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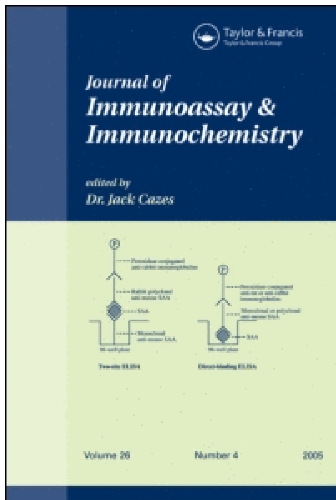

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VALIDATION OF AN ELISA FOR THE QUANTITATION OF LANOTEPLASE, A NOVEL PLASMINOGEN ACTIVATOR

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ABSTRACT

An ELISA was developed and validated for the quantitation of lanoteplase in human citrated plasma. The ELISA employed a monoclonal anti-lanoteplase antibody absorbed onto 96-well microtiter plates to capture lanoteplase in citrated human plasma samples containing PPACK, a protease inhibitor. The captured lanoteplase was detected using a biotinylated rabbit anti-lanoteplase polyclonal antibody. The standard curve range in human plasma for the ELISA was 7-100 ng/ml. Assessment of individual standard curve variability indicated reproducible responses with r^2 values of $\geq$ 0.985. The accuracy (% DEV) and precision (%RSD) estimates for the ELISA based on the predicted values from quality control (QC) samples were within 7.3% and 11.1%, respectively. Cross-reactivity with t-PA was determined to be less than 11% by ELISA. The stability of lanoteplase was established in human citrated PPACK plasma for 24 hours at 4^0C, for 2 months at -20°C, for 22 months at -70^0C, three weeks at room temperature, and through four freeze/thaw cycles. To quantify lanoteplase plasminogen activator (PA) activity, a commercially available chromogenic activity assay was also validated. This method and its application is described briefly here. The lanoteplase ELISA as well as the commercial activity method were successfully employed to evaluate the pharmacokinetic parameters of lanoteplase in support of clinical Phase II/III studies.

(KEY WORDS: ELISA, activity, lanoteplase, thrombolytic, human plasma.)

INTRODUCTION

Tissue-type plasminogen activator (t-PA), first cloned and expressed in 1983 (1), is currently used in thrombolytic therapy for patients with acute myocardial infarction. t-PA acts enzymatically at the site of thrombosis by binding to fibrin, where it cleaves inactive plasminogen to plasmin, which in turn lyses the fibrin clot. In an effort to improve on the fibrinolytic potency and short half-life of t-PA, lanoteplase was developed (2). Lanoteplase is a novel molecule distinct from t-PA. It

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does not possess the finger and epidermal growth factor domains that exist in t-PA, and has different amino acids present at important sites which are involved in the glycosylation pattern of the molecule. The presence of Glu at a position in lanoteplase that corresponds to the Asn at position 117 in t-PA results in lanoteplase lacking glycosylation at this site. Lanoteplase also showed improved thrombolytic efficacy, with little or no change in fibrin specificity.

Commercial assay kits are available for the analysis of t-PA (3-4) and for plasminogen activator activity (5,6). However, specific assays to quantify lanoteplase in human plasma samples are not available. The objective of the present study was to develop and validate an ELISA for lanoteplase, in order to evaluate the pharmacokinetics of lanoteplase in patients with acute myocardial infarction.

