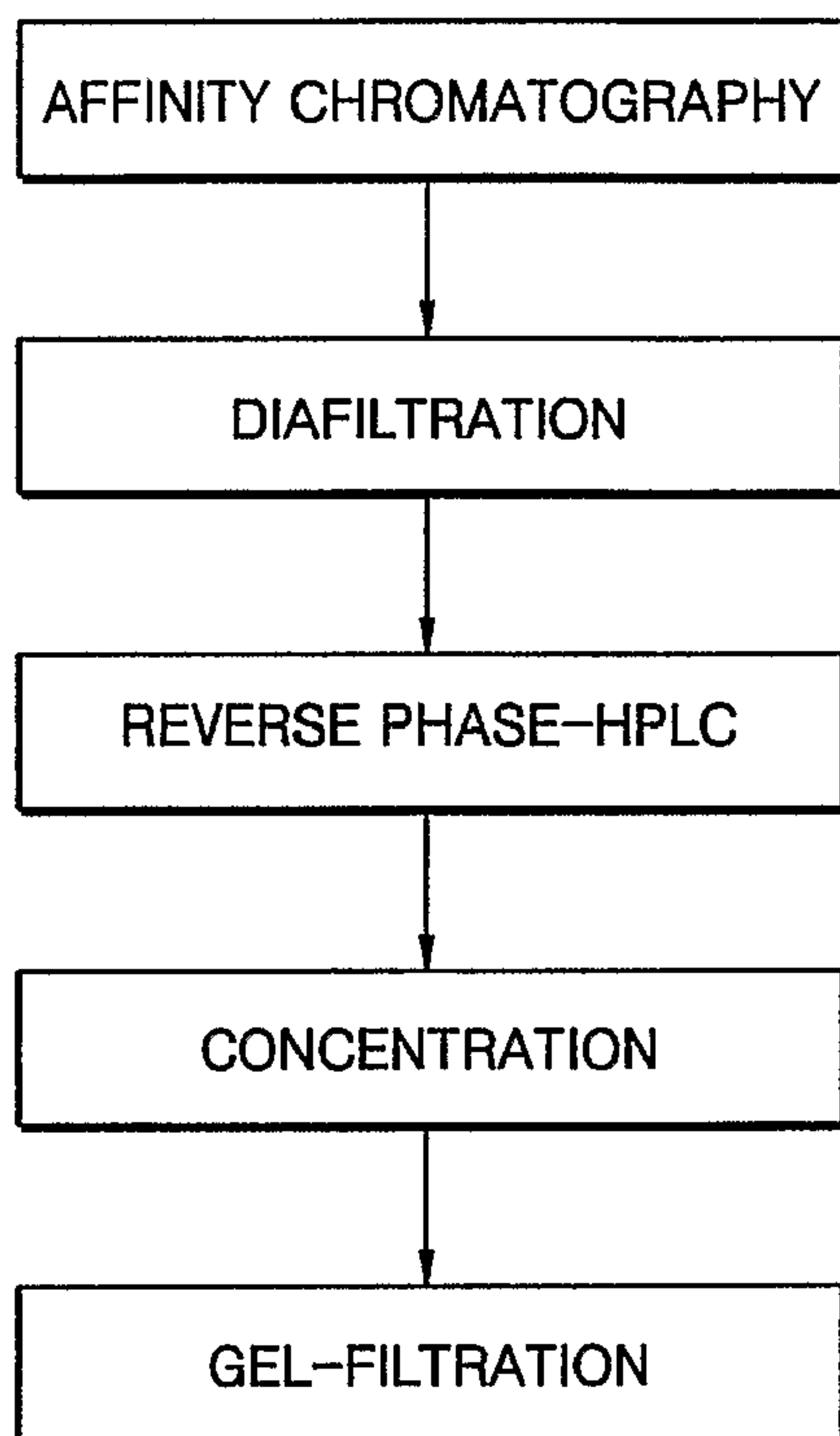




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 (54) Title: PROCESS FOR PURIFYING INTERFERON BETA



(57) **Abrégé/Abstract:**

Provided is a process for purifying human interferon beta from a recombinant human interferon beta-containing culture comprising performing affinity chromatography and cation exchange chromatography, wherein the affinity chromatography includes: adsorbing the interferon beta-containing culture to an equilibrated affinity chromatography column, followed by washing with an equilibration buffer solution; washing the column with a washing buffer solution A of pH 6.5-7.5 containing 30-60 wt% of propylene glycol and a washing buffer solution B of pH 6.5-7.5 containing 10-30 wt% of propylene glycol and 1-2M NaCl; and eluting a human interferon beta-containing fraction with a buffer solution of pH 6.5-7.5 containing 40-60 wt% of propylene glycol and 1-2M NaCl.

