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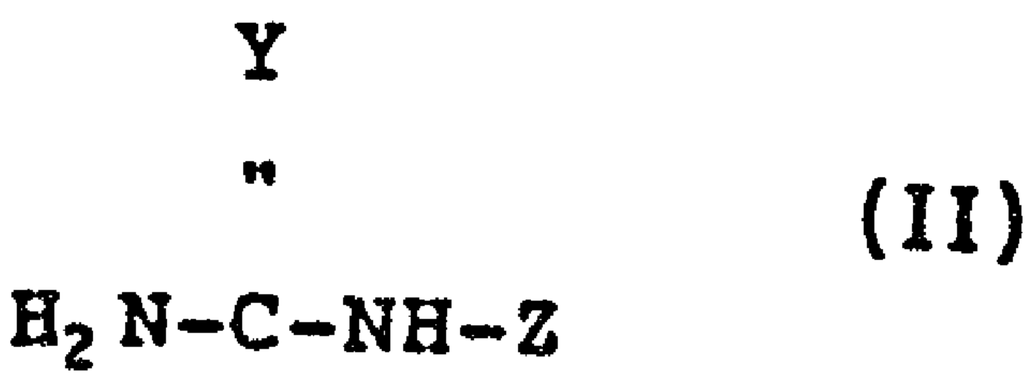
(54) **STABILISATION DU K2P PRO**

(54) **K2P PRO STABILISATION**



(II)

(57) Une composition pharmaceutique d'un dérivé K2P pro de t-PA non glycosylé, ayant une activité enzymatique d'au moins 1,4 MU/ml et une valeur de pH comprise entre 4,5 et 6,5, contient du citrate et au moins un composé du groupe formé par: a) l'acide ascorbique; b) l'EDTA; c) des composés amine ayant la formule R^1R^2N - R - X, dans laquelle X = SO_3H, $CH(NH_2)$-CO_2H, CO_2H, H, NH_2 ou OH, R = C_1-C_9-alkylène, de préférence C_4-C_7-alkylène, C_3-C_6-cycloalkylène ou benzylidène et R^1 et R^2 représentent indépendamment l'un de l'autre H ou C_1-C_3-alkyle; d) des composés analogues de la guanidine ayant la formule (II), dans laquelle Y = H_2N + ou 0; Z = H ou $(CH_2)_m$ V, $(CH_2)_mCH(NH_2)$-CO_2H, $CH(CO_2H)$-$(CH_2)_mCO_2H$; V = NH_2 ou CO_2H et m = 1 à 4; e) des acides carboxyliques substitués par un ou plusieurs groupes hydroxyles, cétoniques et/ou des groupes carboxyles additionnels; f) la diméthylbiguanide; g) des nucléosides et des nucléotides de pyrimidine; h) le tréhalose et la glucosamine. L'invention concerne également un médicament à base du dérivé K2P pro de t-PA comme principe actif et son procédé de production.

(57) The invention relates to a pharmaceutical preparation of a non-glycosylized t-PA derivative, K2P pro, with an enzymatic activity of at least 1.4 MU/ml and a pH of 4.5 to 6.5, containing citrate and at least one compound from the following group: a) ascorbic acid, b) EDTA, c) amino compounds of formula R^1R^2N - R - X, where X = SO_3H, $CH(NH_2)$-CO_2H, CO_2H, H, NH_2 or OH, R = C_1-C_9 alkylene, preferably C_4-C_7 alkylene, C_3-C_6 cycloalkylene or benzylidene and R^1 and R^2, independently of each other, are H or C_1-C_3 alkyl, d) guianidine analogs of formula (II), where Y = H_2N + or 0, Z = H or $(CH_2)_m$ V, $(CH_2)_mCH(NH_2)$-CO_2H, $CH(CO_2H)$-$(CH_2)_mCO_2H$, V = NH_2 or CO_2H and m = 1 to 4, e) carboxylic acids substituted with one or more hydroxyl, keto and/or further carboxyl groups, f) dimethylbiguanide, g) pyrimidine nucleosides and pyrimidine nucleotides, h) trehalose, glucosamine. The invention also relates to a drug based on the t-PA derivative K2P pro as the active ingredient and a process for producing it.

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(54) Title: STABILIZATION OF K2P PRO

(54) Bezeichnung: K2P PRO-STABILISIERUNG

$$H_2N-\overset{\overset{Y}{\|}}{C}-NH-Z \qquad (II)$$

(57) Abstract

The invention relates to a pharmaceutical preparation of a non-glycosylized t-PA derivative, K2P pro, with an enzymatic activity of at least 1.4 MU/ml and a pH of 4.5 to 6.5, containing citrate and at least one compound from the following group: a) ascorbic acid, b) EDTA, c) amino compounds of formula R^1R^2N - R - X, where X = SO_3H, $CH(NH_2)$-CO_2H, CO_2H, H, NH_2 or OH, R = C_1-C_9 alkylene, preferably C_4-C_7 alkylene, C_3-C_6 cycloalkylene or benzylidene and R^1 and R^2, independently of each other, are H or C_1-C_3 alkyl, d) guianidine analogs of formula (II), where Y = H_2N+ or 0, Z = H or $(CH_2)_m$ V, $(CH_2)_mCH(NH_2)$-CO_2H, $CH(CO_2H)$-$(CH_2)_mCO_2H$, V = NH_2 or CO_2H and m = 1 to 4, e) carboxylic acids substituted with one or more hydroxyl, keto and/or further carboxyl groups, f) dimethylbiguanide, g) pyrimidine nucleosides and pyrimidine nucleotides, h) trehalose, glucosamine. The invention also relates to a drug based on the t-PA derivative K2P pro as the active ingredient and a process for producing it.