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MATERIALS AND METHODS

Materials

Lanoteplase reference standard, the anti-lanoteplase monoclonal (clone no. H3wt/1.17.20) and rabbit anti-lanoteplase (R251) polyclonal antibodies (IgG fraction, in PBS) were obtained from Genetics Institute, Cambridge, MA. Streptavidin-horseradish-peroxidase (SA-HRP) was obtained from Zymed Laboratories, S. San Francisco, CA., TMB Microwell Peroxidase Substrate System was obtained from Kirkegaard and Perry Laboratories, Gaithersburg, MD., and tissue plasminogen activator (t-PA) was obtained from American Diagnostica Inc., Greenwich, CT. Immulon IV, 96-well flat-bottomed microtiter plates for the ELISA were purchased from Dynatech Laboratories Inc., Alexandria, VA. Human citrate plasma was purchased from Biological Specialty Corp., Colmar, PA. Gelatin was purchased from Biorad, Richmond, CA. Two-chain lanoteplase was produced by BMS using plasmin enzymatic cleavage of single chain lanoteplase. PAI-1 (plasminogen activator inhibitor-1) was obtained from American Diagnostica, Inc., Greenwich, CT. D-Phe-Pro-Arg Chloromethyl Ketone, Dihydrochloride (PPACK-HCl) was purchased from Calbiochem, La Jolla, CA. All reagents for the chromogenic activity assay were purchased as a kit from American

Diagnostica Inc. Each kit contained the following items: human glu-plasminogen, DESAFIB®, Spectrozyme® PL, 2-chain t-PA, 10x Tris buffer, acetate buffer, and stop solution (containing SDS).

Methods

Preparation of the ELISA standard curve

Samples for preparation of the standard curve were prepared by serial dilution of the lanoteplase stock with the appropriate volume of human plasma to produce standards at concentrations of 100, 75, 50, 25, 15, 10, and 7 ng/ml plasma. For the assay, working standard solutions were further diluted with TBST to achieve a final plasma concentration of 20%.

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Preparation of quality control samples

To monitor accuracy and precision of the assay, stock quality control (QC) samples were prepared at concentrations of 15, 50, 75 and 500 ng/ml in plasma, aliquoted and stored frozen at -70^0 C. For the assay, working QC samples were prepared by dilution with TBST to achieve a final plasma concentration of 20%. Both working standards and QC samples for the assay were prepared daily.

Biotinylation of purified rabbit anti-lanoteplase polyclonal antibody (R251).

Two nmoles of R251 were added to a 5-ml cryovial to which was added 385 µl of 1 M HEPES buffer for a final buffer concentration of 0.1 M. Nine mg of biotin (long arm) NHS: N-hydroxysuccinimidyl 6-(biotinamido) hexanoate (Vector Laboratories) was dissolved in 1.98 mL DMSO, and 50 µl of this was added to the R251 solution. Milli-Q water was added to obtain a final volume of 2.25 ml for the reaction. The tube was capped, wrapped in foil, and placed on a rotator at 4°C for 90 minutes. The reaction mixture was dialyzed overnight in 2 liters of 100 mM HEPES buffer at 4°C to remove free biotin. Aliquots of the biotinylated antibody (1 mg/mL) were stored

frozen at -70°C. Biotinylated antibody was diluted 1:2000 in PBS at the time of the assay. New lots of biotinylated antibodies were titrated before use.

ELISA procedure

All steps were carried out at room temperature except where noted. Immulon IV microtiter plates were prepared by adding 100 µl of anti-lanoteplase antibody (H3wt/1.17.20; 2 µg/ml) in saline to each well. The plates were covered with a plate sealer, and stored at 4°C for a minimum of 16 hours. The antibody solution was aspirated, and remaining sites were blocked with the addition of 200 µl TBS-gelatin blocking solution, (0.05mM Tris-base, 0.15 mM NaCl, 1 mM glycine, pH 8.0 containing 5% gelatin) for 1.5 hours at 37°C. This was followed by five 300 µl washes with PBS, pH 7.4±0.2 containing 0.05% Tween 20. Standards and QC samples (50 µl) in 20% human plasma/TBST buffer were then added. The plates were incubated covered for 2 hours, washed as before, and followed by the addition of 50 µl of biotinylated rabbit polyclonal anti-lanoteplase R251 (500 ng/mL) to each well. The plates were further incubated covered for 2 hours and washed, after which 50 µl of HRP-streptavidin conjugate (1:25,000) was added to each well. The plates were then incubated 45 minutes, washed, and 100 µl of TMB Chromogen/Substrate working solution was added to each well. Color was allowed to develop for 4-8 minutes, and the reaction stopped by the addition of 100 µl of 1 M H_3PO_4. The absorbance was then measured within 30 minutes using a Tecan SLT 340 ATTC plate reader equipped with a 450 nm test and a 630 nm reference filter.

