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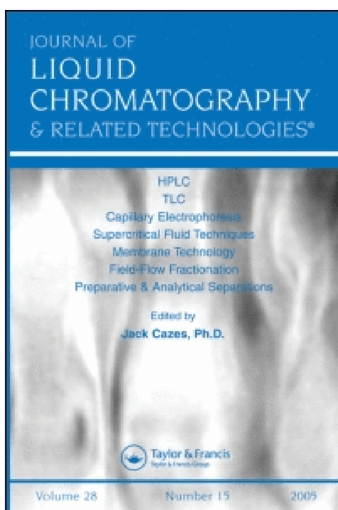

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A REVERSED-PHASE HPLC ASSAY FOR PLASMINOGEN ACTIVATORS

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A REVERSED-PHASE HPLC ASSAY FOR PLASMINOGEN ACTIVATORS

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ABSTRACT

ACTIVASE® is the recombinant form of human tissue-type plasminogen activator (r-tPA), used in the management of acute myocardial infarction and pulmonary embolism. ACTIVASE® is also now approved for treating ischemic stroke. It is produced by expressing the complementary DNA (cDNA) for natural human tPA in Chinese hamster ovary (CHO) cells. TNK-tPA is a genetically engineered variant of r-tPA with enhanced efficacy and lower incidence of bleeding compared with activase. It was created by three site-directed mutations (T103N, N117Q and KHRR296-299AAAA) and, is also cloned and expressed in CHO cells. CHO cells biosynthesize endogenous hamster tPA called CHO-PA. The amino acid sequence of CHO-PA is highly homologous (80% identical) to that of r-tPA. All three thrombolytic proteins exist as heterogeneous isoforms, mainly due to proteolysis/hydrolysis and differential glycosylation. In this report, a reversed-phase HPLC method was developed to support manufacturing process development. This method not only has the ability to resolve the three plasminogen activators from each other, but also is capable of identifying and quantifying different isoforms of each molecule.

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INTRODUCTION

Tissue-type plasminogen activators (tPA) are endogenous serine proteases involved in a cascade of events leading to the dissolution of a blood clot.[1-4] ACTIVASE® is the recombinant form of human tPA (r-tPA), used in the management of acute myocardial infarction and pulmonary embolism.[5] ACTIVASE® is also now approved for treating ischemic stroke.[6-7] It is a glycoprotein produced by expressing the complementary DNA (cDNA) for natural human tPA in Chinese hamster ovary (CHO) cells. TNK-tPA is a genetically engineered variant of human tPA cloned and expressed in CHO cells.[8] Site-directed mutations were introduced at three specific sites of human tPA to create the TNK-tPA variant. They are Thr103 to Asn (T103N), Asn 117 to Gln (N117Q), and Lys-His-Arg-Arg 296-299 to Ala-Ala-Ala-Ala (KHRR296-299AAAA). When compared to tPA, TNK-tPA exhibits similar *in vitro* biological activity, an increased resistance to plasminogen activator inhibitor, and an enhanced fibrin specificity, and is cleared more slowly from plasma.[8-11] It is currently awaiting regulatory approval as a single bolus administered form of r-tPA. CHO cells biosynthesize endogenous hamster tPA called CHO-PA.[12] CHO-PA has a similar fibrinolytic activity to human tPA as determined by the clot lysis assay. The amino acid sequence of CHO-PA is 80% identical to that of human tPA. Many of the substitutions are semi-conservative such as: Arg<—>Lys, Glu<—>Asp, Phe<—>Tyr, Val<—>Ala, Ile<—>Leu, or Thr<—> Ser. Using a model of the human tPA protease domain based upon the bovine chymotrypsin structure, it is observed that virtually all of the substitutions in CHO-PA are localized at or near the protein surface.

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r-tPA, TNK-tPA, and CHO-PA are all single polypeptide chains composed of 527 amino acids with 17 disulfide bonds.[12-13] For all three proteins, the peptide bond between Arg_{275} and Ile_{276} is particularly susceptible to protease cleavage. The cleavage results in two fragments: one consisting of the N-terminal 275 amino acids and the other consisting of the C-terminal 252 amino acids. The N-terminal chain contains regions which are homologous to the kringle regions found in plasminogen and prothrombin and, therefore, is often referred to as the "kringle fragment."[13-14] The C-terminal chain contains the catalytically active site and, therefore, is commonly referred to as the "protease fragment."[15] The cleaved two chains are linked by a single disulfide bond formed between Cys_{264} and Cys_{395}.[12] The cleaved molecule is commonly referred to as "two-chain tPA" as opposed to "single-chain tPA" or the intact form.