(57) Zusammenfassung

Pharmazeutisches Präparat eines nicht glykosylierten t-PA-Derivats K2P pro mit einer enzymatischen Aktivität von mindestens 1,4 MU/ml und einem pH-Wert von 4,5 bis 6,5, das Citrat und mindestens eine Verbindung aus der aus a) Ascorbinsäure, b) EDTA, c) Aminoverbindungen der Formel R^1R^2N - R - X, wobei X = SO_3H, $CH(NH_2)$-CO_2H, CO_2H, H, NH_2 oder OH ist, R = C_1-C_9-Alkylen, vorzugsweise C_4-C_7-Alkylen, C_3-C_6-Cycloalkylen oder Benzyliden ist und R^1 und R^2 voneinander unabhängig H oder C_1-C_3-Alkyl sind, d) Guanidin-analoge Verbindungen der Formel (I) wobei Y = H_2N+ oder 0 ist, Z = H oder $(CH_2)_m$ V, $(CH_2)_mCH(NH_2)$-CO_2H, $CH(CO_2H)$-$(CH_2)_mCO_2H$ ist, V = NH_2 oder CO_2H und m = 1 bis 4 ist, e) mit einer oder mehreren Hydroxy-, Keto- oder/und weiteren Carboxylgruppen substituierten Carbonsäuren, f) Dimethylbiguanid, g) Pyrimidinnukleosiden und Pyrimidinnukleotiden, h) Trehalose, Glucosamin bestehenden Gruppe enthält sowie Arzneimittel auf Basis des t-PA-Derivats K2P pro als Wirkstoff und Verfahren zu seiner Herstellung.

DESCRIPTION

The human tissue plasminogen activator (t-PA) possesses a great therapeutic importance in the case of the dissolving of blood coagula, e.g. in the case of heart infarcts. t-PA brings about the dissolving of the blood coagula by the activation of plasminogen to plasmin. Plasmin in turn dissolves fibrin, the main component of the protein matrix of coagulated blood.

Natural t-PA is composed of several functional domains F, E, K1, K2 and P. The domain P contains the proteolytically-active centre which brings about the cleavage of plasminogen to plasmin. The gene technological production of t-PA or of different t-PA muteins, in which some of the domains F, E, K1 and K2 are deleted, in eukaryotic and prokaryotic cells is already known. In contradistinction to natural t-PA, t-PA derivatives are thereby synthesised in non-glycosylated form.

Furthermore, it is known that the sugar part has a considerable influence in the solubility and aggregation of proteins (J. Biol. Chem. 263 (1988), 8832-8837). It was now ascertained that a non-glycosylated t-PA mutein with the domain composition K2P possesses a substantially poorer solubility than, perhaps, glycosylated t-PA derivatives. This non-glycosylated t-PA variant dissolves to only a small

extent in the buffers usually employed for the solubilisation of proteins, such as e.g. 50 mmol/l. Na citrate, pH 6, 50 mmol/l. phosphate buffer or physiological NaCl solution. However, for the use as therapeutically active material, the non-glycosylated t-PA derivative K2P pro should be present with a distinctly higher enzymatic activity of at least 1.4 MU/ml., preferably of 1.4 to 10 MU/ml.

From EP-A-0 217 379, it is known to increase the solubility of t-PA from prokaryotes (t-PA pro) by means of neutral or slightly alkaline arginine formulations. However, a disadvantage of this process is that good solubilities of t-PA pro can only be achieved with very high arginine concentrations. Furthermore, the stability of the highly concentrated t-PA derivative K2P pro is low under neutral or slightly alkaline conditions.

Consequently, it is the task of the invention to develop formulations which contain the non-glycosylated t-PA derivative K2P pro with an enzymatic activity of at least 1.4 MU/ml., whereby the stability of the t-PA derivative is to remain over a comparatively long period of time.

The task according to the invention is solved by a pharmaceutical preparation of a non-glycosylated t-PA derivative K2P pro with an enzymatic activity of at least 1.4 MU/ml. and a pH value of 4.5 to 6.5,

whereby this composition contains at least one compound from the group consisting of

a) ascorbic acid,

b) EDTA,

c) amino compounds of the formula

$$R^1R^2N - R - X$$

whereby X = SO_3H, $CH(NH_2)-CO_2H$, CO_2H, H, NH_2 or OH, R = C_1-C_9-alkylene, preferably C_4-C_7-alkylene, C_3-C_6-cycloalkylene or benzylidene and R^1 and R^2, independently of one another, are H or C_1-C_3-alkyl,

d) guanidine analogues of the formula

$$H_2N - \overset{\overset{Y}{\|}}{C} - \overset{\overset{H}{|}}{N} - Z$$

whereby Y = H_2N^+ or O, Z = H or $(CH_2)_mV$, $(CH_2)_mCH(NH_2)-CO_2H$, $CH(CO_2H)-(CH_2)_mCO_2H$, V = NH_2 or CO_2H and m = 1 to 4,

e) carboxylic acids substituted with one or more hydroxyl, keto and/or further carboxyl groups,

f) dimethylbiguanide,

g) pyrimidine nucleosides and pyrimidine nucleotides,

h) trehalose, glucosamine, N-methylglucamine.

By K2P pro according to the present invention, one understands a t-PA derivative which consists of the kringle 2- and of the protease domain and thus begins at one of the amino acids 174-180 and ends with the amino acid 527. In addition, K2P pro can also contain partly or wholly the amino acids -3 (Gly) to +5 (Ile).

A protein is thereby preferred which begins at amino acid 176 and possibly from the region -3 to +5 also contains the amino acids Ser, Tyr, Gln added. This designation follows the nomenclature given in T.J.R. Harris, Protein Engineering, Volume 1 (1987) 449-458 for t-PA. The production of such a t-PA derivative K2P pro is described in EP-A 0 382 174. The enzymatic activity for K2P pro is given as standard unit U according to the definition of the WHO for t-PA. The determination of the activity takes place according to H. Lill, ZGIMAL 42 (1987), 478-486.