Validation Procedures

While both the ELISA and the commercial method for PA activity were validated for internal purposes, only the validation of the ELISA for lanoteplase is described here. Validation of the ELISA included assessments of standard curve fit, lower limit of quantitation, precision and accuracy, specificity, long- and short-term sample stability, stability in whole blood, and linearity of dilution.

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Lower limit of quantitation

The sensitivity, expressed as the lower limit of quantitation (LLQ), was assessed using plasma samples from at least 8 different human donors spiked with lanoteplase. Accuracy at the LLQ level (7 ng/ml) was calculated as the % deviation (%DEV) of the observed concentrations from the nominal concentration of each spiked sample. The acceptance criteria for the LLQ determination was set such that 80% of the observed concentrations of the LLQ replicates had to be within 20% of the nominal value.

Accuracy and precision

The assessment of accuracy and precision of the ELISA used predicted QC concentrations that were back calculated from individual standard curves. In each of three analytical runs, three replicates of each standard concentration were assayed, along with 6 replicates of QC samples at concentrations of 15, 50, 75, and 500 ng/ml. Accuracy and precision of the assay were therefore based on the predicted concentrations 18 replicates at each concentration, a total of 72 QC samples. The accuracy (%DEV) is defined as the deviation of the overall mean predicted concentration from its nominal value. To determine assay precision, the predicted QC concentrations were evaluated using an ANOVA model (7). Variance component analysis was utilized to calculate an estimate of between-run and within-run precision from the factors in the ANOVA model. Estimates of precision were expressed as a percent relative standard deviation (%RSD) relative to the overall mean predicted concentration for all analytical runs at that concentration level. Inter-assay (between-day) and intra-assay (within-day) precision were calculated by ANOVA as follows:

$$\text{Inter-assay precision} = (SD_{Day} / GM) \times 100$$

$$\text{Intra-assay variability} = [(ErrMS)^{0.5} / GM] \times 100$$

$$SD_{Day} = [(DayMS - ErrMS)/n]^{-0.5}$$

where DayMS is the day mean square, ErrMS is the error mean square, GM is the grand mean, and n is the number of replicates.

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Stability

For long-term storage stability, QC samples were spiked with lanoteplase and stored at -70°C. These samples were assayed for lanoteplase for a period of up to 22 months. Additionally, short-term storage stability of lanoteplase in human plasma was evaluated at -20°C for up to 2 months, at 4°C for 24 hours, room temperature for three weeks, and through 4 freeze/thaw cycles. To assess freeze/thaw stability, QC samples were frozen at -70°C and thawed in tap water in a manner consistent with typical sample analysis. All stability QC samples were analyzed in triplicate (mean result reported).

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Stability in whole blood

The objective of the whole blood stability experiment was to ascertain that no appreciable degradation of the molecule occurred during the time taken to prepare plasma samples. Fresh blood was drawn from a donor using Vacutainer tubes containing citrate and PPACK, a serine protease inhibitor. Immediately after obtaining the blood sample, lanoteplase was added to a final concentration of 200 ng/ml blood. The blood was mixed gently and aliquots were stored on ice or room temperature for 15, 45 minutes, and 1, 2, 4, 8 and 24 hours before being centrifuged at 2400 rpm (1000 x g) for 15 minutes at 4°C. Plasma was collected and stored at -70°C until analysis. Stability of lanoteplase kept at 4°C and room temperature was expressed as the %DEV of the predicted concentration from the 15 minute (4°C) value. All stability QC samples were analyzed in triplicate (mean result reported).

