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Evaluation on the Analytical Methods for Tissue Plasminogen Activator (tPA) in Pharmaceutical Formulations

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ABSTRACT

Tissue plasminogen activator, tPA, is a thrombolytic agent for treatment of the brain or myocardium infarction. Clot lysis assay is a functional determination method of tPA described in the pharmacopeia. However, the accuracy of clot lysis assay is insufficient because the lysis-time is not determined by the end-point detection. Therefore, an alternative analytical method for the post-marketing quality survey of tPA pharmaceutical formulations is needed. This study was aimed to establish a rapid, precise and cost-saving analytical method for tPA routine test. We have compared methods of capillary zone electrophoresis (CZE), size-exclusion high performance liquid chromatography (SE-HPLC), *in vitro* clot lysis, enzyme-linked immunosorbent assay (ELISA), and chromogenic substrate assay for the evaluation of recombinant tPA drug products. The results showed that S-2288^{TM}, as substrate of chromogenic assay for tPA, exhibited a good linear relationship at tPA concentration of 5-50 μg/mL (correlation coefficient: 0.9988). The intraday precision ranged between 0.5% - 4.3%. Results obtained from our comparative study, suggested that the chromogenic substrate assay is among the best for tPA assay. The features of precision, rapidity and inexpensiveness of this method are suitable for a routine test for tPA in pharmaceutical formulations.

Key words: tissue plasminogen activator, capillary zone electrophoresis, SE-HPLC, chromogenic substrate assay

INTRODUCTION

Tissue plasminogen activator (tPA) is a polypeptide consisting of 527 amino acid residues with relative molecular mass of 59,050 Dalton without consideration of the carbohydrate moieties attached at positions Asn 117, Asn 184 and Asn 448. The total relative molecular mass is approximately 65,000 Dalton. tPA binds to fibrin clots and activates plasminogen, leading to the generation of plasmin and the degradation of fibrin clots or blood coagulates[1]. Clinically, tPA has deemed as a major therapeutic thrombolytic agent for the treatment of myocardial infarction, and is also approved for the treatment of ischaemic stroke. The clarification of patenting issues has been seen nowadays, resulting in a single therapeutic product of full length natural tPA in the market place, produced by two manufactures[2]. Since the patents of most therapeutic proteins have expired, and the first biosimilar product has already been approved in the market[3], the quality compliance of those products from non-brand-name manufacturers becomes a critical issue. Potency assay of tPA provided in the pharmacopeia monograph of alteplase[1,4] was clot lysis assay. However, considering the purpose of routine test, a rapid, precise, and inexpensive method would be demanded and built to analyze tPA directly in pharmaceutical formulations. This study was aimed to compare the reference method (clot lysis), size-exclusion high performance liquid chromatography (SE-HPLC), capillary zone electrophoresis (CZE), enzyme-linked immunosorbent assay (ELISA) and chromogenic substrate assay, and employ a suitable method for routine testing intent.

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MATERIALS AND METHODS

I. *Materials and Reagents*

Tenectaplase reference standard and commercial product, Metalyse®, were obtained from Boehringer Ingelheim (Germany), tPA International Standard (98/714) including approximately 20 μg tPA and 5 mg human albumin from National Institute for Biological Standards and Control (UK), plasminogen, tPA, arginine, phosphoric acid, and polysorbate 20 from Sigma (USA), human thrombin and fibrinogen from Calbiochem

(Germany), molecular weight standards from Bio-Rad Laboratories (USA). The water used was Milli-Q grade (Millipore, USA). In the following analytical methods, standards (Tenectaplase reference standard) and drug product (Metalyse®) were prepared in serial dilution with buffer or water to conduct linearity, range, precision, and accuracy (or recovery) test. Placebo solution consisting of 522 mg arginine, 164 mg phosphoric acid and 4 mg polysorbate 80 was dissolved in 10 mL water. Individual testing sample concentrations were described in each testing method.

