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# UROKINASE: STABILITY STUDIES IN SOLUTION AND LYOPHILIZED FORMULATIONS

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## Abstract

Urokinase - a serine protease - is used clinically as a thrombolytic agent to dissolve blood clots. Low molecular weight Urokinase (33,000 dalton) isolated from human kidney cells using tissue culture techniques was used in the stability studies. Quantitative determination of Urokinase was accomplished by either amidolytic or fibrinolytic activity assay methods. The degradation of Urokinase in solution at 55 °C follows pseudo-first order kinetics at several pH values. The pH range for maximum stability has been determined to be about 6 to 7.

The stability of Urokinase is very sensitive to the quantity of residual moisture in the lyophilized formulation. Rubber stoppers used as closures for the glass vials were identified as a major source of moisture. The loss of activity from freeze dried formulations was inversely related to the specific activity of tissue culture derived Urokinase. Similar relationship was also observed for the adsorption of Urokinase from 5% dextrose diluent to the surface of polyvinyl chloride large volume parenteral diluent bags. Initial degradation rates (zero order) for freeze dried urokinase formulations with and without the addition of human serum albumin (HSA) as a stabilizer determined at 50, 40 and 30 °C demonstrated that loss of urokinase followed the Arrhenius relationship with an apparent energy of activation (Ea) of 15 kcal per mol. The addition of HSA resulted in an increase in stability by about a factor of four. However, the apparent Ea for the formulations with and without HSA was not significantly different as evident from parallel slopes in the Arrhenius plots.

## INTRODUCTION

Urokinase, a naturally occurring plasminogen activator enzyme (protein), is used as a thrombolytic agent in clinical practice (1). Urokinase acts on the endogenous fibrinolytic system to convert plasminogen to plasmin, which ultimately dissolves blood clots. It has been used in deep vein thrombosis, pulmonary embolism and arterial thrombosis (2); and may also be useful in

myocardial infarction. Urokinase enzyme has been prepared and isolated from urine, kidney cell, and lung adenocarcinoma cell line cultures; and by expression in both bacterial and human cells using recombinant DNA techniques (3). Structurally, urokinase has two peptide chains, designated A and B, linked by disulfide bonds. There are two forms of urokinase differing in molecular weight (MW) but having similar clinical effects (4). Both molecular forms of urokinase are used clinically as thrombolytic agents (5). The high and low MW forms of urokinase have been assigned values of 33,000 and 54,000 dalton, respectively (6). Limited proteolysis of the high molecular weight form yields low molecular weight urokinase in vitro (4). The specific activities of highly purified high and low MW urokinase have been reported (3) to be about 120,000 and 240,000 International Units (IU) per mg.

## ANALYTICAL METHODS

**AMIDOLYTIC ASSAY:** Urokinase activity is quantitatively related to its ability to hydrolyze the synthetic chromogenic substrate, *(pyro)*-glutamyl-glycyl-arginyl-*p*-nitro-anilide, (S-2444), to a yellow colored product, *p*-nitroaniline. Quantitation of samples was achieved using a standard curve with urokinase standards at 129-516 IU/ml. The assay has a linear response for Urokinase in the 30 to 1000 IU/ml range. The amidolytic activity assay measures the ability of urokinase to

catalyze the cleavage of the peptide linkage between arginine and *p*-nitroaniline in the synthetic substrate S-2444. Thus, it is indicative of the integrity of the serine protease active site of the enzyme (7, 8). S-2444 is also a substrate for a number of other serine proteases, including trypsin, thrombin, Factor Xa and plasmin (9). However, bovine pancreatic trypsin inhibitor, aprotinin, which is added to the assay solutions is an excellent inhibitor of these potentially interfering enzymes (10).

**FIBRINOLYTIC ASSAY:** A modification of an *in vitro* clot lysis assay (11, 12) in which a mixture of fibrinogen is clotted with thrombin in the presence of plasminogen and various dilutions of urokinase test samples and reference standards was used. The following sequence of reactions form the basis of this analytical method.

| | | | |
|---|---|---|---|
| HUMAN PLASMA | —THROMBIN→ | FIBRIN CLOT | (1) |
| PLASMINOGEN | —UROKINASE→ | PLASMIN | (2) |
| FIBRIN CLOT | —(PLASMIN)→ | DEGRADATION PRODUCTS | (3) |

In reaction 1, thrombin catalyzes the conversion of soluble fibrinogen in human plasma to an insoluble fibrin clot in a test tube in an ice-water bath. A glass bead is placed over the clot. Urokinase activation of the primary substrate plasminogen (reaction 2) at 37 °C, results in the formation of plasmin which catalyzes the dissolution of the fibrin clot. The amount of