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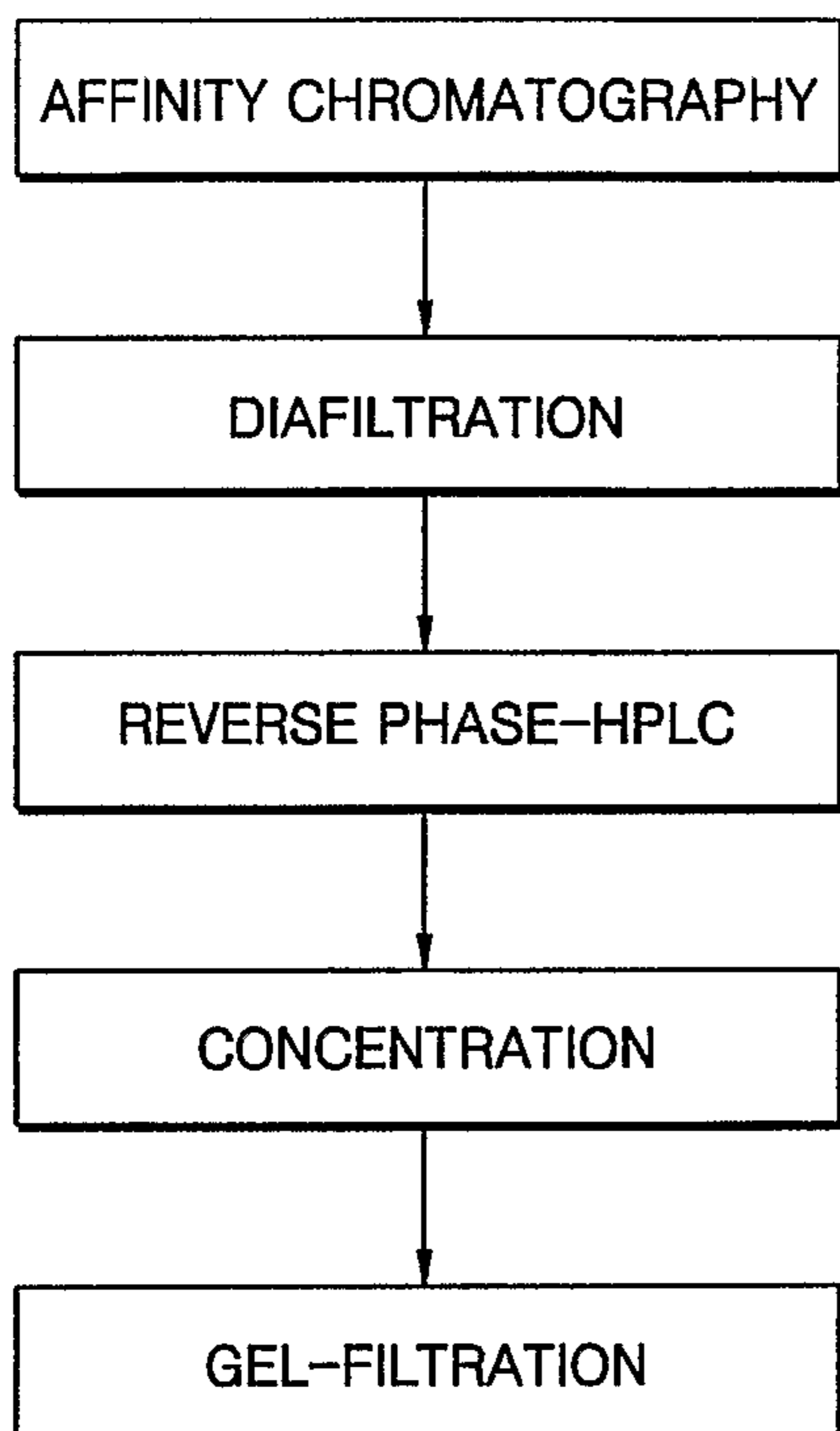
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(54) Title: PROCESS FOR PURIFYING INTERFERON BETA



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PROCESS FOR PURIFYING INTERFERON BETA

5 Technical Field

The present invention relates to a process for purifying a human interferon beta from a recombinant human interferon beta-containing culture using affinity chromatography and cation exchange chromatography.

10 Background Art

Interferons in a broad meaning are extracellular messengers mediating reactivity of hosts and evolutionally conserved protein families that are released in a relatively small size from cells. Interferons are released from interferon-producing cells in response to stimulation by viruses, double-stranded RNAs, various microorganisms, or cytokines such as TNF or IL1, and then bind to surfaces of neighboring cells with
15 interferon receptors. Thereafter, interferons induce synthesis of various proteins so that reactivity and homeostasis of hosts are maintained by consecutive signaling in—the cells. Therefore, interferons act as antiviral, antiproliferative, and immune signaling proteins in the bodies and have direct antiproliferation effects on cancer cells, and thus,
20 have received much attention as therapeutic agents [Postka S., Langer J. A. and Zoon K. C. (1987) Interferons and their actions, Annu. Rev. Biochem. 56:727-777].

Interferons belong to the class of helical, physiologically active substances. According to physicochemical characteristics and functionalities, there are two classes of interferons: type 1 and 2. Interferon- alpha, -beta, -tau, and -epsilon are members
25 of the type 1 interferon [Weissman C. and Weber H. (1986) The Interferon genes, Prog. Nucleic Acid Res. Mol. Biol. 33:251-300] and interferon gamma is a member of the type 2 interferon. Among them, interferon betas belonging to the type 1 interferon are proteins that exhibit species specificity. Interferon betas are also called as fibroblast interferons considering their sources and as pH2-stable interferons considering
30 biological characteristics. Interferon betas bind to the same receptors of cell surfaces, together with interferon alphas belonging to the type 1 interferon, and then induce transcription of antiviral factors in response to a consecutive cell signaling system.

