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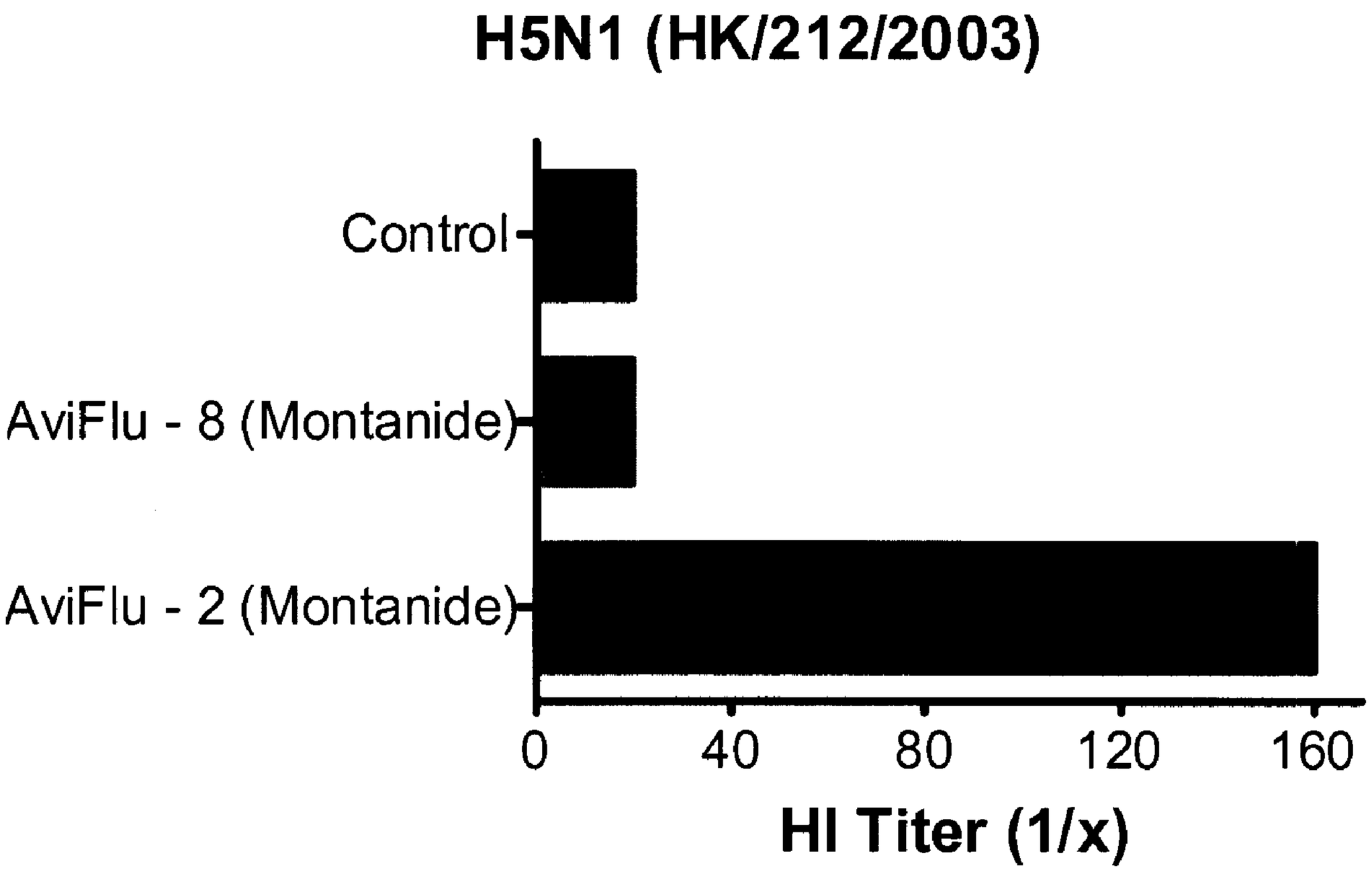
(54) **Titre : FORMULATIONS DE VACCIN CONTRE L'INFLUENZA**
(54) **Title: INFLUENZA VACCINE FORMULATION**



(57) **Abrégé/Abstract:**
Peptide-based anti-influenza formulations against influenza are disclosed. The peptides are derived from influenza-based epitopes. The formulations are based on peptide mixtures which may be formulated so that variability is present at particular residues. The formulations can be used to prepare vaccines for preventing influenza, particularly avian influenza.

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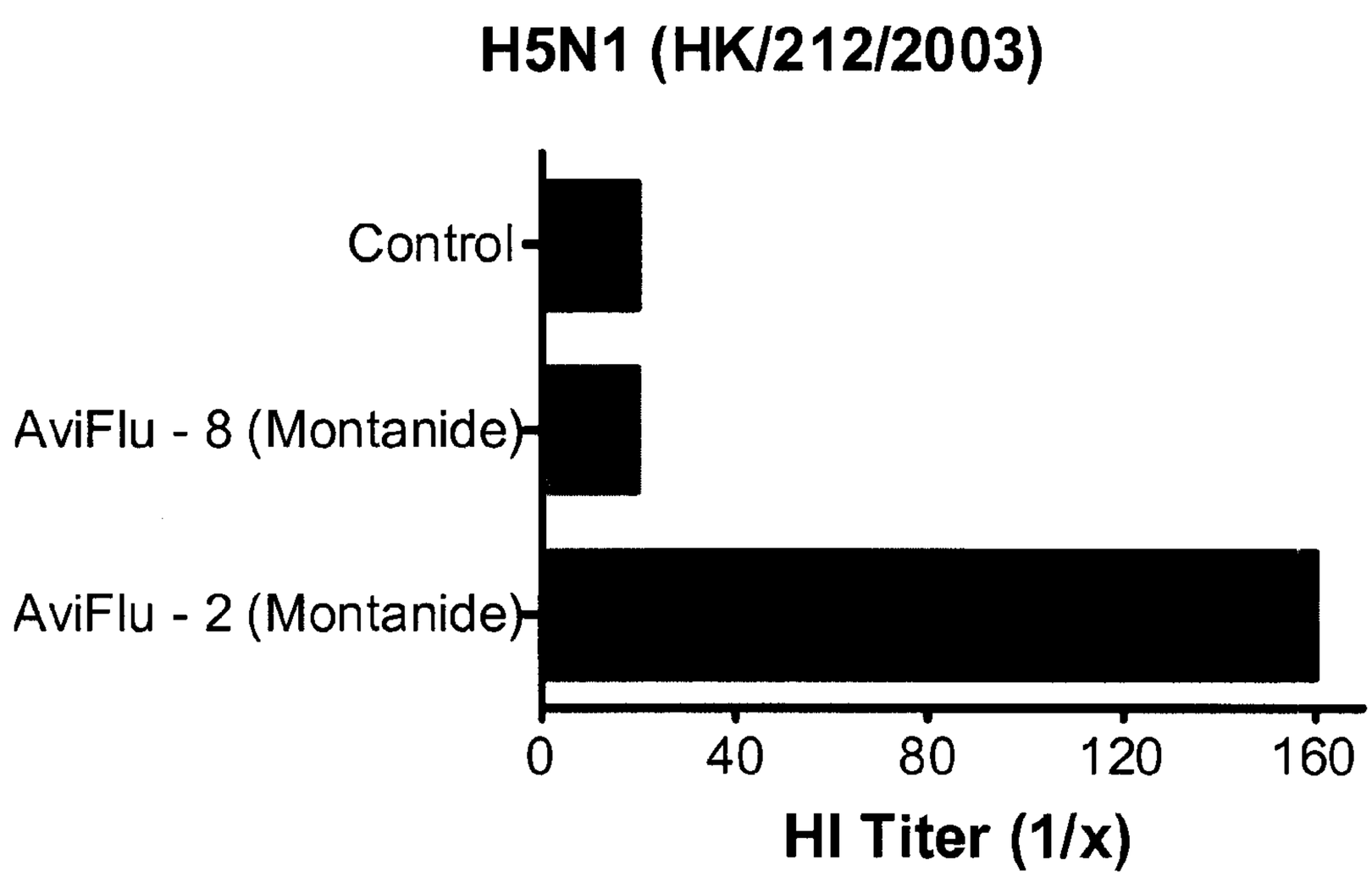

(57) Abstract: Peptide-based anti-influenza formulations against influenza are disclosed. The peptides are derived from influenza-based epitopes. The formulations are based on peptide mixtures which may be formulated so that variability is present at particular residues. The formulations can be used to prepare vaccines for preventing influenza, particularly avian influenza.

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INFLUENZA VACCINE FORMULATION

FIELD OF THE INVENTION

The present invention relates generally to an anti-viral formulation, and in particular relates to a peptide-based influenza vaccine formulation, more particularly to an avian influenza peptide-based vaccine formulation.

BACKGROUND OF THE INVENTION

Avian influenza is an infectious disease of birds caused by type A strains of the influenza virus. The disease, which was first identified in Italy more than 100 years ago, occurs worldwide. Sixteen subtypes of influenza virus are known to infect birds, thus providing an extensive reservoir of influenza viruses potentially circulating in bird populations. To date, all known outbreaks of the highly pathogenic form have been caused by influenza A viruses of subtypes H5 and H7.

Of the 16 avian influenza virus subtypes, H5N1 is of particular concern for several reasons. H5N1 mutates rapidly and has a documented propensity to acquire genes from viruses, thereby facilitating infection of other animal species. Indeed, its ability to cause severe disease in humans has now been documented. Laboratory studies have demonstrated that isolates from this virus have a high pathogenicity and can cause mortality in humans.

Two other avian influenza viruses have recently been found to cause illness in humans: H7N7 and H9N2.

All type A influenza viruses are genetically labile and well adapted to elude host defences. Influenza viruses lack mechanisms for the “proofreading” and repair of errors that occur during replication. As a result of these uncorrected errors, the genetic composition of the viruses changes as they replicate in humans and animals, and new antigenic variants emerge. These constant, permanent and usually small changes in the antigenic composition of influenza A viruses are known as antigenic “drift”.

Influenza viruses are typed as A or B on the basis of relatively stable intracellular nucleoproteins and envelope associated matrix proteins. Virus subtypes are based on two proteins in the viral envelope, hemagglutinin (HA) and neuraminidase (NA), which undergo constant antigenic change. 16 distinct subtypes of HA and 9 subtypes of NA are recognized for influenza A viruses. The sudden appearance of a new subtype (antigenic shift) has caused three major pandemics in the past century: 1918 (Spanish Flu, H1N1), 1957 (Asian Flu, H2N2) and 1968 (Hong Kong Flu, H3N2).

Influenza viruses have a second characteristic of great public health concern: influenza A viruses can swap or "re-assort" genetic materials between subtypes of any species resulting in novel subtypes. This reassortment process, known as antigenic "shift," has resulted in worldwide pandemics in humans.

Influenza pandemics have occurred, on average, three to four times each century when new virus subtypes have emerged that are readily transmitted from person to person. In the 20th century, the great influenza pandemic of 1918 -1919, which caused an estimated 40 to 50 million deaths worldwide, was followed by pandemics in 1957-1958 and 1968 -1969. Experts surmise that another influenza pandemic is inevitable and possibly imminent. Given the unpredictable behaviour of influenza viruses, neither the timing nor the severity of the next pandemic can be predicted with any certainty.

Seven variable B-cell epitopes, and one variable T-cell epitope collectively represent the antigenic drift sites found on the hemagglutinin HA1 protein of Influenza A (subtype H5). Each of the B-cell variable epitopes represents a conformational epitope, and four of them are comprised of two discontinuous stretches of amino acids. There are two extended antigenic sites on the HA1 proteins, and each of them is represented by two distinct peptide sequences. The nonadjacent segments (stretches of amino acids) that are artificially joined together to represent the discontinuous epitopes are selected using the three-dimensional structure of A/duck/Singapore/3/97 hemagglutinin (PDB ID code: 1JSM). Use of crystallographic data aids in design of linear peptides that can mimic the native conformational epitopes of proteins. The T-cell eptiope is represented by a linear peptide sequence which may also be lipidated.

To date, no effective peptide-based vaccine against avian influenza is commercially available.

Current antiviral therapies may be clinically effective against influenza A virus strains in otherwise healthy adults and children; however, these therapies have limitations. Some of these drugs are expensive and supplies are limited. The vaccine composition must also change each year to account for changes in the virus circulating in the population due to antigenic drift. At least four months of development time is required to produce a new effective vaccine in significant quantities.

Processes for preparation of an immunogenic peptide mixture are described by Torres in U.S. Patent No. 7,118,874, and in PCT application PCT/CA06/000891. According to one of these processes, the variability of immunogenic epitope sequences of a pathogen are evaluated. A peptide mixture is synthesized comprising a plurality of peptides representative of the frequency with which different amino acids are found at variable residues of selected epitopes.

Thus, there is a need to develop a vaccine formulation effective against multiple subtypes and multiple variants of avian influenza.

SUMMARY OF THE INVENTION

It is an object of the present invention to obviate or mitigate at least one disadvantage of previous influenza vaccine formulations.

In a first aspect, the present invention provides a peptide-based anti-influenza formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 1 to 496 and analogues thereof. Particularly, the present invention provides a peptide-based anti-influenza formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 1 to 248 and analogues thereof. In addition, the present invention provides a formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 249 to 496 and analogues thereof. In exemplary embodiments, the present invention provides a formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 1 to 212, a formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 249 to 460, a formulation comprising peptides selected from the group consisting of: a) SEQ ID NOs: 1 to 212, and b) SEQ ID NOs: 249 to 460; a formulation comprising peptides selected from the group consisting of: a) SEQ ID NOs: 213 to 248, and b) SEQ ID NOs: 461 to 496; a formulation comprising peptides

selected from the group consisting of: a) SEQ ID NOs: 1 to 248, and b) SEQ ID NOs: 249 to 496; or a formulation comprising peptides selected from the group consisting of: a) SEQ ID NOs: 1 to 40, and b) SEQ ID NOs: 249 to 288.

In another exemplary embodiment of the present invention, the formulation comprises at least one peptide sequence from at least one of the following groups: a)SEQ ID NOs: 1 to 24; b) SEQ ID NOs: 25 to 40; c) SEQ ID NOs: 41 to 64; d) SEQ ID NOs: 65 to 88; e) SEQ ID NOs: 89 to 120; f) SEQ ID NOs: 121 to 144; g) SEQ ID NOs: 145 to 176, h) SEQ ID NOs: 177 to 212; i) SEQ ID NOs: 249 to 272; j) SEQ ID NOs: 273 to 288; k) SEQ ID NOs: 289 to 312; l) SEQ ID NOs: 313 to 336; m) SEQ ID NOs: 337 to 368; n) SEQ ID NOs: 369 to 392; o) SEQ ID NOs: 393 to 424; or p) SEQ ID NOs: 425 to 460.

In yet another exemplary embodiment of the present invention, the formulation comprises 2^n peptide sequences from at least one of the following groups: a) SEQ ID NOs: 1 to 24; b) SEQ ID NOs: 25 to 40; c) SEQ ID NOs: 41 to 64; d) SEQ ID NOs: 65 to 88; e) SEQ ID NOs: 89 to 120; f) SEQ ID NOs: 121 to 144; g) SEQ ID NOs: 145 to 176, h) SEQ ID NOs: 177 to 212, i) SEQ ID NOs: 249 to 272; j) SEQ ID NOs: 273 to 288; k) SEQ ID NOs: 289 to 312; l) SEQ ID NOs: 313 to 336; m) SEQ ID NOs: 337 to 368; n) SEQ ID NOs: 369 to 392; o) SEQ ID NOs: 393 to 424; or p) SEQ ID NOs: 425 to 460, wherein n is 1 to 4.

The formulation can further comprise at least one peptide sequence from SEQ ID NOs: 213 to 248, or SEQ ID NOs: 461 to 496.

In a further aspect of the present invention there is provided a vaccine comprising the formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 1 to 496 and analogues thereof, together with a pharmaceutically-acceptable diluent or carrier. The vaccine can further comprise an adjuvant. In one example, the adjuvant is alum.

The anti-viral formulation can be an anti-influenza formulation. More particularly, the anti-influenza formulation can be an avian anti-influenza formulation.

In a further aspect of the present invention, there is provided a use of the formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 1 to 496 and analogues thereof, for the preparation of a vaccine. The vaccine can be used for preventing or treating influenza in an animal in need thereof. In one exemplary embodiment, the influenza is avian influenza. The present invention further relates to a method for

inducing an immune response in humans or animals and conferring protection against avian influenza, or novel subtypes of influenza derived from avian influenza, which comprises administering to humans or other animals a peptide-based vaccine as described herein.