Specificity

The specificity of the ELISA assay for lanoteplase was assessed in the presence of t-PA, the 2-chain form of lanoteplase and PAI-1. Tissue plasminogen activator (t-PA) was spiked into blank human plasma at 71.6 and 119 ng/ml, the 2-chain lanoteplase was spiked into plasma at 25 ng/ml and 50 ng/ml. PAI-1 was added at concentrations of 25 and 16 ng/ml into QC samples containing

50 and 75 ng/ml of lanoteplase, respectively, in order to see if this specific inhibitor would interfere with the quantitation of lanoteplase in plasma samples. All specificity samples were analyzed in triplicate.

Linearity of dilutions

The objective of the experiment was to demonstrate that samples with lanoteplase concentrations greater than the upper limit of the ELISA standard curve can be accurately predicted when diluted into any region of the curve. A 500 ng/ml QC sample was diluted at five different dilutions selected such that the resulting absorbencies fell into all four quartiles of the standard curve range. The samples were then analyzed by ELISA.

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Data Analysis

A 4-parameter logistic regression model of the form $\mathbf{Y = max + [(min\text{-}max)/(1+conc/ED_{50})^{B}]}$, was used to describe the relationship between the absorbance readings and nominal concentrations of lanoteplase standards on each plate, where "max" is the estimated maximum of the function, "min" is the estimated minimum of the function, "conc" is the value of the nominal concentrations of the standard curve range, ED_{50} is the estimated midpoint of the regression line, and B is the slope of the apparent linear region of the curve. Evaluation of potential outliers in the individual plate standard curves was performed according to the procedure described by Dixon and Massey (8). Statistical evaluation of standards and QCs was done using a SAS software package (9).

RESULTS

Standard curve characteristics

The associated r^2 of the four-parameter fit for the three ELISA accuracy and precision validation runs ranged from 0.986 to 0.997 (data not shown). The overall background (blank plasma)

for those runs was low, with average ODs ranging from 0.057 to 0.113. A typical ELISA standard curve is shown in Figure 1 and summarized in Table 1. The % RSD for the standard ODs for this run (n=3) was within 14.4 %. Predicted values of the standards for this run were within 1.3 % of nominal concentrations.

Lower limit of quantitation

The results indicated that all of the eight individually prepared samples predicted with a % DEV of 17% or less. Based on these data, the LLQ for the ELISA was established at 7 ng/ml in 100% human plasma.

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Precision and accuracy

The mean QC concentrations, calculated from individual ELISA runs, are summarized in Table 2. The between-run precision and within-run precision for the ELISA, calculated from the predicted QC values, were within 11.1% and 7.2%, respectively. The overall accuracy across concentrations was within 7.3%. These results indicated that assay variability was low.

Long and short-term stability

Predicted concentrations by ELISA of lanoteplase prepared in human plasma were within 15% of their nominal values for up to 22 months at -70°C except for the 500 ng/ml QC concentration, which was within 18%. The results of the short-term stability experiments indicated that lanoteplase was stable for at least 2 months at -20°C, 24 hours at 4°C, 3 weeks at room temperature, and through 4 freeze/thaw cycles in human plasma.

Stability of lanoteplase in whole blood

The results indicated that the concentrations of the test samples at various time points up to 24 hours predicted within 6.1% of the concentration at the initial time point (15 minutes) at both