For the solubilisation of K2P pro, a citrate buffer has proved to be especially suitable. The citrate concentration is to amount e.g. to at least 5 mmol/l., preferably to 5 to 100 mmol/l., especially preferably to 50 mmol/l. The pH value is adjusted according to basicity of the added compound, preferably with HCl or a base, such as e.g. NaOH or KOH.

Surprisingly, it was ascertained that the solubility of non-glycosylated K2P pro in other buffer systems, e.g. phosphate buffer, at equal ionic strength and equal pH value, is substantially smaller. It has proved to be suitable to adjust the pH value of alkaline citrate solutions with HCl, i.e. that the composition additionally also contains chloride ions. In the presence of chloride ions, surprisingly, highly concentrated solutions of non-glycosylated K2P pro

are, namely, substantially more stable than e.g. in the presence of phosphate ions. The pH value of acidic citrate solutions is usually adjusted with naOH.

Suitable for a pharmaceutical preparation according to the invention is a pH value between 4.5 and 6.5, a pH value of 5 to 6 being preferred. Surprisingly, in the case of the formulations usually employed for native t-PA with a pH value of > 7, the stability of the non-glycosylated t-PA derivative K2P pro decreases considerably in the solution. Thus, the single-chain form of K2P pro is only stable for a few days at room temperature or comparatively high temperatures at pH 7.2 and pH 8 in arginine-buffered solutions.

The pharmaceutical preparation according to the invention contains ascorbic acid, preferably of 0.1 to 1 mol/l., especially preferably of 0.2 to 0.3 mol/l.

The concentration of EDTA should preferably amount to 1 to 200 mol/l., especially preferably 10 to 100 mmol/l.

For a composition according to the invention, as amino compounds are preferred taurine, ε-aminocaproic acid, tranexamic acid, lysine, ornithine, δ-aminovaleric acid, p-aminomethylbenzoic acid, 8-aminooctanoic acid and/or 7-aminoheptanoic acid. Especially preferred is the use of ε-aminocaproic acid, p-aminomethylbenzoic acid, 7-aminoheptanoic acid, 8-aminooctanoic acid,

tranexamic acid and/or lysine. The preferred concentrations amount to 0.5 to 20 mmol/l., especially preferably to 1 to 10 mmol/l.

Also suitable are 4-aminobutanol-1, 5-aminopentanol-1, 6-aminohexanol-1, 1,9-diaminononane, 1,8-diaminooctane, 1,7-diaminoheptane, 1,6-diaminohexane, 1,5-diaminopentane, 1,4-diaminobutane and/or 1,3-diaminopropane. The concentrations of the α,ω-diamines and α,ω-aminoalcohols suitable for a preparation according to the invention preferably amount to 10 to 100 mmol/l.

Taurine and analogous compounds are preferably used with 0.1 to 0.5 mol/l., especially preferably with 0.1 to 0.3 mol/l.

Furthermore, for a composition according to the invention, as guanidine-analogous compounds are preferably used urea, guanidinobutyric acid and/or arginine. The concentration of urea preferably amounts to 0.1 to 4 mol/l., especially preferably to 0.5 to 2 mol/l. For other guanidine-analogous compounds, the concentration preferably amounts to 10 to 200 mmol/l., especially preferably to 50 to 100 mmol/l.

As carboxylic acids which are substituted with hydroxyl, keto and/or further carboxyl groups are suitable e.g. malic acid, lactic acid, fumaric acid and/or 2-oxoglutaric acid. Their concentration preferably amounts to 0.001 to 1 mol/l., especially preferably to 0.01 to 0.5 mol/l.

Dimethylbiguanide is preferably used in concentrations of 50 to 400 mmol/l., especially preferably with 100 to 300 mmol/l.

As pyrimidine nucleosides or pyrimidine nucleotides are suitable e.g. thymidine, cytosine and uridine or the corresponding nucleotides. These substances are preferably used in concentrations of 1 to 300 mmol/l., especially preferably of 10 to 300 mmol/l.

Trehalose, glucosamine and N-methylglucamine are preferably used in concentrations of 1 to 500 mmol/l., especially preferably with 10 to 300 mmol/l.

Furthermore, a subject of the invention is a composition according to the invention which additionally contains one or more α-aminocarboxylic acids, especially histidine.

In the following is set out a series of especially preferred preparations according to the present invention. One formulation contains 50 mmol/l. Na citrate/HCl, pH 6, 2 mol/l. urea. A further formulation contains 50 mmol/l. Na citrate, pH 6, and 0.5 mol/l. to 1 mol/l. guanidine. Furthermore, a further formulation contains 50 mmol/l. Na citrate, pH 6, and 0.3 mol/l. taurine. Furthermore, a further formulation contains 50 mmol/l. Na citrate, pH 6, and 0.2 mol/l. to 0.3 mol/l. ascorbic acid. Furthermore, a further formulation contains 50 mmol/l. Na citrate/HCl, pH 6, and 300 mmol/l. dimethylbiguanide. A further

formulation contains 50 mmol/l. Na citrate/HCl, pH 6, and 10 to 300 mmol/l. thymidine, uridine or cytosine or 10 to 100 mmol/l. of one of the above-mentioned α,ω-diamines or one of the above-mentioned α,ω-aminoalcohols. A further formulation contains 50 mmol/l. Na citrate/HCl, pH 6, and 10 to 300 mmol/l. trehalose, glucosamine or N-methylglucamine.

Furthermore, an especially preferred formulation according to the invention contains 50 mmol/l. Na citrate/HCl, pH 6, and 1 mmol/l. to 10 mmol/l. ε-aminocaproic acid, δ-aminovaleric acid, 7-aminoheptanoic acid, 8-aminooctanoic acid, p-aminomethylbenzoic acid, L-lysine, ornithine or tranexamic acid. Surprisingly, already in small molar excess (10 to 40 fold), these substances bring about an outstanding solubility of K2P pro.

