A Rapid Two-Step Iduronate-2-Sulfatase Enzymatic Activity Assay for MPSII Pharmacokinetic Assessment

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Abstract Clinical studies involving enzyme replacement therapies (ERTs) have increasingly utilized enzymatic activity assays to monitor efficacy and biofunction of the drug; as a result, these assays have become an important part of pharmacokinetic (PK) and pharmacodynamic assessments in ERT trials. This paper presents a two-step enzymatic activity assay for iduronate-2-sulfatase (I2S) (EC 3.1.6.13) which we have optimized to fit in 1 day and to complete in less than 6 h. The rapid assay presented here is a significant improvement over the original two-step method with run time of 24 h which spanned 2 days. The resulting 1 day assay is efficient, robust, reproducible, and better suited for use in pharmacokinetic studies. The method was fully validated in accordance with regulatory agency guidelines so that it could be implemented in PK studies. Validation of the method required additional modifications to circumvent limitations surrounding the calculation of accuracy. This challenge was overcome by developing strategies to determine both the expected and the measured values of validation samples in activity units. Subsequently, the method was validated in accordance with the FDA guidance for the validation of quantitative ligand binding assays (LBAs). Results of method development and optimization with focus on evaluations aimed at reducing the total assay run time as well as a summary of method validation performance are presented in this publication.

Introduction

Mucopolysaccharidosis type II (MPSII), or Hunter Syndrome, is a genetically inherited lysosomal storage disease characterized by the deficiency of enzyme I2S. As an X-linked recessive disease, Hunter Syndrome primarily affects males at the approximate rate of 1 out of every 70,000 live births. The deficiency affects the body's ability to break down glycosaminoglycans (GAGs) (Muenzer et al. 2006, 2007), resulting in accumulation of GAGs which causes progressive damage impacting physical appearance, range of motion and mobility, organ function, and in some, cognitive abilities. Recombinant human I2S drugs such as Elaprase (Shire, USA) and Hunterase (Green Cross, South Korea) have been approved as ERTs for the treatment of MPSII. A novel brain penetrating I2S-anti-human insulin receptor IgG fusion compound, AGT-182, which is a chimera of two I2S and one IgG molecules (Lu et al. 2010) is also in evaluation and in a phase I clinical trial (NCT02262338).

Fluorometric enzyme assays have long been employed to measure the activity of I2S as part of pharmacodynamic and pharmacokinetic studies of this enzyme (Tolun et al. 2012; Johnson et al. 2013). This paper presents a two-step I2S enzymatic activity assay, modified from a previously published method (Voznyi et al. 2001) which is also fluorometric but differs from most existing assays in a number of ways. The method presented here utilizes 4-methylumbelliferyl α -L-iduronide-2-sulfate (4-MUS) as substrate (Voznyi et al. 2001). 4-MUS has greater specificity towards I2S. Use of 4-MUS is an advantage over other I2S enzymatic assays which utilize 4-methylumbelliferyl-sulfate (4-MS) (Inoue et al. 1982), a substrate that is hydrolyzed by all sulfatases (Inoue et al. 1982; Hopwood 1979).

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In the first of the two steps, 4-MUS is hydrolyzed by I2S to 4-methylumbelliferyl α -L-iduronide (MUBI). The first step reaction is completely stopped by the phosphate present in the second step reaction buffer (Voznyi et al. 2001), and subsequently in the second step, another lysosomal enzyme, α -L-iduronidase (IDUA) (EC 3.2.1.76), hydrolyzes MUBI to the final product, 4-methylumbelliferone or 4-MU. 4-MU emits fluorescence, and the signal is quantified against a calibration curve prepared with synthetic 4-MU. In a series of evaluations presented in this paper, both reagent concentrations and the incubation times of the two-step assay were optimized to yield a method that is robust, can be performed in only a few hours, costs less, and is hence more suitable for clinical and nonclinical studies.