II. *In Vitro Clot Lysis*

The potency of tenectaplase was determined by comparing its ability to activate plasminogen to form plasmin with the same capacity of a reference preparation calibrated in International Units. The formation of plasmin was measured by the determining the lysis time of a fibrin clot in given conditions. Testing procedures were as followed: add 600 µL plasminogen-fibrinogen (1:50, v/v) mixture solution to each cuvette, add 60 µL thrombin, add 60 µL standard or sample solution, mix well and gently, read the absorbance at 340 nm, and record the time (second) when the end point (OD_{340} < 0.03 and Δ OD_{340} < 0.003) was reached. Assay buffer was 0.06 M phosphate buffer/0.02% (w/v) NaN_3/0.01% (v/v) Tween 80. Assay range was performed from 0.4 to 1.2 µg/mL. The precision was performed on three different concentrations (0.6, 0.8, and 1.0 µg/mL).

III. *Chromogenic Substrate Assay*

Coaset® S-2288^{TM} tPA kit for determining the activity of tPA was purchased from Chromogenix-Instrumentation Laboratory SpA (Italy). The structure of S-2288 was H-D-Ile-Pro-Arg-*p*NA•2HCl. The tPA could induce dissociation of *p*-nitroaniline (*p*NA), and its activity was thus determined by the rate at which *p*NA is released. The formation of *p*NA could be followed spectrophotometrically at 405 nm. The procedure was modified from original insert: for each well of 96-well plate, add Tris buffer 50 µL, sample or standard 50 µL, and S-2288 50 µL, put in 37°C incubator for 3 minutes. Then, add 50 µL stop solution (20% acetic acid) and read by ELISA reader. Assay range was performed from 5 to 50 µg/mL. For determining the tPA only or tPA with albumin (tPA International Standard), intra-day precision was performed on three different concentrations (15, 25, and 35 µg/mL). The accuracy of the method was provided by spiking various concentrations of tPA (15, 25, and 35 µg/mL) into the placebo solution.

IV. *Size-Exclusion High Performance Liquid Chromatography (SE-HPLC)*

The SE-HPLC analysis was performed using a GL Sciences HPLC pump with an absorbance detector. A TSK G2000 SWXL column (7.8 mm × 300 mm i.d., 5 µm, from Tosoh, Japan) was used. Chromatographic data were calculated using a Shiunn Haw computing integrator, SISC v4.0 software. The mobile phase consisted of 120 mM ammonium sulfate containing 10% 2-propanol was adjusted to pH 7.3. The flow rate was 1.0 mL/min, the detecting wavelength was 280 nm, and the injection volume was 20 µL. Assay range was performed from 100 to 500 µg/mL. Intra-day and inter-day precision were performed on three different concentrations (200, 300, and 400 µg/mL). The accuracy of the method was provided by spiking various concentrations of tPA (100, 200, and 300 µg/mL) into the placebo solution.

V. *Capillary Zone Electrophoresis (CZE)*

An Agilent G1600A CE system (Germany) was equipped with UV-VIS detector (photodiode array, 190-600 nm), cassette temperature control (4-60°C), and ChemStation software. The uncoated fused-silica capillary (58.5 cm × 50 µm i.d., 50 cm to detector, from Polymicron, USA) was used with the CZE separation buffer consisted of 100 mM boric acid/40 mM LiCl, pH 5.0, capillary temperature of 25°C, and detector set at 214 nm. Sample was pressure-injected for 10 sec and the applied voltage was set at 20 kV across the capillary. Assay range was performed from 20 to 100 µg/mL. Intra-day and inter-day precision were performed on three different concentrations (40, 60, and 80 µg/mL). The accuracy of the method was provided by spiking various concentrations of tPA (40, 60, and 80 µg/mL) into the placebo solution.