Further preferred are also formulations which contain 50 mmol/l. Na citrate/HCl, pH 6, and a guanidine-analogous compound, especially arginine and guanidinobutyric acid, in a concentration of 50 to 100 mmol/l. A further formulation contains 50 mmol/l. Na citrate, pH 6, and 10 to 500 mmol/l. malic acid, lactic acid, fumaric acid or 2-oxoglutaric acid. Furthermore, a further formulation contains 50 mmol/l. Na citrate, pH 6, and 10 to 100 mmol/l. EDTA.

As is to be seen from the Examples, combinations of several of the above-mentioned compounds with citrate

also bring about a very good solubility of K2P pro. Suitable are e.g. combinations of lysine with arginine, ornithine, glucosamine and/or thymidine or of EDTA with ε-aminocaproic acid, lysine, arginine, glycosamine and/or thymidine. However, also suitable are also other combinations of at least 2 of the above-mentioned compounds with citrate.

Finally, a subject of the invention is also a medicament based on K2P pro as active material in solution or as lyophilisate with the above-mentioned substances and possibly still further pharmaceutically compatible additive, adjuvant, carrier and filling materials.

The pharmaceutical preparations according to the invention are preferably used as injection and infusion solutions. This can take place in that a solution already ready for injection is made available which possesses the composition according to the invention. However, it is also possible to make available the pharmaceutical preparations in the form of lyophilisates. These are then reconstituted with per se known agents or solutions suitable for injection purposes. As injection medium, water is preferably used which contains the additives usual in the case of injection solutions, such as stabilising agents, solubilising agents, buffers and isotonic additives, for example a physiological NaCl concentration. Such additives are,

for example, mannitol, tartrate or citrate buffers, ethanol, complex formers, such as e.g. ethylenediaminetetraacetic acid and its non-toxic salts, as well as high molecular polymers, such as liquid polyethylene oxide, for viscosity regulation. Liquid carrier materials for injection solutions must be sterile and are preferably filled into ampoules.

Finally, the present invention also comprises the use of K2P pro for the production of the pharmaceutical preparations according to the invention.

The following Examples are to explain further concrete embodimental formulations of the invention.

Example 1.

Influence of urea on the solubility of a non-glycosylated t-PA mutein with the domain composition K2P.

In this Example is described the influence of urea on the solubility of K2P pro (production according to EP-A 0 382 174) in citrate-buffered solutions at pH 6.0. As is to be seen from Table 1, K2P pro is of only limited solubility in 50 mmol/l. citrate buffer at pH 6.0. By addition of urea, the solubility can be considerably improved. The optimum thereby lies at about 2 mol/l. urea.

Carrying out

170 ml. purified K2P pro (dissolved in 0.5 mol/l. arginine/H_2PO_4, pH 7.2) are concentrated by ultra-

filtration over an Amicon YM 10 membrane. In each case, 1 ml. of the concentrate (activity 5.8 MU/ml.) is dialysed against the buffers set out in Table 1. After centrifuging of the samples, the enzymatic activity is measured in the clear supernatant.

The enzymatic activity is given as volume activity in MU/ml. and as total activity in MU.

The measurement of the K2P activity can thereby be determined in the usual way by cleavage of a chromogenic substrate (H. Lill, ZGIMAL 42 (1987), 478-486). The unit U is a unit of the activity for t-PA according to the definition of the WHO, National Institute for Biological Standards and Control.

Table 1

buffer	activity MU/ml.	activity MU
50 mmol/l. Na citrate/HCl, pH 6.0 8 mol/l. urea	1.03	1.24
50 mmol/l. Na citrate/HCl, pH 6.0 6 mol/l. urea	2.76	3.59
50 mmol/l. Na citrate/HCl, pH 6.0 4 mol/l. urea	3.46	4.67
50 mmol/l. Na citrate/HCl, pH 6.0 2 mol/l. urea	4.20	5.67
50 mmol/l. Na citrate/HCl, pH 6.0 1 mol/l. urea	2.62	3.27
50 mmol/l. Na citrate/HCl, pH 6.0	0.32	0.93

Example 2

Influence of various substances on the solubility of K2P pro

In this Example is described the influence of various substances on the solubility of K2P pro in citrate-buffered solutions at pH 6. Outstanding solubilities (> 2 MU/ml.) were achieved with taurine, dimethylbiguanide, glucosamine, trehalose, N-methyl-glucamine, uridine, cytidine, p-aminomethylbenzoic acid, fumaric acid and oxoglutaric acid. Furthermore, it is to be seen that the citrate buffer in the case of equal molar concentration, brings about a better solubility of the t-PA derivative than an NH_4HCO_3, Tris or phosphate buffer.

Carrying out:

see Example 1

Concentrate:

activity: 5.8 MU/ml.

Table 2

buffer	activity MU/ml.	activity MU
50 mmol/l. Na citrate/Hcl, pH 6.0 50 mmol/l. ornithine	1.92	2.11
50 mmol/l. Na citrate/NaOH, pH 6.0 0.3 mol/l. taurine	2.67	4.00
50 mmol/l. Na citrate/NaOH, pH 6.0 0.3 mol/l. ascorbic acid	4.00	4.20
50 mmol/l. Na citrate/HCl, pH 6.0 10 mmol/l. EACA	3.88	5.80