An earlier version of this assay was validated and used in preclinical studies of AGT-182 in primates (Boado et al. 2014). The primate assay was based on the original method published by Voznyi et al. (2001), without the optimizations presented in this paper. Some of the challenges in the validation of the two-step activity assay for use in regulated studies stemmed from the absence of a typical quantitative calibration curve prepared from the study matrix fortified with the AGT-182. This posed a significant limitation on validating the method since it did not allow for the extrapolation of the validation sample (VS) values in the same units used to spike them. The resulting disparity between the units for the expected versus measured values of VSs (ng/mL or μ g/mL versus nmol/h/mL or nmol/h/ μ g) made it difficult to assess method accuracy. We addressed this limitation by establishing expected values in activity units for each VS which subsequently allowed for the calculation of accuracy as percent recovery of measured versus expected value for each validation parameter. This publication presents optimization strategies used to reformat the assay into a rapid method as well as a summary of method validation performance including accuracy and precision, selectivity, and dilutional linearity.

Materials and Methods

Preparation of Assay Quality Controls

Assay quality controls were prepared at high, medium, and low levels (HQC, MQC, and LQC at 800, 400, and 100 ng/mL, respectively) by adding AGT-182 into a qualified pool of human K_2 EDTA plasma. For accuracy and precision runs, VSs were prepared at five levels of upper limit of quantitation (ULOQ, 1,000 ng/mL), HQC, MQC, LQC, and lower limit of quantitation (LLOQ, 50 ng/mL) to span the targeted quantitative range of the assay.

Sample Storage and Preparation

The study plasma samples as well as the assay quality controls were stored at -80°C . In preparation for the activity assay, plasma samples and the quality controls were thawed on ice and subsequently diluted ten fold to the minimum required dilution (MRD) of the assay, in ABST buffer (acetate-buffered saline with 0.001% polysorbate 80, pH 6.0). MRD is defined as the minimum predilution of plasma sample needed in order to overcome matrix interference and to achieve acceptable performance during evaluation of validation parameters. For this two-step activity assay, MRD was established during method development at 1/10 as that was the minimum dilution at which plasma samples generated acceptable selectivity results (% recovery within 75–125%). Diluted samples were stored on ice until they were loaded onto the reaction plate.

Substrate, 4-MUS

The optimal concentration of the substrate, 4-MUS substrate (4-methylumbelliferyl α -L-iduronide-2-sulfate, C16H15O12S $\cdot$ 2Na, molecular weight 477.33, Santa Cruz Biotechnologies, catalog no. sc-210122) was determined by side-by-side comparison of 1.2, 0.6, 0.3, 0.12, and 0.06 mg/mL concentrations in the same assay. Selection of 0.6 mg/mL was based on (a) the best dilutional linearity within the quantitative range of the assay (range of 50–1,000 ng/mL of AGT-182 plasma samples), (b) separation of signal amongst individual AGT-182 plasma samples ranging from 50 to 1,000 ng/mL, as well as (c) the highest signal/noise which was the ratio of the ULOQ at 1000 ng/mL over the blank plasma sample. These factors lead to the selection of 0.6 mg/mL as the optimal concentration of 4-MUS in this assay. At substrate concentration higher than 0.6 mg/mL, for example, 1.2 mg/mL, the assay background was elevated to 7,000–9,000 fluorescence units (FUs) which had suboptimal separation from the targeted LLOQ of the assay (data not shown).

α -L-Iduronidase

Recombinant human α -L-iduronidase (Shire) was expressed in stable human cell line (HT1080) using G418 selection. Production of IDUA was performed in a wave reactor seeded at 50,000 cells/mL, cultured for 10 days at 37°C . Culture media was collected by perfusion and stored at 4°C . At the end of the production run, the cell viability was maintained at $\geq 94\%$. The culture media of three wave runs were pooled and purified using a Ni-Sepharose Fast Flow column method. The fractions were pooled and dialyzed into 50 mM Na Acetate, 500 mM NaCl, pH 5.0 storage

buffer. The purity of the final product was determined to be 94%.

