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(54) **Title:** A METHOD FOR REDUCING POTENTIAL VIRUS BURDEN IN A SAMPLE BY CYANATE TREATMENT

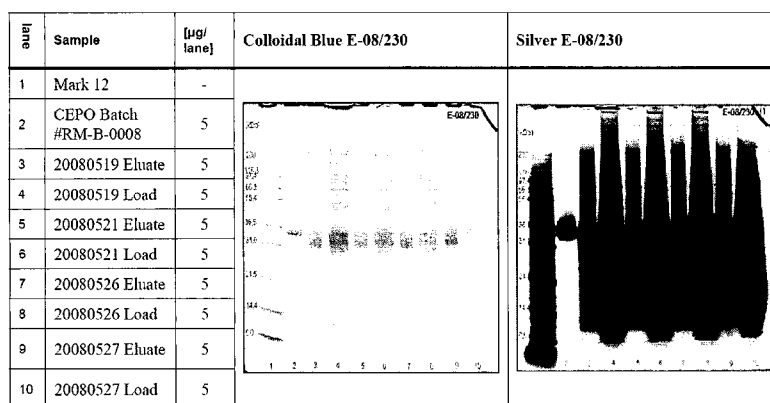


Fig. 1

(57) **Abstract:** The invention relates to a method for reducing viral contamination in a sample comprising the step of adding cyanate to said sample as well as a method for carbamylating erythropoietin obtained from a crude harvest by using this virus reducing step.

A method for reducing potential virus burden in a sample by cyanate treatment

FIELD OF THE INVENTION

- 5 The present invention relates to a method for reducing virus contamination in a sample using cyanate. In particular, this method is suitable in the carbamylation process of making carbamylated erythropoietin from a crude harvest which potentially can contain virus contamination.

10 BACKGROUND OF THE INVENTION

- Carbamylated erythropoietin (CEPO) is a cytoprotective compound currently in development for acute ischemic stroke and Friedreich's Ataxia. It is chemically modified erythropoietin (EPO) by carbamylation of lysine residues (Leist et al. Science. 2004;305(5681):239-42 hereby incorporated by reference) and does
15 therefore not bind to the erythropoietin receptor whereby the haematopoietic side-effects are avoided. Despite the lack of binding to the erythropoietin receptor CEPO retains full cytoprotective properties, demonstrating that CEPO mediates its beneficial effects via a mechanism different from that via the classical erythropoietin receptor.

- 20 The impairment of biological hematopoietic activity of CEPO has been shown by Satake, R. et al. (1990) Biochimica et Biophysica Acta; 1038: 125-129 and Mun, K-C. and Golper, TA. (2000) Blood Purif.; 18: 13-17. Brines et al. 2003, US patent application 20030072737, showed that the loss of the hematopoietic activity did
25 not interfere with the tissue protective properties.

- Carbamylation of proteins is widely known when using urea in purification of proteins and as a result of high urea serum levels. This is caused by spontaneously decomposition of urea to cyanate. Cyanate is responsible for the carbamylation
30 of the primary amines of a protein, hence the N-terminal end and lysines of a protein are susceptible to carbamylation. Additionally, other potential amino acid residues susceptible to carbamylation are arginine, cysteine, tyrosine, aspartic acid, glutamic acid and histidine. The reaction is, however, pH dependent and does not proceed as readily as with the N- terminal and lysine residues.

Carbamylation and purification of CEPO has been described in the prior art. For example in WO 2006/002646 (hereby incorporated by reference) is described a method using purified EPO for carbamylation, followed by a purification by the use of gel filtration, anion-exchange chromatography and finally ultra- and diafiltration.

According to an aspect of the invention a method is provided for removal or reducing the load of virus contamination by cyanate treatment in a sample containing EPO or a derivative or an analogue thereof. In particular this treatment is beneficial when the final product, such as a protein, needs to be carbamylated and the start material can be contaminated by virus. According to one embodiment, this method uses EPO a derivative or an analogue thereof from a crude harvest as a starting material for the cyanate treatment in order to generate CEPO.

SUMMARY OF THE INVENTION

The present invention provides a method for reducing viral contamination in a sample comprising EPO a derivative or an analogue thereof, wherein said method comprises the step of adding cyanate to said sample in a concentration from about 0.05 M to about 2 M and for time period of about 1 hour or more.

The invention additionally provides a method for carbamylating EPO or a derivative or an analogue thereof comprising the steps of

- a) harvesting EPO or a derivative or an analogue thereof from a cell culture of host cells expressing said EPO or a derivative or an analogue thereof,
- b) treating the mixture obtained in step a) with cyanate, and
- c) optionally, stopping the carbamylation process.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 SDS-PAGE analysis and silver staining (5µg/lane) from crude harvest obtained before and after volume reduction. Lane 1 (Mark 12) is showing pure EPO purchased commercially, whereas lanes 2-10 shows, in addition to the band from

EPO, the various impurities from the cell culture or fermentation process. Lane 2 is showing the load obtained from the harvest, lanes 3, 5, 7 and 9 shows the eluate after volume reduction and lanes 4, 6, 8 and 10 shows composition at loading to an anion-exchange column.

5

Fig. 2 shows the volume reduction of the crude harvest performed in an anion-exchange column (CaptoQ from GE Healthcare). A step elution resulted in eight well comparable chromatograms with a typical elution profile of two peaks. The fraction F3 represented the EPO containing eluate.

10

Fig. 3 shows the HIC step with the resin Toyopearl Butyl 600M (Tosoh Biosciences) as a the subsequent step after carbamylation. The media containing the carbamylated EPO were conditioned with 3M ammonium sulphate to a final concentration of 0.6 mol/L. After a wash step with a lower ammonium sulphate concentration of 0.3 mol/L the elution was induced by a Tris buffer without AS at pH 7.5. The fraction between F4 and F5 represented the CEPO containing eluate.