In a further aspect of the present invention, there is provided a method for preparing an anti-viral formulation, such as the anti-viral formulation as described herein. According to one embodiment, there is provided a method for preparing a peptide from SEQ ID NOs: 1 to 212 comprising the steps of determining a linear sequence representative of primary sequences of discontinuous epitopes of an avian influenza viral protein, wherein the epitopes are in proximity to each other when the protein is in a folded conformation; and synthesizing a peptide representative of the linear sequence. In another embodiment, there is provided method for preparing a peptide mixture comprising any two peptide sequences from SEQ ID NOs: 1 to 212 comprising the steps of: determining a linear sequence representative of primary sequences of discontinuous epitopes of an avian influenza viral protein, the epitopes being in proximity to each other when the protein is in a folded conformation; said discontinuous epitopes comprising variable residues, and synthesizing a peptide mixture including at least two different amino acids at a variable residue.

In yet another aspect, the present invention relates generally to an anti-influenza vaccine comprising a mixture of peptides containing at least one hemagglutinin (HA) antigen of influenza virus. Hemagglutinin (HA) is a potent immunogen, and viral neutralizing antibodies are directed against the variable regions of HA. The isolated peptide mixture represents variants of multiple variable regions of hemagglutinin. Thus, in accordance with one aspect of the present invention, there is provided an anti-viral formulation comprising a mixture of isolated peptides, said mixture being formulated on the basis of the variable region of the avian influenza virus HA protein and said isolated peptide mixture representing variants of a variable region of the HA or HA1 protein, wherein each of said variable regions comprising a plurality of variable amino acid residues, at least one of which is represented by two or more amino acids.

In one embodiment, the plurality of variable amino acid residues in the anti-viral formulation comprises three or more residues. One or more of said Avian influenza proteins can be an HA or HA1.

Other aspects and features of the present invention will become apparent to those ordinarily skilled in the art upon review of the following description of specific embodiments of the invention in conjunction with the accompanying Figures.

BRIEF DESCRIPTION OF THE DRAWINGS

Embodiments of the present invention will now be described, by way of example only, with reference to the attached Figures, wherein:

Fig. 1 shows an analytical HPLC chromatogram of crude INFA-H5-1-V1 peptide sequences (corresponding to SEQ ID NOs: 249 to 272).

Fig. 2 shows an analytical HPLC chromatogram of crude INFA-H5-1-V2 peptide sequences (corresponding SEQ ID NOs: 273 to 288).

Fig. 3 shows an analytical HPLC chromatogram of crude INFA-H5-1-V3 peptide sequences (corresponding to SEQ ID NOs: 289 to 312).

Fig. 4 shows an analytical HPLC chromatogram of crude INFA-H5-1-V4 peptide sequences (corresponding to SEQ ID NOs: 313 to 336).

Fig. 5 shows an analytical HPLC chromatogram of crude INFA-H5-1-V5 peptide sequences (corresponding to SEQ ID NOs: 337 to 368).

Fig. 6 shows an analytical HPLC chromatogram of crude INFA-H5-1-V6 peptide sequences (corresponding to SEQ ID NOs: 369 to 392).

Fig. 7 shows an analytical HPLC chromatogram of crude INFA-H5-1-V7 peptide sequences (corresponding to SEQ ID NOs: 393 to 424).

Fig. 8 shows an analytical HPLC chromatogram of crude INFA-H5-1-V8 peptide sequences (corresponding to SEQ ID NOs: 425 to 460).

Fig. 9 shows a MALDI-TOF spectrum of crude INFA-H5-1-V1 peptide sequences (corresponding to SEQ ID NOs: 249 to 272).

Fig. 10 shows a MALDI-TOF spectrum of crude INFA-H5-1-V2 peptide sequences (corresponding to SEQ ID NOs: 273 to 288).

Fig. 11 shows a MALDI-TOF spectrum of crude INFA-H5-1-V3 peptide sequences (corresponding to SEQ ID NOs: 289 to 312).

Fig. 12 shows a MALDI-TOF spectrum of crude INFA-H5-1-V4 peptide sequences (corresponding to SEQ ID NOs: 313 to 336).

Fig. 13 shows a MALDI-TOF spectrum of crude INFA-H5-1-V5 peptide sequences (corresponding to SEQ ID NOs: 337 to 368).

Fig. 14 shows a MALDI-TOF spectrum of crude INFA-H5-1-V6 peptide sequences (corresponding to SEQ ID NOs: 369 to 392).

Fig. 15 shows a MALDI-TOF spectrum of crude INFA-H5-1-V7 peptide sequences (corresponding to SEQ ID NOs: 393 to 424).

Fig. 16 shows a MALDI-TOF spectrum of crude INFA-H5-1-V8 peptide sequences (corresponding to SEQ ID NOs: 425 to 460).

Fig. 17 shows a MALDI-TOF spectrum of crude lipidated INFA-H5-1-V8 peptide sequences (corresponding to SEQ ID NOs: 461 to 496).

Fig. 18(a)-(h) shows the different variosite peptide sequences of the present invention, with variable residues beneath the consensus sequence. Fig. 18(a) is INFA-H5-1-V1; Fig. 18(b) is INFA-H5-1-V2; Fig. 18(c) is INFA-H5-1-V3; Fig. 18(d) is INFA-H5-1-V4; Fig. 18(e) is INFA-H5-1-V5; Fig. 18(f) is INFA-H5-1-V6; Fig. 18(g) is INFA-H5-1-V7; Fig. 18(h) is INFA-H5-1-V8.

Fig. 18(i) shows different lipidated variosite peptide sequences based on the consensus sequence in Fig. 18(h).

Fig. 19 illustrates induction of humoral immunity by a vaccine of the present invention after immunization. Blue bar (top bar) = AviFlu vaccine INFA-02L + alum; Red bar (2nd bar from top) = AviFlu vaccine INFA-02L without adjuvant; Purple bar (middle bar) = AviFlu vaccine INFA-02P + montanide; Green bar (2nd bar from bottom) = AviFlu vaccine INFA-02P + alum; Black bar (bottom bar) = control.

Fig. 20 illustrates a survival plot of vaccinated mice against challenge with H5N1. Legend from top: Black = control; Green = INFA-02P + alum; Purple = INFA-02P + montanide; Red = INFA-02L without adjuvant; Blue = INFA-02L + alum.

Fig. 21 shows induction of humoral immunity by INFA-01P (INFA-HA-1-(V1-V2)) versus INFA-02P (INFA-HA-1-(V1-V8)) after vaccination in mice as measured by HAI titres. Blue bar (bottom bar) = INFA-01P + montanide; Purple bar (middle bar) = INFA-02P + montanide; Black bar (top bar) = control.

Fig. 22 shows a survival plot of mice, vaccinated by INFA-01P (INFA-HA-1-(V1-V2)), against challenge with H5N1. Legend from top: Black = control; Blue – INFA-01P + montanide.

DETAILED DESCRIPTION

Generally, the present invention provides an anti-influenza formulation, and, more specifically, a vaccine for Influenza A, including avian subtypes.

In a first aspect, the present invention provides a peptide-based anti-influenza formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 1 to 496 and analogues thereof. Particularly, the present invention provides a peptide-based anti-influenza formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 1 to 248 and analogues thereof. In addition, the present invention provides a formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 249 to 496 and analogues thereof. In exemplary embodiments, the present invention provides a formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 1 to 212, a formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 249 to 460, a formulation comprising peptides selected from the group consisting of: a) SEQ ID NOs: 1 to 212, and b) SEQ ID NOs: 249 to 460; a formulation comprising peptides selected from the group consisting of: a) SEQ ID NOs: 213 to 248, and b) SEQ ID NOs: 461 to 496; a formulation comprising peptides selected from the group consisting of: a) SEQ ID NOs: 1 to 248, and b) SEQ ID NOs: 249 to 496; or a formulation comprising peptides selected from the group consisting of: a) SEQ ID NOs: 1 to 40, and b) SEQ ID NOs: 249 to 288.

In another exemplary embodiment of the present invention, the formulation comprises at least one peptide sequence from at least one of the following groups: a)SEQ ID NOs: 1 to 24; b) SEQ ID NOs: 25 to 40; c) SEQ ID NOs: 41 to 64; d) SEQ ID NOs: 65 to 88; e) SEQ ID NOs: 89 to 120; f) SEQ ID NOs: 121 to 144; g) SEQ ID NOs: 145 to 176, h) SEQ ID NOs: 177 to 212; i) SEQ ID NOs: 249 to 272; j) SEQ ID NOs: 273 to 288; k) SEQ ID NOs: 289 to 312; l) SEQ ID NOs: 313 to 336; m) SEQ ID NOs: 337 to 368; n) SEQ ID NOs: 369 to 392; o) SEQ ID NOs: 393 to 424; or p) SEQ ID NOs: 425 to 460.

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The formulation can further comprise at least one peptide sequence from SEQ ID NOs: 213 to 248 or SEQ ID NOs: 461 to 496.

In a further aspect of the present invention there is provided a vaccine comprising the formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 1 to 496 and analogues thereof, together with a pharmaceutically-acceptable diluent or carrier. The vaccine can further comprise an adjuvant. In one example, the adjuvant is alum.

The anti-viral formulation can be an anti-influenza formulation. More particularly, the anti-influenza formulation can be an avian anti-influenza formulation.

In a further aspect of the present invention, there is provided a use of the formulation comprising at least one peptide selected from the group consisting of SEQ ID NOs: 1 to 496 and analogues thereof, for the preparation of a vaccine. The vaccine can be used for preventing or treating influenza in an animal in need thereof. In one exemplary embodiment, the influenza is avian influenza. The present invention further relates to a method for inducing an immune response in humans or animals and conferring protection against avian influenza, or novel subtypes of influenza derived from avian influenza, which comprises administering to humans or other animals a peptide-based vaccine as described herein.

In a further aspect of the present invention, there is provided a method for preparing an anti-viral formulation, such as the anti-viral formulation as described herein. According to one embodiment, there is provided a method for preparing a peptide from SEQ ID NOs: 1 to 212 comprising the steps of determining a linear sequence representative of primary sequences of discontinuous epitopes of an avian influenza viral protein, wherein the epitopes are in proximity to each other when the protein is in a folded conformation; and synthesizing a peptide representative of the linear sequence. In another embodiment, there is provided

method for preparing a peptide mixture comprising any two peptide sequences from SEQ ID NOs: 1 to 212 comprising the steps of: determining a linear sequence representative of primary sequences of discontinuous epitopes of an avian influenza viral protein, the epitopes being in proximity to each other when the protein is in a folded conformation; said discontinuous epitopes comprising variable residues, and synthesizing a peptide mixture including at least two different amino acids at a variable residue.

In yet another aspect, the present invention relates generally to an anti-influenza vaccine comprising a mixture of peptides containing at least one hemagglutinin (HA) antigen of influenza virus. Hemagglutinin (HA) is a potent immunogen, and viral neutralizing antibodies are directed against the variable regions of HA. The isolated peptide mixture represents variants of multiple variable regions of hemagglutinin. Thus, in accordance with one aspect of the present invention, there is provided an anti-viral formulation comprising a mixture of isolated peptides, said mixture being formulated on the basis of the variable region of the avian influenza virus HA protein and said isolated peptide mixture representing variants of a variable region of the HA or HA1 protein, wherein each of said variable regions comprising a plurality of variable amino acid residues, at least one of which is represented by two or more amino acids.

In one embodiment, the plurality of variable amino acid residues in the anti-viral formulation comprises three or more residues. One or more of said Avian influenza proteins can be an HA or HA1.

The vaccine may be formulated with or without representing variation at specific residues for each peptide. When variation is not represented, the peptide formed may be referred to herein as a Discotope™ construct. A discotope construct is a linear sequence synthetic construct that approximates the position of primary sequence sections that compose discontinuous epitopes. The individual sections are constructed in sequence to elicit immune responses that recognize the discontinuous epitopes found in the original intact protein.

Discontinuous epitopes are composed of two or more segments of the primary sequence of a protein that when properly folded come together and are bound by specific antibodies. They are not recognized by antibodies when the secondary structure is lost and therefore have not been represented by a continuous linear peptide.

When variation is present at particular residues that are known to have different amino acids represented according to different sequences for that particular pathogen, the formulation comprises a number of peptides, which may be collectively referred to herein as a Discosite™ construct.

Design of eptiopes

Hemagglutinin is the major surface glycoprotein of influenza virus and a potent immunogen against which viral neutralizing antibodies are directed. We have designed eight peptides that mimic discontinuous B- and T-cell epitopes on antigenic sites of HA. The sequences of these peptides are determined based on analysis of the crystal structure of influenza hemagglutinin (HA) protein to determine peptide epitopes. Hemagglutinin is the major surface glycoprotein of influenza virus and a potent immunogen against which viral neutralizing antibodies are directed. The linear peptide epitopes in the cocktail mimic discontinuous epitopes on the HA protein surface. Using bioinformatics software that analyzes the antigenic variation of HA proteins from thousands of human influenza isolates, degenerative peptide cocktails based on these epitopes can be prepared which represent the antigenic variation of HA within these epitopes. Thus, the influenza vaccine formulations of the present invention comprise a cocktail of peptides that represent major epitopes of the HA protein.

HA is the major envelope glycoprotein of influenza virus, and mediates the penetration of virus into host cells. The native HA is formed by the association of three HA monomers which, as a precondition of virus infectivity, are cleaved enzymatically into the amino-terminal HA1 and carboxy-terminal HA2. Based on the three dimensional structure of HA1, antigenic sites have been mapped by determining the amino acid changes of antigenic variants. The antigenic variations were mostly seen surrounding the receptor binding region of HA. including residues around the antibody inaccessible receptor binding pockets. Monoclonal antibodies to these antigenic sites neutralize influenza virus infectivity when the exact sequences are present. Both T and B cell epitopes are found on these sites.

All amino acids changes documented in virus escape mutants, selected by MAB or other methods, were analyzed. The proteins were aligned, and position of those amino acids was mapped onto 3-D structure of hemagglutinin H5. The location of the epitope was

roughly predicted in hemagglutinin H5 protein as the area surrounds amino acids that undergo the immune pressure. Antigenic sites were then redefined using the three-dimensional structure of A/duck/Singapore/3/97 hemagglutinin (PDB ID code: 1JSM) in a sense that antigenic determinants must be freely accessible for B-cell antibodies, and that different segments of same epitope must be in close proximity to each other (for example, within 20 A).

The occurrence of amino acids at variable sites within constructed epitopes was assessed by analyzing hemagglutinin HA1 strains of Influenza A (subtype H5) virus, available in the Los Alamos Data Base as of June 28, 2005. Either 460 from all hosts or only 38 human hemagglutinin HA1 strains were used for analysis. A variable residue was defined as a position in which the occurrence of the most frequent amino acid at that position is less than 85% among all viral sequences examined.

A plurality of variable amino acid residues may comprise three or more residues, with two or more different amino acids at each variable position.

Part or all of the peptides comprising an influenza vaccine may be lipidated.

Discontinuous epitopes are composed of two or more segments of the primary sequence of a protein that exist in close proximity when in a native, three-dimensional conformation. They are not recognized by antibodies when the secondary or tertiary structure is lost; thus, linear peptides cannot traditionally be used to represent discontinuous epitopes. Crystallographic data from influenza hemagglutinin was used to design linear sequences that represent at least five conformational epitopes.

From each variable epitope, the peptide length is selected, and within the peptide, a plurality of variable residues is selected. Each variable residue has at least two optional amino acids, found naturally occurring in sequenced versions of the virus. In this way, a high degree of variability is represented. For example, three or four variable residues may be represented in the mixture of peptides, each having two or more different amino acids represented in the sequenced database records for influenza variants. If two variable residues occur in a variable region, then 2^2 different peptides would be used in the mixture representing that particular region. If three or four variable residues are indicated in a hypervariable region, the number of peptides in the resulting mixture would be 2^3 and 2^4, respectively. Generally, if variable regions consist of A, B, C, and so on variable sites, with a,

b, c, and so on different amino acids at respective site, the total number of peptides would be $A^axB^bxC^c$ and so on.

Once the proteins, variable epitopes, peptide lengths, and variable residues are selected, the synthesis of the peptide mixtures occurs, according to any acceptable method of peptide synthesis.

Peptide mixtures are synthesized with each different peptide sequence represented in roughly equimolar quantities. However, there is no requirement to provide equimolar quantities of the individual peptides.