t-tPA contains four potential sites for N-linked glycosylation identified by the sequence Asn-X-Ser/Thr.[13] These are Asn_{117}, Asn_{184}, $Asn_{218,}$ and Asn_{448}. rt-PA exists as two glycosylation isozymes designated type I and type II. Type I rt-PA is glycosylated at Asn_{117}, Asn_{184}, and Asn_{448}; whereas type II rt-PA is glycosylated only at Asn_{117} and Asn_{448}. Asn_{218} is not glycosylated in either isoforms. TNK-tPA has the same glycosylation pattern as rt-PA, except that the Thr103 to

Asn and Asn117 to Gln mutations effectively moved the glycosylation site from position 117 to 103.[8] The glycosylation pattern for CHO-PA is not fully characterized.

To support the manufacturing process development, a reversed-phase HPLC method was developed for the analysis of the three thrombolytic molecules. This method is not only capable of identifying and quantifying different isomers of each plasminogen activator, but also has the ability to resolve rt-PA, TNK-tPA, and CHO-PA from each other.

EXPERIMENTAL

Materials

ACTIVASE® (r-tPA), TNK-tPA, and monoclonal antibody #354 for CHO-PA were produced and purified using proprietary procedures at Genentech, Inc. (South San Francisco, CA). Plasminogen was obtained from Fluka (Switzerland). HPLC-grade acetonitrile was obtained from Burdick & Jackson (Muskegon, MI) and trifluoroacetic acid (TFA) was from Pierce (Rockford, IL). Water for the HPLC mobile phase and sample solutions was purified with a Milli-Q system from Millipore (Milford, MA). All other chemicals were of reagent grade from Sigma (St. Louis, MO).

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Methods

Purification of CHO-PA

CHO-PA was isolated from CHO cell culture fluid by affinity chromatography followed by immunoabsorption. Lyine hyper D resin from BioSepra (Paris, France) was used for the affinity chromatography, as lysine binds to the kringle 2 region of plasminogen activators.[17] The immunoabsorption was conducted by using CHO-PA specific monoclonal antibody #354 (MAb#354). MAb#354 was coupled to CNBr-activated Sepharose 4B gel according to the vendor's protocol (Pharmacia Biotech, Piscataway, NJ). About 10 mg of MAb #354 was coupled to per mL of the CNBr-activated Sepharose 4B gel. After coupling, a MAb#354-Sepharose 4B column was packed.

CHO cell culture fluid containing secreted CHO-PA was loaded onto a lysine-affinity column pre-equilibrated with an equilibration buffer containing 50 mM sodium phosphate and 0.01% polysorbate 80 at pH 7.5. After loading, the lysine-affinity column was washed three times: first with the equilibration buffer, followed by a buffer containing 40 mM Tris, 800 mM NaCl, and 0.008% polysorbate 80 at pH 8.0, and finally with the equilibration buffer. CHO-PA

was then eluted from the lysine affinity column with a buffer containing 50 mM sodium phosphate, 200 mM L-arginine, and 0.01% polysorbate 80 at pH 7.5.

After equilibrating with phosphate-buffered saline (PBS, 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4 at pH 7.4), the MAb#354-Sepharose 4B column was loaded with the lysine-affinity column elution pool. After loading, the column was washed with a buffer containing 9.5 mM Na_2HPO_4, 1 M NaCl, and 5% propylene glycol (v/v) at pH 7.4. The bound CHO-PA was eluted from the column with 0.2 M glycine-HCl at pH 2.5. The elution was monitored spectrophotometrically at 280 nm and the CHO-PA containing fractions were neutralized with 0.14 volumes of 1.5 M arginine-phosphate (pH 8.0) immediately upon collection. The identity and purity of the eluted CHO-PA was confirmed by SDS-PAGE and amino acid sequence analysis.

DTT/Urea Treatment

The solution containing plasminogen activator(s) was diluted 1:1 (v/v) with the denaturation buffer (8 M urea, 0.5 M Tris, and 3.2 mM EDTA at pH 8.4). Dithiothreitol (DTT) was added from a 1 M stock solution to a final concentration of 20 mM and, the mixture was incubated at 37°C for 30 min.

