

METHOD FOR PURIFYING VIRUSES BY CHROMATOGRAPHY

The invention relates to the field of purification of viruses, and more particularly to the
5 purification of viruses obtained by culturing on cell lines.

Harvests of viruses obtained from culturing on cell lines such as Vero cells contain not only the desired viruses but also proteins and DNA originating
10 from the culture cells. However, when the viruses are intended for certain uses, such as the manufacture of vaccines, it is essential for them to be as pure as possible. This is because standards exist which limit, to $100 \cdot 10^{-12}$ g/vaccinal dose, the maximum authorized
15 amount of cellular DNA in vaccines comprising products obtained from continuous and heteroploid cell lines.

Methods for purifying viruses are known in the prior art. Thus, US patent 4,664,912 discloses in particular a method for purifying rabies virus by zone
20 centrifugation in a sucrose gradient. However, such a method has the drawback of being difficult to automate; in addition, a prior inactivation step is necessary, which may lead to interactions between the virus and the cellular DNA which then make the step for removal
25 of this DNA more difficult.

This reference also discloses a purification method combining a step of gel filtration and a step of ion-exchange chromatography. However, such a purification method does not allow maximum removal of
30 the cellular DNA.

One aim of the invention is thus to propose a novel method for purifying viruses in very high yield which is easy to automate.

Another aim of the invention is to propose a
35 purification method which allows whole, non-degraded viruses to be obtained.

To achieve these aims, the subject of the invention is a method for purifying viruses obtained from a cell line culture, this method consisting in

separating by ion-exchange chromatography the viruses from the cell proteins and DNA originating from the culture, characterized in that it comprises at least one step of anion-exchange chromatography and one step
5 of cation-exchange chromatography.

According to a particular characteristic of the method according to the invention, the step of cation-exchange chromatography is carried out after the step of anion-exchange chromatography. Optimized
10 purification yields are thus obtained, compared with inversion of the order of the steps.

According to a particular embodiment, the method according to the invention also comprises a step of affinity chromatography by metal chelation. Thus,
15 the amounts of residual DNA are truly reduced to a minimum.

According to another preferred embodiment, the method according to the invention also consists in carrying out all the chromatography steps at the same
20 pH value. It is thus possible to automate the method easily and to reduce its costs appreciably, while at the same time maintaining its efficiency.

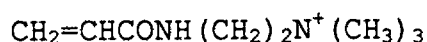
The invention will be better understood on reading the detailed description which follows.

25 The viruses to be purified according to the method of the invention are viruses obtained by means of cell lines, in particular continuous and heteroploid cell lines, which are thus liable to be combined with cellular DNA. These may be, in particular, rabies
30 virus, Japanese encephalitis virus or influenza virus. These viruses to be purified may have been obtained by culturing on Vero cells on microsupports. This concerns, for example, rabies virus obtained by culturing, as is described in US patent 4,664,912.

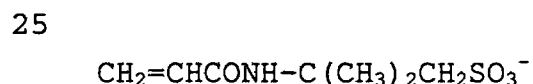
35 The viral harvest is filtered and then processed, according to the invention, by anion-exchange chromatography and by cation-exchange chromatography.

The anion-exchange chromatography can be an exchange chromatography of weak anions carried out, for example, using gel containing the diethylaminoethyl radical: DEAE Spherox gel sold by Sepracor, DEAE
5 Sepharose gel sold by Pharmacia, EMD DEAE gel sold by E. Merck. Although this is not common for the removal of nucleic acids, it is preferred to use a strong anion exchanger such as one of the following gels: High Q gel sold by Bio-Rad, Q Sepharose gel sold by Pharmacia, EMD
10 TMAE gel sold by E. Merck.

All of these gels can be used in Tris buffer or in phosphate buffer. Advantageously, a gel is used which allows great availability of the cationic group, as is the case in EMD TMAE from E. Merck, which has
15 chains of 15 to 25 units of the following monomer:



Similarly, according to the method of the
20 invention, the step of cation-exchange chromatography is preferably carried out using a gel which allows great accessibility of the anionic group, as is the case in EMD SO_3 sold by E. Merck, which has chains of 15 to 25 units of the following monomer:



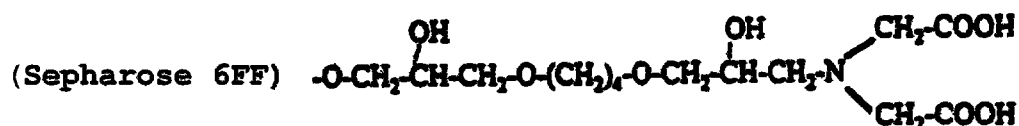
This gel can also be equilibrated using Tris buffer or phosphate buffer.