Lipidation of peptides may be conducted by any conventional or acceptable route, as would be known to those of skill in the art. Peptides need not be lipidated, but it may be advantageous for certain peptides to be lipidated with any acceptable lipid, such as palmitic acid, so as to allow a peptide to pass through a cell membrane. Peptides incorporating lipid may benefit from placement of a KSS motif at the C-terminal. The peptides incorporating lipid may contain 1 or more lipid moieties, for example, two lipid moieties per peptide. Immunization with lipidated peptides may result in an enhanced cytotoxic T lymphocyte (CTL) response.

Peptides in accordance with one aspect of the present invention (i.e., corresponding to SEQ ID NOs. 1-212 and SEQ ID NOs. 249-460) form 8 groups derived from H5 antigenic sites on hemagglutinin. These groups are identified as INFA-H5-1-V1, INFA-H5-1-V2, INFA-H5-1-V3, INFA-H5-1-V4, INFA-H5-1-V5, INFA-H5-1-V6, INFA-H5-1-V7 and INFA-H5-1-V8.

The groups contain the following sequences:

Groups INFA-H5-1-V1 (SEQ ID NOs 1-24 and SEQ ID NOs. 249-272), INFA-H5-1-V3 (SEQ ID NOs 41-64 and SEQ ID NOs. 289-312), INFA-H5-1-V4 (SEQ ID NOs 65-88 and SEQ ID NOs. 313-336) and INFA-H5-1-V6 (SEQ ID NOs 121-144 and SEQ ID NOs. 369-392) consist of 24 peptide variants.

Groups INFA-H5-1-V5 (SEQ ID NOs 89-120 and SEQ ID NOs. 337-368) and INFA-H5-1-V7 (SEQ ID NOs 145-176 and SEQ ID NOs. 393-424) consist of 32 peptide variants.

Group INFA-H5-1-V2 (SEQ ID NOs 25-40 and SEQ ID NOs. 273-288) consists of 16 peptide variants.

Group INFA-H5-1-V8 (SEQ ID NOs 177-212 and SEQ ID NOs. 425-460) consists of 36 peptide variants.

During typical preparation of the peptide sequences, an additional residue (such as a glycine residue) may be added at an end of sequence. Sequences corresponding to peptides having an additional glycine residue are shown in SEQ ID NOs: 249 to 496. The additional glycine residue has no material effect on the function of the peptide, and the presence of the glycine residue is merely a product of peptide synthesis which would be well understood to the person of ordinary skill in the art. In addition, therefore, peptides normally synthesized in this manner would represent typical examples of "analogues" (as described below) of peptides used in the preparation of formulations in accordance with one aspect of the present invention.

Figs. 1 and 9 are related to peptide group INFA-H5-1-V1. Fig. 1 shows an analytical HPLC chromatogram of crude INFA-H5-1-V1 peptides. Fig. 9 shows a MALDI-TOF spectrum of crude INFA-H5-1-V1 peptides.

Figs. 2 and 10 are related to peptide group INFA-H5-1-V2. Fig. 2 shows an analytical HPLC chromatogram of crude INFA-H5-1-V2 peptides. Fig. 10 shows a MALDI-TOF spectrum of crude INFA-H5-1-V2 peptides.

Figs. 3 and 11 are related to peptide group INFA-H5-1-V3. Fig. 3 shows an analytical HPLC chromatogram of crude INFA-H5-1-V3 peptides. Fig. 11 shows a MALDI-TOF spectrum of crude INFA-H5-1-V3 peptides.

Figs. 4 and 12 are related to peptide group INFA-H5-1-V4. Fig. 4 shows an analytical HPLC chromatogram of crude INFA-H5-1-V4 peptides. Fig. 12 shows a MALDI-TOF spectrum of crude INFA-H5-1-V4 peptides.

Figs. 5 and 13 are related to peptide group INFA-H5-1-V5. Fig. 5 shows an analytical HPLC chromatogram of crude INFA-H5-1-V5 peptide. Fig. 13 shows a MALDI-TOF spectrum of crude INFA-H5-1-V5 peptides.

Figs. 6 and 14 are related to peptide group INFA-H5-1-V6. Fig. 6 shows an analytical HPLC chromatogram of crude INFA-H5-1-V6 peptides. Fig. 14 shows a MALDI-TOF spectrum of crude INFA-H5-1-V6 peptides.

Figs. 7 and 15 are related to peptide group INFA-H5-1-V7. Fig. 7 shows an analytical HPLC chromatogram of crude INFA-H5-1-V7 peptides. Fig. 15 shows a MALDI-TOF spectrum of crude INFA-H5-1-V7 peptides.

Figs. 8 and 16 are related to peptide group INFA-H5-1-V8. Fig. 8 shows an analytical HPLC chromatogram of crude INFA-H5-1-V8 peptide. Fig. 16 shows a MALDI-TOF spectrum of crude INFA-H5-1-V8 peptides.

Fig. 17 shows a MALDI-TOF spectrum of crude lipidated INFA-H5-1-V8 peptide sequences (corresponding to SEQ ID NOs: 213 to 248). SEQ ID NOs 213-248 are lipidated versions of SEQ ID NOs 177-212.

Fig. 18 shows the variosites of the present invention, including variable amino acid residues. Fig. 18 (a) shows the peptides of group INFA-H5-1-V1. Fig. 18 (b) shows the peptides of INFA-H5-1-V2. Fig. 18 (c) shows the peptides of INFA-H5-1-V3. Fig. 18 (d) shows the peptides of INFA-H5-1-V4. Fig. 18 (e) shows the peptides of INFA-H5-1-V5. Fig. 18 (f) shows the peptides of INFA-H5-1-V6. Fig. 18 (g) shows the peptides of INFA-H5-1-V7. Fig. 18 (h) shows the peptides of INFA-H5-1-V8.

Design of vaccine formulations

In the context of the present invention, a vaccine formulation is a cocktail of peptides that are used in the preparation of an influenza vaccine. The vaccine can comprise the cocktail of peptides and other substituents known in the art that would be found acceptable for inclusion. These substituents can include, but are not limited to, adjuvants, diluents and/or carriers.

As used in the present application, a peptide "analogue" can include a variant in which one or more residues are added, deleted, inserted or substituted, while having no material effect on the function of the peptide. That is, a peptide analogue in accordance with one aspect of the present invention should be capable of inducing an antibody or T-cell response to HA. A residue (or residues) may be added or deleted from either end of the peptide, deleted from within the peptide, inserted within the peptide, or substituted for one or more of the residues within the peptide. As would be understood by a person of ordinary skill in art, one or more peptide residues may be added, deleted, inserted or substituted while still maintaining the function of the peptide. For example, as many as five or more residues may be added to or removed from either end of a peptide, or inserted into a peptide, and be considered a peptide analogue within the context of the present invention. In a further example, a conservative substitution of one or more residues within a peptide may result in a

peptide analogue. As would be well understood to the skilled artisan, a conservative substitution includes a substitution of one amino acid residue with another amino acid residue having one or more similar chemical properties, such as polarity, charge, hydrophobicity, or aromaticity, for example.

The vaccine formulations of the present invention are particularly suitable for preparing vaccines in the treatment of avian influenza. However, it will be appreciated that any combination of peptide sequences, or formulations comprising these peptide sequences, may be used in other influenza phenotypes.

A vaccine of the present invention may be formulated from a peptide mixture with or without variation at specific residues within each peptide. When variation is not present, the peptide formed is referred to herein as a consensus epitope. When variation is present at particular residues that are known to have different amino acids represented according to different sequences for that particular viral variant or subtype, the formulation comprises a number of peptides, collectively referred to as a variable epitope or "variosite".

Peptide vaccines can be prepared with a pool of one or more peptide sequences from SEQ ID NOs: 1 to 212 or SEQ ID NOs: 249 to 460 representing epitopes contained in the three-dimensional structure of HA. The vaccines may further comprise one or more lipidated peptides, including one or more peptides from SEQ ID NOs: 213 to 248 or SEQ ID NOs: 461 to 496. The vaccines may comprise one or more discotope constructs (peptides containing non-variable amino acid residues) or one or more discosite constructs (peptides containing variable amino acid residues). A discosite construct of the present invention is derived from one of these epitopes. Thus, a discosite construct formulation comprises one or more peptide sequences derived from the epitope containing the variable residues.

Each discosite construct of the present invention represents 2^x possible peptide sequences based on x varied residues. For example, a discosite construct having 3 or 4 variable residues represents $2^3 = 8$ or $2^4 = 16$ sequences, respectively. Therefore, in the context of the present invention, a discosite construct as referred to herein includes the epitope sequence containing the variable residues and the one or more possible sequences derived therefrom. It will be appreciated by the person of ordinary skill in the art that additional sequences may or may not be added as required.

The vaccine may be prepared by any methodology acceptable to one skilled in the art. For example, oligonucleotides encoding these peptides may be inserted in viral or non-viral vectors for delivery. Peptides may be synthesized individually in and mixed together to accomplish an acceptable formulation. Any variety of different modes by which these peptide antigens may be prepared is acceptable for use with the invention.

In order to formulate a vaccine that is subtype-specific, the proteins selected may contain variable regions with selected variable amino acids that are characteristic of the variability found within the subtype of interest. This allows the vaccine to be subtype specific, which may have the advantage of better representing the antigenic variation among variants within said subtype. To formulate a vaccine that has less subtype distinctiveness, the final peptide formulation may comprise the different subtype specific formulations. For example, a vaccine formulated against avian flu could target variable residues particular to subtype H5 sequences. A vaccine formulated against human flu could target variable residues characteristic of subtypes 1, 2 and/or 3 sequences.

In order to formulate a vaccine that is species-specific, the proteins selected may contain variable regions with selected variable amino acids that are characteristic of the variability found within the species of interest. This allows the vaccine to be species specific, which may have the advantage of better representing the antigenic variation among variants within said species. For example, a vaccine formulated against avian flu could target variable residues particular to avian H5 sequences. A vaccine formulated against human flu could target variable residues characteristic of both, human and avian H5 sequences.

As a specific example, the anti-INF vaccine may include the following isolated peptides: SEQ ID NOs: 1 to 40, SEQ ID NOs: 249 to 288, or peptide analogues thereof, in combination with a pharmaceutically acceptable carrier.

An exemplary anti-INF formulation may comprise one or more of, or all of SEQ ID NOs: 1 to 212, SEQ ID NOs. 249 to 460 or peptide analogues thereof; in combination with a pharmaceutically acceptable carrier. The formulation may also comprise one or more lipidated peptides of SEQ ID NOs: 1 to 212 or SEQ ID NOs: 249 to 460, such as, for example, one or more of SEQ ID NOs: 213 to 248 or SEQ ID NOs: 461 to 496.

Although all peptides of SEQ ID NOs: 1 to 212 or SEQ ID NOs: 249 to 460 may be used in combination as the vaccine formulation, sub-groups of these peptides could be used

together according to the invention. For example, a formulation may comprise at least one peptide sequence from at least one of the following groups: a) SEQ ID NOs: 1 to 24; b) SEQ ID NOs: 25 to 40; c) SEQ ID NOs: 41 to 64; d) SEQ ID NOs: 65 to 88; e) SEQ ID NOs: 89 to 120; f) SEQ ID NOs: 121 to 144; g) SEQ ID NOs: 145 to 176, h) SEQ ID NOs: 177 to 212, i)SEQ ID NOs: 249 to 272; j) SEQ ID NOs: 273 to 288; k) SEQ ID NOs: 289 to 312; l) SEQ ID NOs: 313 to 336; m) SEQ ID NOs: 337 to 368; n) SEQ ID NOs: 369 to 392; o) SEQ ID NOs: 393 to 424; or p) SEQ ID NOs: 425 to 460. Further, a vaccine of the present invention may comprise a formulation comprising 2^n peptide sequences from at least one of the following groups: a) SEQ ID NOs: 1 to 24; b) SEQ ID NOs: 25 to 40; c) SEQ ID NOs: 41 to 64; d) SEQ ID NOs: 65 to 88; e) SEQ ID NOs: 89 to 120; f) SEQ ID NOs: 121 to 144; g) SEQ ID NOs: 145 to 176, h) SEQ ID NOs: 177 to 212, i) SEQ ID NOs: 249 to 272; j) SEQ ID NOs: 273 to 288; k) SEQ ID NOs: 289 to 312; l) SEQ ID NOs: 313 to 336; m) SEQ ID NOs: 337 to 368; n) SEQ ID NOs: 369 to 392; o) SEQ ID NOs: 393 to 424; or p) SEQ ID NOs: 425 to 460, wherein n is 1 to 4.

EXAMPLES

Peptide synthesis

The peptides were synthesized by solid phase peptide synthesis (SPPS) using 9-fluoroenylmethoxycarbonyl (Fmoc) chemistry on Pioneer™ automated peptide synthesizer, utilizing pre-loaded Fmoc protected NovaSyn™ TGT resin (NovaBiochem) as described. Where variability at a given position is desired, mixture of two amino acids is placed at that position. This is repeated each time during the synthesis wherever the variability is desired. While 1M solution of 2-(1H-Benzotriazole-1-yl)-1, 1,3,3tetramethyluronium tetrafluoroborate (TBTU) and N-Hydroxybenzotriazole (HOBt) in dimethylformamide (DMF), and 1 M solution of diisopropylethyl amine (DIPEA) in OMF was used for coupling amino acids, 20% piperidine in DMF was used for deblocking amino acids during the synthesis. Coupling was allowed to occur for one hour at room temperature. After the last amino acid was coupled, the resin was taken out from synthesizer and washed on a sintered glass funnel several times with OMF, with 2-propanol and with dichloromethylene (DCM), and dried under high vacuum. The peptide mixtures are cleaved and deprotected by the addition of a solution containing TFA / water / phenol / thioanisole / EDT /TIS [82:5:5:5:2:1]. The resin was

incubated at room temperature for 4 hours. Cleavage mixture was then filtered under reduced pressure into a flask containing a 10-fold volume of cold ether. Resin was also rinsed twice with TFA into the same ether solution. Following incubation for 30 minutes in a freezer to further assist precipitation, the sample was centrifuged at 1 ,000Xg for 5 minutes, and the ether removed. This extraction process was repeated three times. Following a final ether extraction, the residual organic solvent was evaporated under nitrogen gas, and the peptide mixture was redissolved in water and purified by using high performance liquid chromatography (HPLC). Excess of the solvent was removed by using a rotor evaporator, and finally lyophilized to dry powder. Mass spectrometry and amino acid analysis were performed on all the Discotopes to ensure that they have the appropriate peptide content.

Lipidation is performed as follows. Upon completion of the synthesis of a mixed peptide formulation on the synthesiser, the resin is removed from the column and placed into a vial. Dissolve 10 eq. of the Palmitic Acid, 10 eq. of TBTU and 10 eq. HOBT (all relative to the resin) in DMF (10 ml/0.1 mmol resin). Add the solution to the peptidyl resin in the vial. Add 20 eq. (relative to the resin) of the DIPEA. Adjust pH to 8-9 by adding DIPEA drop-wise. Seal the vial with a screw cap and shake the mixture overnight (at least 12 hours).

Vaccine efficacy

Vaccine formulations comprising the peptide sequences of the present invention were tested in mice. The vaccines used are as follows:

INF-01P consists of two variosites INFA-H5-1-V1 and INFA-H5-1-V2 (SEQ ID NOs: 249-288), also referred as AviFlu-2(Montanide).

INF-02P consists of 8 variosites INFA-H5-1-V1 to INFA-H5-1-V8 (SEQ ID NOs: 249-460), also referred as AviFlu-8(Montanide), AviFlu(Montanide), AviFlu(Alum).

INF-02L consist of 9 variosites INFA-H5-1-V1 to INFA-H5-1-V8L (SEQ ID NOs: 249-496), also referred as AviFlu(Lipidated), AviFlu(Lipidated/Alum).

Fig. 19 illustrates induction of humoral immunity by a vaccine of the present invention after immunization.

Fig. 20 illustrates a survival plot of mice vaccinated with a vaccine of the present invention against challenge with H5N1.

Fig. 21 shows induction of humoral immunity by INFA-01P (INFA-HA-1-(V1-V2)) versus INFA-02P (INFA-HA-1-(V1-V8)) after vaccination in mice as measured by HAI titres.

Fig. 22 shows a survival plot of mice, vaccinated by INFA-01P (INFA-HA-1-(V1-V2)), against challenge with H5N1.

The above-described embodiments of the present invention are intended to be examples only. Alterations, modifications and variations may be effected to the particular embodiments by those of skill in the art without departing from the scope of the invention, which is defined solely by the claims appended hereto.

