutes on a shaker to extract the TCA. To avoid ether interfering with HPLC separation, effective removal was achieved by exposing the matrix to the air for 5 minutes to allow evaporation of the ether. A sample volume of 10 µl was injected into the HPLC.

2.7 Chromatographic conditions

Chromatographic separation of substrate and product was achieved using reversed phase chromatography with isocratic elution using a Waters Alliance HPLC 2795 system. A pre-packed C18 column (Spherisorb ODS 125 mm x 4.6 mm ID, 5 µm particle size, Waters) was used as the stationary stage. Analytes were eluted using a mobile phase of ammonium acetate (40 mM) with the ion-pairing agent tetrabutyl ammonium hydrogen sulphate (5 mM) adjusted to pH 2.70 with HCl, delivered at a flow rate of 1.0 ml/min, with a run time of 8 minutes. UV detection was at 254 nm and 0.1 absorbance units full scale. Metabolites were identified by comparing spectra with pure standards.

2.8 Method validation

The analytical method was validated in agreement with ICH Harmonised Tripartite Guidelines [10] using the following analytical parameters: specificity linearity, range, detection and quantification limits, precision, accuracy, robustness and stability.

Specificity of the method was verified by analysing potential interference by the lysed erythrocyte matrix; chromatograms of incubations of sham-loaded erythrocyte lysates without thymidine, sham-loaded erythrocyte lysates spiked with thymine QC standards, and an incubation of EE-TP with thymidine (final incubation concentration 1.67mmol/l) were compared. The linearity of the method was evaluated by constructing calibration curves from eleven thymine standards in the range of 5 to 500 nmol/ml in a matrix of sham-loaded erythrocyte supernatant (see section 2.4). Each standard was analysed eight times. Peak area (response) against thymine concentration was plotted and a linear least-squares regression analysis was conducted to determine slope, intercept, residual sum of squares, and correlation coefficient (*r*2) for the determination of the linearity of the method. The minimally acceptable *r2*for the calibration curve was 0.99 or greater. The limit of detection (LOD) was defined as the lowest detectable thymine concentration, taking into consideration a signal to noise ratio of three. The lower limit of quantification was the lowest thymine concentration on the calibration curve that could be determined with a precision within ± 20%, as indicated by the coefficient of variation (CV%), and an accuracy within 20%, as indicated by the relative error (RE%). Accuracy, which determines the proximity between the obtained experimental results and the predicted results, was calculated as follows: [(calculated QC concentration – predicted QC concentration/predicted QC concentration) x 100]. Precision was measured as repeatability and intermediate precision. Reproducibility (the use of the analytical procedure in different laboratories) was outside the scope of this study. Repeatability (intra-day precision) was assessed from the results of 20 analyses of each validation QC sample on a single day. Intermediate precision (inter-day precision) was determined by from the analysis of the same QC samples six times on five consecutive days. The criteria for acceptability of precision were that the CV for each QC concentration should not exceed ± 20%. The criteria for acceptability of accuracy were that the averaged value should be within ± 20% of the nominal concentration. Assay robustness was investigated by evaluating the influence of variations in the pH of the assay buffer on the concentrations of validation QC samples at low, middle and high concentrations. Thymine QC samples were prepared in sodium phosphate buffer which had been adjusted to pH 5.4, 6.0, 6.5, 7.0 and 7.4 and were each analysed 8 times on the same day. The assay is considered as robust where the final accuracy criteria within ± 20% were observed at the pH tested.

Stability after exposure to different experiment conditions was determined for both thymidine phosphorylase activity and thymine (the analyte) contained within the erythrocyte matrix.

Thymidine phosphorylase stability in the erythrocyte matrix at ambient temperature was evaluated by thawing seven different EE-TP samples at room temperature for 30 minutes and assaying for thymidine phosphorylase activity at 9 different time points (in duplicate) up to 7 hours.

The effect of six freeze-thaw cycles (-80oC/ambient temperature) on thymidine phosphorylase stability in the erythrocyte matrix was assessed. For each cycle two aliquots of EE-TP samples (stored at -80oC) were thawed, unassisted for 30 minutes at room temperature and then refrozen for at least 12 hours. All six sets of samples were analysed in duplicate in one assay.

Thymidine phosphorylase stability in the erythrocyte matrix during storage was determined by analysing EE-TP samples for enzyme activity at 4 and 12 months of storage at -80oC. Four samples were analysed in duplicate at baseline (0 time) and after the assigned storage period.

Thymidine phosphorylase activity at baseline was used as a reference to determine the relative stability in the experiments described above and was considered stable when the mean precision (CV%) was within ± 20%, and the mean accuracy (RE%) within ± 20% of the baseline activities.

Thymine stability in the erythrocyte matrix was assessed by analysis of QC samples at low, middle and high concentrations. The effect of ambient temperature was assessed by thawing aliquots of each QC at room temperature for one and three hours (three hours being the expected maximum duration test samples will thawed before analysis (n=3 in duplicate).