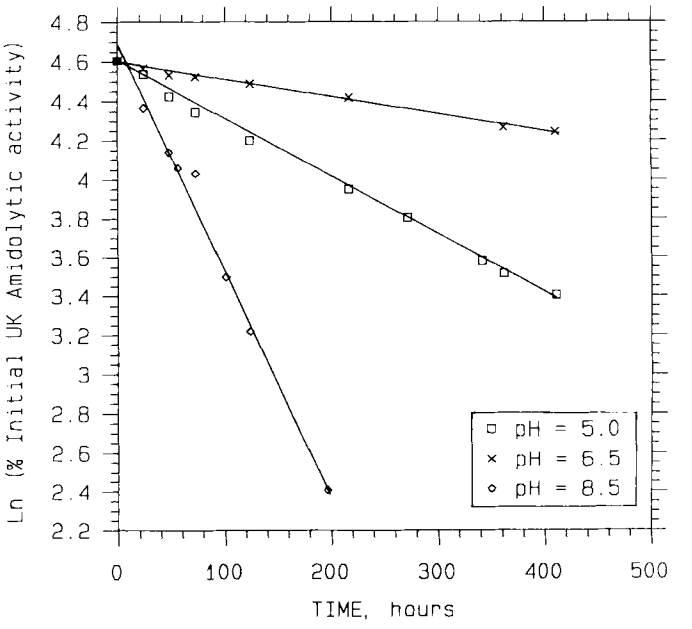


FIGURE 1

Urokinase stability in solution as a function of pH at 55 °C.

plasmin produced in each assay tube is dependent on the urokinase dilution used and is related to the time required for the glass bead to fall to the bottom of the tube. The clot lysis time is related to the concentrations of urokinase in the test sample and reference standards using a parallel line biological assay approach (13) relating log lysis time to log dose (log dilution level). The two regression lines for the reference standard and test samples are parallel and therefore the horizontal distance between the lines is related to the relative strength of the two materials.

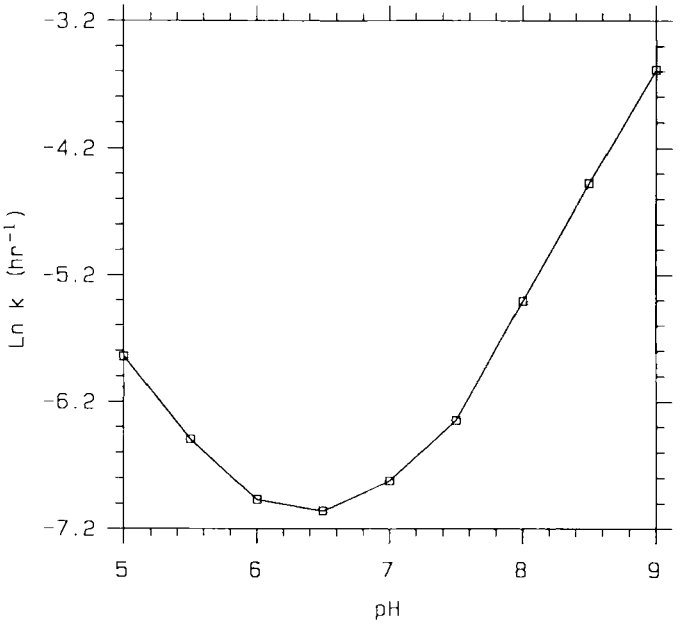


FIGURE 2

pH - Rate profile of Urokinase at 55 °C.

## SOLUTION STABILITY STUDIES

**pH Rate Profile:** The loss in amidolytic activity of Urokinase from solution at 55 °C follows pseudo first-order kinetics from pH 5-9 (Figure 1). The pH rate profile is shown in Figure 2. These studies were done in unbuffered solutions at an initial urokinase concentration of 50,000 IU/ml. The pH of maximum stability is 6.5. Miwa and co-workers (14) have reported pH of about 9 for the maximum stability of urine derived low MW Urokinase. Additionally, these researchers indicated that high MW Urokinase was equally stable in a broad pH range of 3-9. This