References:

1. Philpott, M. et al., *Journal of virology* (1990), 64(6), 2941-2947.
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3. Hioe, C. et al., *Journal of Virology* (1990), 64(12), 6246-6251.
4. Ha, Y. et al., *Proceedings of the National Academy of Sciences, USA* (2001), 98, 11181-11186.
5. Macken, C. et al., "The value of a database in surveillance and vaccine selection." in *Options for the Control of Influenza IV*. A.D.M.E. Osterhaus, N. Cox & A.W. Hampson (Eds.) Amsterdam: Elsevier Science, 2001, 103-106.

CLAIMS:

1. A peptide-based immunogenic composition comprising a peptide which is SEQ ID NO:1.

2. The composition of claim 1 further comprising at least one peptide selected from the group consisting of SEQ ID NOs:2 to 24.

3. The composition of claim 1 further comprising a pharmaceutically-acceptable diluent or carrier.

4. The composition of claim 3 further comprising an adjuvant.

5. The composition of claim 4 wherein the adjuvant is alum.

6. An immunogenic composition comprising a mixture of isolated peptides selected from the group consisting of SEQ ID NOs:1 to 24, said peptides representing variants of at least one variable region of an avian influenza virus HA or HA1 protein, wherein each of said variable regions comprises one or more variable amino acid residues, at least one of said variable amino acid residues is represented by two or more amino acids.

7. The immunogenic composition of claim 6, wherein said one or more variable amino acid residues is represented by three or more residues.

8. The immunogenic composition of claim 6, further comprising a peptide selected from the group consisting of SEQ ID NOs:25 to 40.