VI. *Enzyme-linked Immunosorbent Assay (ELISA)*

Coaliza® ELISA Kit for tPA was purchased from Chromogenix-Instrumentation Laboratory SpA (Italy). Microplate wells coated with a monoclonal anti-tPA antibody were used to capture tPA in the sample. A second antibody, conjugated to horse-radish peroxidase (HRP), was added. After incubation and removal of untreated conjugate, enzyme substrate (tetramethylbenzidine, TMB) was added. Incubation with enzyme substrate produced a blue color, which turned yellow when the reaction was stopped by adding sulphuric acid. The absorbance at 450 nm was then read with ELISA reader (MRXII, Dynex Technologies, USA). Assay range was performed from 0 to 15 ng/mL.

RESULTS AND DISCUSSION

I. *In Vitro Clot Lysis*

The standard curve of tPA analyzed by *in vitro* clot lysis was established by plotting the Log second (y) versus Log concentration of tPA (x). The regression

Journal of Food and Drug Analysis, Vol. 17, No. 1, 2009

equation was y = -0.735x + 4.4806 (r^2 = 0.9888). The satisfactory linearity ranged from 0.4 μg/mL (0.08 U/mL) to 1.2 μg/mL (0.24 U/mL). However, several reagents used in the method were extremely expensive. In addition, the precision and repeatability were not satisfying enough (RSD 5.3-9.8%).

Clot lysis is a standard method. Before testing, the protein contents of samples must be determined. In a collaborative study for establishment of tPA international standard, several laboratories conducted their in-house developed methods rather than this pharmacopeia adopted standard method [2].

II. *Chromogenic Substrate Assay*

The chromogenic substrate assay was carried in 96-well plate and finished around one hour. The results showed that the linearity was much better than clot lysis and detection range (ranging from 5 μg/mL to 50 μg/mL of tPA) was also acceptable. The regression equations obtained for tPA only and tPA with bovine serum albumin were y = 0.0165x – 0.0156 (r^2 = 0.9966) and y = 0.0148x + 0.0245 (r^2 = 0.9952), respectively. In addition, the RSD of intra-day precision showed 0.5%- 4.3% (Table 1) and it was within acceptable criteria (< 5%).

tPA is one of serine proteases. Several chromogenic substrates could be used in analysis of tPA. S-2251 was one substrate of plasmin used for determining tPA activity, which has been reported and measured in absolute units [5,6]. The principle was the same as the clot lysis assay. However, it was an indirect method. In this study, a commercial available substrate S-2288 was introduced. tPA directly catalyzed the S-2288 to *p*NA. The data was read by ELISA reader and the loading time control of samples or reagents was a critical step in the assay.

III. *Size-Exclusion High Performance Liquid Chromatography (SE-HPLC)*

The ideal column, TSK G2000 SWXL, for protein separation was introduced. The chromatogram was shown in Figure 1. The data showed excellent linearity (r^2 >0.999) and reproducibility ranging from 100 μg/mL to 500 μg/mL of tPA concentration. Furthermore, the RSD of intra-day and inter-day precisions was 0.3-1.8% (Table 1), the recoveries of spiked tPA of 100-300 μg/mL were 98.9-103.5%, and CV value was 0.4-2.0% (data not shown). However, the resolution of main peak was interfered with human serum albumin, a common excipient in pharmaceutical formulation (e.g. the tPA international standard including 20 μg tPA and 5 mg albumin).

The main purpose of SE-HPLC was impurity determination of reduced tPA, monomer and dimer aggregate. The protein content (specific activity) could not reflect the real potency.

