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Development and implementation of tPA clot lysis activity assay using ACL TOP™ hemeostasis testing system in QC laboratories

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ABSTRACT

This report describes the design, development, validation and long-term performance of tPA clot lysis activity assay using Advanced Chemistry Line Total Operational Performance (ACL TOP)™ Homeostasis Testing System. The results of the study demonstrated robust and stable performance of the analytical method. The accuracy of the assay, expressed by percent recovery is 98–99%. The intermediate precision and repeatability precision, expressed as Relative Standard Deviation (RSD), was 3% and less than 2% respectively. The validated range is from 70% to 130% of the target potency of 5.8×10^5 IU/mg. The linearity of this range, expressed in correlation coefficient, is 0.997. After the assay is transferred to a QC laboratory, the assay retained high accuracy and precision with a success rate of > 99%.

1. Introduction

Recombinant human tissue Plasminogen Activator (rt-PA) is a serine protease with a molecular weight of about 60,000–110,000 Da [1,2]. It proteolytically converts plasminogen to active plasmin that in turn degrades insoluble fibrin to soluble by products [3,4]. This property has been shown to provide both clinical and cost effectiveness for the treatement of stroke [5]. Since the registration of rt-PA, many variants that confer the clot lysis therapeutic activity have been reported [6]. The activity of rt-PA can be determined by measuring the lysis of a synthetic fibrin clot over time [7,8]. The *in vitro* potency measurement relies on simultaneous clot formation and clot lysis reactions triggered by mixing fibrinogen, plasminogen, thrombin and tPA together at once (Fig. 1). In practice, this is achieved by sequential addition of rtPA, plasminogen, thrombin and finally, fibrinogen, which triggered the clot formation and the following clot lysis cascade. The change in turbidity of the clot is monitored and the time needed to achieve a predetermined level of reduction in turbidity is used as a measurement of potency. Different forms of potency assays using the kinetic method have been developed; including a microcentrifugal analyzer (MCA, also referred to as the Monarch, manufactured by Instrumentation Laboratory) based semi-automated method [9], and plate-based methods [10]. The accuracy and precision of the protein activity determination is crucial to ensure the efficacy and safety of the therapeutic product, because the thrombolytic drugs can cause serious bleeding in the brain, which can be fatal (for a recent review: [11]).

A new type of homeostasis analyzer with automation capability was introduced by Instrumentation Laboratory. The ACL TOP system is a fully-automated stand-alone random-access multiparameter coagulation analyzer [12] The optical reading unit allows 16 simultaneous reaction readings at two currently available wavelengths i.e. 405 and 671 nm. The cuvette loading area, located on the left side of the instrument, can be filled, even while running, with up to 20 clips of 10 cuvette-strips each for a total of 800 cuvettes (4 cuvettes per strip). A conveyor belt moves the cuvette-strips to a cuvette shuttle that places them in position to be used by the analyzer for sample handling. Up to 120 samples can be loaded at once using the rack system (12 racks of 10 tubes each). Technical evaluation has indicated the reliability of ACL TOP analyzer for clinical homeostasis testing [13].

A potency assay intended for lot release must meet pre-defined validation acceptance criteria of specificity, accuracy, precision, linearity, and range in accordance with ICH guidelines. For potency assays supporting marketed products, method comparability to the current assay must be demonstrated. Lastly, to reduce human error and ergonomic risk, new analytical methods should be automated if possible. This report describes the development and validation of an automated clot lysis activity assay using ACL TOP analyzer, with characterization of critical parameters and assessment of robustness of assay. Since the development, validation and transfer of the assay, it was accepted by multiple health authorities and proven to be highly consistent and reliable in supporting GMP activities. Although the method was developed for enzymatic clot lysis, the principle and setups can be applied to establish other functional assays as well.

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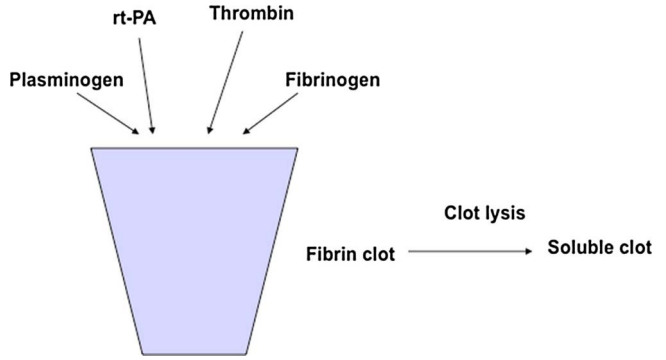

Fig. 1. Clot lysis assay design. Potency of rt-PA was determined by simultaneous clot formation and clot lysis. In this reaction, rt-PA (sample or standard) was mixed with plasminogen, thrombin and fibrinogen together to allow the formation of fibrin clot by thrombin and fibrinogen and subsequent lysis by plasmin converted from plasminogen by rtPA. The change in turbidity was used to determine the kinetics of enzyme reaction.