9. The composition of claim 1, further comprising a peptide selected from the group consisting of SEQ ID NOs:25 to 40.

10. The composition of claim 1, comprising peptides of SEQ ID NOs:1 to 24.

11. The composition of claim 1, comprising peptides of SEQ ID NOs:1 to 40.

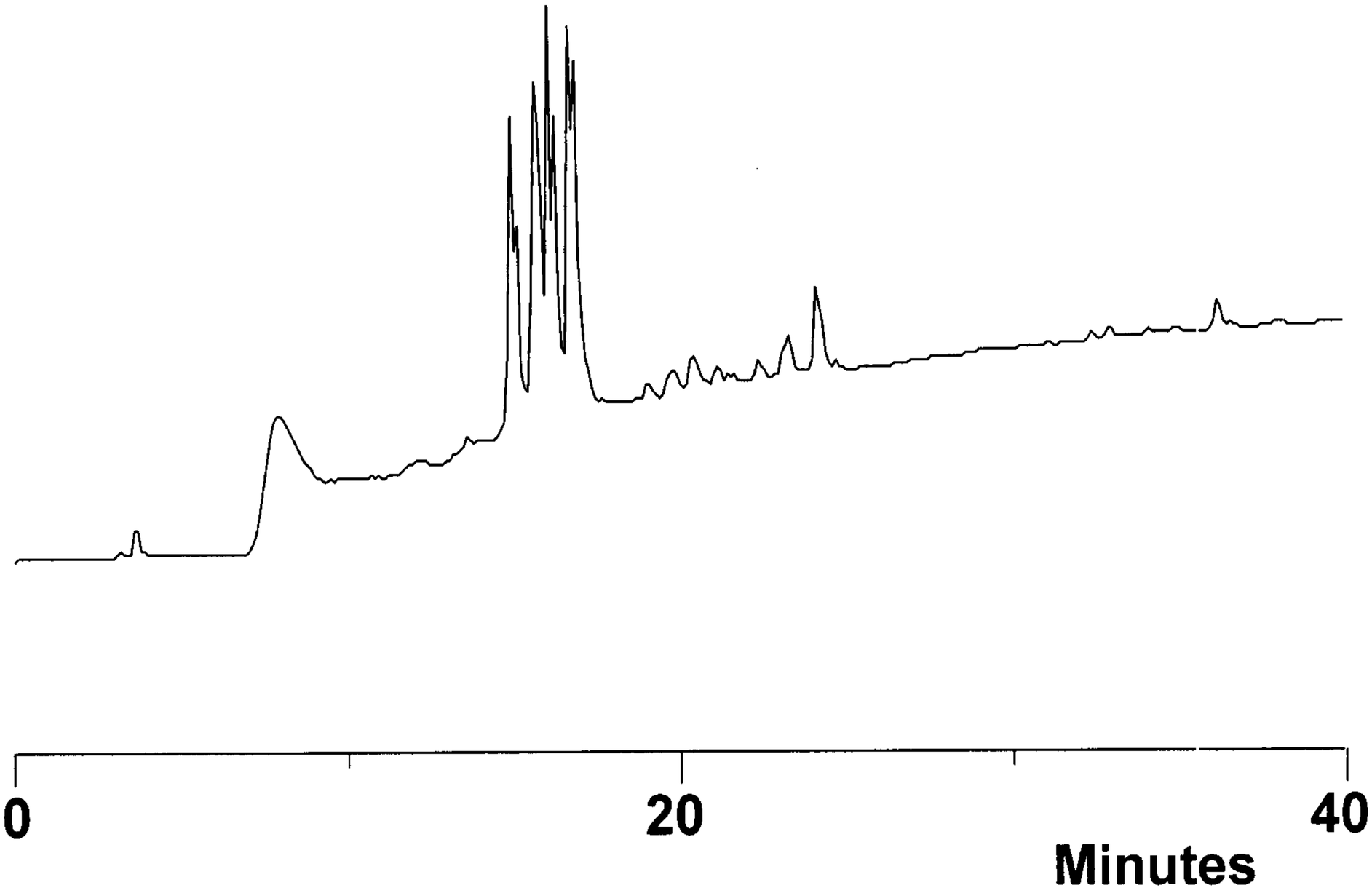


FIG. 1

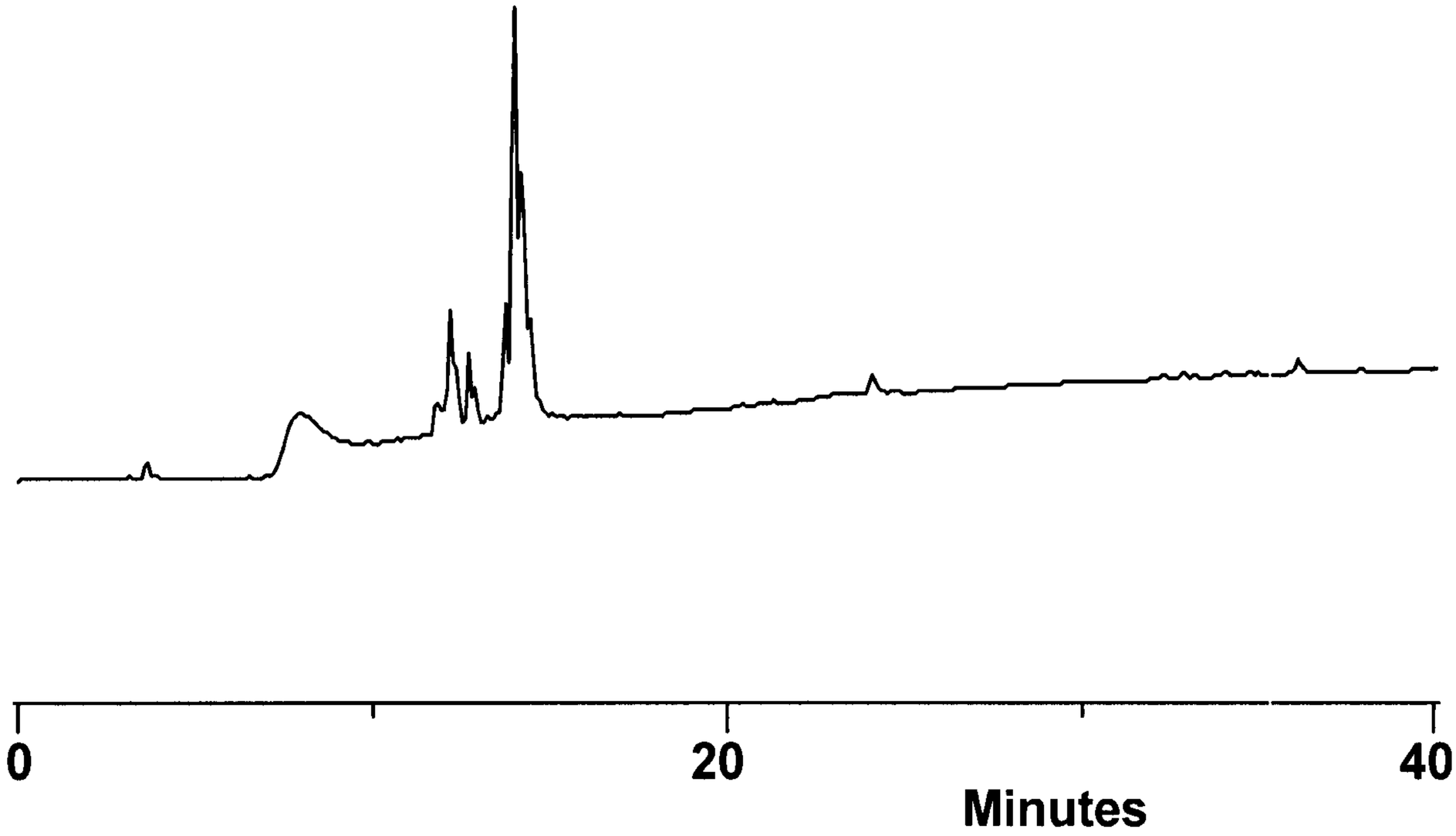


FIG. 2

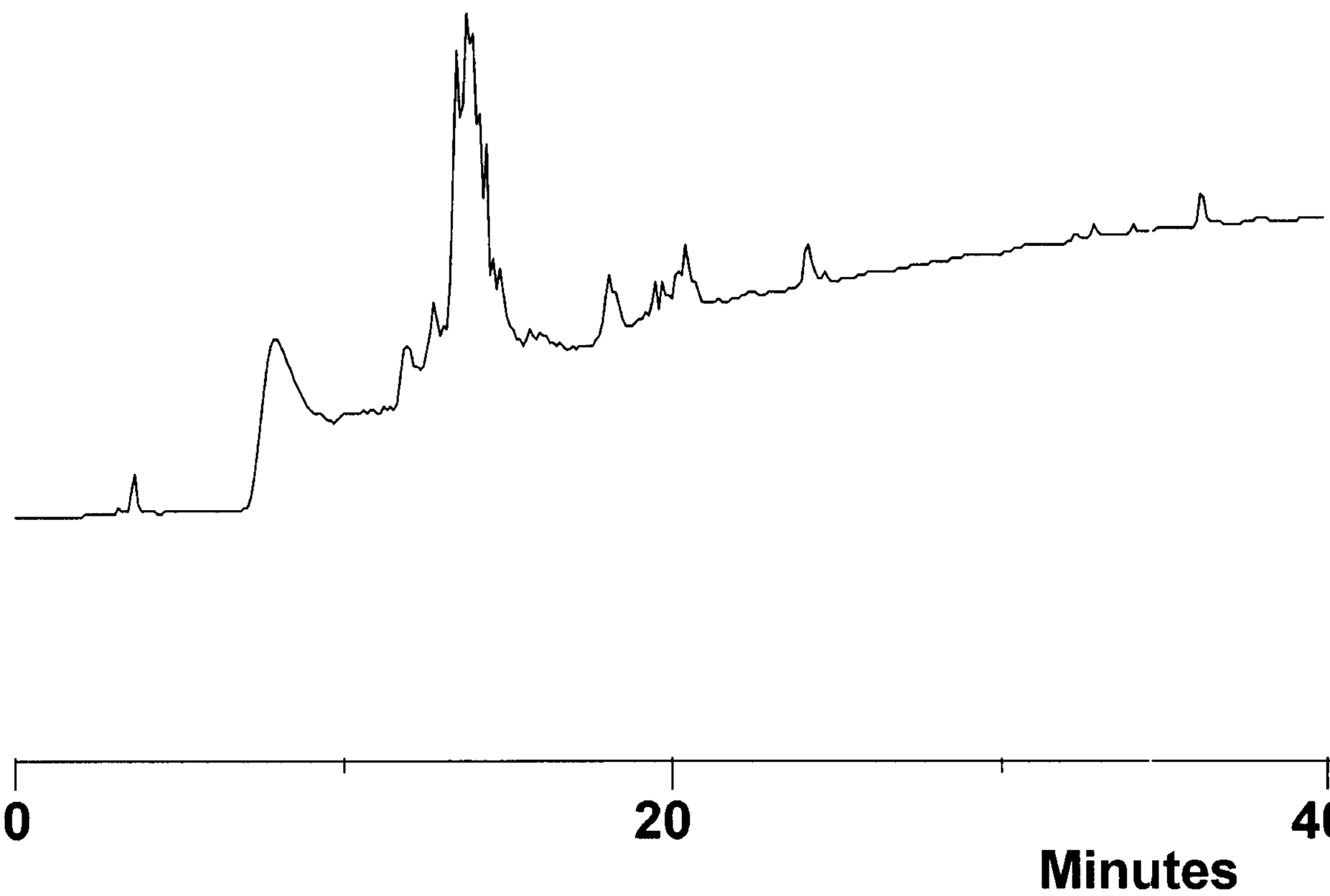


FIG. 3

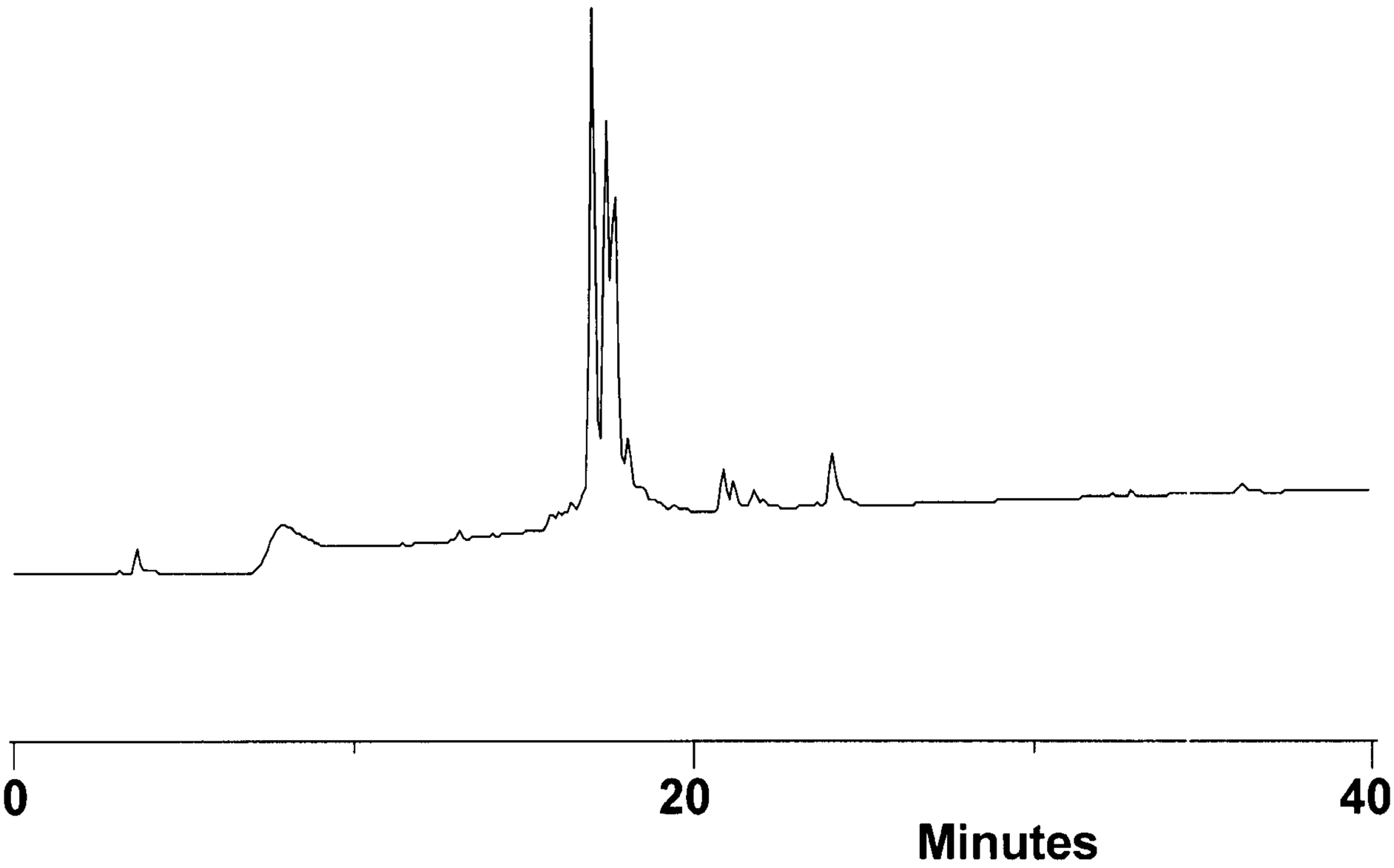


FIG. 4

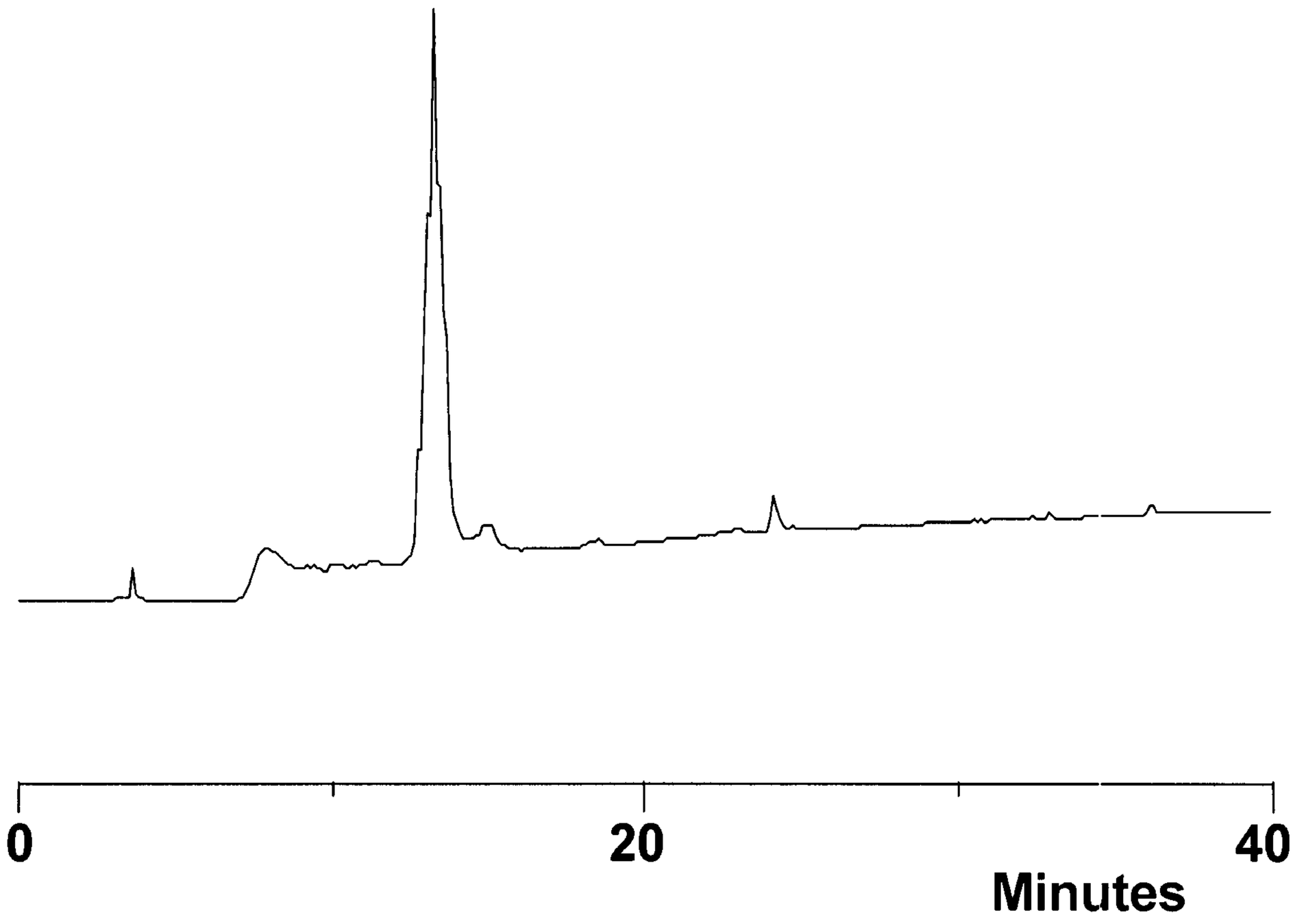


FIG. 5

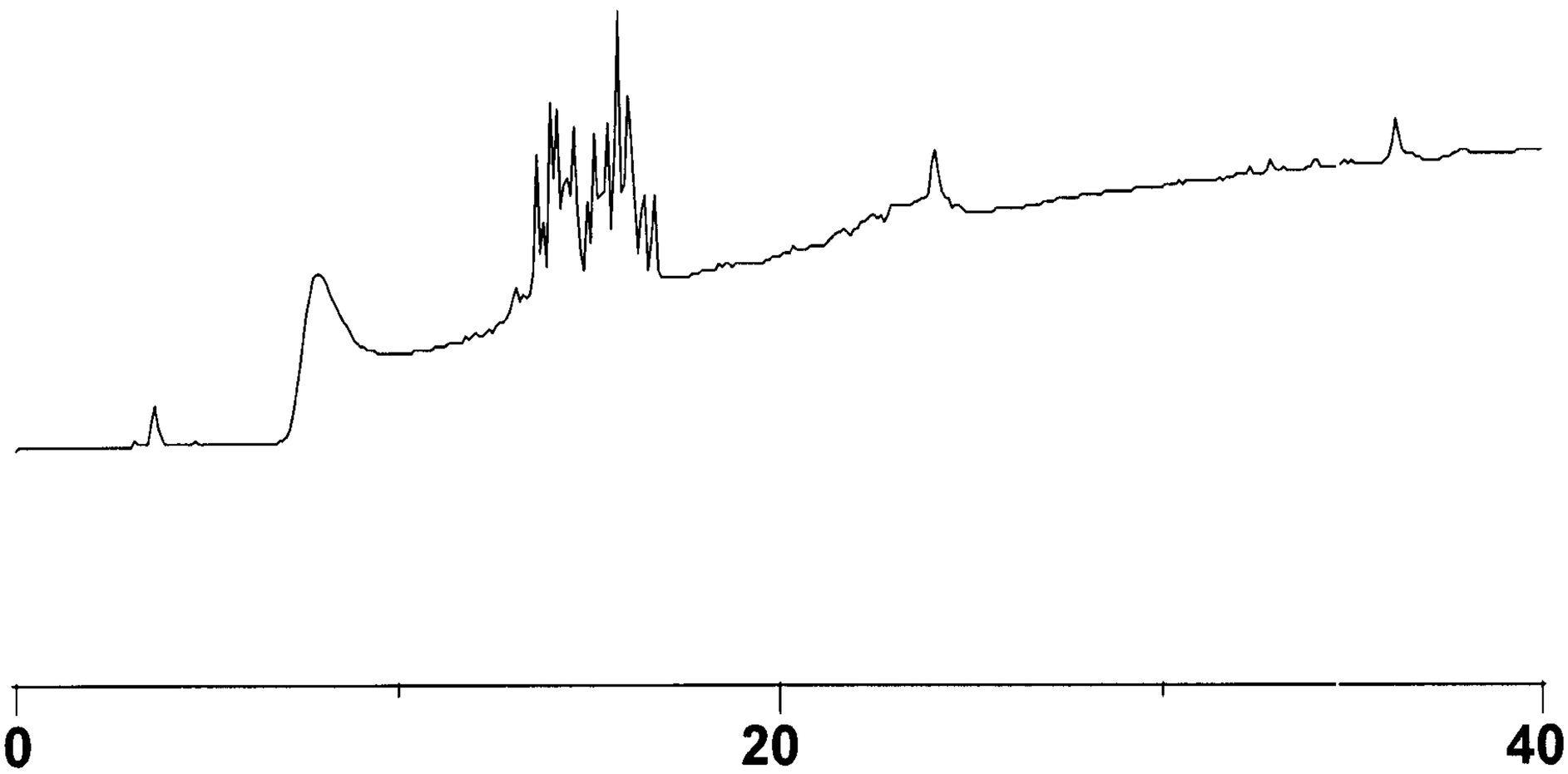


FIG. 6

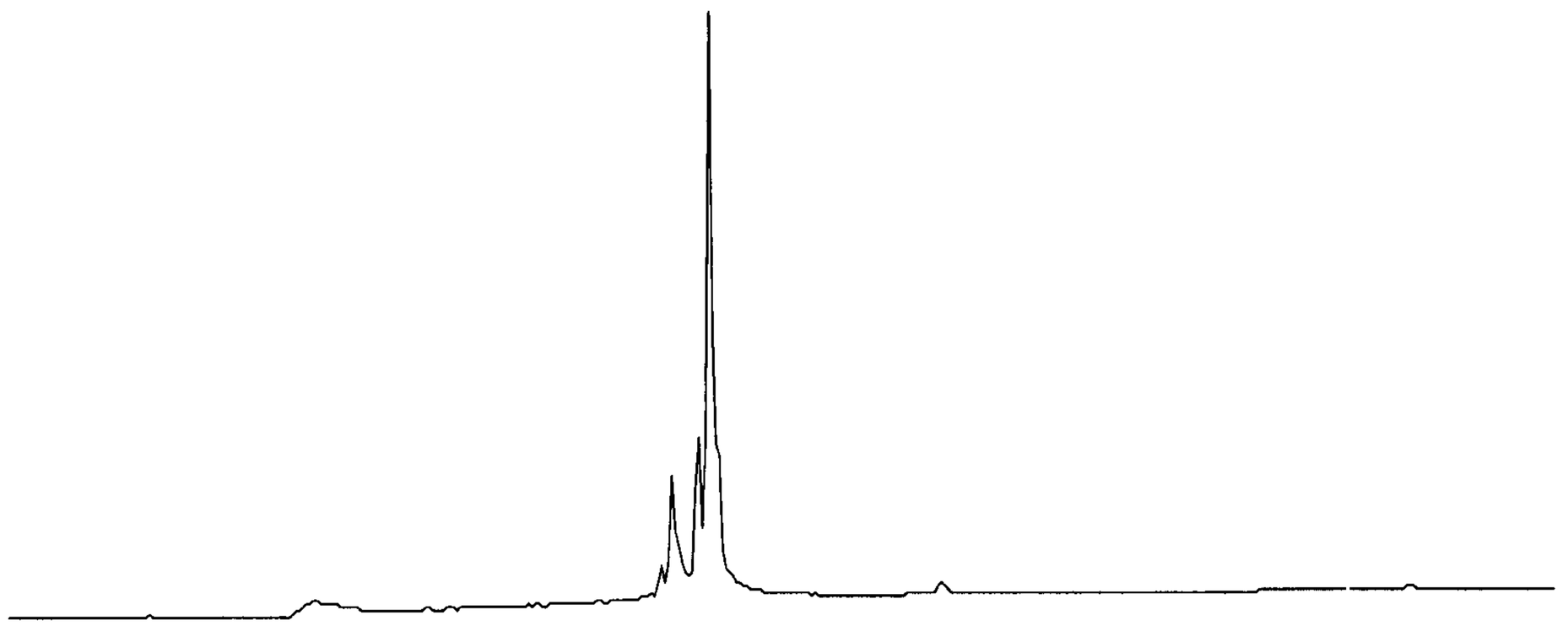


FIG. 7

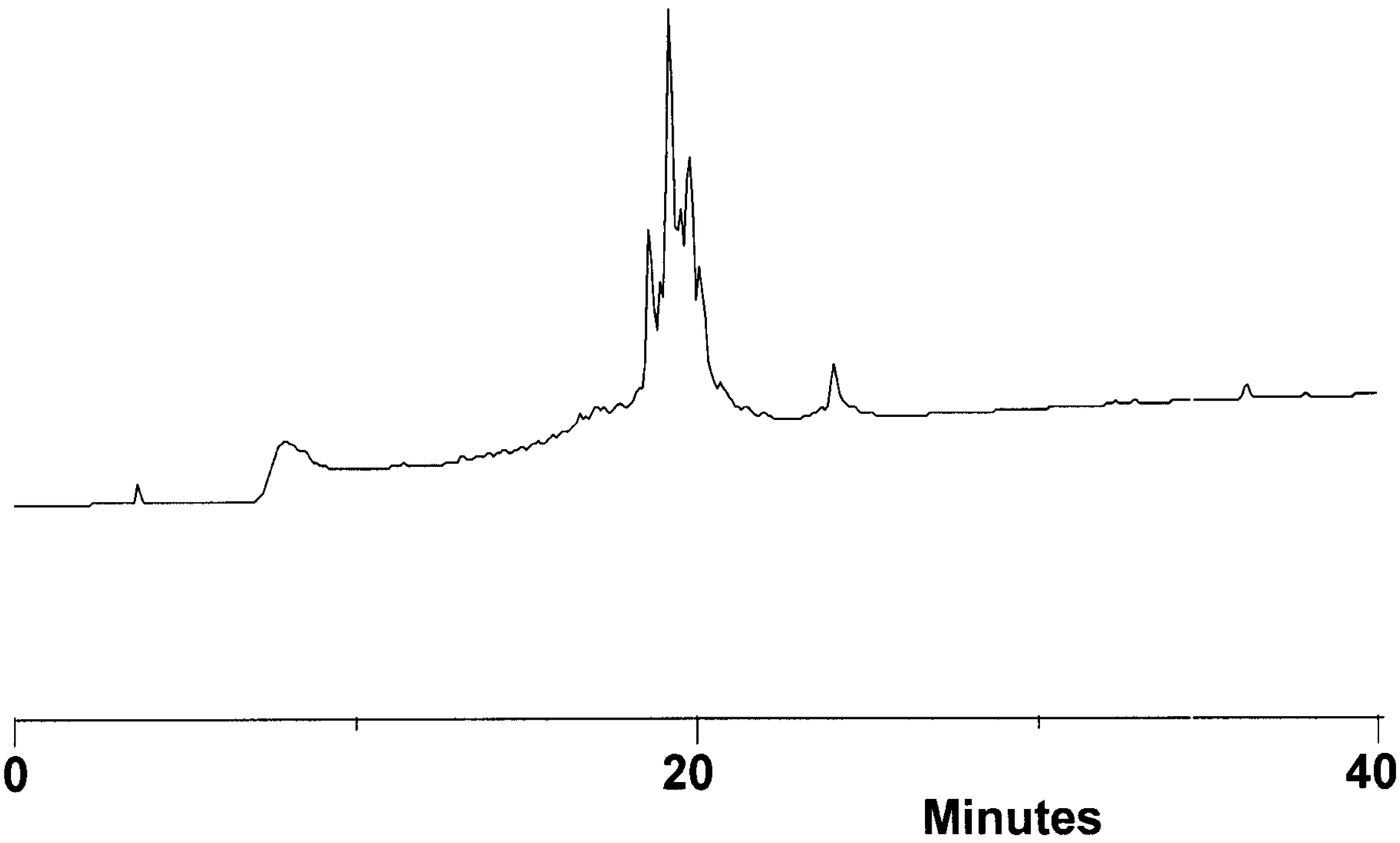
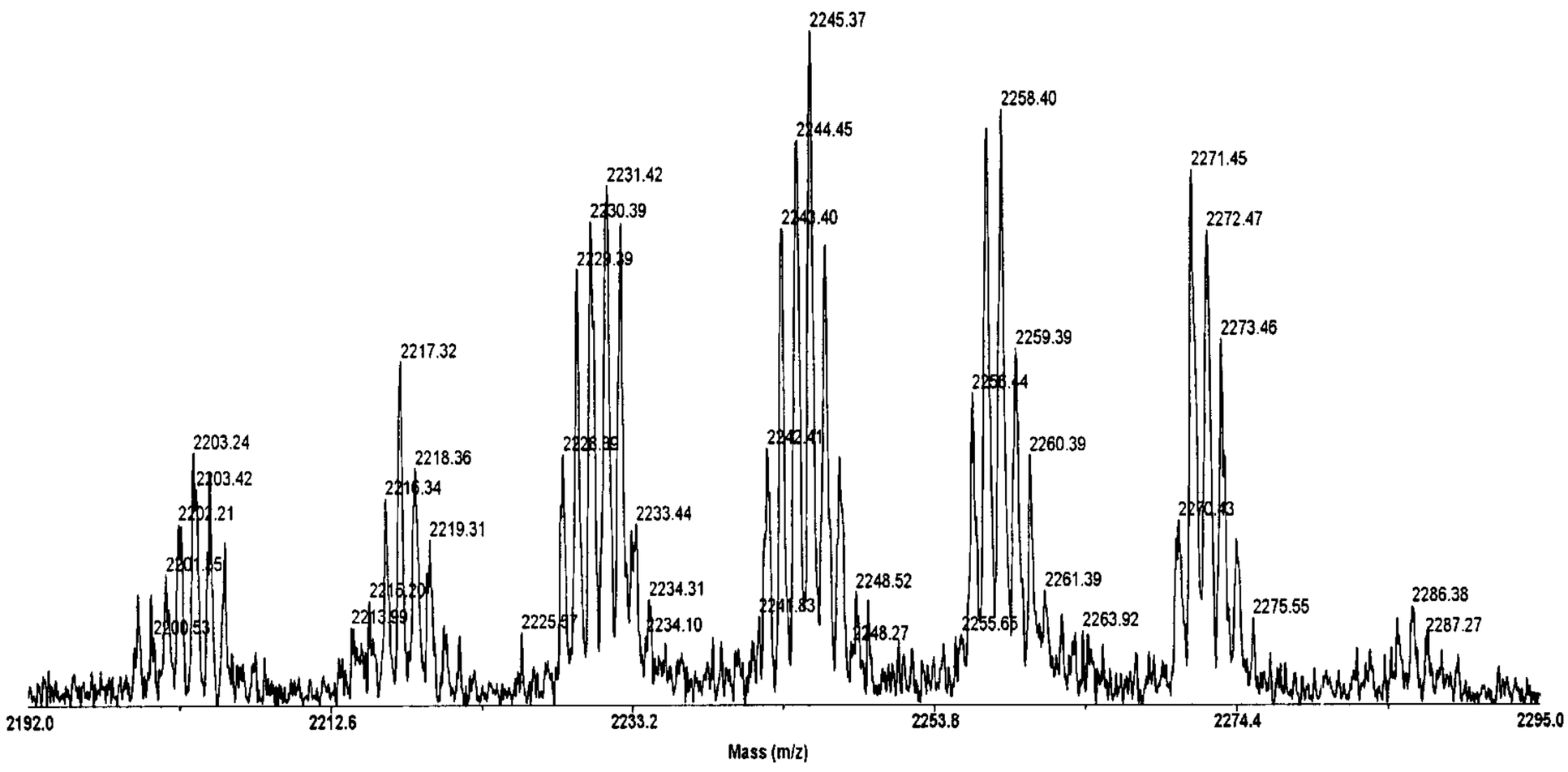