30 According to a preferred embodiment of the invention, the anion-exchange chromatography is carried out before the cation-exchange chromatography, the eluate from the first chromatography being, after optional dilution, introduced directly into the second
35 chromatography column. This order of operation allows optimization of the removal of the cellular DNA to be obtained.

The viral suspension obtained by elution of the cation-exchange chromatography column has a degree of

purity which is already satisfactory with regard to the standards established for the use of viruses in the manufacture of vaccines.

However, according to a preferred embodiment of the invention, an additional step of affinity chromatography by metal chelation using, for example, the Ca^{2+} ion as metal ion is added to the purification method described above. This chromatography can be carried out using an agarose gel as chelation support, such as the Sepharose® FF chelating gel sold by Pharmacia, which has the following structure:



According to the method of the invention, this chelation step is performed in line with the previous two ion-exchange chromatography steps, the eluate from the cation exchanger being introduced directly into the chelation chromatography column.

During this step, the cellular DNA remains bound whereas the virus passes through the gel without being retained.

This additional step makes it possible to bind at least another half of the residual DNA which is, admittedly, already in very low amount but which is, on the other hand, at this stage, very difficult to remove.

The viral suspension thus purified is diluted with a stabilizer and then inactivated according to any known method of the prior art, for example with β -propiolactone, before being concentrated and freeze-dried, so as then to be mixed with the other harvests obtained from the same Vero cell culture and which will constitute the same manufacturing batch.

The vaccines manufactured from such viruses were injected into humans and were well tolerated. In addition, their protective activity in animals was

verified by virtue of a test carried out on mice, the results of which were positive.

According to the invention, it is possible, surprisingly and very advantageously, to carry out the entire purification at room temperature in line and always at the same pH, which makes the method simple, easy to automate and reliable; it is especially possible to carry out the entire method under good conditions of sterility.

By virtue of this purification method, whole, non-degraded viruses are obtained whose spicules are visible by electron microscopy, in a very high degree of purity.

15 Example 1

An anion-exchange chromatography column is prepared by introducing EMD TMAE gel sold by E. Merck into a column, through which 1 M sodium hydroxide NaOH is first passed for the purposes of disinfection. The column is then equilibrated with 20 mM Tris buffer at pH 7.5.

A cation-exchange chromatography column is prepared by introducing EMS SO₃ gel sold by E. Merck into a column, through which 1 M sodium hydroxide is also passed, followed by 20 mM Tris buffer at pH 7.5.

A metal-chelation affinity chromatography column is prepared by introducing Sepharose® FF chelating gel sold by Pharmacia into a column, through which is passed 1 M sodium hydroxide and then water, followed by loading it with CaCl₂ at 3 g/l; the column is then rinsed with water, after which 0.5 M NaCl 20 mM Tris buffer at pH 7.5 is passed through.

Example 2

A harvest of rabies virus cultured on Vero cells is filtered using a membrane whose cutoff threshold is 0.65 µm and then on a membrane whose cutoff threshold is 0.45 µm.

This harvest consists of a viral suspension in culture medium also comprising cellular DNA as well as proteins originating both from the Vero cells and from the culture medium. The filtered crude harvest is
5 passed through the anion-exchange chromatography column prepared as described in Example 1. The non-bound part, containing proteins and DNA, is collected at the column outlet for assay purposes. The column is rinsed with 0.1 M NaCl 20 mM Tris buffer at pH 7.5 and the bound
10 viruses are then eluted by passing 0.4 M NaCl 20 mM Tris buffer at pH 7.5 through the column until the characteristic peak for the viruses has passed. The eluate from the first column is diluted to 1/4 in line with 20 mM Tris buffer at pH 7.5 and then introduced
15 into the cation-exchange chromatography column prepared according to Example 1.

The non-bound part of this second column is collected for assay purposes. When all of the eluate from the first column containing the virus has passed
20 through the second column, it is rinsed with 0.1 M NaCl 20 mM Tris buffer at pH 7.5 and the bound viruses are then eluted by passing 0.5 M NaCl 20 mM Tris buffer through until the characteristic peak for the viruses has passed.

25 The eluate from this second column is introduced directly, without dilution, into the metal-chelation affinity chromatography column prepared according to Example 1. This time, the DNA complexes with the Ca^{++} ions present in the column, whereas the
30 virus passes through the column and is collected at the outlet in order to be combined with a stabilizer and inactivated. The DNA complexed with the Ca^{++} ions is eluted by passing 50 mM EDTA solution through the column.

35 The ion-exchange chromatography columns are regenerated by passing 1 M NaCl 20 mM Tris buffer at pH 7.5 through and are disinfected with 1 M sodium hydroxide before being used for the purification of the other harvests originating from the same culture, which

will all be pooled to constitute a single manufacturing batch.