The effect of three freeze-thaw cycles on thymine stability in the erythrocyte matrix was assessed. For each cycle three aliquots of each QC concentration (stored at -80oC) were thawed, unassisted for 2 hours at room temperature. The aliquots were then refrozen for at least 12 hours. All three sets of QC samples were analysed to determine stability (n=3 in duplicate) in one assay.

The evaluated stability QC samples were considered stable when the mean precision (CV%) was within ± 20%, and the mean accuracy (RE%) is within ± 20% of the predicted concentrations.

2.9 Application of the method

The method was applied to the determination of thymidine phosphorylase activity in clinical batches of EE-TP which had been manufactured for the treatment of two patients with MNGIE. Samples of pre-dialysis in-process control suspension (thymidine phosphorylase mixed with washed and packed erythrocytes) and EE-TP were prepared for analysis as described in section 2.6.

The stability of thymidine phosphorylase was assessed over the proposed maximum anticipated time delay between EE-TP formulation and infusion in the clinical setting by analysis of enzyme activity after 0, 24, 48, 72 and 96 hours of storage at 4oC and 22oC. One hundred microliter aliquots of 8 different preparations of EE-TP were stored in closed microtubes at the appropriate temperature and then frozen after the appropriate incubation time until analysis.

2.10 Statistical methods

Data are expressed as mean ± SD or mean ± SEM. Linearity was calculated by linear regression analysis. Cell lysate sonication data was analysed using student’s t-test, and clinical EE-TP batch stability data was analysed using Anova (two-Factor with replication).

3. Results and Discussion

3.1 Method validation

Using the described HPLC method, the chromatograms of sham-loaded incubations demonstrated no endogenous peaks that co-eluted with thymine, thus confirming the absence of interference from components of the erythrocyte lysate, and demonstrating that the method is specific (data not shown). Chromatograms from incubations of EE-TP with thymidine (final concentration 1.67 mmol/l) demonstrated that thymine and thymidine were well resolved and completely separated at mean retention times of 3.3 and 7.4 minutes, respectively (data not shown). Calibration curves for thymine demonstrated good linearity over the concentration range of 5 to 500 nmol/ml, with a mean regression equation (± SD) of *y* = (0.0040 ± 4.4 x 10-5)*x* + (0.0001 ± 0.0100), a correlation coefficient of 0.992 ± 0.005 and residual sum of squares of 0.397 ±0.005. The LOD of thymine was 1.2 nmol/ml, the LOQ of thymine quantification was 10 nmol/ml (CV% of 9.8 and RE% 10.1), this being the lowest concentration on the calibration curve that could be determined with a precision error and accuracy within 20%. A chromatogram representing the thymine peak at the LOQ is shown in Fig. 1.

Table 1 summarizes the results for intra-day and inter-day precision. In the range of 10 to 400 nmol/ml, intra- and inter-day precision (CV) ranged from 3.3 to 5.9 and 6.0 to 9.6%, respectively. The intra- and inter-day accuracy of thymine concentrations in the range of 10 to 400 nmol/ml ranged from 7.2 to 10.7 and 10.4 to 12.0, respectively. This data is within the acceptable ranges and therefore indicates that the proposed method has good precision and accuracy. Changing the pH of the buffer in which the QC samples were prepared had no marked effect on the performance of the assay and robustness is thus verified for the pH range tested (data not shown).

Thymidine phosphorylase was stable in the erythrocyte matrix at ambient temperature for 7 hours, after storage for 12 months and following 6 freeze/thaw cycles (Table 2).

Values for the validation QC samples (expressed as mean RE%) after standing at ambient temperature for 3 hours, and after three freeze/thaw cycles were all within ± 20%, demonstrating the stability of thymine in the erythrocyte matrix under these conditions (Table 3).

3.2 Application of the method

The proposed method was applied to batches of EE-TP manufactured for clinical use. There was a good agreement between the measured activity and the expected activity in the pre-dialysis in-process controls (Table 4). For each patient a dose escalation was successfully achieved by doubling the volume of erythrocytes and activity of thymidine phosphorylase employed in the dialysis process (EE-TP batches 3 and 4, Table 4). Storage of EE-TP for up to 24 hours at 4oC and 22oC had no significant effect on thymidine phosphorylase activity (Fig. 2) demonstrating stability over the proposed maximum anticipated time delay between EE-TP formulation and infusion into the patient.

4. Conclusion

This study describes a validated reverse phase HPLC method for the determination of thymidine phoshorylase according to the Guidelines of the ICH. All analytical parameters are within the limits proposed by those guidelines for pharmaceutical formulations indicating that the method is specific, precise, accurate and robust with low detection and quantification limits. The method was successfully applied to measure thymidine phosphorylase during in-process testing and in batches of EE-TP formulated for clinical use. The method is sufficiently simple and quick to enable in-house testing, an expeditious quality control release of EE-TP under good manufacturing practice (GMP) conditions after completion of the fill/finish step, and a timely shipment to clinical trial sites.

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