FIG. 8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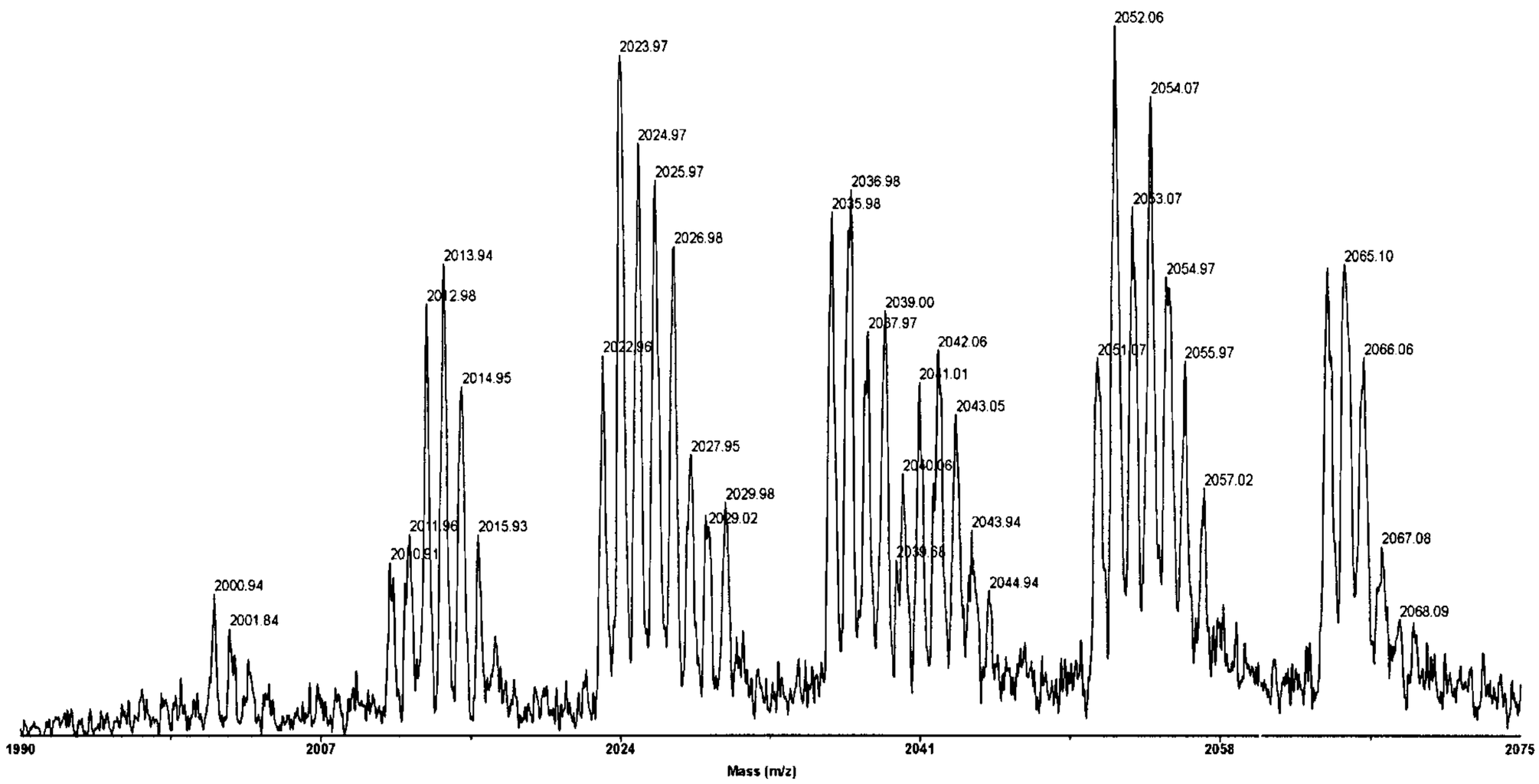
Mass (m/z)

FIG. 9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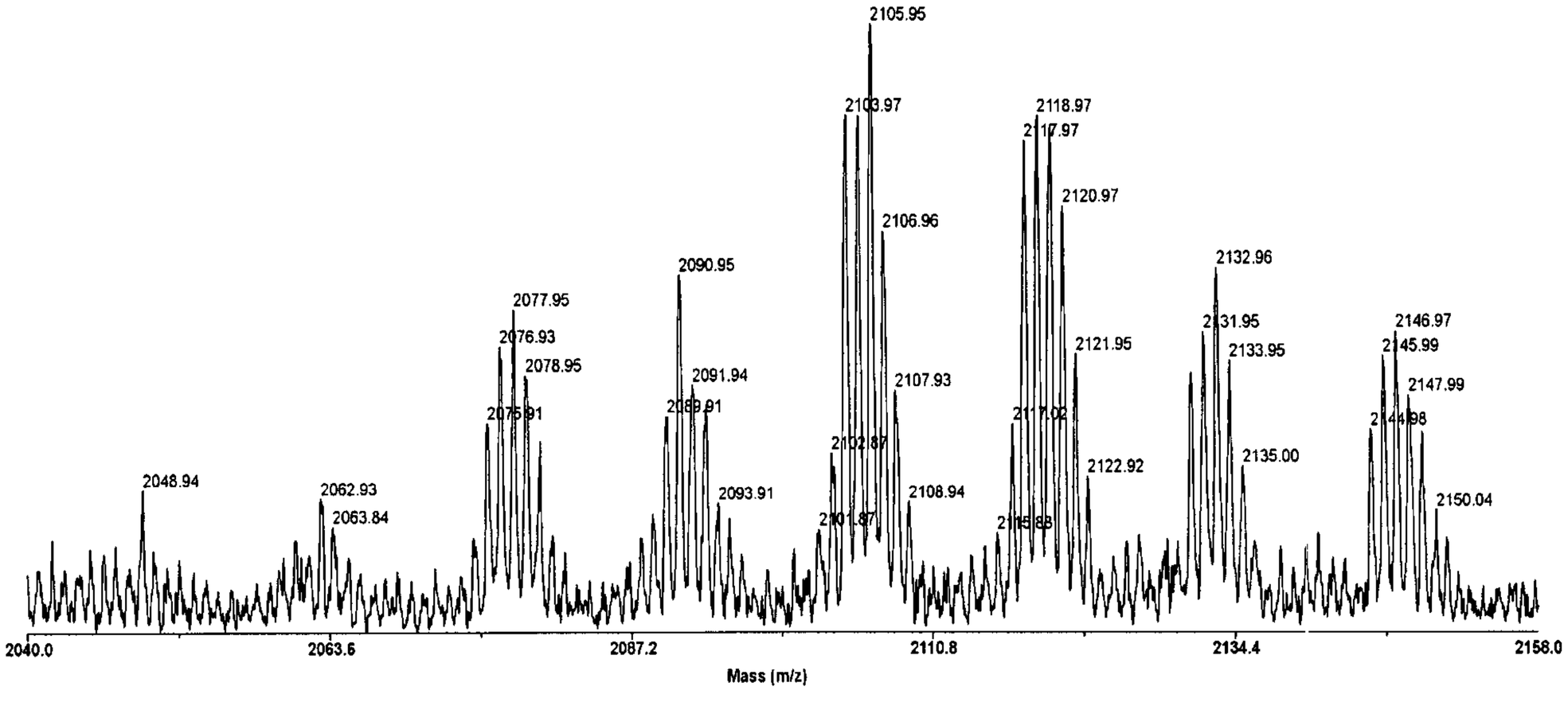
Mass (m/z)

FIG. 10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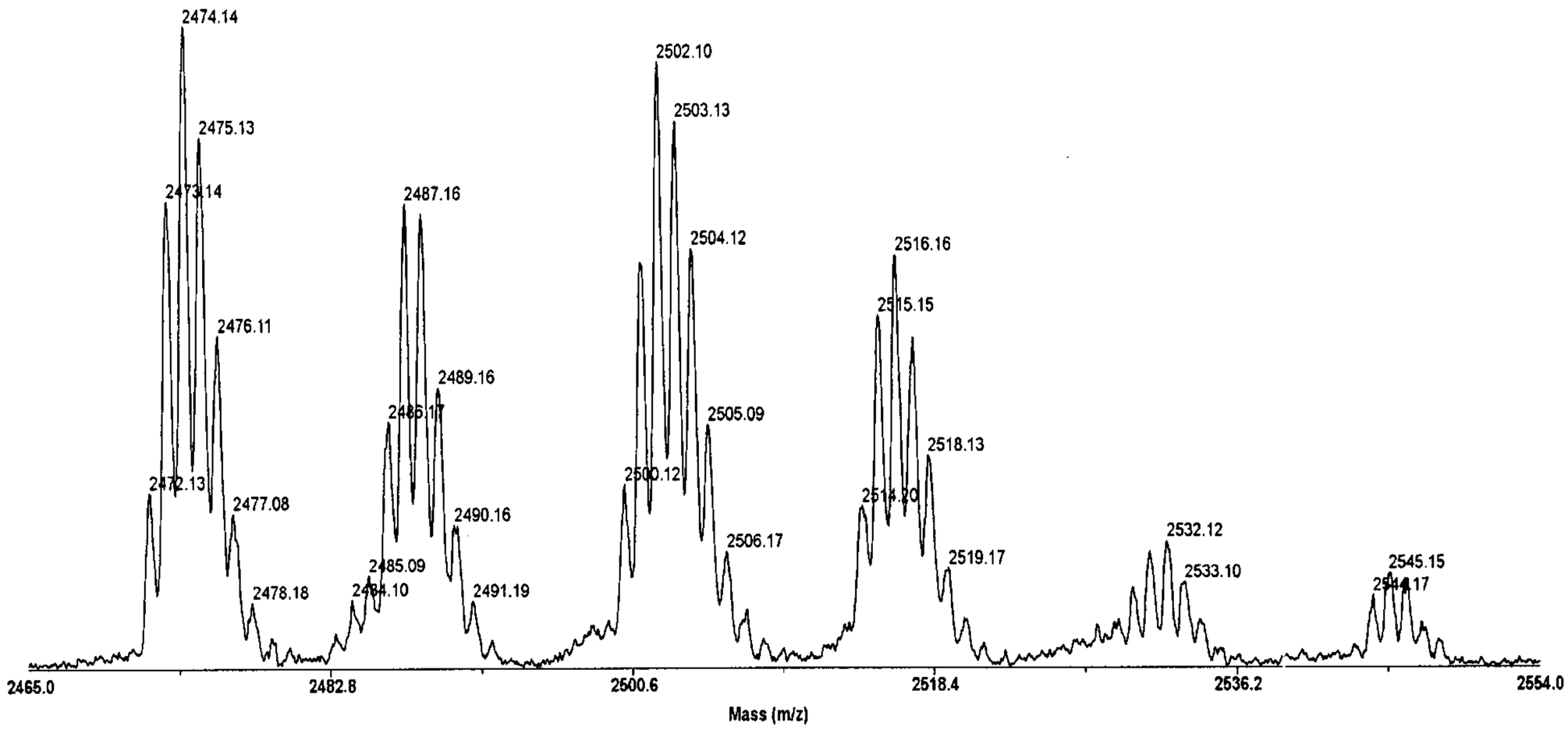
Mass (m/z)

FIG. 11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Mass (m/z)

FIG. 12

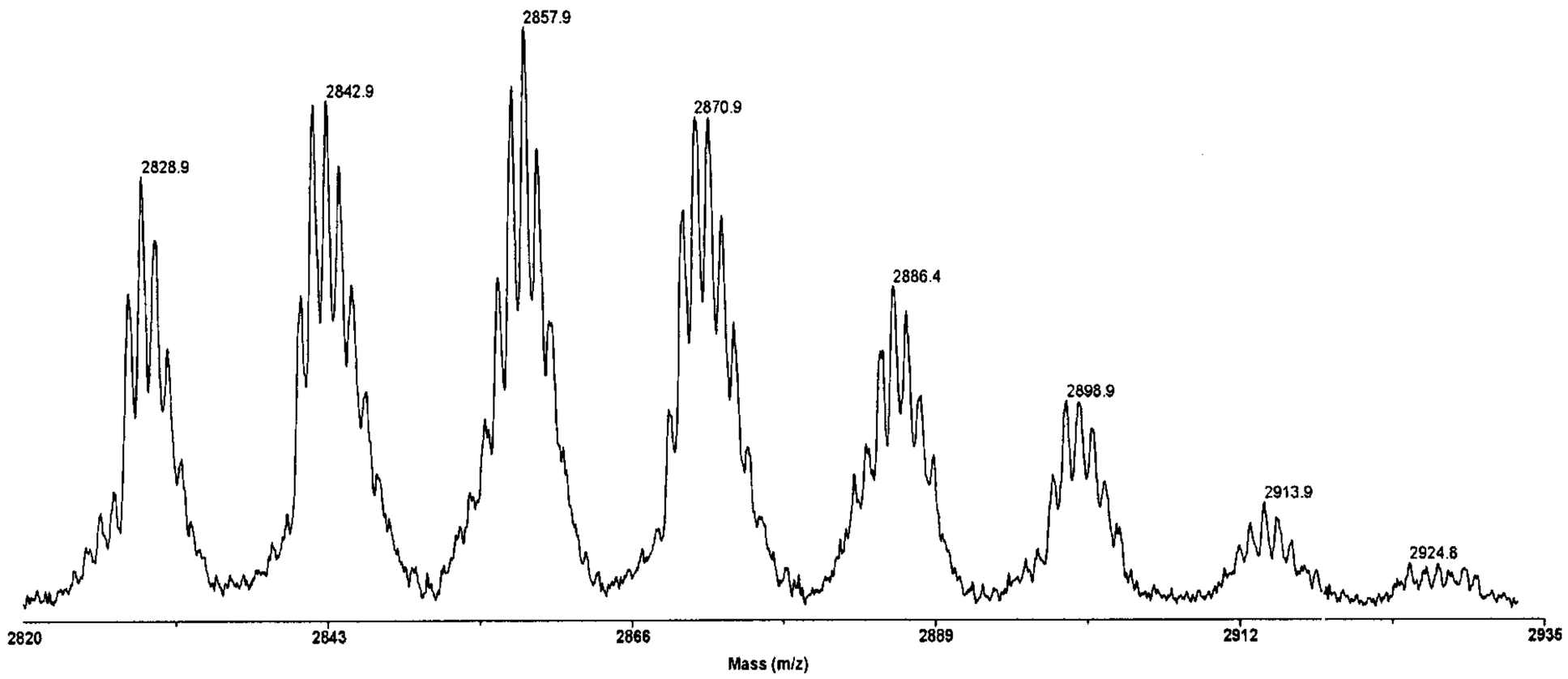
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FIG. 13