Plasmin Treatment

The solution containing plasminogen activator(s) was diluted 1:3 (v/v) with the digestion buffer (125 mM Na_2HPO_4, 200 mM arginine, and 0.01% NaN_3 at pH 7.5). One hundredth (w/w) of plasminogen was added, and the mixture was incubated at 37°C for 30 min.

Reversed-Phase HPLC Assay for Plasminogen Activators

The assay was performed on a Hewlett-Packard 1090M HPLC system (Hewlett Packard, Avondale, PA) with a 4.6 mm x 250 mm, 5 µ particle size, 300 Å pore resin, Zorbax SB-C8 column (Mac-Mod, Chadds Ford, PA). The column was equilibrated for at least 15 minutes prior to sample injection. The initial mobile phase composition was 70/30/0.1 (v/v/v) of water/acetonitrile/TFA.

After a five-minute initial hold, a linear gradient was performed in 80 minutes to 50/50/0.1 (v/v/v) of water/acetonitrile/TFA. Immediately following the gradient, the column was regenerated for 10 minutes with 100/0.1 (v/v) of acetonitrile/TFA. The composition was then brought back to the initial conditions in 5 minutes, and the system was re-equilibrated for the next injection.

The injection volume was 250 µL, and the flow rate was 1 mL/min. The chromatography was conducted at 40°C. Fluorescence was measured with a Hewlett-Packard 1046A programmable fluorescence detector (Ex = 275 nm and

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Em = 340 nm). The chromatograms were recorded and analyzed with Hewlett-Packard ChemStation software.

RESULTS AND DISCUSSION

Due to high sequence homology, ACTIVASE® (r-tPA), TNK-tPA, and CHO-PA have very similar biochemical/biophysical properties. An analytical method capable of resolving these three plasminogen activators was needed to support the recovery process development, as well as, to estimate the purity of each molecule for clinical studies. In this report, we describe a simple reversed-phase HPLC method which accomplishes this goal.

With a shallow gradient ramp at 0.25% acetonitrile per minute, reversed-phase HPLC was not able to separate the native form of r-tPA from native CHO-PA (Figure 1). However, the native form of TNK-tPA was resolved very well

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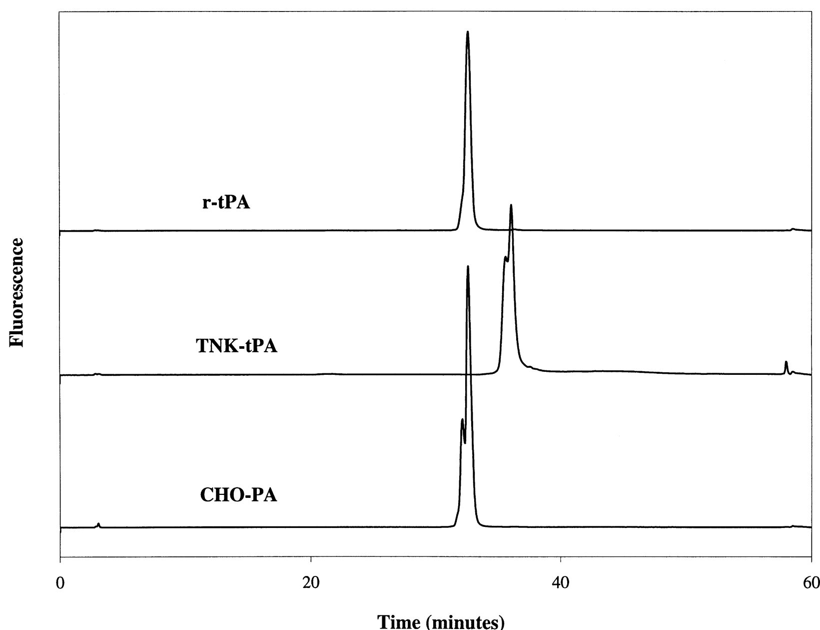

Figure 1. Reversed-phase HPLC analysis of native r-tPA, TNK-tPA and CHO-PA. Chromatographic conditions: column, Zorbax SB-C8 column (4.6 nm X 250 nm, 5 μ particle size); injection volume, 250 μL; temperature, 40°C; detection, Ex = 275 nm and Em = 340 nm; flow rate, 1 mL/min; gradient, linear from 70/30/0.1 (v/v/v) of water/acetonitrile/TFA to 50/50/0.1 (v/v/v) of water/acetonitrile/TFA in 80 minutes.

from both native r-tPA and CHO-PA. Next, DTT/urea treatment was performed to reduce the disulfide bonds and denature the proteins. For all three proteins, the peptide bond between Arg_{275} and Ile_{276} is very susceptible to protease cleavage. Over time, this susceptibility leads to heterogeneity for r-tPA, TNK-tPA, and CHO-PA in solution. A small amount of the single-chain form is converted to the two-chain form due to the protease cleavage.