Interferon betas are glycoproteins (about 20% sugar moiety) with a molecular

mass of about 20 kDa and single-chain proteins consisting of 166 amino acids. One N-glycosylation site is known to play a role in increasing material stability or solubility as physicochemical functions, rather than participating in biological activity or antigenicity [Karpusas M., Whytty A., Runkel L., and Hochman P. The structure of human interferon- β : implications for activity CMLS, 54:1203-1216 1998].

Advance in genetic recombination technology enabled determination of the amino acid sequence of human interferon beta and cloning and expression of human interferon beta in *E. coli* [Taniguchi, Gene 10:11-15, 1980]. Furthermore, expression of interferon beta in Chinese hamster ovary (CHO) cells was also reported [USP4,966,843, USP5,376,567, and USP5795779].

Currently, interferon betas are manufactured by gene recombination technology and commercially available under the trade name of Betaseron[®], Avonex[®], and Rebif[®]. Recombinant interferon betas are known to be effective in delaying the progression of multiple sclerosis in patients with the signs of the disease and relieving the pains of the disease. Furthermore, recombinant interferon betas are widely used as therapeutic agents for multiple sclerosis, and at the same time are effective in nonspecific regulation of human immune response, immune response to viral infection, and anti-proliferation of cancer cells.

Currently available purification technologies of recombinant interferon betas expressed in CHO cells involve 3-5 purification procedures including primary purification by affinity chromatography (USP4,278,661, USP4,289,689, USP4,541,952, USP4,808,523, etc.), metal-chelate chromatography (USP4,257,938, USP4,359,389, USP4,541,952, USP5,244,655, etc.), CPG (controlled pore glass) chromatography (USP4,359,389, USP5,066,786, USP5,244,655, etc.), or Concanavalin A chromatography (USP4,289,689, USP4,658,017, etc.) followed by cation exchange chromatography and reversed-phase chromatography.

In the above-described common purification technologies, metal-chelate chromatography may cause environmental contamination due to use of heavy metal. CPG or Concanavalin A chromatography has poor purification specificity. That is, Concanavalin A chromatography based on selective binding with many sugar-chain proteins contained in a CHO cell culture exhibits low specificity. A CPG column allows separation by molecular size after binding with a protein. However, separation efficiency and purity of interferon betas are lower than those by affinity chromatography

(e.g., Blue Sepharose column chromatography).

Furthermore, common purification technologies by affinity chromatography involve washing and elution with ethylene glycol using a monoclonal antibody and/or a dye-resin. However, affinity chromatography using a monoclonal antibody separately
5 requires the removal of the nonglycosylated form of interferon beta, which renders mass production difficult. In particular, ethylene glycol used in washing and elution is very toxic in the body, which restricts actual purification application.

Meanwhile, U.S. Patent No. 4,483,849 discloses a process for purifying and stabilizing interferon beta using propylene glycol, instead of toxic ethylene glycol, by
10 affinity chromatography. The process disclosed in this patent document includes applying an interferon-containing culture to a dye-affinity column such as equilibrated Affi-Gel Blue, washing the column with a 1.0M NaCl/PO₄ buffer solution and a 1.0M NaCl/PO₄ buffer solution containing 40% propylene glycol, and eluting interferon with
15 50% propylene glycol. Even though the process of this patent document involves column washing and elution, a desired final product peak and an impurity peak coexist, thereby lowering purity.

Disclosure of the Invention

The present invention provides a process for purifying interferon beta, which
20 includes recovering a high-purity primary purification product of interferon beta by enhanced affinity chromatography using nontoxic propylene glycol followed by cation exchange chromatography.

Therefore, the present invention provides a process for purifying human interferon beta from a recombinant human interferon beta-containing culture comprising
25 performing affinity chromatography and cation exchange chromatography, which includes washing and elution with a specific buffer solution.

According to an aspect of the present invention, there is provided a process for purifying human interferon beta from a recombinant human interferon beta-containing culture comprising performing affinity chromatography and cation exchange
30 chromatography, wherein the affinity chromatography includes: adsorbing the interferon beta-containing culture to an equilibrated affinity chromatography column, followed by washing with an equilibration buffer solution; washing the column with a washing buffer solution A of pH 6.5-7.5 containing 30-60 wt% of propylene glycol and a washing buffer

solution B of pH 6.5-7.5 containing 10-30 wt% of propylene glycol and 1-2M NaCl; and eluting a human interferon beta-containing fraction with a buffer solution of pH 6.5-7.5 containing 40-60 wt% of propylene glycol and 1-2M NaCl.