15

DETAILED DESCRIPTION OF THE INVENTION

The manufacturing of many pharmaceutical proteins originate from biological sources wherein a cell culture of host cells expresses the desired protein. These cells, as well as the medium in which they grow, can be contaminated with viruses. For proteins used as pharmaceuticals, and in particular those for parental administration, it is mandatory to effectively remove and/or inactivate such a potential contamination.

25

The present inventors have found that by treating a virus contaminated sample with cyanate viruses are effectively removed and/or inactivated.

As the cyanate treatment also carbamylates primary amines of a protein, the invention is most useful when the carbamylation is desired, such as in a process of carbamylating EPO or derivatives or an analogue of EPO.

30

In another aspect of the invention, the inventors have discovered that it is possible to carbamylated EPO effectively from a crude harvest in spite of the impurities

from the cell culture or fermentation process. The invention thus also relates to a method for producing CEPO from a such harvest, which method at the same time ensures an effective virus removal step.

- 5 Erythropoietin can be produced in a variety of eukaryotic hosts including yeasts such as *Saccharomyces cerevisiae* and *Pichia Pastoria* and insect cells such as *Drosophila* cells and lepidopteran cells. Eukaryotic cells for expression also include mammalian cells lines such as Chinese hamster ovary (CHO) cells or Baby hamster kidney (BHK) cells. In an embodiment the starting material for the
10 present carbamylation is EPO or EPO derivatives or an analogue harvested from a transfected cell culture of CHO cells using a fed batch or a continuous process, such as a perfusion process.

- The starting material for the present invention may thus comprise EPO or deriva-
15 tives or an analogue of EPO either directly obtained from the cell culture or fermentation and/or subjected to a limited volume reduction, e.g. by filtration, centrifugation or one or more steps of chromatography. In the present invention, the term "harvest" is thus intended to refer to a solution comprising EPO or derivatives or an analogue of EPO obtained from a cell culture in which solution
20 essentially all of the naturally-occurring materials from the cell culture are present and potentially also a virus contamination. According to one embodiment of the invention about 10% or more of the naturally-occurring materials from the cell culture are present in the, such as e.g. about 15 %, about 20%, about 25% or about 30% or more of the naturally-occurring materials from the cell culture are
25 present in the solution comprising EPO an EPO derivate or an EPO analouge.

- In the present invention, EPO is intended to include any variant or derivative EPO which may be carbamylated (e.g described in US 2004157293 or Science, Vol. 35, pp 239-242 or WO 2006/050819 hereby incorporated by reference in its en-
30 tirety) by carbamylating at least one of the primary-amino groups (the lysines and the N-terminal group) of the protein. In particular, the invention relates to EPO with an amino acid sequence as depicted below in table 1 (SEQ ID NO 2) or comprising an additional arginine in the C-terminal end (SEQ ID NO 1), or a sequence which is 95%, 98% or 99% identical to SEQ ID NO 1 or 2.

Table 1

1	A	P	P	R	L	I	C	D	S	R	V	L	E	R	Y	L	L	E	A	K
21	E	A	E	N	I	T	T	G	C	A	E	H	C	S	L	N	E	N	I	T
41	V	P	D	T	K	V	N	F	Y	A	W	K	R	M	E	V	G	Q	Q	A
61	V	E	V	W	Q	G	L	A	L	L	S	E	A	V	L	R	G	Q	A	L
81	L	V	N	S	S	Q	P	W	E	P	L	Q	L	H	V	D	K	A	V	S
101	G	L	R	S	L	T	T	L	L	R	A	L	G	A	Q	K	E	A	I	S
121	P	P	D	A	A	S	A	A	P	L	R	T	I	T	A	D	T	F	R	K
141	L	F	R	V	Y	S	N	F	L	R	G	K	L	K	L	Y	T	G	E	A
161	C	R	T	G	D															

Table 1: Potential carbamylation sites are shown in bold and conventional amino acids in arial font.

There are nine potential carbamylation sites as shown in table 1: Alanine at position 1 and Lysine at positions 20, 45, 52, 97, 116, 140, 152 and 154. Accordingly, the invention relates to CEPO in which at least one or more, such as at least two, three, four, five, six, seven, eight or nine, of the of the amino acids selected from the group comprising alanine at position 1 and lysine at positions 20, 45, 52, 97, 116, 140, 152 and 154 (as shown in table 1) are carbamylated. Other potential amino acids that can be carbamylated are arginine, cysteine, tyrosine, aspartic acid, glutamic acid and histidine, thus, according to one embodiment the CEPO include these forms of carbamylated EPO as well.

Several analogues and derivatives of EPO are known from the literature (e.g. Leist et al., Science (2004). Vol. 305. no. 5681, pp. 239–242 and WO2009/094172 hereby incorporated by reference). According to one embodiment of the invention these may be used in the present invention.

Identity: The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "identity". For purposes of the present invention, the degree of identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch,

1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends in Genetics 16: 276-277), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension
5 penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows: (Identical Residues x 100)/(Length of Alignment - Total Number of Gaps in Alignment). In all cases, the accepted IUPAC single letter or triple letter amino acid abbrevia-
10 tion is employed.

According to an aspect of this invention is provided a method for reducing viral contamination in a sample from a cell culture process or fermentation process comprising the step of adding cyanate to said sample. According to one embodi-
15 ment the sample may comprise EPO or a derivative or analogue thereof.

The concentration of cyanate may be from about 0.05 M to about 2 M, such as from about 0.5 to about 1.5 M, or about 1 M.

20 The pH may be adjusted to a range from about 7 to about 11, such as about 8 to about 10, or about 9, using an appropriate buffer such as a borate buffer e.g. potassium tetraborate. The concentration of borate buffer may be from about 0.05 to about 2 M but in a preferred embodiment about 0.25 M as potassium tetraborate is used.