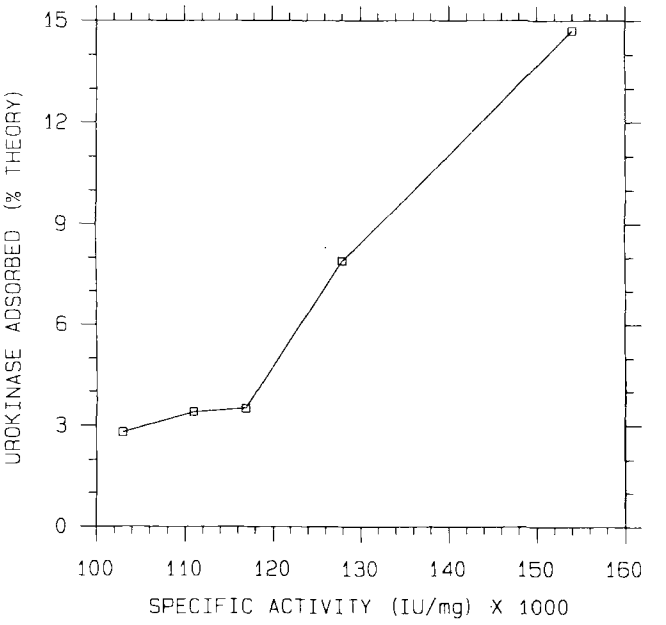


FIGURE 3

Adsorption dependence of Urokinase on protein specific activity.

suggests that the pH for maximum stability is dependent on the source and the MW of the enzyme.

**Adsorption Losses:** Lyophilized Urokinase formulations containing mannitol and sodium chloride as excipients were dissolved in Water for Injection, USP and diluted to 1500 IU/ml in 5% Dextrose for Injection, USP. Some loss due to adsorption to polyvinyl chloride (PVC) infusion bags occurs almost instantaneously. Figure 3 indicates that the percent adsorbed is dependent on the specific activity of the urokinase. In the preparation, isolation and purification of Urokinase by the tissue

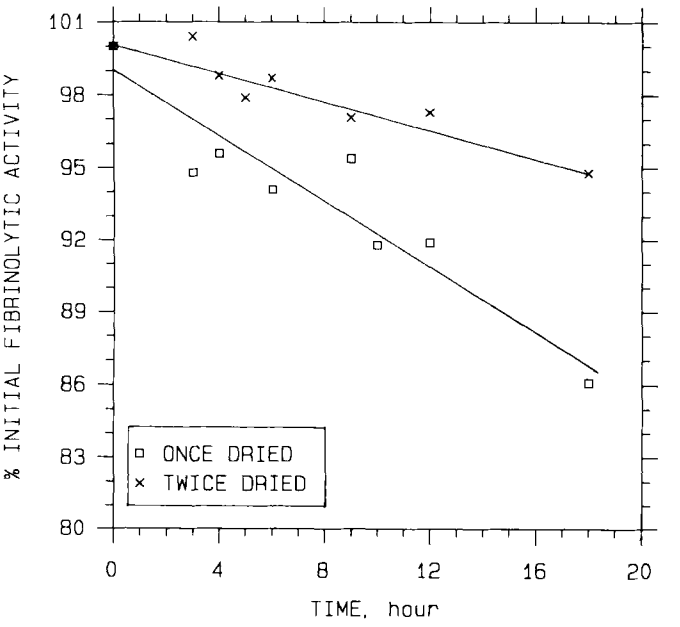


FIGURE 4

Rubber closure drying process effects on lyophilized Urokinase stability at 5 °C.



culture technique, bulk drug solutions undergo a "heat treatment" process for virus inactivation resulting in some loss of urokinase activity. From the adsorption study results it appears that this active and inactive urokinase compete for the same sites on the PVC container.

## LYOPHILIZED FORMULATION STABILITY

**Residual Moisture Effects:** The incomplete drying of rubber stoppers used as closures for lyophilized proteins may decrease the stability of the product. Figure 4 shows the average potency

**TABLE 1**

Moisture Content of West-867 Stoppers Processed with Two Drying Cycles.

| Drying Cycle | Stoppers/Tray | Moisture Content, % | Visual Inspection |
|---|---|---|---|
| 1 X 6-Hour | 1600 | 0.19<br>0.26 | Wet |
| 2 X 6-Hour | 1600 | 0.04<br>0.04 | Dry |
| | 1000 | 0.01<br>0.01 | Dry |

values and the regression lines for the stability (5 °C) from several batches of Urokinase formulations. These stability lots used rubber stoppers prepared by two drying processes. The cycle consists of vacuum drying at 50 °C for six hours after the rubber stoppers have been cleaned and sterilized in an autoclave. The results in Table 1 indicate that single dried stoppers contain considerable amount of moisture which can diffuse into the formulation during storage.