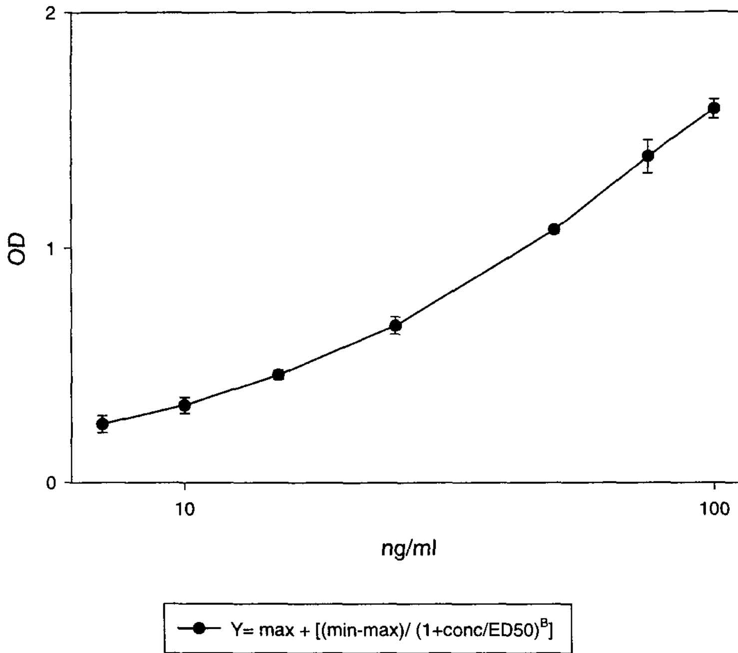

FIGURE 1

Representative Standard Curve of the ELISA Method for Quantitation of Lanoteplase in Human Plasma.

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room temperature and at 4°C. These results demonstrate that lanoteplase is stable in whole blood up to 24 hours at room temperature and at 4°C before being centrifuged for plasma separation.

Specificity

The specificity of the ELISA for lanoteplase was evaluated in the presence of t-PA, 2-chain lanoteplase and PAI-1. Cross-reactivity with t-PA was determined by ELISA to be less than 11%. These results indicate that the ELISA will quantify lanoteplase, with negligible interference from endogenous t-PA. The 2-chain form of lanoteplase was also analyzed and determined to be 100%

TABLE 1

Representative Standard Curve Statistics for the ELISA Method for Quantitation of Lanoteplase in Human Plasma.

Standard Concentration (ng/ml)	Mean OD (n=3)	%RSD OD	Mean Predicted Concentration (ng/ml)	% DEV
100	1.586	2.6	99.5	-0.5
75	1.386	5.2	76.0	1.3
50	1.079	1.6	49.5	-1.1
25	.674	5.0	25.0	0.0
15	.459	5.5	15.2	1.1
10	.328	10.5	9.9	-0.5
7	.248	14.4	7.0	-0.4
0 (NSB)*	.113	-	-	-

* non-specific binding

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TABLE 2

Summary of the Accuracy and Precision Data of the ELISA for Lanoteplase in Human Plasma.

Nominal Concentration (ng/ml)	15	50	75	500
Mean Observed	13.9	48.3	73.5	525
% DEV	-7.31	-3.40	-1.96	5.05
Between-Run Precision (%RSD)	6.78	0.39	0.54	11.1
Within-Run Precision (%RSD)	5.81	5.59	7.24	5.16
N	18	18	18	18

TABLE 3

Evaluation of the Specificity of the ELISA Method for the Quantitation of Lanoteplase in Human Plasma.

Cross-Reactant	Cross-Reactant Conc. (ng/ml)	Lanoteplase Nominal Conc. (ng/ml)	Predicted Conc. (ng/ml)	% Recovery
t-PA	71.6	-	7.74	10.8
t-PA	119	-	9.56	8.03
2-chain lanoteplase	25.0	-	29.3	117
2-chain lanoteplase	50.0	-	48.6	97.2
PAI-1	25.0	50.0	41.8*	83.6
PAI-1	16.0	75.0	80.8*	108

* Predicted concentration of lanoteplase.

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cross-reactive compared to the 1-chain lanoteplase reference standard. More importantly, PAI-1 added to lanoteplase samples showed no significant effect on the predicted values (Table 3).

Linearity of dilutions in plasma

The predicted concentrations of the individually diluted test sample for the ELISA were within 13.4% of the nominal value with a RSD of 5.2% for the 5 dilutions. This demonstrates that lanoteplase samples can be diluted in normal human citrate plasma without affecting accuracy and precision of sample prediction.