25 As shown in Example 5, the virus reduction is effective within the first hour, and becomes increasingly effective as the time period increases. Already after 1 hour a reduction of hybrid moloney amphotrophic murine leukemia virus (Mo/A-MuLV) is obtained. After about 44 hours the reduction factors for both virus types were
30 found to be $\geq 4.24 \pm 0.18 \log_{10}$ for Mo/A-MuLV and $\geq 5.80 \pm 0.30 \log_{10}$ for Minute Virus of Mice (MVM), which is a very effective virus removal.

The cyanate treatment can thus be carried out for time period of about 1 hour or more, such as from about 6 hours or more, about 24 hours or more, about 40 hours or more, or about 45 hours or more.

- 5 The temperature may be range from about 20°C to 50°C, preferably about 30°C to about 37°C, or about 30°C.

Combining the cyanate treatment with additional virus removal steps further will increase the virus reduction. By further including a membrane absorber step
10 (such as Sartobind S) and a nanofiltration step (such as Planova 15 N), the overall virus reduction obtained can be as much as $\geq 12.88 \pm 0.43 \log_{10}$ for Mo/A-MuLV and $\geq 12.66 \pm 0.56 \log_{10}$ for MVM. This means that even if the crude harvest carried as much as 69,000 retrovirus like particles per mL the probability of one virus particle to be present in a single dose is $10^{-7.09}$. Therefore less than
15 one out of 12.3 million doses might theoretically be contaminated with one single virus particle.

The virus removal step as outlined above, may be included as a carbamylation step in the second aspect of the invention, namely the manufacturing CEPO from
20 a crude harvest.

According to an aspect of the invention the crude harvest of EPO, or a derivative or an analogue thereof is subjected to a carbamylation step which results in a carbamylated product such as CEPO. Using a crude harvest instead of using pu-
25 rified EPO has the advantage that no prior purification steps needs to be performed before the carbamylation step. On an industrial scale this is a much more effective and cost saving way of producing e.g. CEPO.

According to the an aspect of the invention, the method comprises the steps of
30

- a) harvesting EPO or a derivative or an analogue thereof from a cell culture of host cells expressing said EPO, EPO derivative or EPO analogue,
- b) treating the mixture obtained in step b) with cyanate, and

c) optionally, stopping the carbamylation process.

Harvesting and volume reduction - Step a)

According to one embodiment of the invention the cell medium from the culture of
5 cells expressing EPO an analogue or a derivative thereof may be used directly
for the cyanate treatment or carbamylation in step b).

However, on a large scale production where the crude harvest is harvested over
several days from a fermentation it may be advantageous to reduce the volumes
10 of the crude harvest. The volume reduction may result in an increased concentra-
tion of EPO, an analogue or a derivative thereof, compared to the other compo-
nents. The volume reduction can be done, for example, by subjecting the crude
harvest to one or more steps of chromatography, such as *e.g.*, two, three, four,
five, six, seven or more steps. According to one embodiment anion-exchange
15 chromatography is used and preferably an anion-exchange chromatography with
strong anions, such as a quaternary amino group, *e.g.* $-N^+(CH_3)_3$, carried out, for
example, coupled to an agarose matrix such as Capto Q sold by GE Healthcare.

It is envisaged that the harvested cell medium may be subjected to centrifugation
20 and/or filtration whereby the cell debris and many proteins precipitate or is fil-
trated off. This step may be performed either before the volume reduction or af-
ter, or even as the only step for volume reduction.

Cyanate treatment of the crude harvest - Step b)

25 The crude harvest obtained from step a) is then subjected to a step of cyanate
treatment.

The concentration of cyanate may be from about 0.05 M to about 2 M, such as
from about 0.5 to about 1.5 M, or about 1 M.

30

The pH may be adjusted to a range from about 7 to about 11, such as about 8 to
about 10, or about 9, using an appropriate buffer such as a borate buffer *e.g.* po-
tassium tetraborate. The concentration of borate buffer may be from about 0.05

to about 2 M but in a preferred embodiment about 0.25 M as potassium tetraborate is used.

According to an embodiment, the crude harvest is mixed with potassium borate
5 tetra hydrate and potassium cyanate with a pH in the range of about 7 to about 11, preferably a pH about 9.0, and incubating the mixture at about 30°C to about 37°C, preferably about 30°C, for a time window of about 40 to about 60 hours, preferably at about 48 hours.

10

Stopping the cyanate process - Step c)

It is envisaged that the cyanate treatment or carbamylation in step b) may be stopped by adding a solution containing excess primary amines before subjecting
15 the mixture to further chromatography to remove unreacted cyanate and reaction products. For example, 3M ammonium sulfate in an appropriate buffer, such as a 150mM Tris buffer at a pH between 6 and 8 may be used for this purpose.

To stop the cyanate treatment or carbamylation process the reaction mixture ob-
20 tained in step b) can also be subjected to one or more chromatography steps whereby the cyanate is removed.

The chromatographic step used to remove the cyanate may be performed using gel filtration with Sephadex™ which is a bead-formed gel prepared by crosslink-
25 ing dextran with epichlorohydrin e.g. G-25 Fine (GE Healthcare) or by using a medium to high hydrophobic resin in hydrophobic interaction chromatography (HIC), such as a methacrylic polymer e.g. Butyl-600 resin sold by Tosoh Biosciences (Toyopearl® Butyl-600M).

30 Further purification of the cyanate treated product

The cyanate or carbamylated product may be further purified to the desired purity, and as stated earlier subjected to further virus removal steps, such as one or more membrane adsorber steps (e.g. Sartobind S) and/or one or more nanofiltration steps (e.g. Planova 15 N).

Additionally, the further purification may be as outlined in WO2006/002646 (hereby incorporated by reference) where the carbamylated product was desalted using gel filtration and subsequently purified using an anion step. However, it is envisaged that other chromatographic steps may be used, in combination with e.g. ultra- and diafiltration.