In the purification process of the present invention, non-limiting examples of the recombinant human interferon beta-containing culture used as a sample include
5 interferon beta-producing cells and strains. For example, the recombinant human interferon beta-containing culture may be a culture obtained by a known method disclosed in Carter and Horoszewicz, Pharm. Ther. 8, 359-377, 1980; Strander and Cantell, Ann. Med. Exp. Fenn. 44, 265-273, 1966; Wheelock, Science, 149, 310-311,
10 1965, and the like. Preferably, the recombinant human interferon beta-containing culture is a serum-free culture derived from recombinant human interferon beta-producing Chinese hamster ovary (CHO) cells.

In the purification process of the present invention, the affinity chromatography column used in the affinity chromatography may be a common dye-affinity column, for
15 example a column (e.g., XK-50 column, Amersham biosciences, Sweden) packed with Blue-Sepharose 6 (Amersham biosciences, Sweden) or an Affi-Gel Blue column (Bio-Rad, America). The equilibration buffer solution for the affinity chromatography column may be a sodium phosphate-EDTA buffer solution (about pH 7.2). The affinity chromatography column may be equilibrated with 3 column volumes (CV) of the
20 equilibration buffer solution, for example at a linear velocity of about 15-30 cm/hr.

In the purification process of the present invention, the affinity chromatography includes adsorbing the interferon beta-containing culture to the equilibrated affinity chromatography column and removing a nonspecifically bound protein by washing with the equilibration buffer solution.

The affinity chromatography also include multi-step washing, i.e., washing the
25 column with a washing buffer solution A of pH 6.5-7.5 containing 30-60 wt% of propylene glycol and with a washing buffer solution B of pH 6.5-7.5 containing 10-30 wt% of propylene glycol and 1-2M NaCl. Preferably, the affinity chromatography further includes washing with a washing buffer solution C of pH 6.5-7.5 containing 1-2M
30 NaCl. Preferably, each washing is performed with 2-4 CV of each buffer solution.

In the purification process of the present invention, there is no limitation on use sequence of the washing buffer solutions. That is, the washing may be performed using the washing buffer solution A and then the washing buffer solution B or using the

washing buffer solution B and then the washing buffer solution A. Further, the washing may be performed using the washing buffer solution A, the washing buffer solution C, and then the washing buffer solution B, or using the washing buffer solution B, the washing buffer solution C, and then the washing buffer solution A. The washing
5 with the washing buffer solution A effectively removes impurities with high hydrophobicity, the washing with the washing buffer solution C removes hydrophilic impurities, and the washing with the washing buffer solution B removes impurity proteins.

Interferon beta recovery may be performed by eluting a human interferon
10 beta-containing fraction with a buffer solution of pH 6.5-7.5 containing 40-60wt% of propylene glycol, preferably 50wt%, and 1-2M NaCl.

Preferably, each buffer solution used in the washing or elution may be a sodium phosphate buffer solution or a potassium phosphate buffer solution.

In the purification process of the present invention, the washing with a buffer
15 solution containing about 50% propylene glycol enables efficient removal of impurity peaks, which is in contrast to the process disclosed in U.S. Patent No. 4,483,849 in which washing and elution are performed with a graded propylene glycol concentration gradient.

In the purification process of the present invention, the above-described affinity
20 chromatography is followed by cation exchange chromatography. Preferably, prior to performing the cation exchange chromatography, an eluted solution from the affinity chromatography undergoes diafiltration with an ultrafiltration membrane of the molecular weight cut-off of 10,000. By the diafiltration, interferon beta with relatively high salt concentration can be adjusted to an appropriate salt concentration.

The cation exchange chromatography is performed as follows: a sample
25 obtained by the diafiltration is loaded on a column and then a human interferon beta-containing fraction is eluted at pH 5-7 by a concentration gradient of NaCl. In detail, interferon beta-containing fractions are eluted by a linear concentration gradient from a sodium phosphate buffer solution (pH 5-7), which is an equilibration buffer
30 solution, to a NaCl-containing sodium phosphate buffer solution (pH 5-7).

A column for the cation exchange chromatography may be CM-Sepharose FF (Amersham biosciences, Sweden) column (XK-50, 150 ml CV, Amersham biosciences, Sweden) and may be equilibrated with about 3 CV of a sodium phosphate buffer

solution at a flow rate of 5 ml/mn. In the cation exchange chromatography, the sample obtained by the diafiltration is allowed to flow through the equilibrated column at an appropriate flow rate and eluted by a linear salt concentration gradient of a NaCl-containing sodium phosphate buffer solution (about 12 CV). As a result, interferon beta with no sugar chains is removed and interferon beta with a sugar chain is selectively obtained.

An interferon beta-containing fraction obtained by the cation exchange chromatography may be further subjected to replacement with a fresh buffer solution. The replacement with a fresh buffer solution may be performed by gel-filtration or concentration and diafiltration.

For example, in the case of performing gel-filtration, the interferon beta-containing fraction obtained by the cation exchange chromatography is concentrated to, for example about 200-1,000 $\mu\text{g/ml}$, dialyzed with 10-50 mM sodium acetate buffer solution (pH 3.5~5.5), and loaded on a gel-filtration chromatography column (e.g., Sephacryl S-200, Amersham biosciences) equilibrated with 10-50 mM, preferably 20 mM sodium acetate buffer solution (pH 3.5~5.5). 10-50 mM sodium acetate buffer solution (pH 3.5-5.5) is then allowed to flow through the column at an appropriate flow rate, thereby resulting in solution replacement for target proteins and separation and removal of polymers.