DISCUSSION

The objectives of these studies were to develop and validate an ELISA for lanoteplase to support clinical pharmacokinetic studies. Towards this end, a sensitive, precise, accurate and robust assay was developed for the quantitation of lanoteplase in human plasma.

The ELISA method utilizes a murine monoclonal anti-lanoteplase capture antibody with a biotinylated rabbit polyclonal anti-lanoteplase antibody for detection. A streptavidin-HRP conjugate followed by TMB chromogen substrate was used to develop color. The range of reliable response of the ELISA was from 7 to 100 ng/ml in human citrate plasma, with an LLQ of 7 ng/ml. Evaluation of QC data in all validation runs carried out by two analysts showed that the ELISA was robust, precise, accurate, and reproducible with %RSDs of <11% and %DEV within $\pm7\%$ of nominal values. QC samples predicted from individual curves were within $\pm15\%$ of nominal values. As determined by ELISA, lanoteplase was shown to be stable after 22 months at -70°C, 2 months at -20°C, 24 hours at 4°C, 3 weeks at RT and through 4 freeze/thaw cycles. These stability data are needed to provide guidelines to the various clinical sites to ensure proper collection, storage and shipment of clinical samples to the analytical site.

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Because of the similarities between lanoteplase and t-PA, it was important to evaluate the specificity of the ELISA for t-PA, the various forms of t-PA and lanoteplase, and endogenous inhibitors. The results from the specificity experiments indicated that the ELISA was specific for lanoteplase, with less than a 11% cross-reactivity with t-PA (Table 3). This low cross-reactivity towards t-PA permitted the accurate determination in human plasma of lanoteplase concentrations in the presence of endogenous t-PA. Endogenous levels of t-PA in men are reported to be approximately 5 ng/ml, with levels in women slightly lower. Levels of t-PA are reported to rise in both sexes with age (3). However, with the low cross-reactivity observed, it is not expected that age or any physiological condition would affect our ability to quantify lanoteplase in human plasma. It is known that t-PA can be cleaved *in vivo* and *in vitro* into a 2-chain form by the serine protease

plasmin, both forms having been shown to be biologically active (10). Similarly, lanoteplase also exists as a mixture of both 1-chain and 2-chain entities, albeit predominantly as the 1-chain moiety. Since it is possible the 1-chain moiety could convert to the biologically active 2-chain form, it is important that the ELISA be able to accurately quantify both forms of lanoteplase. From the results summarized in Table 3, the ELISA method was shown to be 100% cross-reactive with the 2-chain form of lanoteplase. The addition of PAI-1 to lanoteplase quality control samples, presumably forming the lanoteplase-PAI complex, did not affect the ability of the ELISA to quantify lanoteplase, indicating that the ELISA could measure both free lanoteplase, and lanoteplase conjugated to PAI-1.

The experiments to evaluate the linearity of dilutions were carried out to ensure that clinical samples which contain lanoteplase concentrations exceeding that of the highest standard, could be accurately quantified following appropriate dilutions in normal human into any portion of the standard curve. The results indicate that accurate and precise quantitation was observed when a sample containing lanoteplase at a concentration greater than the highest reference standard was diluted in human plasma at five different dilutions. The observed ODs all predicted accurately when diluted into the different quartiles of the standard curve.

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A plasminogen activator activity assay was developed from a commercially available chromogenic activity assay as previously described (5, 6). In this 96-well method, the plasminogen activator (lanoteplase or t-PA) activates plasminogen to plasmin, which in turn cleaves the plasmin substrate, Spectrozyme® PL. This reaction, which is enhanced by fibrin and plasminogen added to the mixture, generates the free chromophore, p-nitroanilide from Spectrozyme® PL, which is then measured directly with a spectrophotometer. A linear response is detected, which is directly related to amount of lanoteplase or t-PA in the sample.