CEPO in a pharmaceutical composition

CEPO may be comprised in a pharmaceutical composition. The pharmaceutical compositions of the invention may comprise a therapeutically effective amount of CEPO and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized foreign pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as saline solutions in water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. A saline solution is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. The compounds of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, fer-

ric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of CEPO, preferably in purified form,
5 together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

Pharmaceutical compositions adapted for parenteral administration include
10 aqueous and non-aqueous sterile injectable solutions or suspensions, which may contain antioxidants, buffers, bacteriostats, and solutes that render the compositions substantially isotonic with the blood of an intended recipient. Other components that may be present in such compositions include water, alcohols, polyols, glycerine and vegetable oils, for example. Compositions adapted for parenteral
15 administration may be presented in unit-dose or multi-dose containers, for example sealed ampules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of a sterile liquid carrier, e.g., sterile saline solution for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, and tablets.
20 In one embodiment, an autoinjector comprising an injectable solution of a compound of the invention may be provided for emergency use by ambulances, emergency rooms.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water-free concentrate in a hermetically-sealed container such as an ampule or sachette indicating the quantity of active agent.
25
30 Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where

the composition is administered by injection, an ampule of sterile saline can be provided so that the ingredients may be mixed prior to administration.

5 EXAMPLES

Example 1

Upstream

Erythropoietin (EPO) was expressed from a CHO-derived cell line in a perfusion
10 process. Media was harvested over 21 days, with accumulated harvested media sent for capture of EPO every third day. Each harvest pool was stored at 4°C before capture. A total of 216 L of harvest was collected in the 7 pools. The protein concentration may optionally be diluted e.g. 1:2 to 1:3.

15 Example 2

Volume reduction

EPO was captured by passage of media from 3 days of harvest over a 70 x 200 mm (diameter x height) of CaptoQ anion-exchange chromatography resin (GE Healthcare). After washing of the column using 20mM Tris/30mM NaCl, pH 7.5,
20 the cell harvest (Example 1) was loaded to the column and bound protein was eluted using a buffer containing 190mM NaCl, 20 mM Tris, pH 7.5. A total of 8 rounds of capture were performed, with EPO loads between 1.5 and 5.6 mg per mL of resin. Chromatography was performed at ambient temperature, and eluates were stored at 2 – 8°C after sterile filtration until all EPO-containing harvests
25 had been processed. Figure 3 shows chromatograms with the typical elution profile and figure 4 shows the volume reduction obtained.

Example 3

Carbamylation

30 Carbamylation of EPO was achieved by adding, to the pooled capture eluates, an equal volume of 1M potassium cyanate/0.25M potassium tetraborate, pH 9.0, and incubating at 29°C for 48 hours. The reaction was stopped by cooling to room temperature, adding 3M ammonium sulphate/150mM Tris-HCl, pH 7.5 (300 mL per L of reaction solution) and hydrophobic interaction chromatography (HIC).

A typical protocol for the carbamylation could be as shown in Table II

5 **Table II**

Step	Buffer description	Temperature [°C]	Duration[h]
Buffer pre- paration	1 M Potassium cyanate, 0.25 M Potassium Tetraborate, pH 9.0	29 °C	4 h
Start	Capture eluate pool diluted with 1 M Potassium cyanate, 0.25 M Potassium Tetraborate, pH 9.0 in ratio of 1:2 (w/w)	29 °C	< 0.5 h
Incubation	---	29 °C	48 h
Stop	3 M Ammonium sulphate, 150 mM Tris, pH 7.5	Room temperature	< 2 h

Example 4

Stopping the carbamylation process

The carbamylation process may be stopped by adding ammonium sulfate mixture as in Example 3, such as about 3M ammonium sulfate in an appropriate buffer, such as a 150 mM Tris buffer at a neutral pH (e.g. pH 6 – 8). Alternatively, or in combination herewith, a desalting step may be performed by using gel filtration (e.g. as disclosed in WO2006/002646 hereby incorporated by reference) by for example employing a running buffer of: 0.3% Tris (25mM), 0.3 % (50 mM) NaCl, pH 8.5; and a elution buffer of: 0.3% Tris (25mM), 5.8 % (1 M) NaCl, pH 8.5. The gradient may be 0-30% over 20 column volumes. The G-25 fine (Amersham Biosciences) may be employed.

Alternatively, hydrophobic interaction chromatography may used in order to stop the carbamylation, preferably in combination with the ammonium sulfate treatment described above. The carbamylation batch may thus be diluted with the 3 M ammonium sulfate in a buffer such as 150 mM Tris, for example at a pH of 7 - 8. Elution of the carbamylated protein from the column can be performed using a similar buffer lacking ammonium sulphate.

Figure 5 shows chromatograms with the typical elution profile using hydrophobic interaction chromatography (Toyopearl, Butyl-600M (Tosoh Biosciences).)

5 Example 5

Determination of virus removal and inactivation by carbamylation

In order to demonstrate the safety of pharmaceutical proteins derived from biological sources it is mandatory for the manufacturer of such products to demonstrate the effective inactivation and/or removal of pathogenic viruses during the manufacturing process. Usually, this is done by the deliberate spiking of a down-scaled version of the manufacturing process with relevant and/or model viruses.

The purpose of this example was to test for the effectiveness of carbamylation (Example 3), to remove/inactivate viruses during the manufacturing process CEPO. Appropriate model viruses for this process are Murine Leukemia Virus (Mo/A-MuLV) (ATCC VR-1450), an enveloped RNA retrovirus and Minute Virus of Mice (MVM) (ATCC VR-1346), a non-enveloped small DNA virus. These viruses vary in their biophysical and structural features and they display a variation in resistance to physical and chemical agents or treatments.