buffer	activity	
	MU/ml.	MU
50 mmol/l. Na citrate/HCl, pH 6.0 10 mmol/l. L-lysine	2.46	3.32
50 mmol/l. Na citrate/HCl, pH 6.0 10 mmol/l. tranexamic acid	5.54	7.36
50 mmol/l. Na citrate/HCl, pH 6.0 0.3 mol/l. dimethylbiguanide	2.96	3.40
50 mmol/l. Tris/HCl, pH 7.2	0.04	0.06
50 mmol/l. NH_4HCO_3	0.12	0.19
50 mmol/l. Na_2HPO_4/H_3PO_4, pH 7.2	0.15	0.23
50 mmol/l. Na citrate/HCl, pH 6.0	0.32	0.93
50 mmol/l. Na citrate/HCl, pH 6 0.3 mol/l. glucosamine	2.02	2.02
50 mmol/l. Na citrate/HCl, pH 6 0.3 mol/l. trehalose	3.52	3.17
50 mmol/l. Na citrate/HCl, pH 6 0.1 mol/l. thymidine	1.57	1.88
50 mmol/l. Na citrate/HCl, pH 6 0.3 mol/l. uridine	6.32	7.58
50 mmol/l. Na citrate/HCl, pH 6 30 mmol/l. cytosine	3.8	4.94
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. p-aminomethylbenzoic acid	3.46	5.03
50 mmol/l. Na citrate/NaOH, pH 6 0.3 mol/l. malic acid	1.53	1.68
50 mmol/l. Na citrate/NaOH, pH 6 0.3 mol/l. lactic acid	1.59	1.98
50 mmol/l. Na citrate/NaOH, pH 6 0.3 mol/l. fumaric acid	4.32	5.16
50 mmol/l. Na citrate/NaOH, pH 6 0.3 mol/l. 2-oxoglutaric acid	4.24	4.66
50 mmol/l. Na citrate/HCl, pH 6 0.3 mol/l. N-methylglucamine	3.36	3.70

Example 3

Influence of ε-aminocaproic acid (EACA) on the solubility of K2P pro

Carrying out:

see Example 1

Concentrate:

activity: 6.3 MU/ml.

Table 3

buffer	activity MU/ml.	activity MU
50 mmol/l. Na citrate/HCl, pH 6.0	0.34	0.48
50 mmol/l. Na citrate/HCl, pH 6.0 10 mmol/l. EACA	3.81	4.57
50 mmol/l. Na citrate/HCl, pH 6.0 5 mmol/l. EACA	3.52	4.86
50 mmol/l. Na citrate/HCl, pH 6.0 1 mmol/l. EACA	2.70	3.83
50 mmol/l. Na citrate/HCl, pH 6.0 0.1 mmol/l. EACA	0.58	0.75

Table 3 shows that EACA, even in a concentration of 1 - 10 mmol/l. (10 - 40 fold molar excess in comparison with K2P pro), brings about a considerable improvement of the solubility in citrate-buffered solutions at pH 6.0.

Example 4

Influence of ascorbic acid on the solubility of K2P pro

Carrying out:

see Example 1

Concentrate:

activity: 6.3 MU/ml.

Table 4

buffer	activity MU/ml.	activity MU
50 mmol/l. Na citrate/HCl, pH 6.0	0.34	0.48
50 mmol/l. Na citrate/NaOH, pH 6.0 0.3 mol/l. ascorbic acid	4.04	4.24
50 mmol/l. Na citrate/NaOH, pH 6.0 0.2 mol/l. ascorbic acid	2.40	2.93

The results of Table 4 show that, in citrate-buffered solution, the solubility of K2P pro is further increased by addition of ascorbic acid.

Example 5

pH Dependency of the stability of K2P pro in arginine-containing solutions

Purified K2P pro is dialysed against the 0.5 mol/l. Arg/H_3PO_4 buffer set out in Table 5 and stored in portions at -20, 25 and 37°C. After 3, 7, 14 and 21 days, in each case 1 sample is tested for activity and stimulatability by addition of fibrin and analysed SDS electrophoretically (starting values: activity: 1.3 MU/ml., stimulatability: 28). The data summarised in Table 5 show that samples stored at pH 8 and pH 7.2 display, after 3 and 7 days storage, respectively, at 37°C., a distinct increase of the activity with simultaneous decrease of the stimulatability. At 25°C., it also results, however chronologically delayed, in

an increase of the activity and decrease of the stimulatability. After 14 to 21 days, a reduction of the activity is then also observed.

Table 5

time	activity (MU/ml.)/stimulatability -20°C.	25°C.	37°C.
pH 8.0			
3 days	1.22/36	1.47/24	1.45/15
7 days	1.23/44	1.87/15	2.38/13
14 days	1.37/33	2.08/9	1.1/10
21 days	1.37/ -	2.14/10	1.0/7
pH 7.2			
3 days	1.12/39	1.5/27	1.7/20
7 days	1.16/45	1.7/21	2.1/13
14 days	1.38/32	1.7/9	1.9/9
21 days	1.22/25	1.7/11	1.52/8
pH 6.0			
3 days	0.93/34	1.43/31	1.54/34
7 days	1.05/46	1.27/42	1.58/36
14 days	1.23/39	1.04/20	1.45/14
21 days	1.43/26	0.95/16	1.00/8
pH 5.5			
3 days	0.85/32	1.47/40	1.08/39
7 days	1.4/47	1.53/68	1.54/48
14 days	1.2/32	1.3/26	1.3/24
21 days	1.36/26	0.95/25	0.95/21

Parallel to these changes, a decrease of the molecular weight of K2P pro (electrophoresis with SDS-PAGE) is observed.

Example 6

Influence of chloride and phosphate ions on the stability of K2P pro in arginine (Arg)-containing solutions

Purified K2P pro is dialysed against the arginine-containing buffers set out below and stored in portions at -20, 25 and 37°C. After 2, 7 and 14 days, in each case 1 sample is analysed SDS-electrophoretically. It is shown that, in the presence of chloride ions, the single-chain form is substantially more stable than in the presence of phosphate ions. Whereas in chloride ion-containing solutions, after 7 days storage at 37°C. at pH 7.2 and 8, only 10% to 20% of the sample are cleaved, in the phosphate-buffered solutions the proportion of the cleaved material amounts to 60% to 90%.