IV. *Capillary Zone Electrophoresis (CZE)*

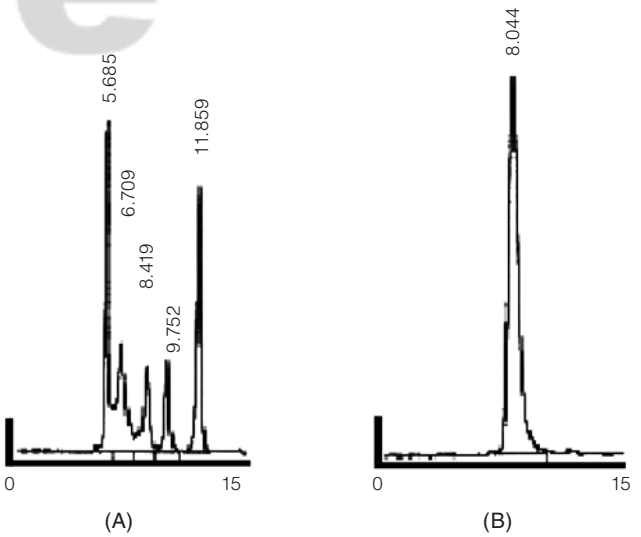

Figure 1. The typical size-exclusion chromatogram of tissue plasminogen activator, (A) the molecular weight marker: thyroglobin 670 KDa, bovine gamma globulin 158 KDa, chicken ovalbumin 44 KDa, equine myoglobin 17.5 KDa, and vitamin B12 1.35 KDa, (B) tPA, 300 μg/mL, retention time: 8.044 minute. Detection was performed at absorbance 280 nm.

Table 1. Intra-day and Inter-day precision of tPA determined by chromogenic substrate assay, SE-HPLC, and CZE

		Theoretical conc. (μg/mL)	Actual conc. Mean ± SD (μg/mL)	RSD (%) (n = 3)
Chromogenic substrate assay	Intra-day (tPA without albumin)	15.0	16.6 ± 0.6	3.9
		25.0	26.2 ± 1.0	3.7
		35.0	36.0 ± 0.4	1.0
	Intra-day (tPA with albumin)	15.0	16.8 ± 0.7	4.3
		25.0	25.9 ± 0.7	2.8
		35.0	36.2 ± 0.2	0.5
SE-HPLC	Intra-day	200	196.6 ± 2.2	1.12
		300	307.7 ± 1.0	0.34
		400	410.8 ± 2.5	0.61
	Inter-day	200	200.4 ± 1.9	0.93
		300	300.2 ± 5.5	1.82
		400	405.4 ± 5.4	1.32
CZE	Intra-day	40	39.9 ± 1.3	3.27
		60	60.1 ± 1.7	2.75
		80	79.9 ± 1.5	1.92
	Inter-day	40	39.1 ± 1.9	4.75
		60	60.2 ± 1.3	2.20
		80	80.7 ± 1.5	1.86