Summary of the results

The enveloped virus **MuLV** is efficiently removed/inactivated by carbamylation treatment and nanofiltration.

The non-enveloped virus **MVM** is efficiently removed/inactivated by carbamylation treatment and nanofiltration.

The following minimum Log₁₀ reduction factors were obtained in the final product containing fractions:

Table III

Virus	MuLV	MVM
Process		
Carbamylation (after 44 hours incubation)	≥ 4.24	≥ 5.80
Membrane adsorber S	3.36	1.44
Nanofiltration	≥ 5.28	≥ 5.42

Methodology

Cultivation of cell lines

- 5 Cells were subcultivated once or twice a week. The cells were seeded subconfluently into cell culture flasks and incubated at $37.0 \pm 0.5^\circ\text{C}$ and $5.0 \pm 0.5\%$ CO_2 in a humidified atmosphere for a cell specific incubation period. After reaching confluence cells were
- 10 trypsinated, centrifuged, counted and seeded into fresh culture flasks at a defined cell density.

- 15 PG-4 cells (ATCC CRL-2032) were kept in McCoys' cell culture medium, supplemented with 5% fetal calf serum, and 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin.

- 20 M. Dunni cells (ATCC CRL-2017) were kept in RPMI-1640 medium, supplemented with 5% fetal calf serum, 20 mM L-glutamine and 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin.

- 25 A9 cells (ECACC 85011426) were kept in MEM cell culture medium, supplemented with 10% fetal calf serum, and 50 U/ml penicillin and 50 $\mu\text{g}/\text{ml}$ streptomycin.

Preparation of virus stock solutions:

The virus-containing cell culture supernatants which also include 5-10 % (v/v) fetal calf

serum were centrifuged at 2000 ± 100 rpm for 10 ± 2 min and then filtered using a $0.45 \mu\text{m}$

filter to remove cell debris. The virus filtrates were then stored at -75°C to -85°C in aliquots

- 5 until use. The titer of the virus stock was determined in duplicate according to the Spearman-Kärber method ("The method of 'right or wrong cases' (constant stimuli) without Gauss's formulae"; Spearman, c.; Brit. J. of Psychology, 2 (1908), 227; "Beitrag zu kollektiven Behandlung pharmakologischer Reihenversuche"; Karber, G. Naunyn Schmiedeberg's Arch. Exp. Path. Pharmacol. 162, (1931), 480-
10 483).

Calculation of the reduction factor was done according to the formula

$$R = \log_{10} A_0 - \log_{10} A_n$$

15

For Chromatographies and carbamylation treatment:

R: reduction factor

A_0 : virus load in the load sample

A_n : virus load in the process samples and the hold sample

20

For Nanofiltration:

Reduction factor of hold and prefiltrate sample:

R: reduction factor

A_0 : virus load in the load sample

- 25 A_n : virus load in the hold / prefiltrate sample

The virus titer ($\text{TCID}_{50}/\text{ml}$) which causes a positive result in 50% of the infected cultures

(TCID_{50}) was calculated according to the method of Spearman and Karber (mode

30 A of

calculation):

$$\text{TCID} = 10^{-(Y_0 + d/2 - d \cdot \sum P_i)}$$

Y_0 : decade logarithm of the highest dilution (expressed as reciprocal of the dilution factor),

which effects the infection of all parallel cultures

5 d: decade logarithm of dilution step

$\sum P_i$: total number of all positive samples starting with Y_0

Virus inactivation by cyanate treatment

Preparation of cyanate and buffer

10 3.82 g Potassium Tetraborate Tetra Hydrate were dissolved in 30 mL water. The pH was measured and adjusted to pH 9.1 with 1 M HCl. 4.06 g Potassium Cyanate were added and the buffer was filled up to 50 mL with water. Conductivity was determined to be
15 114 - 117 mS/cm.

Preparation of the MuLV virus stock solution by ultracentrifugation

35 mL virus stock solution were centrifuged at 65,000 x g at a temperature of 4°C
20 for 90 min. The virus pellet was resuspended in 1500 µL PBS. Frothing was avoided during the resuspension process. After the resuspension the vessel was filled up with PBS to a final
25 volume of 3.5 mL. The resuspended MuLV pellet was filtered through a 0.45 µm filter. A sample was withdrawn and analysed for the viral titer by endpoint titration (ultracentrifuged virus stock solution).

30 Inactivation assay

20.0 mL of the sample to be tested were spiked with 2.0 mL of the virus stock solution in a water bath at 28.0 °C ± 1.0 °C. After mixing a sample was withdrawn and analysed for the viral titer (load). A further sample was withdrawn and kept in

a water bath at $28.0\text{ }^{\circ}\text{C} \pm 1.0\text{ }^{\circ}\text{C}$ until the end of the inactivation process (hold). Then, a sample was withdrawn, diluted with cell culture medium and titrated. 20 mL virus spiked medium were added to 20 mL of carbamylation buffer. As soon as the carbamylation buffer had been added the incubation period was started.

- 5 After 15 min a sample was withdrawn, diluted with cell culture medium and analysed for the virus titer by endpoint titration. Further samples were taken after 1 hour, 6 hours, 24 hours and 44 hours of incubation at $28.0\text{ }^{\circ}\text{C} \pm 1.0\text{ }^{\circ}\text{C}$. All samples were diluted with cell culture medium and analysed for the virus titer by endpoint titration.