Enzymatic Activity Assay

For the standard assay, reaction mixtures consisting of 10 μL of MRD-diluted plasma samples or controls and 20 μL of 0.6 mg/mL of substrate 4-MUS in substrate diluent (0.1 M sodium acetate, 10 mM lead acetate, 0.2% sodium azide, pH 5.0) were loaded in duplicates onto black polystyrene, non-treated plates (COSTAR, part no. 3915). Assay plates were immediately covered with foil sealers and placed in the 37 °C shaking incubator set at 350 RPM for 1 h. For the second reaction step, the IDUA working solution was prepared at 22 $\mu\text{g/mL}$ in McIlvaine's buffer (0.4 M sodium phosphate dibasic, 0.2 M sodium citrate, 0.2% sodium azide, pH 4.5) and added at 45 μL per reaction to bring its final reaction concentration to 13.2 $\mu\text{g/mL}$. Plates were once again covered with foil sealers and placed in the 37 °C shaking incubator set at 350 RPM for 4 h. Reactions were stopped by the addition of 200 μL of Stop Solution (0.5 M sodium carbonate, 0.025% Triton X-100, pH 10.7). The final hydrolysis product, 4-MU, was quantitated against a standard curve prepared fresh in the Stop Solution using commercial 4-MU (MP Biomedicals, part no. 152475). 275 μL of each standard was loaded per well to match the final volume in the sample reaction wells. Fluorescence was measured at 365 nm (Ex), 450 nm (Em), 435 nm (Cutoff, defined as the wavelength for the filter at which the transmission is 50%) using a SpectraMax M5 (Molecular Devices) plate reader.

Calculation of Sample Activity

Calculations of sample activity were based on the mean of fluorescence unit of the duplicate wells extrapolated against the 4-MU standard curve. The 4-MU concentration of every sample was adjusted for the MRD as well as for any additional predilutions that the sample had been subjected to. Activity for unknown samples was calculated in nmol/h/mL using the following equation: Activity (nmol/h/mL) = $[27.5 \times (4\text{-MU Result}/1,000)] / 1$, where the denominator 1 represents the 1 h first step reaction time. Activity for the assay controls and some VSs was calculated in nmol/h/ μg as appropriate for each particular validation parameter. This was done by dividing activity in nmol/h/mL by the $\mu\text{g/mL}$ concentration of the control or the spiked VS. All activity results were corrected for the appropriate background; for example, the assay plasma controls were corrected for the unfortified plasma pool (the 0 ng/mL control), the validation selectivity samples were corrected for their matched unfortified individual plasma

samples, and unknown samples were corrected for their respective pre-dose or baseline samples.

Results

Method Development and Optimization

4-MU Standard Curve

The 4-MU standard curve was prepared by fortifying the assay Stop Solution with commercial 4-MU. The calibration curve range which was optimized to attain maximal sensitivity; the final quantitative range was from 31.25 to 4,000 nM.

I2S Reaction Time

The first step reaction involved catalysis of the substrate, 4-MUS, by I2S. According to previously published work (Voznyi et al. 2001), different laboratories carry out this step at either 1 or 4 h. We investigated whether 1 or 4 h constituted an optimal reaction time for this step. Figure 1a presents a comparison of activity for AGT-182-spiked samples after 1 or 4 h of first step. The data suggest that activity is in the linear

Fig. 1 Optimization of I2S reaction time. (a) A comparison of 1 and 4 h reactions times using plasma samples spiked with increasing concentrations of AGT-182. (b) Comparison of 45, 60, and 75 min to establish the acceptance limits of the 1 h reaction time

Fig. 2 Optimization of α -L-iduronidase. α -L-iduronidase was used in the second reaction step at ten-fold increasing concentrations ranging from 0.00132 to 132 $\mu\text{g/mL}$ for the analysis of plasma samples fortified with AGT-182

Fig. 3 Optimization of α -L-iduronidase reaction time. Evaluation of the second step reaction catalyzed by α -L-iduronidase and carried out for 4, 18, and 24 h using plasma samples containing varying concentrations of AGT-182

range after 1 h incubation but is diminished if the first step is carried out for 4 h potentially due to the exhaustion of the substrate. This comparison was repeated and confirmed in a separate experiment. Subsequently in a separate experiment, the first step reaction time was further optimized at the proximity of 1 h so that limits of this time window could be assessed. A comparison of 45, 60, and 75 min reaction times is shown in Fig. 1b; no significant difference was observed amongst the three conditions. It was therefore concluded that the first step could be carried out for anywhere within the 45–75 min window with optimal activity yields.

Optimization of α -L-Iduronidase Activity

To assess potency, efficiency, and maximal enzymatic activity of the Shire IDUA, this enzyme was evaluated at a multitude of concentrations which started with 132 $\mu\text{g/mL}$ followed by serial ten-fold dilutions. The results are presented in Fig. 2 where activity in nmol/h/mL at each enzyme concentration is plotted against the concentration of AGT-182 in the plasma sample in $\mu\text{g/mL}$ units. Data suggested that maximal enzymatic activity was achieved with IDUA concentrations ranging from 1.32 to 132 $\mu\text{g/}$

mL. Based on these results, 13.2 $\mu\text{g/mL}$ which was ten folds higher than the activity saturation dose (1.32 $\mu\text{g/mL}$) was selected to avoid exhaustion of this enzyme during the second step reaction. In a separate but similar assay, the IDUA used in this study was compared to IDUA from a commercial source (R&D Systems, Minneapolis, MN). The activity and potency of IDUA from the two sources were shown to be identical when used at equal concentrations (data not shown).