A flowchart illustrating the purification process of the present invention is shown in FIG. 1.

Brief Description of the Drawings

FIG. 1 is a flowchart illustrating a purification process of the present invention.

FIG. 2 is a C4 RP-HPLC (Reversed-Phase High-Performance Liquid Chromatography) analysis chromatogram of interferon beta eluted in affinity chromatography according to a purification process of the present invention.

FIG. 3 is C4 RP-HPLC analysis chromatogram of interferon beta eluted without washing with 50% propylene glycol.

FIG. 4 is a C4 RP-HPLC analysis chromatogram of an eluted solution after gel-filtration.

Best mode for carrying out the Invention

Hereinafter, the present invention will be described more specifically by Examples. However, the following Examples are provided only for illustrations and thus the present invention is not limited to or by them.

Example 1: affinity chromatography

350 ml of Blue-Sepharose 6 (Amersham biosciences, Sweden) was packed in a XK-50 column (Amersham biosciences, Sweden) to make an affinity chromatography column. A 20 mM sodium phosphate buffer solution containing 1mM EDTA was allowed to sufficiently flow through the column to equilibrate the column. Then, 25 L of a Chinese hamster ovary (CHO) cell serum-free culture containing interferon beta was allowed to flow through the column at a flow rate of 5-10 ml/min and then the column was washed with about 3 column volumes (CV) of an equilibration buffer solution.

About 3 CV of a 20 mM sodium phosphate buffer solution (pH 7.2) containing 50 % propylene glycol was allowed to flow through the column at a flow rate of 5 ml/min to remove impurity proteins, followed by washing with about 3 CV of an equilibration buffer solution. Then, about 3 CV of 20 mM sodium phosphate buffer solution (pH 7.2) containing 2M NaCl was allowed to flow through the column at a flow rate of 5 ml/min to remove impurity proteins. Finally, about 3 CV of 20 mM sodium phosphate buffer solution (pH 7.2) containing 2M NaCl and 20% propylene glycol was allowed to flow through the column at a flow rate of 5 ml/min to remove impurity proteins.

About 3 CV of an elution buffer solution (20 mM sodium phosphate buffer solution containing 2M NaCl and 50% propylene glycol, pH 7.2) was allowed to flow through the column at a flow rate of 5 ml/min to thereby recover an interferon beta-containing solution. The purity of the eluted solution thus recovered was measured using C4 HPLC analysis chromatography and the result is shown in FIG. 2. Referring to FIG. 2, the purity of interferon beta was about 85% or more.

As a control, affinity chromatography was performed according to the above-described manner except that washing with a 20 mM sodium phosphate buffer solution (pH 7.2) containing 50% propylene glycol was omitted. The purity of the resultant eluted solution was measured using C4 HPLC analysis chromatography and the result is shown in FIG. 3. It can be seen from FIG. 3 that the absence of the washing with 20 mM sodium phosphate buffer solution (pH 7.2) containing 50% propylene glycol remarkably decreases the purity of interferon beta.

Example 2: cation exchange chromatography

The interferon beta-containing solution obtained according to the present invention in Example 1 underwent diafiltration using an ultrafiltration system (molecular weight cut-off of 10,000) and then was loaded on a CM-Sepharose FF (Amersham biosciences, Sweden) column (XK-50, 150 ml CV, Amersham biosciences, Sweden) at a flow rate of 1.5 ml/min. The column was then equilibrated with about 3 CV of a 50 mM sodium phosphate buffer solution (pH 6.7) at a flow rate of 5 ml/min. Elution of interferon beta was performed by a linear concentration gradient from a 50 mM sodium phosphate buffer solution (pH 6.7) to a 50 mM sodium phosphate buffer solution (pH 6.7) containing 400 mM NaCl to thereby recover sugar chain-linked interferon beta fractions.

Example 3: gel-filtration chromatography

An interferon beta-containing solution obtained in Example 2 was concentrated to 200 $\mu\text{g/ml}$ and ethanol contained in the concentrate was replaced 500 times or more by a 20 mM sodium acetate buffer solution (pH 4.0). The resultant solution was loaded on a Sephacryl S-200 column (1700ml, XK-50/100, Amersham biosciences, Sweden) equilibrated with a 20 mM sodium acetate buffer solution (pH 4.0) to obtain an interferon beta-containing solution.

Example 4: reversed-phase high-performance liquid chromatography (RP-HPLC) analysis

Each solution obtained in Examples 1, 2, and 3 was loaded on a C4 RP-HPLC column (Vydac 214TP54, 4.6mm in inner diameter x 25cm in length, 5 μm in particle size, 300 Å in pore size) at a flow rate of 1 ml/min. Then, 20 CV of a 0.1% trifluoroacetic acid-containing acetonitrile was allowed to flow through the column by a linear concentration gradient from 30% acetonitrile containing 0.1% trifluoroacetic acid to 80% acetonitrile containing 0.1% trifluoroacetic acid, to analyze chromatogram patterns.