10

Results

Log₁₀ reduction factors for the Mo/A-MuLV-spiked carbamylation runs

Description	TCID ₅₀ titer [log ₁₀ /mL]	Total volume [mL]	Total virus load [log ₁₀]	Reduction factor [log ₁₀]
Load	5.39 ± 0.18	20.0	6.69 ± 0.18	-
15 min	5.10 ± 0.34	40.0	6.70 ± 0.34	$- 0.01 \pm 0.39$
1 hour	4.92 ± 0.22	40.0	6.53 ± 0.22	0.16 ± 0.28
6 hours	$\leq 2.83 \pm 0.00$	40.0	$\leq 4.43 \pm 0.00$	$\geq 2.26 \pm 0.18$
24 hours	$\leq 2.83 \pm 0.00$	40.0	$\leq 4.43 \pm 0.00$	$\geq 2.26 \pm 0.18$
44 hours	$\leq 0.85 \pm 0.00$	40.0	$\leq 2.45 \pm 0.00$	$\geq 4.24 \pm 0.18$
Hold	4.61 ± 0.27	20.0	5.91 ± 0.27	0.78 ± 0.33

- 15 The inactivation of Mo/A-MuLV during the carbamylation step was effective. There was no residual infectivity obtained in the final samples. Due to large-volume plating (LVP) the limit of detection was reduced from 2.83 log₁₀/mL to 0.85 log₁₀/mL, so that the final sample (44 hours) revealed a reduction factor of at least $\geq 4.24 \pm 0.18\text{ log}_{10}$.

20

Log₁₀ reduction factors for the MVM-spiked carbamylation run

Description	TCID ₅₀ titer [log ₁₀ /mL]	Total volume [mL]	Total virus load [log ₁₀]	Reduction factor [log ₁₀]
Load	7.33 ± 0.30	20.0	8.63 ± 0.30	-
15 min	7.28 ± 0.22	40.0	8.88 ± 0.22	- 0.25 ± 0.37
1 hour	7.22 ± 0.12	40.0	8.82 ± 0.12	- 0.19 ± 0.32
6 hours	4.71 ± 0.23	40.0	6.32 ± 0.23	2.31 ± 0.32
24 hours	≤ 3.10 ± 0.00	40.0	≤ 4.70 ± 0.00	≥ 3.93 ± 0.30
44 hours	≤ 1.23 ± 0.00	40.0	≤ 2.83 ± 0.00	≥ 5.80 ± 0.30
Hold	7.21 ± 0.25	20.0	8.51 ± 0.25	0.12 ± 0.39

The inactivation of MVM during the carbamylation step was very effective. There was no residual infectivity obtained in the final samples. Due to large-
 5 volume plating (LVP) the limit of detection was reduced from 3.10 log₁₀/mL to 1.23 log₁₀/mL, so that the final sample (44 hours) revealed a reduction factor of at least ≥ 5.80 ± 0.30 log₁₀.

CLAIMS

1. A method for reducing viral contamination in a sample comprising erythropoietin (EPO) or a derivative or an analogue thereof, which method comprises the step of
5 adding cyanate to said sample in a concentration from about 0.05 M to about 2 M and for time period of about 1 hour or more.
2. The method according to claim 1, wherein said concentration of cyanate is from about 0.5 to about 1.5 M, such as about 1 M .
3. The method according to any one of claims 1 or 2, wherei n said cyanate is in the
10 form of potassium cyanate.
4. The method according to any one of the preceding claims, wherein said time period is from about 6 hours or more, about 24 hours or more, about 40 hours or more, or about 45 hours or more.
5. The method according to any one of the preceding claims, wherein the pH is from
15 about 8 to about 10, such as about 9.
6. The method according to any one of the preceding claims, wherein said sample is obtained from a cell culture of host cells expressing EPO or a derivative or an analogue thereof.
7. The method according to claim 6, wherein said sample is obtained directly from the
20 cell culture or subjected to a volume reduction by one or more steps of chromatography, filtration or centrifugation.
8. The method according to claim 7, wherein the chromatography is performed with an anion exchanger.
9. The method according to any one of the preceding claims, wherein said method is
25 performed using a final concentration of about 0.25 M potassium borate tetra hydrate and about 1M potassium cyanate with a pH about 9.0, and incubating the mixture at about 30°C to about 37°C for about 40 to about 50 hours.
10. The method according to any one of the previous claims wherein the cyanate treatment is using a chromatographic column and/or adding a solution with primary
30 amines to the mixture of any one of claims 1-9.
11. The method according to claim 10, wherein the solution with primary amines comprises or consist of a solution of ammonium sulfate, e.g. at concentration at about 3M.
12. The method according to claim 10 or 11, wherein the chromatographic column is gel filtration or hydrophobic interaction chromatography.
- 35 13. The method according to any one of claims 10-12, wherein the resin in the hydrophobic interaction chromatography comprises a methacrylic polymer.

14. A method according to any one of claims 1-13, followed by one or more virus removal steps, such as e.g. one or more membrane adsorber steps and/or one or more nano-filtration steps.
- 5 15. A method for carbamylating EPO or a derivative or an analogue thereof comprising the steps of
- a) harvesting EPO or a derivative or an analogue thereof from a cell culture of host cells expressing said EPO or EPO derivative or EPO analogue,
 - b) treating the mixture obtained in step b) with cyanate, and
 - c) optionally, stopping the carbamylation process.
- 10 16. A method according to claim 15, wherein the harvest in step a) is obtained as defined in any one of claims 6, 7 or 8.
17. The method according to claim 15, wherein step b) is carried out as defined in any one of claims 1-5 or 9.
- 15 18. The method according to claim 15, wherein the carbamylation in step c) is stopped as defined in any one of claims 10-14.
19. CEPO obtained according to any one of claims 1 to 18.
20. CEPO obtained according to any one of claims 1 to 18 for use as a medicament.
21. CEPO obtained according to any one of claims 1 to 18 for use in the treatment of Friedreich's ataxia, stroke or an ischemic event in the brain.
- 20 22. Use of CEPO obtained according to any one of claims 1 to 18 for the preparation of a medicament.
23. Use of CEPO obtained according to any one of claims 1 to 18 for the preparation of a medicament for the treatment of Friedreich's ataxia, stroke or an ischemic event in the brain.
- 25 24. A method for treating Friedreich's ataxia, stroke or an ischemic event in the brain comprising administering a therapeutically effective amount of CEPO according to claim 19.
25. A pharmaceutical composition comprising a therapeutically effective amount of carbamylated erythropoietin obtained using the method according to any one of claims
- 30 1-18, and a pharmaceutically acceptable carrier.
26. The pharmaceutical composition according to claim 25, wherein the carrier is a diluent, an adjuvant or an excipient.
27. A pharmaceutical composition according to claims 25 or 26 for treating Friedreich's ataxia, stroke or an ischemic event in the brain.