2. Materials and methods

2.1. Critical reagents

Recombinant human fibrinogen, thrombin, and plasminogen were purchased from Calbiochem. The recombinant human tissue plasmin activator samples and reference standard were manufactured by Genentech. Instrument specific proprietary cleaning reagents (labeled as cleaning A and B reagents and rinse solutions) used for the ACL TOP instrument were purchased from Instrumentation Laboratory (Bedford, MA).

2.2. Final format

The automated clot lysis method utilizes the pre-dilution function of ACL TOP and each concentration is programmed into each test. Briefly, in house reference standard for the active pharmaceutical ingredient (API), control and samples are first diluted to 40 µg/mL with assay buffer, loaded into the ACL TOP sample chamber and recorded by the ACL TOP software as “patients”. Standards are generated by 5 tests, each of which specifies a dilution level during the pre-dilution step with assay buffer. Control, samples and blank are tested at the three middle dilution levels (corresponding to 3333.3 ng/mL, 2222.2 ng/mL, 1111.1 ng/mL of the reference standard). Twenty µL of diluted standards, samples and control are then loaded into individual cuvettes and mixed with 20 µL of 33 Units/mL thrombin. The clot formation/lysis reaction starts as soon as 200 µL of a cocktail of plasminogen (36 µg/mL)/fibrinogen (2.2 mg/mL) is added into the cuvettes. Absorbance at 405 nm is monitored from 10 s to 700 s after initiation of the reaction. Lysis time is determined using the 50% threshold of the absorbance reading. Standard curve is generated by linear regression of log(lysis time) against log(tPA concentration). The potency of control and samples is then calculated by interpolating lysis time readout on the standard curve.

2.3. Potency calculation and statistics

The clot lysis activities of control and samples using standard curve was calculated by parallel line analysis described in USP Chapter 1032, Design and Development of Biological Assays [14]. The potency values for samples and control were calculated by multiplying the potency estimate from PLA by the specific activity of the reference standard (5.8×10^5 IU/mg, reference [17]) used in the standard curve.

2.4. DOE for range finding and robustness confirmation

All Design of Experiments (DOE) for range finding and robustness studies were created using JMP version 7.0 or 8.01 with the Customer Design function. Statistical significance was determined with $\alpha = 0.05$.

2.5. Comparability study

Forty-four samples were tested using the licensed procedure and the ACL TOP automated method. Samples included drug substance as well as various configurations of drug products under normal storage as well as stressed conditions, including heat, light exposure, low and high pH, and oxidation (with 2,2′-Azobis(2-amidinopropane) dihydrochloride) treatments., as described in ICH Harmonized Tripartite Guideline, Stability Testing of New Drug Substance and Products Q1A(R2), current Step 4 version, dated 6 February 2003. The mean of the paired difference of the results as well as the 95% confidence interval of the mean difference was calculated and compared to a pre-determined maximum allowable difference by Two One sided t Test (TOST). The two methods were considered comparable if the 95% confidence interval falls within the maximum allowable difference [15].

2.6. Validation of the method

Method validation was performed in accordance with the International Conference on Harmonisation (ICH) Guideline on the validation of analytical procedures (ICH Q2[R1]). Briefly, accuracy, intermediate precision, linearity and range were determined using samples of 70%, 85%, 100%, 115% and 130% of the target concentrations from results of 24 assays performed by three analysts on two instruments. Robustness evaluation was based on ANOVA with 95% confidence interval. Results are compared to pre-determined acceptance criteria in% recovery for accuracy, % relative standard deviation for precision, coefficient of determinations (R^2) for linearity. Variant component analysis was performed to determine the contribution of analyst, day, and instrument to variability.

3. Results and discussion

3.1. Design of the clot lysis assay

The purpose of establishing a potency assay was to assess quality attribute of the therapeutic protein, not demonstrating the efficacy. While the efficacy of tPA has been demonstrated by the resolution of preformed clot, the potency assay design was based on simultaneous clot formation and clot lysis for the consistency and reproducibility of the reaction. A semi-automated potency method using the Monarch system and a plate based manual method have been described previously for lot release and stability testing for tPA (references [9] and [10]).

3.2. Adapting ACL TOP for in house clot lysis assay

3.2.1. Selection of wavelength

The ACL TOP instrument is capable of monitoring two wavelengths: 671 nm and 405 nm. The clot lysis curves were monitored using both wavelengths for two tPA concentrations. The results indicate much stronger absorption signals at 405 nm than at 671 nm (Fig. 2). Based on the result, the wavelength of 405 nm was selected for the analytical method.

3.2.2. Selection of rt-PA concentrations

A large linear range of dose response, from 400 ng/mL to 10 µg/mL (corresponding to 30–830 ng/mL rt-PA in the final reaction buffer), was demonstrated for tPA (Data not shown). Several factors were considered to select the tPA concentrations for standard [1] Dilution capability of ACL TOP. The maximum capacity within one single dilution step is 148X by the ACL TOP instrument; [2] Even spacing between concentrations on the standard curve. Because the response curve is