Standards for this method were prepared in buffer as the kit procedure recommended. QC samples, which were prepared in citrated plasma, were diluted at least 9-fold in the same buffer as the standards. The final assay mixture contained plasminogen, study sample, fibrin and a plasmin substrate as dictated by the manufacturer.

The range of reliable response for the activity assay was established from 4.5 to 108 IU/ml in citrate human plasma with a lower limit of quantitation of 4.5 IU/ml (range calculated after correcting for dilution of samples). Evaluation of the QC data in the validation runs showed that the activity assay was precise, accurate and reproducible (data not shown). This activity assay also measured accurately the PA activity from both 1-chain and 2-chain forms of lanoteplase and t-PA. This finding is significant, because now the same method can be used to compare the activity of lanoteplase to t-PA in clinical trials.

In a clinical study designed to characterize the pharmacokinetics of lanoteplase, a single 10 KU/kg dose was administered as an intravenous bolus to 10 young male subjects. Serial blood samples were collected over a 24 h period after dosing. Blood samples were collected in tubes containing sodium citrate/PPACK for determination of lanoteplase antigen, and into tubes containing 0.45 M sodium citrate, pH 4.4, for determination of lanoteplase activity. Plasma samples were analyzed using the methods described herein. A representative plasma concentration-time profiles of lanoteplase antigen and activity are shown in Figure 2.

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The plasma lanoteplase antigen concentration-time profiles were best described by a biexponential model, whereas activity profiles were best fitted to a monoexponential model. The mean total body clearance and steady-state volume of distribution of lanoteplase antigen were 2.5 L/h and 14.2 L, respectively. Lanoteplase antigen was eliminated more slowly with half-lives of about 20 min and 5 h for the α and β phases, respectively. The area under the terminal elimination phase (AUCβ) accounted for ~50% of total AUC suggesting that the exposure in the terminal phase contributes significantly towards the pharmacological effects of lanoteplase.

Lanoteplase activity concentrations declined rapidly, and were generally below the LLQ within 4 h of dosing. The mean total body clearance and steady-state volume of distribution of lanoteplase activity were 4.8 L/h and 3 L, respectively. The elimination half-life of lanoteplase activity was about 30 min.

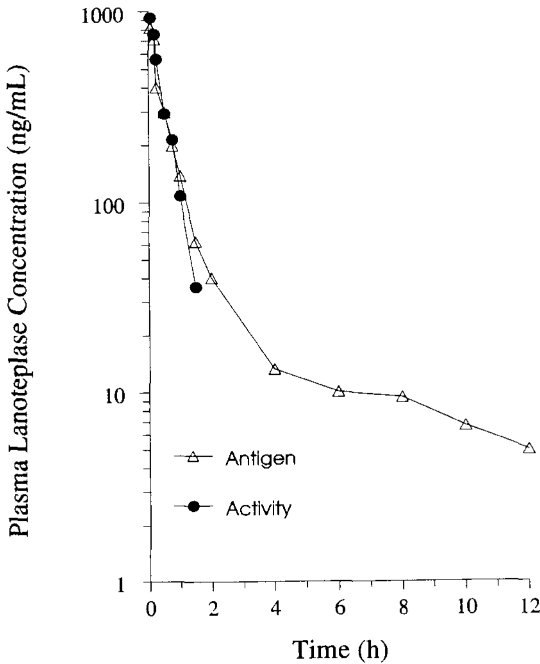

FIGURE 2

Plasma Lanoteplase Antigen and Activity Concentration-Time Profiles in a Young Male Subject Following a Single 10 KU/kg Intravenous Bolus Dose.

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In conclusion, an ELISA assay for the quantitation of lanoteplase in human plasma was developed and validated to support clinical studies for lanoteplase. Additionally, a commercially available method for plasminogen activator activity was also validated for the purpose of measuring the plasminogen activator activity of lanoteplase in human plasma. Both assays were shown to be sensitive, precise and accurate. The utilization of both assays was helpful in evaluating the pharmacokinetics of lanoteplase during Phase II and III clinical trials.

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