DTT/urea treatment reduces the disulfide bond between Cys_{264} and Cys_{395} that holds the two-chain form of the molecule together, resulting in the dissociation of the molecule into two fragments (the kringle fragment and the protease fragment). Figure 2 shows that, under the same gradient ramp, reversed-phase HPLC was able to resolve the single-chain form of the three thrombolytic molecules from each other after DTT/urea treatment. All three proteins exhibited similar elution profiles with the kringle fragment of the two-chain form eluting first, the single-chain form eluting second, and the protease fragment of the

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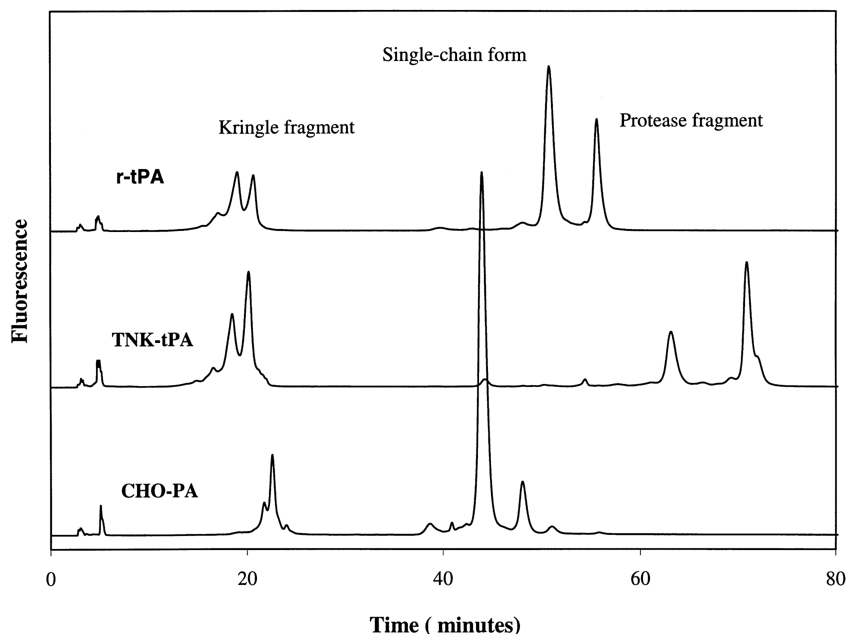

Figure 2. Reversed-phase HPLC analysis of DTT/urea treated r-tPA, TNK-tPA and CHO-PA. Plasminogen activators were treated with DTT/urea prior to chromatography. Chromatographic conditions: column, Zorbax SB-C8 column (4.6 nm X 250 nm, 5 μ particle size); injection volume, 250 μL; temperature, 40°C; detection, Ex = 275 nm and Em = 340 nm; flow rate, 1 mL/min; gradient, linear from 70/30/0.1 (v/v/v) of water/acetonitrile/TFA to 50/50/0.1 (v/v/v) of water/acetonitrile/TFA in 80 minutes.

two-chain form eluting last. The protease fragment of the three plasminogen activators was also well resolved from each other, while the kringle fragments for the three molecules were not well separated. Consequently, this method can be used to detect and quantify the fragmentation of the single-chain form into the two-chain form of the plasminogen activators.

The heterogeneity observed in the r-tPA, TNK-tPA, and CHO-PA profiles (Figure 2) makes the quantification of these molecules very difficult, especially when trying to quantify each individual molecule in a mixture of rtPA, TNK-tPA, and CHO-PA. To eliminate the heterogeneity associated with proteolysis, all of the single-chain form was converted to two-chain form by incubating with plasminogen. Plasminogen is the substrate of plasminogen activator in the natural fibrinolytic system. r-tPA, TNK-tPA, and CHO-PA all have the enzymatic activity of cleaving the Arg_{560}-Val_{561} peptide bond of plasminogen. Such cleavage converts plasminogen into its active form, plasmin.

Plasmin is a serine protease with low specificity and is capable of cleaving the Arg_{275}-Ile_{276} peptide bond in r-tPA, TNK-tPA, and CHO-PA. As a result, incubation with plasminogen converts the single-chain form of the three plasminogen activators to the two-chain form. Following the plasmin treatment, the samples were treated with DTT/urea to reduce disulfide bonds and thus dissociate the two-chain form of the molecule into two discrete fragments.