Buffers:

0.5 mol/l. Arg/H_3PO_4, pH 8.0

0.5 mol/l. Arg/HCl, pH 8.0

0.5 mol/l. Arg/H_3PO_4, pH 7.2

0.5 mol/l. Arg/HCl, pH 7.2.

Example 7

Influence of ε-aminocaproic acid on the solubility of K2P pro in citrate- and phosphate-buffered solutions

Purified K2P pro is concentrated by ultra-filtration to 4.2 MU/ml. and dialysed against the buffers given in Table 8 and centrifuged. After

centrifuging of the samples, the activity is measured in the clear supernatant.

Table 6

buffer	activity	
	MU/ml.	MU
50 mmol/l. Na citrate/HCl, pH 6.0	0.32	0.93
50 mmol/l. Na_2HPO_4/H_3PO_4, pH 6	0.07	0.10
50 mmol/l. Na citrate/HCl, pH 6 2 mmol/l. EACA	3.30	4.60
50 mmol/l. Na_2HPO_4/H_3PO_4, pH 6 2 mmol/l. EACA	0.90	1.39
50 mmol/l. Na citrate/HCl, pH 6 2 mmol/l. EACA 0.15 mol/l. NaCl	2.43	3.52
50 mmol/l. Na_2HPO_4/H_3PO_4, pH 6 2 mmol/l. EACA 0.15 mol/l. NaCl	1.08	1.57

Table 6 shows that the improvement of the solubility due to EACA in citrate-buffered solutions is considerably better than in phosphate buffers.

Example 8

Influence of tranexamic acid (TEA) on the solubility of K2P pro

Carrying out:

see Example 1.

Concentrate:

activity: 4.2 MU/ml.

Table 7

buffer	activity MU/ml.	activity MU
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. TEA	2.66	3.60
50 mmol/l. Na citrate/HCl, pH 6 5 mmol/l. TEA	2.54	3.30
50 mmol/l. Na citrate/HCl, pH 6 1 mmol/l. Tea	2.35	3.29

The results show that with tranexamic acid (TES)[1], a similar solubility of K2P pro is to be achieved as with EACA.

1) trans-4-aminomethylcyclohexanecarboxylic acid

Example 9

Influence of ω-aminocarboxylic acids on the solubility of K2P pro

Carrying out:

see Example 1

Concentrate:

activity: 4.2 MU/ml.

Table 8

buffer	activity MU/ml.	activity MU
50 mmol/l. Na citrate/HCl, pH 6	0.32	0.93
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. 8-aminooctanoic acid	3.12	4.05
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. 7-aminoheptanoic acid	3.54	4.42
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. EACA	4.22	2.90
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. ζ-aminovaleric acid	1.83	2.47

Example 10

Influence of guanidine analogues on the solubility of K2P pro

Carrying out:

see Example 1

Concentrate:

activity: 4.2 MU/ml.

Table 9

buffer	activity	
	MU/ml.	MU
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. arginine	1.42	2.06
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. guanidinobutyric acid	2.21	3.08

The results show that the solubility of K2Ppro is clearly improved with a guanidino group.

Example 11

Influence of EDTA on the solubility behaviour of K2P pro

Carrying out:

see Example 1

Concentrate:

activity: 4.2 MU/ml.

Table 10

buffer	activity MU/ml.	activity MU
10 mmol/l. EDTA/NaOH, pH 6	0.02	0.03
0.3 mol/l. EDTA/NaOH, pH 6	1.95	1.95
50 mmol/l. Na citrate/NaOH, pH 6 50 mmol/l. EDTA	1.80	2.25
50 mmol/l. Na citrate/NaOH, pH 6 100 mmol/l. EDTA	3.36	4.03
50 mmol/l. Na citrate/HCl, pH 6	0.32	0.93

Table 10 shows that the combination of EDTA with citrate has more than an additive effect on the solubility of K2P pro.

Example 12

Influence of amino acids alone or in combination on the solubility of K2P pro

Carrying out:

see Example 1

Concentrate:

activity: 4.2 MU/ml.

Table 11

buffer	activity MU/ml.	activity MU
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. arginine	1.42	2.06
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. L-lysine	2.81	3.94
50 mmol/l. Na citrate/HCl, pH 6 1 mmol/l. L-lysine	2.00	2.6

50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. L-lysine 10 mmol/l. arginine	2.56	3.84
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. lysine 50 mmol/l. arginine	3.27	4.9
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. lysine 10 mmol/l. arginine 10 mmol/l. ornithine	3.18	4.13
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. lysine 50 mmol/l. arginine 10 mmol/l. ornithine	2.70	3.78
50 mmol/l. Na citrate/HCl, pH 6 10 mmol/l. lysine 50 mmol/l. arginine 50 mmol/l. ornithine	3.34	4.30

Example 13

Influence of ω-aminoalcohols on the solubility of

K2P pro

Carrying out:

see Example 1

Concentrate:

activity: 4.9 MU/ml.

Table 12

buffer	activity	
	MU/ml.	MU
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. 4-aminobutanol-1	1.84	2.4
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. 5-aminopentanol-1	2.54	3.55

Example 14

Influence of various substances in combination on the solubility of K2P pro

Carrying out:

see Example 1

Concentrate:

activity: 5.5 MU/ml.