Optimization of IDUA Reaction Time

The second step reaction catalyzed by IDUA is commonly run for 24 h according to published data (Voznyi et al. 2001). This makes for a 2-day assay. For efficiency purposes, shorter incubation times were evaluated to determine the feasibility of a 1-day assay. An activity assay was performed with three identical sets of AGT-182-fortified plasma samples in which the second step reaction was carried out for 4, 18, and 24 h, respectively. The data are presented in Fig. 3 and demonstrate that the activity difference between a 24 h reaction versus either 4 or 18 h reaction ranges from zero to 11%, and that the activity

yields are generally similar for all three reaction times. Based on these results, a 4 h incubation time was selected so as to allow for the completion of the two-step assay in 1 day.

Method Validation

Accuracy and Precision

Inter- and intra-assay accuracy and precision were assessed in six independent runs with three sets of each VS level per run, by two operators using VSs prepared by spiking AGT-182 into qualified human plasma pool at five levels which included 50, 100, 400, 800, and 1,000 ng/mL for LLOQ, low, medium, high, and ULOQ, respectively. VS activity values were reported in nmol/h/μg and were each corrected for the endogenous plasma pool activity. The calculation of inter- and intra-assay precision was based on the ANOVA analysis method of DeSilva et al. (2003). Results are presented in Table 1 and show that relative error (RE) for all five VSs was in the targeted acceptance range of ±20%. RE was calculated using the following equation:

$$\%RE = \frac{\text{Measured Activity of Individual VS} - \text{Mean Activity of A\&P VSs}}{\text{Mean Activity of A\&P VSs}} \times 100$$

where the mean of all VSs assessed in the accuracy and precision runs was used as reference to establish the expected activity value in nmol/h/μg. The range of specific activity for AGT-182 based on accuracy and precision data was 524.0–655.0 nmol/h/μg. The range of specific activity based on all data obtained during method development and validation was 517.3–688.8 nmol/h/μg. The total error was calculated by adding the RE absolute value to the inter-assay CV; it met the targeted specification of ±30%. Inter-

and intra-assay CVs were <20% for all VSs and met the precision acceptance criteria.

Selectivity and Dilutional Linearity

Selectivity was evaluated by spiking ten individual human K₂EDTA plasma samples from normal healthy donors with AGT-182 at low and high levels. The low selectivity spike level was 70 ng/mL to position it between the LLOQ and the LQC of the assay; the high spike level was 800 ng/mL. The qualified plasma pool was also spiked with AGT-182 at the same levels as baseline control. The endogenous I2S activity in all unfortified samples was also evaluated. Percent recovery was determined as the ratio of measured activity in nmol/h/μg over expected activity using the following equation:

$$\% \text{Recovery} = \left(\frac{\text{Measured Activity of Individual Plasma}}{\text{Expected Activity}} \right) \times 100$$

$$\text{Where Expected Activity} = (\text{Activity of Unspiked Sample}) + (\text{Total Expected Activity for the AGT-182 spike level of 70 or 800 ng/mL})$$

$$\text{Expected Activity for the AGT-182 spike level of 70 or 800 ng/mL} = (\text{Reference mean activity of positive controls from accuracy and precision runs in nmol/h/}\mu\text{g}) \times 0.07 (\text{for 70 ng/mL}) \text{ or } 0.8 (\text{for 800 ng/mL})$$

All ten individual plasma samples met the target selectivity recovery acceptance criteria of 75–125% at both high and low spike levels.