Example 3

5 The amounts of purified cellular proteins and DNA are assayed for each harvest, after each chromatography step.

 The total proteins are assayed by means of the Lowry method and the total DNA is assayed using the
10 Threshold machine from Molecular Device.

 The results obtained, taking an average of the yields per step for each culture harvest, expressed as a residual percentage for each of the steps of the purification method, are as follows:

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Yield (%)	Total DNA	Total proteins
Anion-exchange column	0.02	0.056
Cation-exchange column	27	25
Metal-chelation affinity column	80	

 Overall, by means of the purification according to the invention, only 0.004% of the cellular DNA and 1.4% of the proteins present in the crude harvests
20 remain.

 The purification carried out is thus entirely satisfactory.

 The infectivity of the viruses obtained is also verified at each step, by means of measuring their
25 infectious titre in CCID₅₀ and observation by electron microscope. It is observed that the integrity of the viruses is well conserved throughout the method.

 When the viruses thus produced are used in the manufacture of vaccines against rabies, it is possible
30 to prepare doses containing not more than 30 to 40·10⁻¹² g of cellular DNA per dose, which is a considerably smaller amount than the standard of 100·10⁻¹² g.

Claims

1. Method for purifying viruses obtained from a cell culture, which consists in separating by ion-exchange chromatography the viruses from the cell proteins and DNA originating from the culture, characterized in that it comprises at least one step of anion-exchange chromatography and one step of cation-exchange chromatography.
2. Method according to Claim 1, characterized in that the anions are strong anions.
3. Method according to either of the preceding claims, characterized in that the cations are strong cations.
4. Method according to one of the preceding claims, characterized in that the cation exchange is carried out after the anion exchange.
5. Method according to one of the preceding claims, characterized in that it also comprises a step of affinity chromatography by metal chelation.
6. Method according to the preceding claim, characterized in that the metal chelation is carried out using calcium ions.
7. Method according to one of the preceding claims, characterized in that the viruses to be purified are obtained by culturing on continuous and heteroploid cell lines.
8. Method according to one of the preceding claims, characterized in that the viruses to be purified are obtained by culturing on Vero cells.
9. Method according to one of the preceding claims, characterized in that the viruses to be purified are rabies viruses.
10. Method according to one of Claims 1 to 9, characterized in that the viruses to be purified are Japanese encephalitis viruses.
11. Method according to one of Claims 1 to 9, characterized in that the viruses to be purified are influenza viruses.

12. Method according to one of the preceding claims, characterized in that it consists in carrying out all of the chromatography steps at the same pH value.
- 5 13. Method according to one of the preceding claims, characterized in that it is an in-line method.
14. Method according to one of the preceding claims, characterized in that it is carried out at room temperature.
- 10 15. Method according to one of the preceding claims, characterized in that it is carried out under sterile conditions.



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(21) International application number: PCT/FR96/01064 (22) International filing date: 8 July 1996 (08.07.96) (30) Data relating to the priority: 95/09,851 10 August 1995 (10.08.95) FR (71) Applicant (for all designated States except US): PASTEUR MERIEUX SERUMS ET VACCINS [FR/FR]; 58, avenue Leclerc, F-69007 Lyon (FR). (72) Inventors; and (75) Inventors/Applicants (US only): Bernard FANGET [FR/FR]; La Vavre, F-69210 Saint-Germain-sur- l'Arbresle (FR). Alain FRANCON [FR/FR]; La Grand Croix, Brullioles, F-69690 Bessenay (FR). (74) Representative: Danièle KERNEIS; Pasteur Merieux Sérums et Vaccins, Industrial Property Directorate, 58, avenue Leclerc, F-69007 Lyon (FR).		(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO Patent (KE, LS, MW, SD, SZ, UG), Eurasian Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European Patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With the International Search Report.

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(54) Titre: PROCEDE DE PURIFICATION DE VIRUS PAR CHROMATOGRAPHIE

(57) Abstract

A method for purifying viruses from a cell line culture by chromatography, comprising an anion exchange chromatography step followed by a cation exchange chromatography step and optionally a metal-binding affinity chromatography step. The method is particularly suitable for producing viruses for use in vaccines.

(57) Abrégé

L'invention a pour objet un procédé de purification par chromatographie de virus obtenus par culture sur lignées cellulaires. Le procédé consiste à effectuer successivement une étape de chromatographie échangeuse d'anions puis une étape de chromatographie échangeuse de cations, ainsi qu'éventuellement une étape de chromatographie d'affinité par chélation métallique. Le procédé est particulièrement bien adapté à l'obtention de virus pour la fabrication de vaccins.