An analysis result after the gel-filtration chromatography is shown in FIG. 4. From FIG. 4, it can be seen that the present invention can produce a high purity interferon beta.

Industrial Applicability

According to a purification process of the present invention, interferon beta can

be purified with high purity of 99% or more using nontoxic propylene glycol and enhanced affinity chromatography.

CLAIMS

1. A process for purifying human interferon beta from a recombinant human interferon beta-containing culture comprising performing affinity chromatography and cation exchange chromatography,

5 wherein the affinity chromatography comprises:

adsorbing the interferon beta-containing culture to an equilibrated affinity chromatography column, followed by washing with an equilibration buffer solution;

10 washing the column with a washing buffer solution A of pH 6.5-7.5 containing 30-60 wt% of propylene glycol and a washing buffer solution B of pH 6.5-7.5 containing 10-30 wt% of propylene glycol and 1-2M NaCl; and

eluting a human interferon beta-containing fraction with a buffer solution of pH 6.5-7.5 containing 40-60 wt% of propylene glycol and 1-2M NaCl.

15 2. The process of claim 1, wherein the washing step further comprises washing the column with a washing buffer solution C of pH 6.5-7.5 containing 1-2M NaCl.

20 3. The process of claim 1 or 2, wherein each buffer solution used in the washing and the elution is a sodium phosphate buffer solution or a potassium phosphate buffer solution.

25 4. The process of claim 1 or 2, wherein a solution obtained by the affinity chromatography is subjected to diafiltration with an ultrafiltration membrane of molecular weight cut-off of 10,000 before the cation exchange chromatography.

30 5. The process of claim 4, wherein in the cation exchange chromatography, a sample obtained by the diafiltration is loaded on a column and then a human interferon beta-containing fraction is eluted at pH 5-7 by a concentration gradient of NaCl.