To evaluate linearity of dilution, an ultrahigh sample was prepared from the qualified plasma pool freshly spiked with 30 μg/mL of AGT-182. This sample was serially two-fold diluted from 1/2 through 1/ 20,480 in the matrix pool,

Table 1 Method accuracy and precision

	LLOQ 50 ng/mL	LQC 100 ng/mL	MQC 400 ng/mL	HQC 800 ng/mL	ULOQ 1,000 ng/mL
Mean activity (nmol/h/μg)	628.9	655.0	604.1	553.3	524.0
%RE ^a	4.1	8.4	0.0	−8.4	−13.3
Intra-assay precision (%CV)	7.8	7.1	0.8	1.9	1.3
Inter-assay precision (%CV)	10.1	8.7	9.0	7.3	7.1
RE + inter-assay CV	11.9	15.5	0.8	10.3	14.6

Inter- and intra-assay precision were evaluated by spiking the qualified plasma pool with AGT-182 at 50, 100, 400, 800, and 1,000 ng/mL for LLOQ, low, medium, high, and ULOQ, respectively. The resulting activities in nmol/h/μg for each of these validation samples were adjusted by the activity of unspiked plasma pool. The mean in nmol/h/μg of all validation samples was used to establish the expected activity value. Relative error (RE) was calculated as the percent difference between measured activity of individual validation sample and the theoretical value (mean activity of A&P)

^a Mean of all A&P activity, 604.1 nmol/h/μg, was used to establish the expected activity for the calculation of relative error

subsequently subjected to the MRD, and finally analyzed in the activity assay. The recovery of linearity samples was assessed as follows:

$$\% \text{Recovery} = (\text{Mean Activity of the Dilution Sample} / \text{Expected Activity}) \times 100$$

$$\text{Where Expected Activity} = (\text{Activity of Plasma Pool}) + (\text{Activity value associated with the Ultrahigh Sample} / \text{Dilution Factor})$$

$$\text{Activity value associated with the Ultrahigh Sample} = (\text{Reference mean activity of positive controls based on accuracy and precision runs in nmol/h/}\mu\text{g}) \times 30$$

In the above formulae, the dilution factor includes the MRD of assay. Linearity samples with in-range nominal concentrations (expected concentration between 50 and 1,000 ng/mL) produced recoveries that ranged from 100 to 116% and met the targeted acceptance criteria of 75–125%. These data established linearity of sample dilution for up to 1/20,480 (data not shown).

Discussion

Through a series of optimizations and modifications, we were able to improve the I2S two-step enzymatic activity assay from a 2-day format into a 1-day assay which can be completed in less than 6 h and is more robust. The resulting two-step enzymatic activity assay is specific towards I2S as the assay substrate could only be hydrolyzed by I2S. Additionally, this assay leads to complete hydrolysis of the starting substrate; this feature helps reduce variability across runs and across multiple sample measurements.

Application of the activity assay in regulated studies requires full validation of the method, and since the enzymatic activity was intended for pharmacokinetic assessment, it was important that method validation followed the FDA regulatory guidance for validation of quantitative LBAs as closely as possible. The absence of a typical LBA calibration curve to allow for extrapolation of sample values in the same concentration units of ng/mL or $\mu\text{g/mL}$ as used for spiking the VSs was a significant challenge. We devised a methodology to establish the expected (spike) values in activity units of nmol/h/mL or nmol/h/ μg based on the mean of measured activity values for VSs. This allowed for the calculation of the accuracy as percent of measured over expected values in each validation run. Subsequently method validation was conducted in accordance with the agency guidance as part of which, validation parameters including accuracy and preci-

sion, selectivity, dilutional linearity, and stability were assessed using the above approach. All validation parameters successfully passed the acceptance criteria. A summary of method development, optimization, and validation results were presented in this paper.

Enzymatic activity assays have become instrumental in the assessment of pharmacokinetics, pharmacodynamics, efficacy, and biofunction of ERTs. Optimization of such assays into more specific, robust, efficient, and faster methods will improve the quality of the PK data as well as have cost saving impact.

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Synopsis

Effective optimization resulted in an I2S enzymatic activity assay which is rapid, more specific, more robust, highly reproducible, and hence better suited for application in clinical or nonclinical studies.

Compliance with Ethics Guidelines

Conflict of Interest

Mitra Azadeh, Luying Pan, Yongchang Qiu, and Ruben Boado declare that they have no conflict of interest.

Informed Consent/Animal Rights

This article does not contain any studies with human or animal subjects performed by any of the authors.

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Author Contributions

Mitra Azadeh was responsible for the design, planning, conduct, data analysis, and reporting of the work described in this publication; she also drafted this article. Luying Pan, Yongchang Qiu, Ruben Boado, and Mitra Azadeh equally contributed to the interpretation of the data and critical revisions of the article for important intellectual content.

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