Table 13

buffer	activity MU/ml.	activity MU
50 mmol/l. Na citrate, pH 6 50 mmol/l. EDTA 1 mmol/l. EACA 50 mmol/l. glucosamine	4.24	5.08 pH
50 mmol/l. Na citrate, pH 6 10 mmol/l. EDTA 1 mmol/l. EACA 10 mmol/l. glucosamine	3.63	5.08
50 mmol/l. Na citrate, pH 6 10 mmol/l. EDTA 1 mmol/l. EACA 10 mmol/l. glucosamine 50 mmol/l. thymidine	3.70	5.14
50 mmol/l. Na citrate, pH 6 50 mmol/l. EDTA 1 mmol/l. lysine 50 mmol/l. glucosamine	4.00	5.40
50 mmol/l. Na citrate, pH 6 50 mmol/l. EDTA 1 mmol/l. lysine 10 mmol/l. glucosamine	3.04	3.95
50 mmol/l. Na citrate, pH 6 10 mmol/l. EDTA 1 mmol/l. lysine 10 mmol/l. glucosamine 10 mmol/l. thymidine	3.12	4.52

buffer	activity MU/ml.	activity MU
50 mmol/l. Na citrate, pH 6 10 mmol/l. EDTA 1 mmol/l. lysine 10 mmol/l. arginine 10 mmol/l. glucosamine 10 mmol/l. thymidine	2.60	3.77
50 mmol/l. Na citrate, pH 6 10 mmol/l. EDTA 1 mmol/l. lysine 25 mmol/l. arginine 10 mmol/l. glucosamine 10 mmol/l. thymidine	2.94	4.12
50 mmol/l. Na citrate, pH 6 1 mmol/l. lysine 10 mmol/l. arginine 10 mmol/l. glucosamine 10 mmol/l. thymidine	2.80	3.64
50 mmol/l. Na citrate, pH 6 10 mmol/l. EDTA 1 mmol/l. lysine 25 mmol/l. arginine 10 mmol/l. glucosamine 25 mmol/l. thymidine	3.20	4.32
50 mmol/l. Na citrate, pH 6 10 mmol/l. EDTA 1 mmol/l. lysine 25 mmol/l. arginine 10 mmol/l. glucosamine 50 mmol/l. thymidine	4.56	5.90
50 mmol/l. Na citrate, pH 6 1 mmol/l. lysine 25 mmol/l. arginine 10 mmol/l. glucosamine 50 mmol/l. thymidine	3.24	4.54
50 mmol/l. Na citrate, pH 6 1 mmol/l. lysine 25 mmol/l. arginine 10 mmol/l. glucosamine 25 mmol/l. thymidine	3.54	4.95

buffer	activity	
	MU/ml.	MU
40 mmol/l. Na citrate, pH 6 1 mmol/l. lysine 25 mmol/l. arginine 50 mmol/l. glucosamine 25 mmol/l. thymidine	3.60	4.68
50 mmol/l. Na citrate, pH 6 1 mmol/l. lysine 25 mmol/l. arginine 100 mmol/l. glucosamine 25 mmol/l. thymidine	5.85	5.85

Example 15

Influence of α,ω-diamine on the solubility of K2P pro

Carrying out:

see Example 1

Concentrate:

activity: 4.9 MU/ml.

Table 14

buffer	activity	
	MU/ml.	MU
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. 1,9-diaminononane	3.12	4.05
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. 1,8-diaminooctane	2.80	3.64
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. 1,6-diaminohexane	3.42	4.45
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. 1,5-diaminopentane	3.52	4.40
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. 1,4-diaminobutane	3.96	5.15
50 mmol/l. Na citrate/HCl, pH 6 50 mmol/l. 1,3-diaminopropane	3.33	4.23

SUMMARY

Pharmaceutical preparation of a non-glycosylated t-PA derivative K2P pro with an enzymatic activity of at least 1.4 MU/ml. and a pH value of 4.5 to 6.5, which contains citrate and at least one compound from the group consisting of

a) ascorbic acid

b) EDTA,

c) amino compounds of the formula

$$R^1R^2N - R - X$$

whereby X = SO_3H, $CH(NH_2)-CO_2H$, CO_2H, H, NH_2 or OH, R = C_1-C_9-alkylene, preferably C_4-C_7-alkylene, C_3-C_6-cycloalkylene or benzylidene and R^1 and R^2, independently of one another, are H or C_1-C_3-alkyl,

d) guanidine analogues of the formula

$$H_2N - \overset{\overset{Y}{\|}}{C} - NH - Z$$

whereby Y = H_2N^+ or O, Z = H or $(CH_2)_mCH(NH_2)-CO_2H$, $CH(CO_2H)-(CH_2)_mCO_2H$, $(CH_2)_mV$, V = NH_2 or CO_2H and m = 1 to 4,

carboxylic acids substituted with one or more hydroxyl, keto and/or further carboxyl groups,

f) dimethylbiguanide,

g) pyrimidine nucleosides and pyrimidine nucleotides,

h) trehalose, glucosamine,

as well as medicaments based on the t-PA derivative K2P pro as active material and processes for its production.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A pharmaceutical preparation comprising:

a non-glycosylated t-PA derivative K2P pro with an enzymatic activity of at least 1.4 MU/ml., a citrate buffer and at least one compound from the group consisting of:

a) ascorbic acid,

b) EDTA,

c) amino compounds of the formula

$$R^1R^2N\text{-}R\text{-}X$$

whereby X = SO_3H, $(CH(NH_2)\text{-}CO_2H$, CO_2H, H, NH_2 or OH, R = C_1-C_9-alkylene, C_3-C_6-cycloalkylene or benzylidene and R^1 and R^2, independently of one another, are H or C_1-C_3-alkyl,

d) guanidine analogous compounds of the formula

$$H_2N - \overset{\overset{Y}{\|}}{C} - \overset{\overset{H}{|}}{N} - Z$$

whereby Y = H_2N^+ or 0, Z = H or $(CH_2)_mV$, $(CH_2)_mCH(NH_2)\text{-}CO_2H$, $CH(CO_2H)\text{-}(CH_2)_mCO_2H$, V = NH_2 or CO_2H and m = 1 to 4,

e) carboxylic acids substituted with one or more hydroxyl, keto and further carboxyl groups,

f) dimethylbiguanide,

g) pyrimidine nucleosides and pyrimidine nucleotides,

h) trehalose, glucosamine, N-methylglucamine,

said preparation having a pH value of 4.5 to 6.5.