28. A method for treating Friedreich's ataxia, stroke or an ischemic event in the brain using an therapeutically effective amount of a pharmaceutical composition according to claims 25 or 26.
29. A method according to any one of claims 1-18, wherein EPO or CEPO has the sequence as defined in SEQ ID 1 or 2 or a sequence which is 95%, 98% or 99% identical hereto.

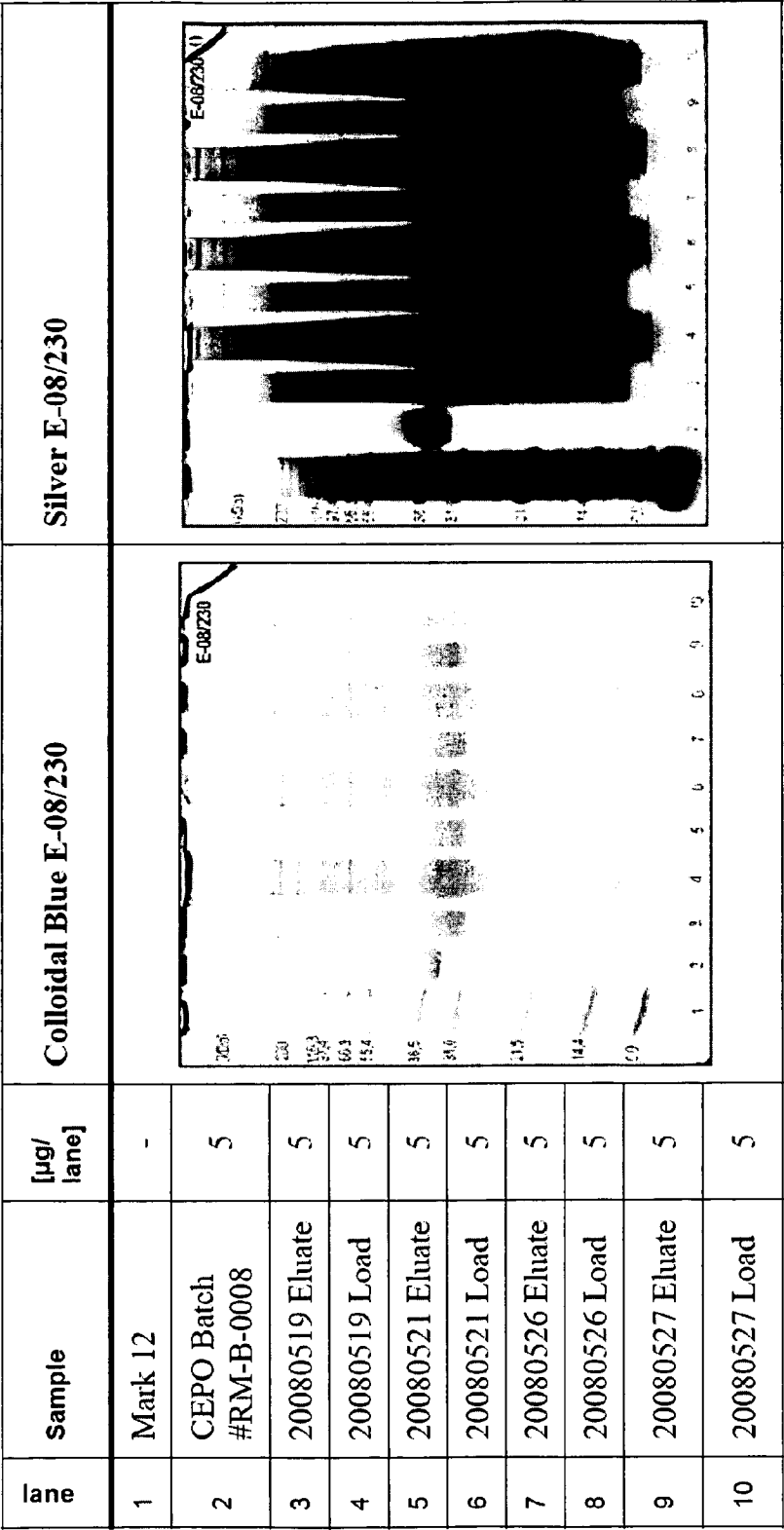


Fig. 1

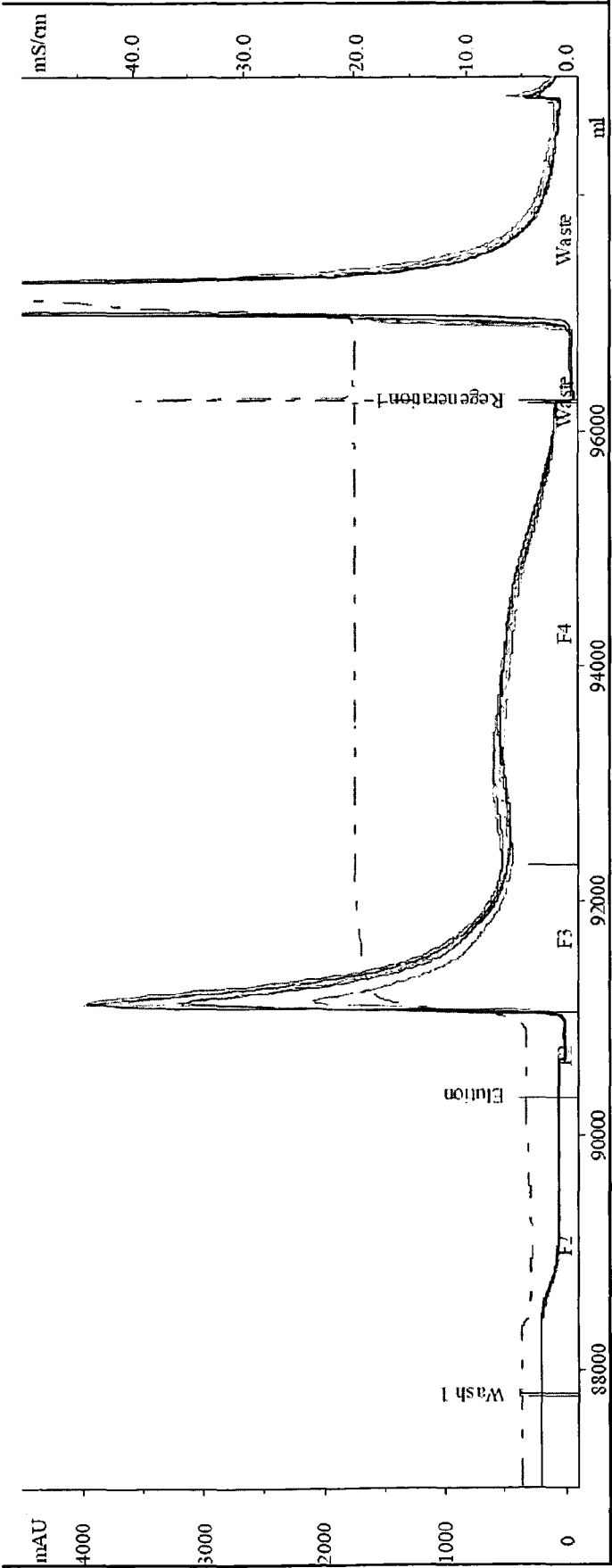


Fig. 2

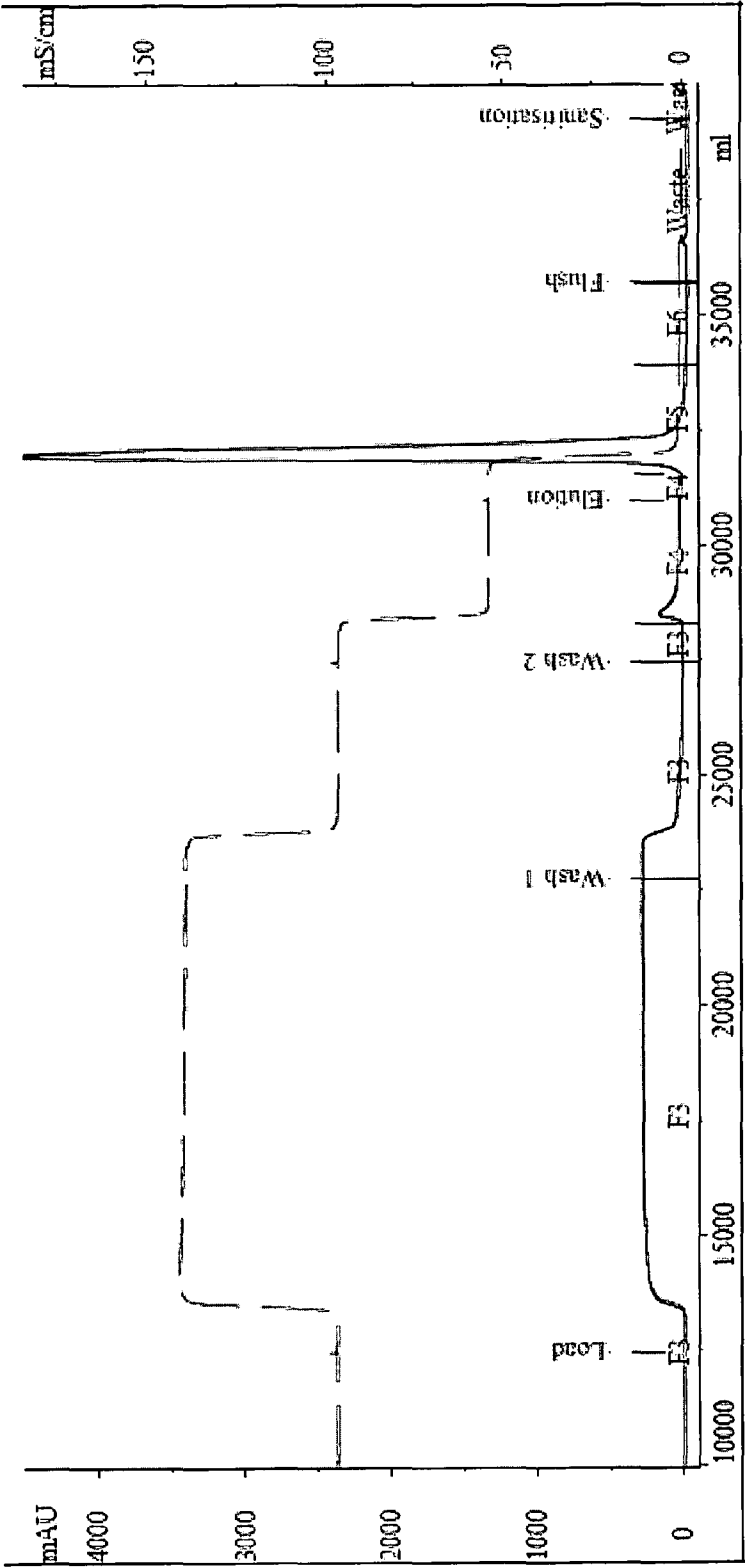


Fig. 3