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FIG. 1

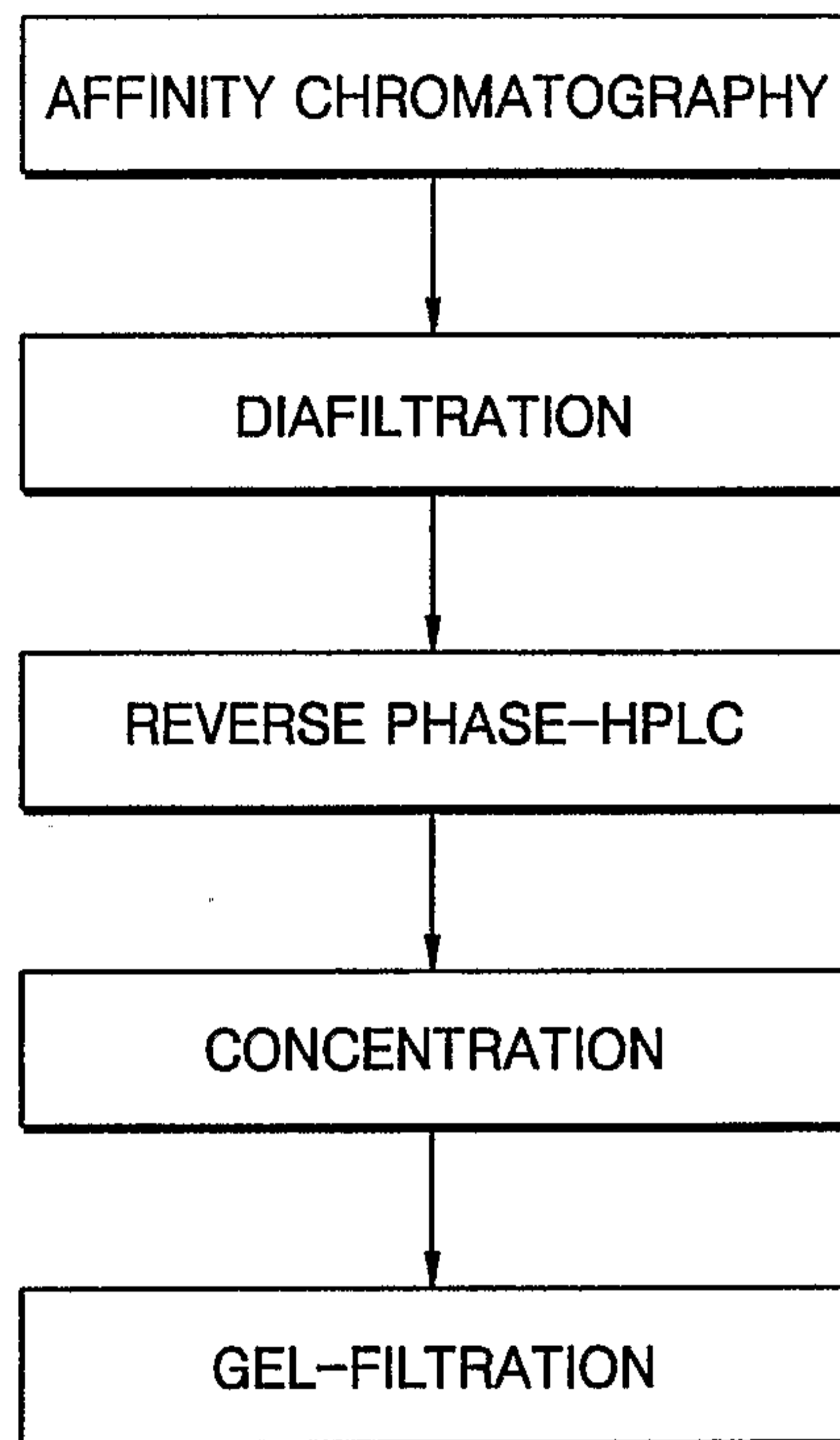


FIG. 2

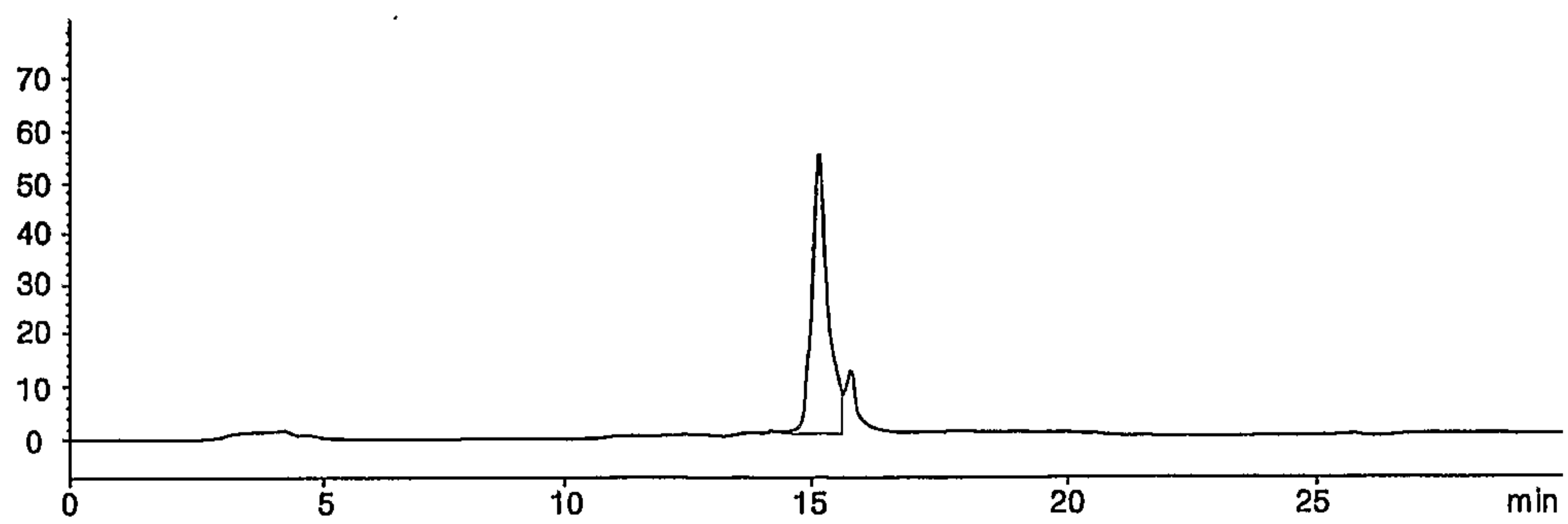


FIG. 3