Figure 3 shows the reversed-phase HPLC profiles for the plasmin and DTT/urea-treated plasminogen activators. The protease fragment of the three proteins was well separated from each other, while the kringle fragment of the three molecules was not resolved. Therefore, the protease fragment was used for the integration and quantification of each plasminogen activator. For both r-tPA and TNK-tPA, the kringle fragment from the type I and type II isozymes was well separated. As a result, this method was also used for the quantification of the type I to type II ratio for both r-tPA and TNK-tPA.

Taken together, a reversed-phase HPLC method was developed for the identification and quantification of r-tPA, TNK-tPA, and CHO-PA. This method not only has the ability to resolve the three plasminogen activators from each other, but also is capable of identifying and quantifying different isoforms of each molecule. When coupled with the DTT/urea treatment to reduce the disulfide bonds and denature the proteins prior to the chromatography, this method can be used to identify r-tPA, TNK-tPA, and CHO-PA, as well as to quantify single chain% of each single species (Figure 2). But it can not be used to quantify each molecule due to the heterogeneity observed. Incubation with plasmin, which converts the single-chain form of the plasminogen activators to the two-chain form, followed by the DTT/urea treatment eliminates such heterogeneity. Consequently, the combined plasmin and DTT/urea treatments prior to the chromatography allow for quantification of each plaminogen activator with the use of the respective reference (Figure 3).

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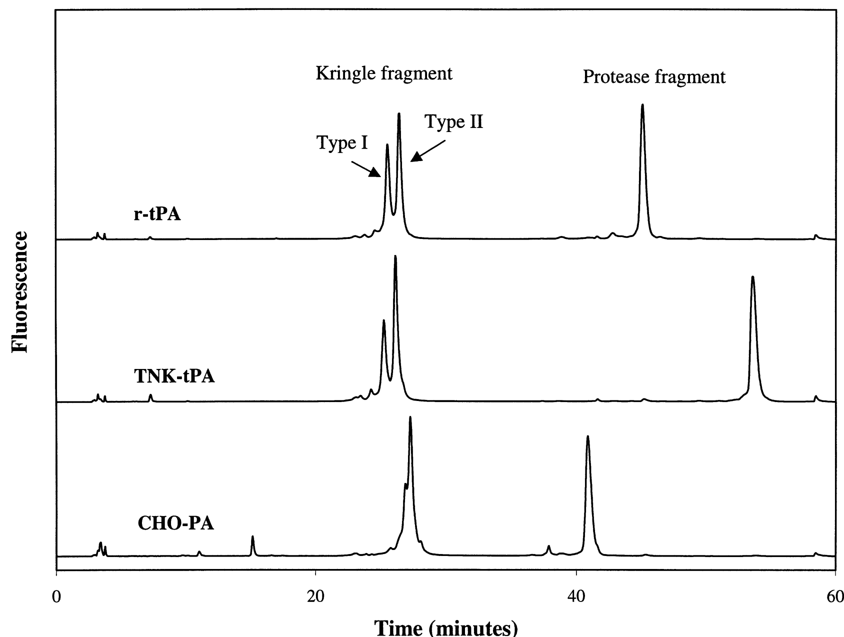

Figure 3. Reversed-phase HPLC analysis of plasmin and DTT/urea treated r-tPA, TNK-tPA and CHO-PA. Plasminogen activators were subjected to plasmin treatment followed by DTT/urea treatment prior to chromatography. Chromatographic conditions: column, Zorbax SB-C8 column (4.6 nm X 250 nm, 5 μ particle size); injection volume, 250 μL; temperature, 40°C; detection, Ex = 275 nm and Em = 340 nm; flow rate, 1 mL/min; gradient, linear from 70/30/0.1 (v/v/v) of water/acetonitrile/TFA to 50/50/0.1 (v/v/v) of water/acetonitrile/TFA in 60 minutes.

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The combined plasmin and DTT/urea treatments also enable the quantification of the type I isozyme to type II isozyme ratio. The availability of this reversed-phase HPLC method greatly facilitated the manufacturing process development. It has been used to evaluate the effect of different fermentation conditions on product quality regarding the integrity of the product (*i.e.* single chain %) and the ratio of type I and type II isozymes. It has also been used to aid the purification process development and to ensure consistency between production batches. All those applications exemplify the crucial role of analytical methods in the development of new pharmaceutics.

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