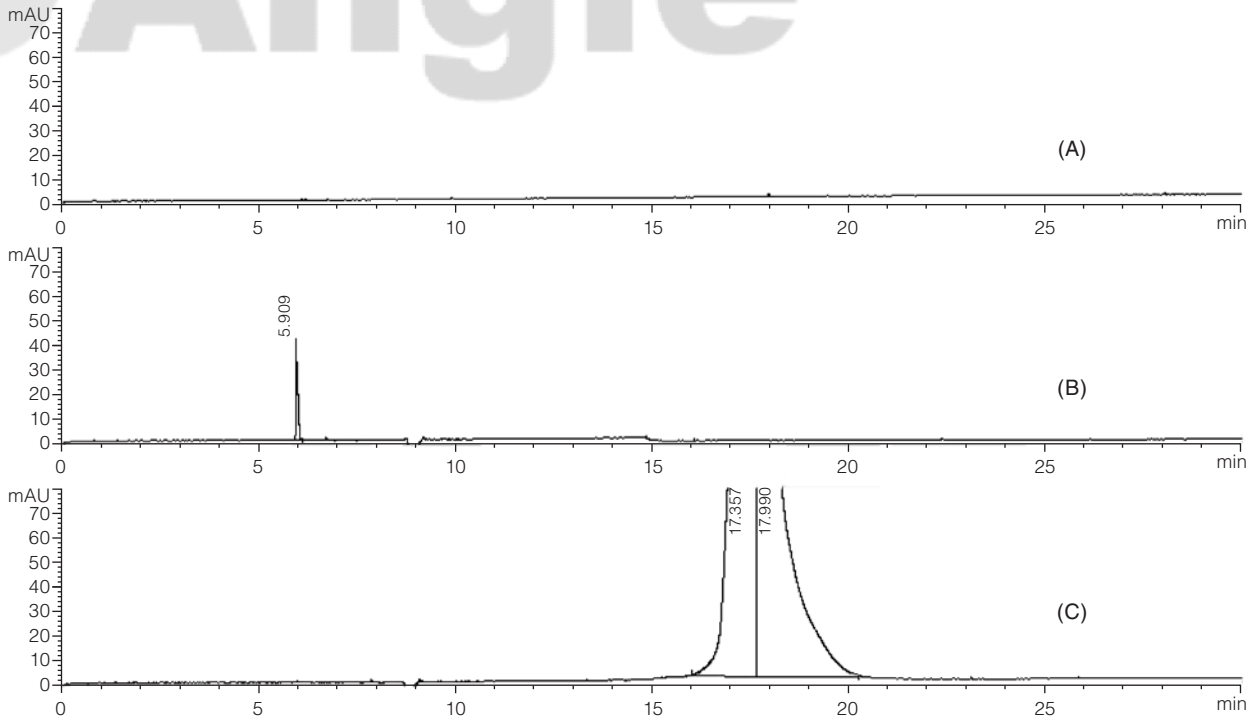

Figure 2. The typical capillary zone electrophoresis of tissue plasminogen activator, (A) blank, (B) tPA 20 μg/mL only, (C) Bovine Serum Albumin 5 mg/mL only.

The CZE result showed good linearity ($r^2 > 0.998$) and reproducibility ranging from 20 μg/mL to 100 μg/mL of tPA concentration (data not shown). The RSD of intra-day and inter-day precisions was 1.9-4.8% (Table 1), which was higher than SE-HPLC. Moreover, the CZE result showed good resolution of tPA and albumin (Figure 2) in the tPA International standard.

Capillary zone electrophoresis or capillary isoelectric focusing (cIEF) for tPA analysis have been developed by several laboratories[7-9]. Compared with SE-HPLC, the advantage of CZE method was to separate the albumin from tPA in pharmaceutical formulation. CZE also showed low sample loading, low solvent consumption, suitable separation of cations, anions and neutrals, and was much more powerful in drug development screening.

V. *Enzyme-Linked Immunosorbent Assay (ELISA)*

The results showed very high sensitivity and good linearity ($r^2 > 0.988$) ranging from 2.5 ng/mL to 15 ng/mL of tPA concentration (data not shown). ELISA is a rapid method for identification and quantitation of tPA[10]. Most commercial ELISA kits are available. ELISA was much suitable for routine or clinical use. However, the quantitation could not reflect the real biological activity and the result would also be non-identical if different anti-tPA epitopes were used.

VI. *Comparison of Analytical Methods Studied*

A comparative analysis of five methods described above showed various precision (Table 2). The clot lysis method adopted by EP and USP, was used as a reference method. It was used to characterize the thrombolytic cascade activity and showed high specificity, but the reagent cost was high and the reproducibility was poor. Chromogenic substrate assay operated by substrate, S-2288, which was suitable for tPA and other serine proteases, showed good accuracy, good reproducibility, time saving, and low cost, but the specificity was less than clot lysis. The bioactivity of tPA could be tested by the above two methods. The instrumental analysis such as SE-HPLC and CZE showed excellent accuracy, reproducibility, and impurity determination, but the protein content could not reflect the real bioactivity and it showed relative low specificity (retention time only). ELISA utilizing antigen-antibody reaction showed the automatic feature, excellent sensitivity, and medium specificity, but the bioactivity and interferences were its main problems. Overall, the time courses for one 96-well plate manipulated in chromogenic substrate assay and ELISA were about 1 and 4.5 hours, respectively. The time consumption for any other three methods was more than 6 hours.