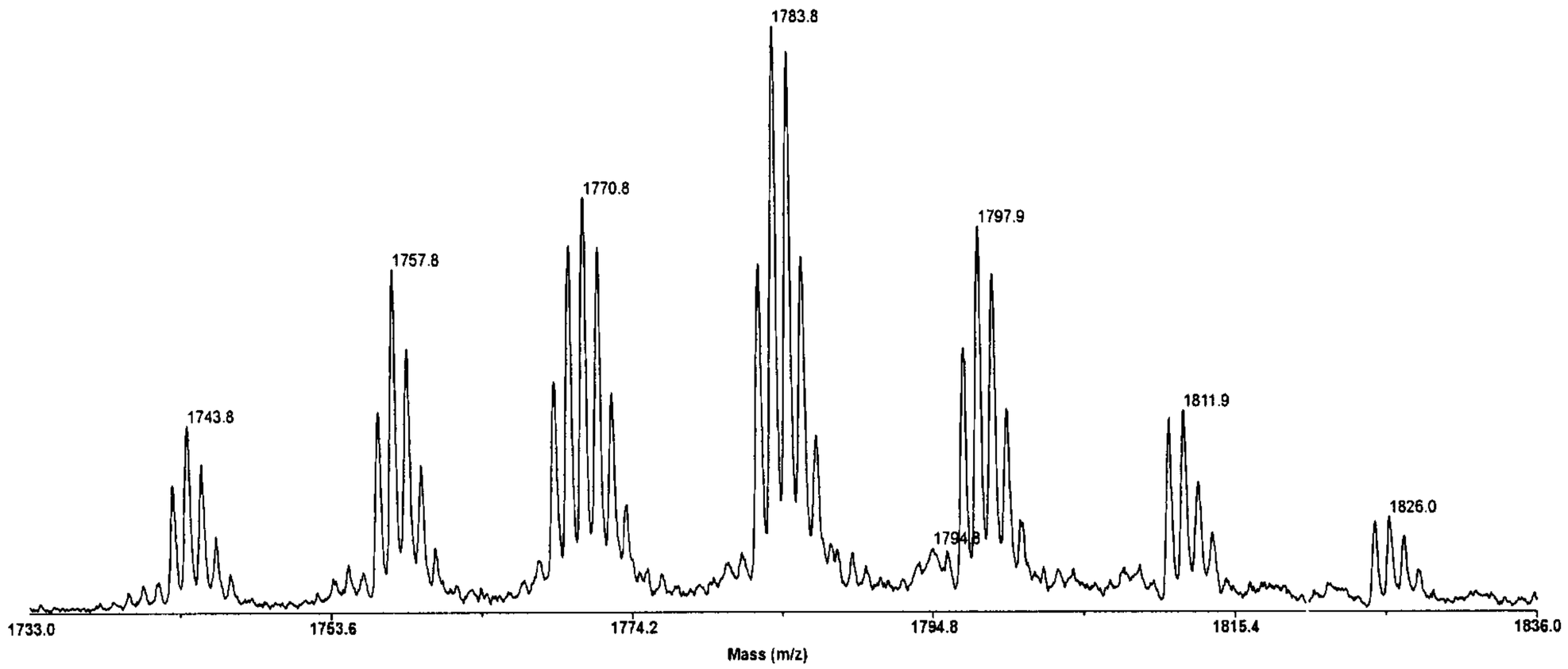
1783.8
1770.8
1797.9
1757.8
1811.9
1743.8
1826.0
1794.8
1733.0
1753.6
1774.2
1794.8
1815.4
1836.0
Mass (m/z)

FIG. 14

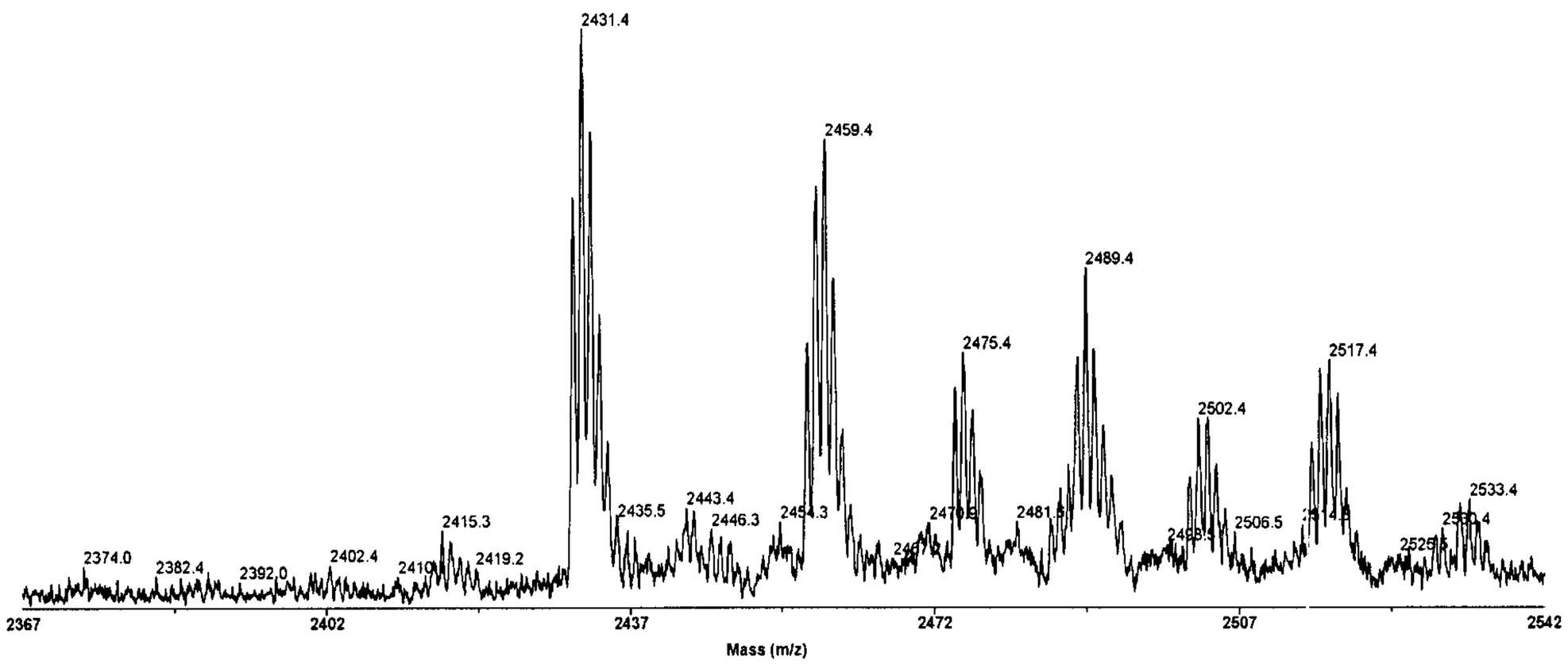
2431.4
2459.4
2489.4
2475.4
2517.4
2502.4
2533.4
2374.0
2382.4
2392.0
2402.4
2415.3
2419.2
2435.5
2443.4
2446.3
2454.3
2506.5
2367
2402
2437
2472
2507
2542
Mass (m/z)

FIG. 15

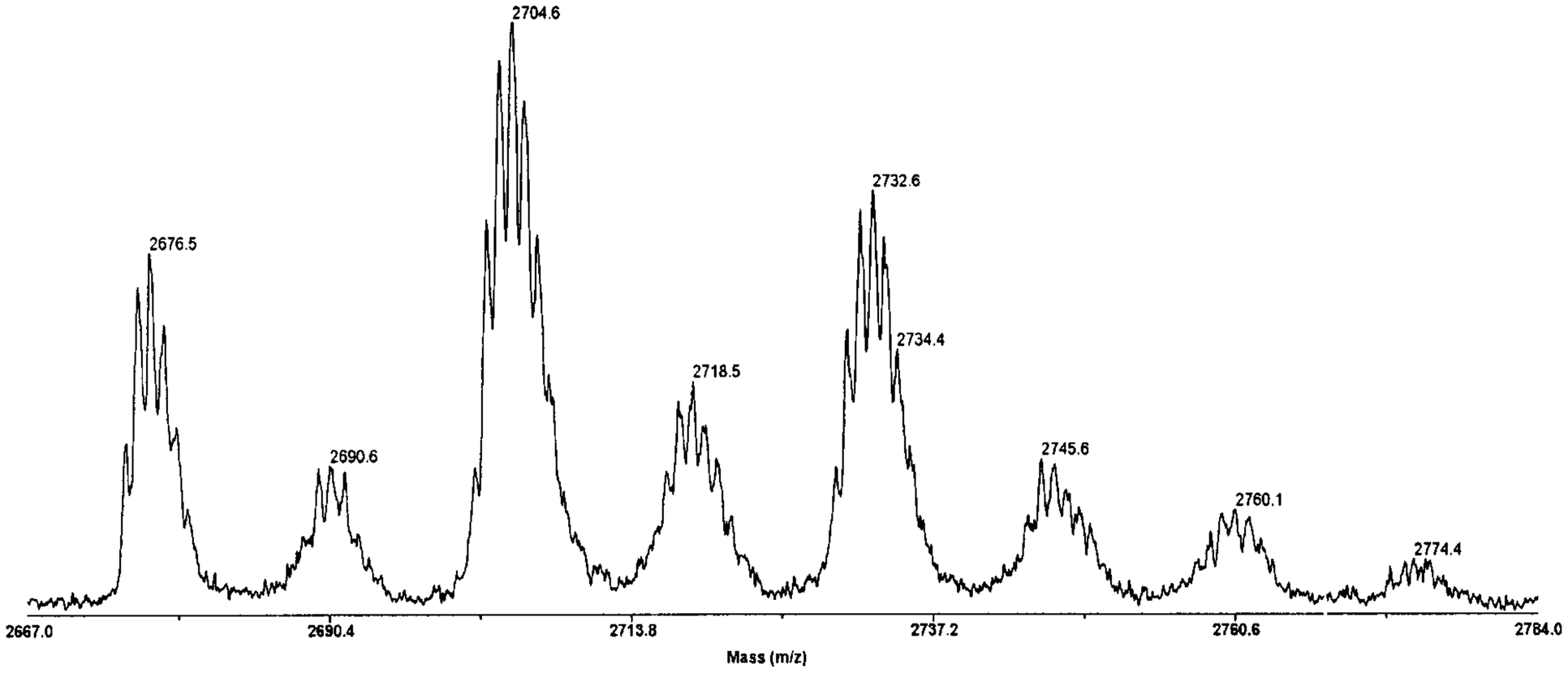
2704.6
2676.5
2690.6
2718.5
2732.6
2734.4
2745.6
2760.1
2774.4
2667.0
2690.4
2713.8
2737.2
2760.6
2784.0
Mass (m/z)

FIG. 16

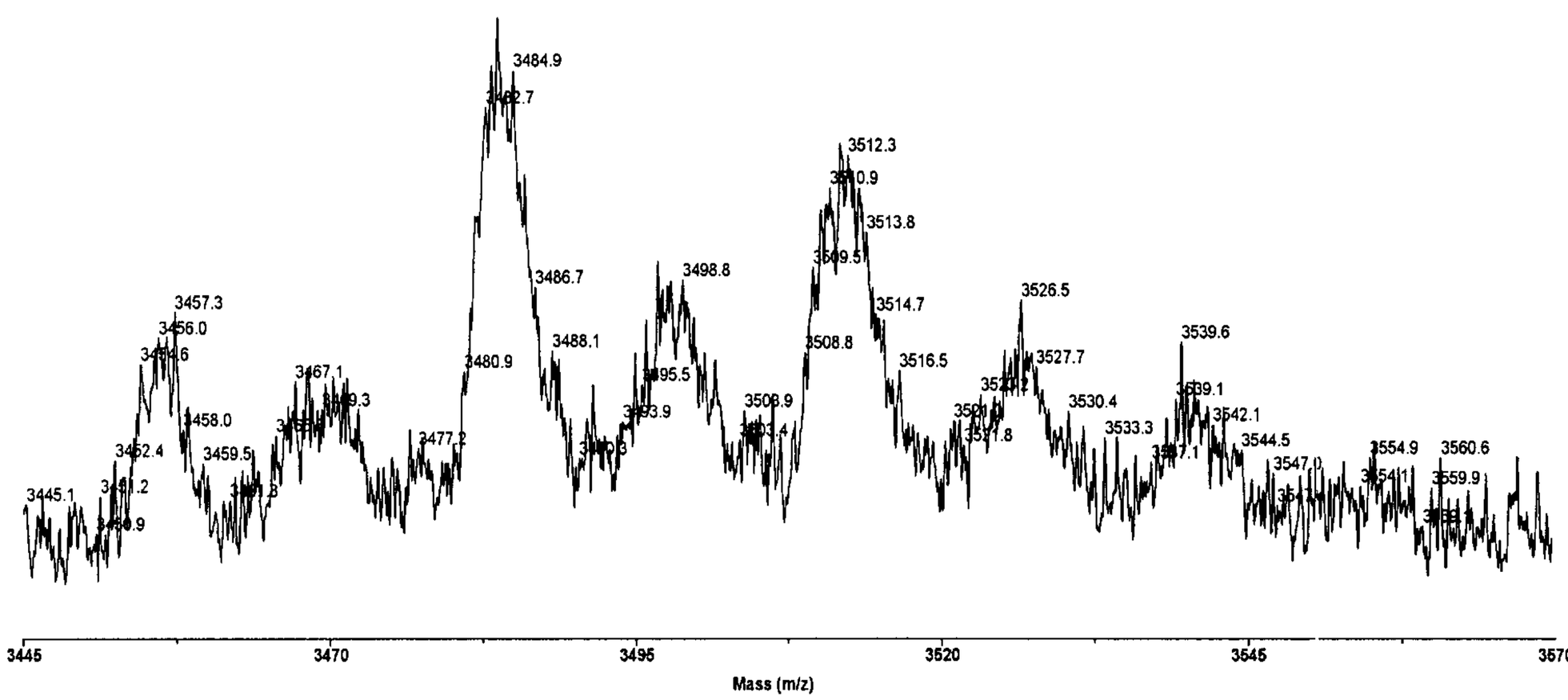
3445.1
3457.3
3456.0
3452.4
3458.0
3459.5
3467.1
3477.2
3480.9
3484.9
3486.7
3488.1
3498.8
3508.8
3512.3
3513.8
3514.7
3516.5
3526.5
3527.7
3530.4
3533.3
3539.6
3542.1
3544.5
3554.9
3560.6
3559.9
3445
3470
3495
3520
3545
3570
Mass (m/z)

FIG. 17

a) INFA-H5-1-V1:

Consensus sequence: NHEASSGVSSASPYQGKSSFF

N	H	E	A	S	S	G	V	S	S	A	S	P	Y	Q	G	K	S	S	F	F
		D			L									N		R				
														L						

b) INFA-H5-1-V2:

Consensus sequence: PNDAAEQTKLYQNPTTY

P	N	D	A	A	E	Q	T	K	L	Y	Q	N	P	T	T	Y
							I	R					S	N		

c) INFA-H5-1-V3:

Consensus sequence: SWSNHEASLIKKNSAYPT

S	W	S	N	H	E	A	S	L	I	K	K	N	S	A	Y	P	T
			S		D								N	T			
														V			

d) INFA-H5-1-V4:

Consensus sequence: GKLSDLDGVKPLILLEYGNSNTK

G	K	L	S	D	L	D	G	V	K	P	L	I	L	L	E	Y	G	N	S	N	T	K
				S		K														D	A	
						N																

e) INFA-H5-1-V5:

Consensus sequence: TIKRSYNNTNQEDKPNDAINFESN

T	I	K	R	S	Y	N	N	T	N	Q	E	D	K	P	N	D	A	I	N	F	E	S	N
				T						V			R				S						T

FIG. 18

f) INFA-H5-1-V6:

Consensus sequence: ISVGTSTLNQRLVPKI

I	S	V	G	T	S	T	L	N	Q	R	L	V	P	K	I
V											S	I		E	
														R	

g) INFA-H5-1-V7:

Consensus sequence: KANPANDLGNPMSDEFINVPEW

K	A	N	P	A	N	D	L	G	N	P	M	S	D	E	F	I	N	V	P	E	W
	D	S		V		G										L					

h) INFA-H5-1-V8:

Consensus sequence: PYQGKSSFFRNVVWLIKKNSAY

P	Y	Q	G	K	S	S	F	F	R	N	V	V	W	L	I	K	K	N	S	A	Y
		N		R															N	T	
		L																		V	

i) INFA-H5-1-V 8L:

Consensus sequence: PYQGKSSFFRNVVWLIKKNSAY

Palm$_2$	K	S	S	P	Y	Q	G	K	S	S	F	F	R	N	V	V	W	L	I	K	K	N	S	A	Y
						N		R															N	T	
						L																		V	

FIG. 18 (continued)

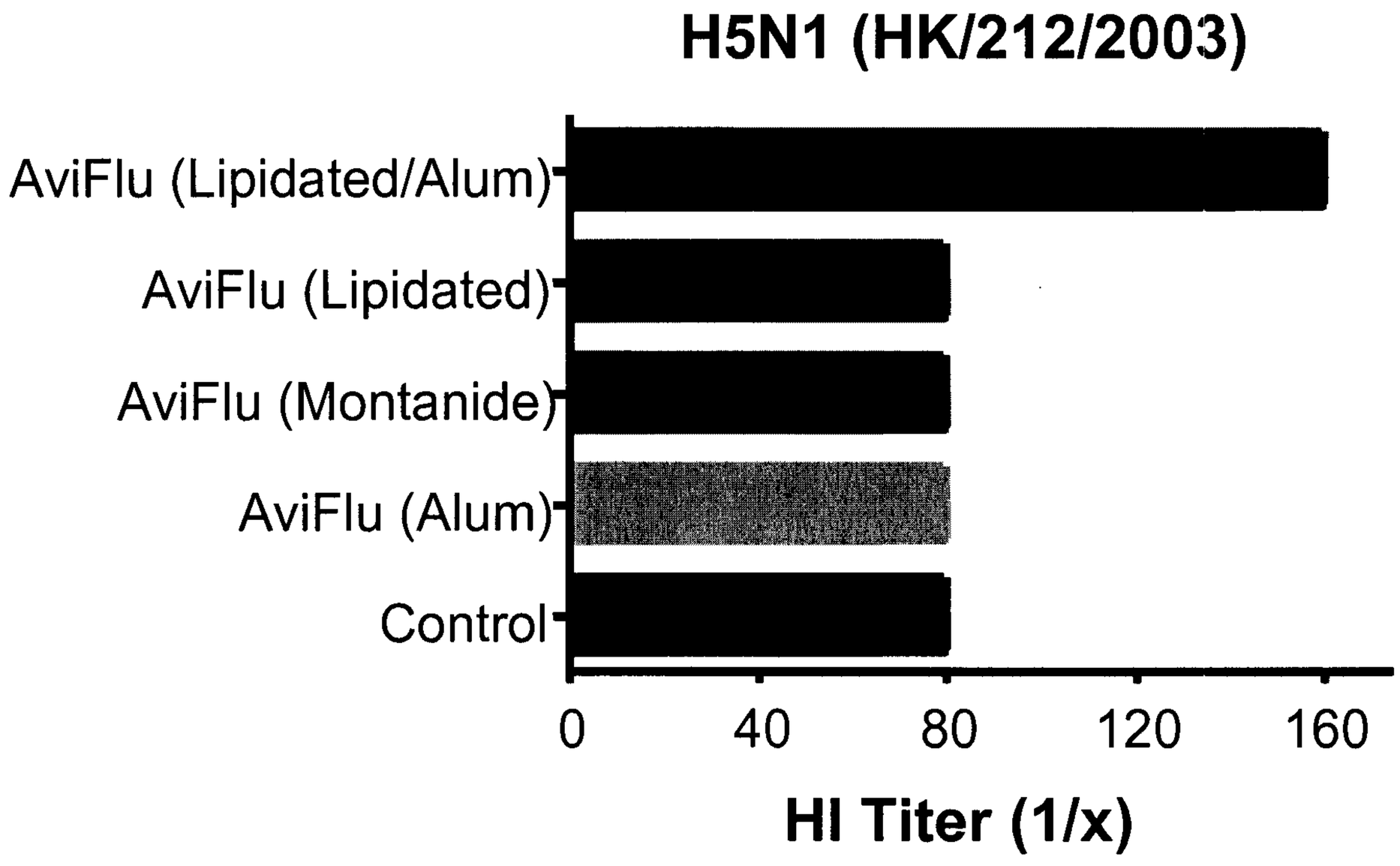
H5N1 (HK/212/2003)
AviFlu (Lipidated/Alum)
AviFlu (Lipidated)
AviFlu (Montanide)
AviFlu (Alum)
Control
0
40
80
120
160
HI Titer (1/x)

FIG. 19

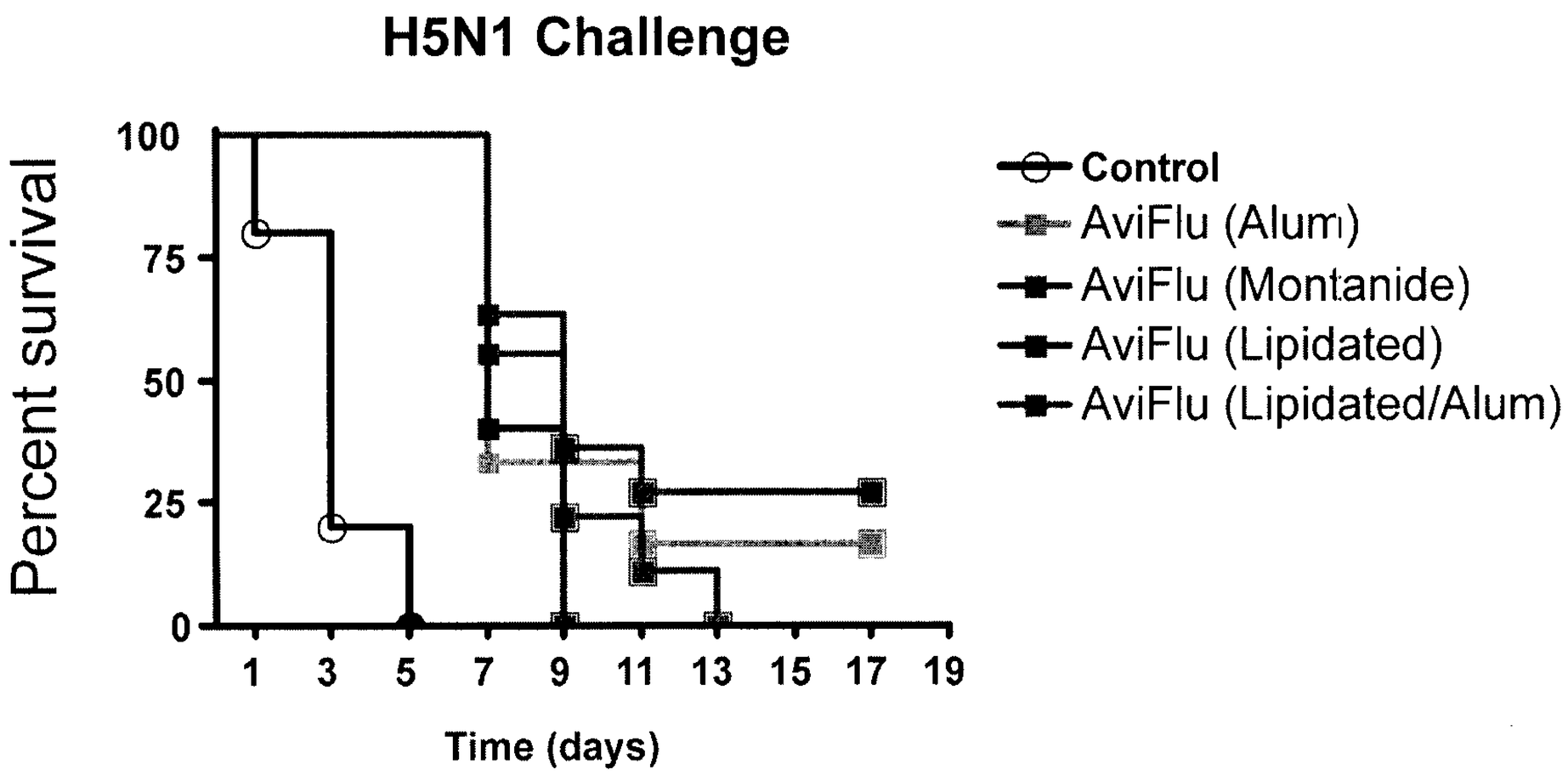
H5N1 Challenge
Percent survival
100
75
50
25
0
1
3
5
7
9
11
13
15
17
19
Time (days)
Control
AviFlu (Alum)
AviFlu (Montanide)
AviFlu (Lipidated)
AviFlu (Lipidated/Alum)

FIG. 20

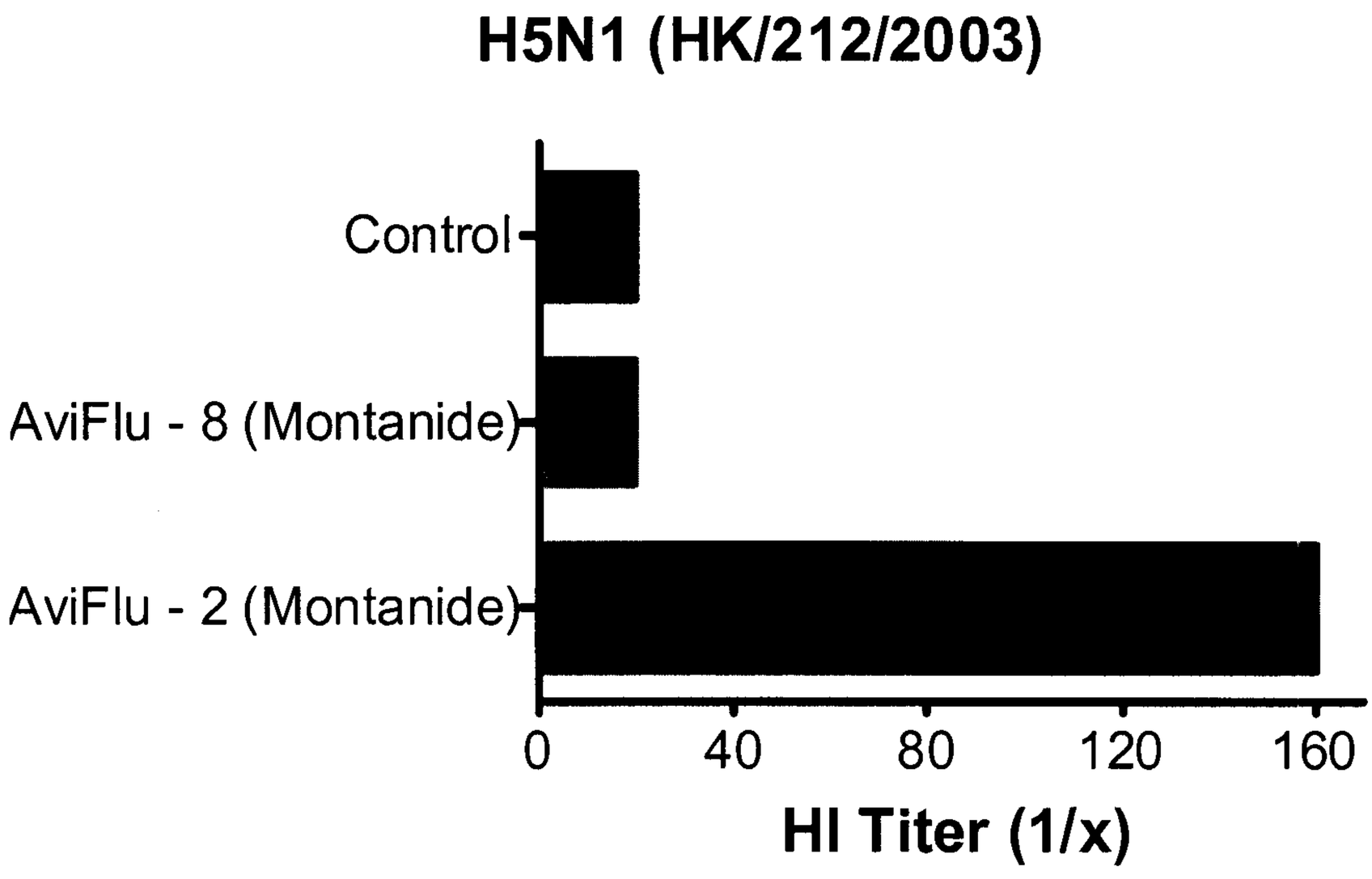
H5N1 (HK/212/2003)
Control
AviFlu - 8 (Montanide)
AviFlu - 2 (Montanide)
0
40
80
120
160
HI Titer (1/x)

FIG. 21

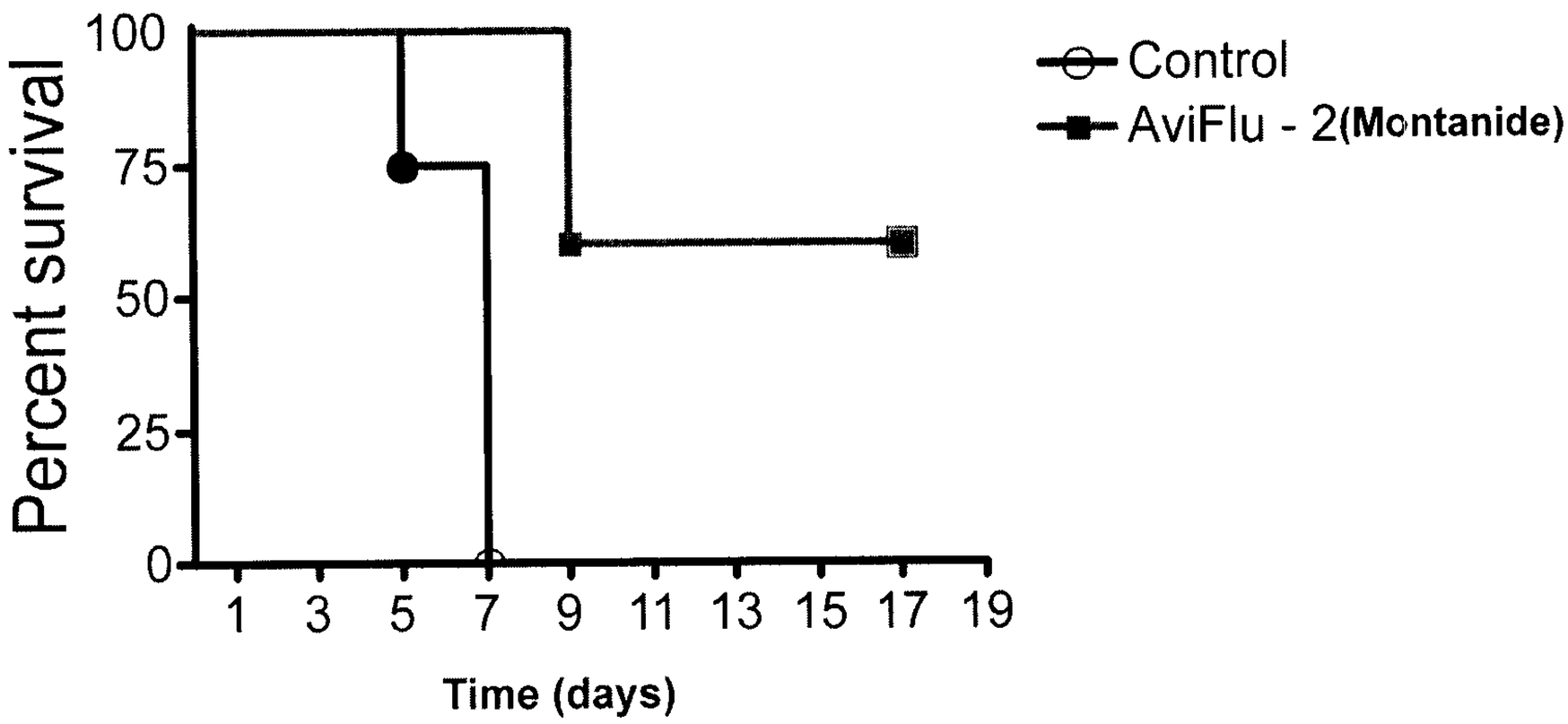
Percent survival
100
75
50
25
0
1
3
5
7
9
11
13
15
17
19
Time (days)
Control
AviFlu - 2(Montanide)

FIG. 22

H5N1 (HK/212/2003)