2. A pharmaceutical preparation according to claim 1, wherein the amino compounds c) are selected from taurine, ε-aminocaproic acid, tranexamic acid, lysine, ornithine, δ-aminovaleric acid, p-aminomethyl-benzoic acid, 4-aminobutanol-1, 5-aminopentanol-1, 6-aminohexanol-1, 1,9-diaminononane, 1,8-diaminooctane, 1,7-diaminoheptane, 1,6-diaminohexane, 1,5-diamino-pentane, 1,4-diaminobutane, 1,3-diaminopropane, 8-aminooctanoic acid and 7-aminoheptanoic acid.

3. A pharmaceutical preparation according to claim 1 or 2, wherein the guanidine analogous compounds d) are selected from urea, guanidinobutyric acid and arginine.

4. A pharmaceutical preparation according to claim 1 or 2, wherein the substituted carboxylic acids e) are selected from malic acid, lactic acid, fumaric acid and oxoglutaric acid.

5. A pharmaceutical preparation according to claim 1, 2, 3 or 4, additionally containing one or more α-aminocarboxylic acids.

6. A pharmaceutical preparation according to claim 1, 2, 3 or 4, additionally containing histidine.

7. A pharmaceutical preparation according to claim 1, 2, 3, 4, 5 or 6, wherein said citrate buffer is in a concentration of 5 to 100 mmol/l.

8. A pharmaceutical preparation according to claim 7, wherein said concentration of citrate buffer is 50 mmol/l.

9. A pharmaceutical preparation according to claim 1, 2, 3, 4, 5, 6 or 8, additionally containing chloride ions.

10. A pharmaceutical preparation according to claim 7, additionally containing chloride ions.

11. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate, 0.1 to 1 mol/l ascorbic acid and having a pH of 6.

12. A pharmaceutical preparation according to claim 11, containing 0.2 to 0.3 mol/l of said ascorbic acid.

13. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate, 1 to 200 mmol/l EDTA and having a pH of 6.

14. A pharmaceutical preparation according to claim 13, containing 10 to 100 mmol/l of said EDTA.

15. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate, 0.1 to 0.5 mol/l taurine and having a pH of 6.

16. A pharmaceutical preparation according to claim 15, containing 0.1 to 0.3 mol/l of said taurine.

17. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate, 0.5 to 20 mmol/l of an amino compound selected from ε-amino-caproic acid, δ-aminovaleric acid, lysine, ornithine, tranexamic acid, p-aminomethylbenzoic acid, 7-amino-heptanoic acid and 8-aminooctanoic acid and having a pH of 6.

18. A pharmaceutical preparation according to claim 17, containing 1 to 10 mmol/l of said amino compound.

19. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate/HCl, 10 to 100 mmol/l 4-aminobutanol-1, 5-aminopentanol-1, 6-aminohexanol-1, 1,9-diaminononane, 1,8-diaminooctane, 1,7-diaminoheptane, 1,6-diaminohexane, 1,5-diaminopentane, 1,4-diaminobutane or 1,3-diaminopropane and having a pH of 6.

20. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate/HCl, 0.1 to 4 mol/l urea and having a pH of 6.

21. A pharmaceutical preparation according to claim 20, containing 0.5 to 2 mol/l of said urea.

22. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate/HCl, 10 to 200 mmol/l guanidinobutyric acid or arginine and having a pH of 6.

23. A pharmaceutical preparation according to claim 22, containing 50 to 100 mmol/l of said guanidinobutyric acid or arginine.

24. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate, 0.001 to 1 mol/l malic acid, lactic acid, fumaric acid or 2-oxo-glutaric acid and having a pH of 6.

25. A pharmaceutical preparation according to claim 24, containing 0.01 to 5 mol/l of said malic acid, lactic acid, fumaric acid or 2-oxoglutaric acid.

26. A pharmaceutical preparation according to claim 1, containing 50 mmol/l dimethylbiguanide and having a pH of 6.

27. A pharmaceutical preparation according to claim 26, containing 100 to 300 mmol/l of said dimethylbiguanide.

28. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate/HCl, 1 to 300 mmol/l thymidine, cytosine or uridine and having a pH of 6.

29. A pharmaceutical preparation according to claim 28, containing 10 to 300 mmol/l of said thymidine, cytosine or uridine.

30. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate/HCl, 1 to 500 mmol/l trehalose, glucosamine or N-methylglucamine and having a pH of 6.

31. A pharmaceutical preparation according to claim 30, containing 10 to 300 mmol/l of said trehalose, glucosamine or N-methylglucamine.

32. A pharmaceutical preparation according to claim 1, containing 50 mmol/l Na citrate and a combination of compounds from the groups a) to h) according to claim 1 and having a pH of 6.

33. A pharmaceutical preparation according to claim 1, 2, 3, 4, 5, 6, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32, further including a pharmaceutically acceptable carrier.

34. A pharmaceutical preparation according to claim 33, further including at least one pharmaceutical additive or adjuvant.

35. A process for the preparation of a pharmaceutical preparation according to claim 10, comprising converting the non-glycosylated t-PA derivative K2P pro, together with at least one substance from the groups a) to h) according to claim 1, into a suitable pharmaceutical form of administration.

36. A process according to claim 35, wherein the pharmaceutical form of administration is an injection solution or a lyophilisate.

37. Use of non-glycosylated t-PA derivative K2P pro for the production of a pharmaceutical preparation according to claim 1, 2, 3, 4, 5, 6, 8, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31 or 32.

38. A pharmaceutical preparation according to claim 1, wherein R is C_4-C_7-alkylene