During the course of these stability studies the lyophilized Urokinase formulations lots which used stoppers prepared by the single drying process had an average residual moisture content of 0.8%, while those that were manufactured using the twice dried stopper had 0.3% residual moisture. The average degradation rate at 5 °C for batches that used the twice dried stopper was 0.3 %

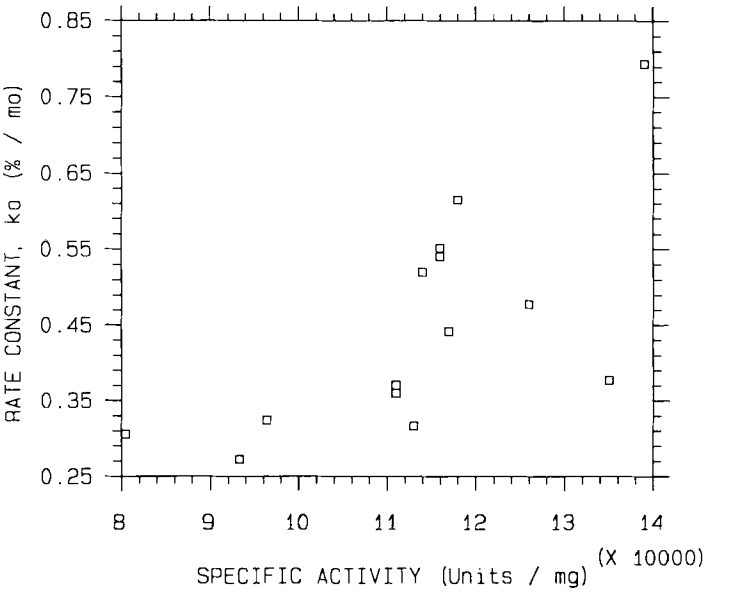


FIGURE 5

Urokinase specific activity effects on lyophilized Urokinase stability at 5 °C.



per month, while lots using single dried stopper degraded at 0.7 % per month (Figure 4) indicating the importance of removing the moisture from rubber stoppers.

Effect of Specific Activity: The stability of lyophilized formulations prepared using urokinase with different specific activity was monitored at 5 °C and the zero order rate constants calculated for each lot. This formulation did not contain any stabilizers (e.g. additional proteins). The residual moisture content for all these lots was similar (average value 0.6%). Figure 5 demonstrates the relationship between zero order rate constant (stability) and Urokinase specific activity. These data

**TABLE 2**

Effects of Temperature and HSA on the Stability of Lyophilized Urokinase Formulations.

| Temperature (°C) | Initial Rate ko, (Percent / Month) No HSA | HSA | Stability Ratio |
|---|---|---|---|
| 50 | 8.19 | 1.66 | 4.9 |
| 40 | 3.43 | 0.70 | 4.9 |
| 30 | 1.46 | 0.34 | 4.3 |

indicate that inactive Urokinase (produced during "heat treatment" of bulk drug) appears to improve the stability of the lyophilized formulation. These results underscore the impact of bulk drug processing and any associated changes on the stability of a protein formulation.

**Effect of Stabilizers:** The stability of lyophilized formulations of urokinase was evaluated at 30, 40 and 50 °C in the presence and absence of human serum albumin (HSA). The stability data follow zero order kinetics. The initial rates for the degradation of Urokinase (Table 2) demonstrate about 4-5 fold improvement in stability of the formulation due to the addition of HSA. The rate constants at different temperatures follow the Arrhenius relationship (Figure 6) with an apparent energy of activation (Ea) of 15 kcal per mol. Although the addition of HSA resulted in an increase in lyophilized urokinase stability by

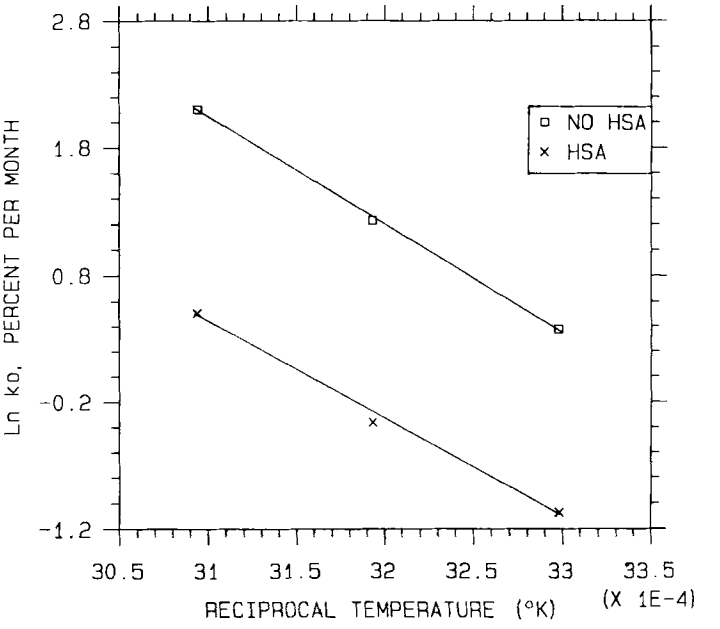


FIGURE 6

Arrhenius plot for lyophilized Urokinase formulations.

about a factor of 4-5 , the apparent Ea for the formulations with and without HSA was not significantly different as evident from parallel slopes in the Arrhenius plots in Figure 6.

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