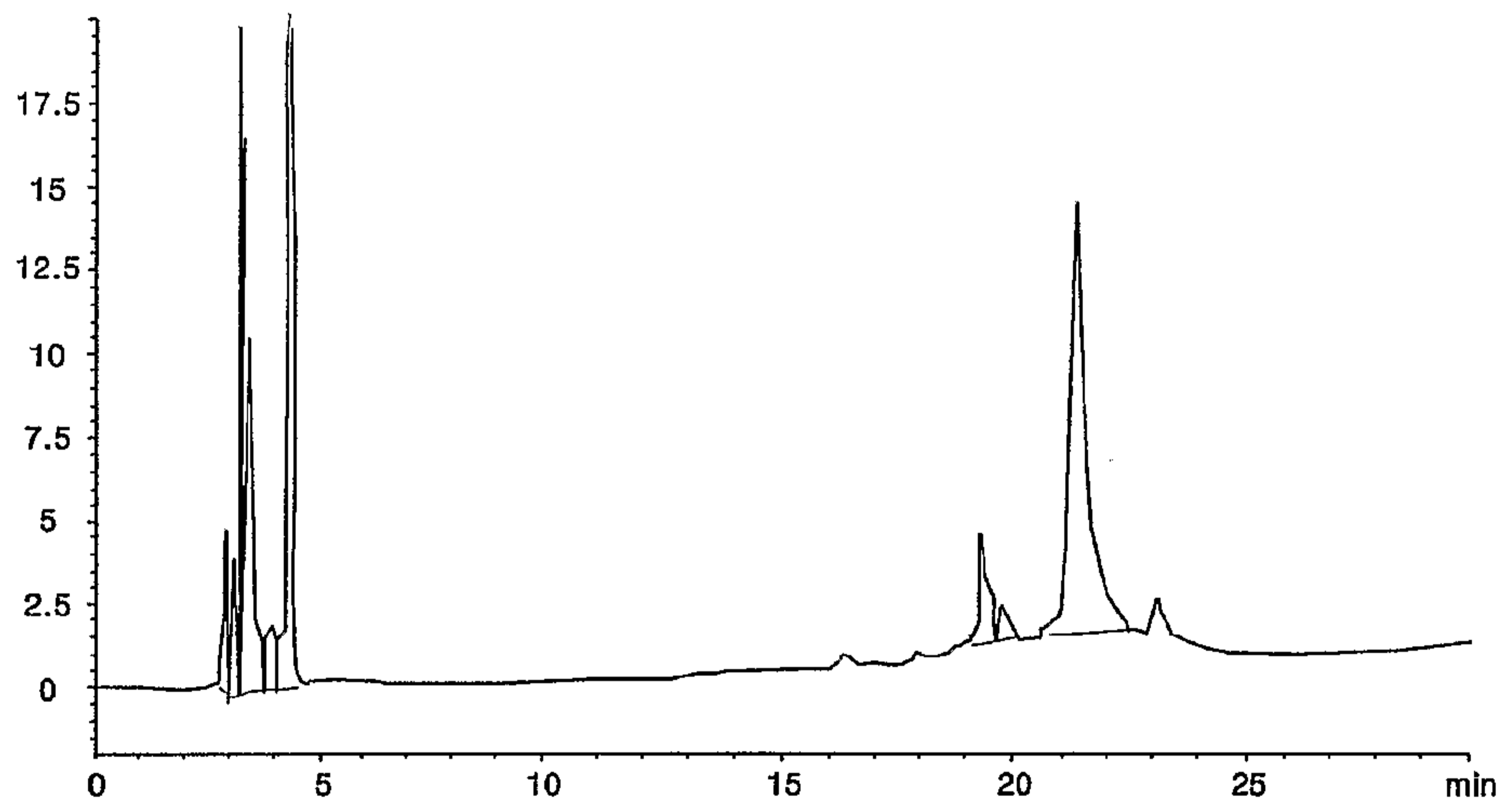
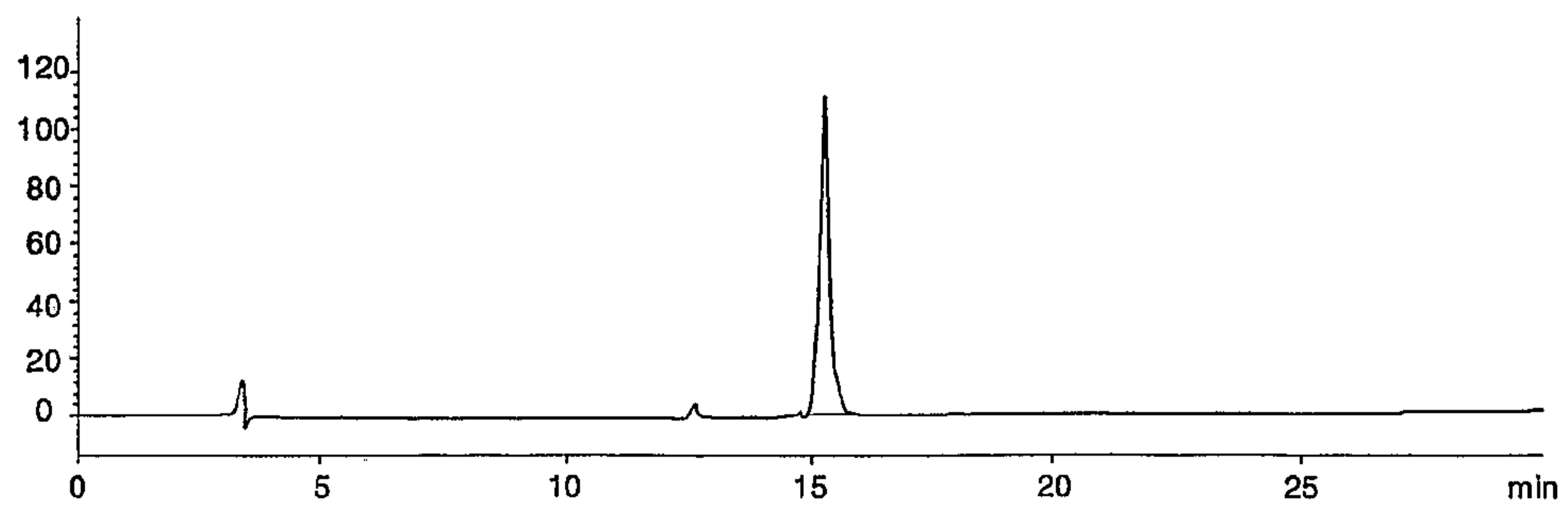


FIG. 4



AFFINITY CHROMATOGRAPHY



DIAFILTRATION



REVERSE PHASE-HPLC



CONCENTRATION



GEL-FILTRATION