CONCLUSIONS

For improving accuracy and precision, five methods have been applied for measuring the activity or content of

Journal of Food and Drug Analysis, Vol. 17, No. 1, 2009

Table 2. The comparison of analytical methods for determination of tPA

	Clot lysis	Chromogenic substrate assay	SE-HPLC	Capillary electrophoresis	ELISA
Specificity	High	Medium	Low	Low	Medium
Range (μg/mL)	0.4~1.2	5~50	100~500	20~100	0.0025~0.015
Linearity (r^2)	0.9888	0.9952	0.9994	0.9979	0.9888
Accuracy (Recovery)	N/A*	RSD< 10%	RSD< 2 %	RSD< 10%	N/A*
Reproducibility (RSD)	<10 %	< 5 %	< 2 %	< 5 %	N/A*
Time course (One plate or 20 tests)	> 6 hours	> 1 hour	> 6 hours	> 6 hours	> 4. 5 hours
Total cost	High	Low	Medium	Medium	Medium

* Not-Available

tPA. Based on the results of our study, the chromogenic substrate assay is the first choice for the assay of bioactivity of tPA in pharmaceutical formulations because of its rapidity, inexpensiveness, accuracy, and enzymatic activity testing. SE-HPLC or CZE method could serve as an alternative choice for a quantitative routine assay of tPA due to its excellent precision.

REFERENCES

1. Council of Europe. 2005. Monograph. Alteplase for injection. In "European Pharmacopoeia". 5th ed. pp. 956-959. Council of Europe. Strasbourg, France.
2. Sands, D., Whitton, C. M., Merton, R. E. and Longstaff, C. 2002. A collaborative study to establish the 3rd International Standard for tissue plasminogen activator. Thromb. Haemost. 88: 294-297.
3. Espicom Business Intelligence. 2006. Biogenerics 2005: an emerging global market? 2nd ed. Espicom Business Intelligence. UK.
4. The United States Pharmacopeial Convention. 2007. Monograph. Alteplase and Alteplase for injection. In "The United States Pharmacopeia". 29th ed. pp. 1327-1330. The United States Pharmaceopeial Convention. Rockville, MD, USA.
5. Longstaff, C. and Whitton, C. 2004. A proposed reference method for plasminogen activators that enables calculation of enzyme activities in SI units. J. Thromb. Haemost. 2: 1416-1621.
6. Longstaff, C., Whitton, C., Thelwell, C. and Belgrave, D. 2007. An international collaborative study to investigate a proposed reference method for the determination of potency measurements of fibrinolytics in absolute units. J. Thromb. Haemost. 5: 412-414.
7. Yim, K. W. 1991. Fractionation of the human recombinant tissue plasminogen activator (rtPA) glycoforms by high-performance capillary zone electrophoresis and capillary isoelectric focusing. J. Chromatogr. A 559: 401-410.
8. Wu, S. L. 1997. The use of sequential high performance liquid chromatography and capillary zone electrophoreses to separate the glycosylated peptides from recombinant tissue plasminogen activator to a detailed level of microheterogeneity. Anal. Biochem. 253: 85-97.
9. Thorne, J. M., Goetzinger, W. K., Chen, A. B., Moorhouse K. G., and Karger, B. L. 1996. Examination of capillary zone electrophoresis, capillary isoelectric focusing and sodium dodecyl sulfate capillary electrophoreses for the analysis of recombinant tissue plasminogen activator. J. Chromatogr. A 744: 155-165.
10. Kondera-Anasz, Z., Michalski, A., Gil, D., Gil, B., Starzewski, J. and Gonciarz, Z. 2000. Accuracy of t-PA, u-PA, PAI- 1 and PAI- 2 estimation in human bile by ELISA kits. Med. Sci. Monit. 6: 616-617.