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(71) Applicant: **NATCO PHARMA LIMITED** [IN/IN]; Natco House, Road No. 2, Banjara Hills, Hyderabad, Andhra Pradesh 500 033 (IN).

(72) Inventors: **BATTULA, Suneel Kumar**; Natco Pharma Limited, Natco House, Road No. 2, Banjara Hills, Hyderabad, Andhra Pradesh, 500033 (IN). **POLAVARAPU, Baby Rani**; Natco Pharma Limited, Natco House, Road No. 2, Banjara Hills, Hyderabad, Andhra Pradesh, 500033 (IN). **ADIBHATLA, Kali Satya Bhujanga Rao**; Natco Pharma Limited, Natco House, Road No. 2, Banjara Hills, Hyderabad, Andhra Pradesh, 500033 (IN). **NANNAPANENI, Venkaiah Chowdary**; Natco Pharma Limited, Natco House, Road No. 2, Banjara Hills, Hyderabad, Andhra Pradesh, 500033 (IN).

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(54) Title: IMPROVED PROCESS FOR ISOLATION AND PURIFICATION OF RAPAMYCIN FROM FERMENTATION BROTH

(57) Abstract: The present invention relates to a novel process for the isolation of water insoluble macrolide antibiotic rapamycin from the fermentation broth through sequential steps of biomass separation, extraction with suitable solvents and concentration. The invention also relates to separating rapamycin from the concentrate containing a mixture of homologs, analogs or isomers thereof by using normal phase chromatography followed by purification.

IMPROVED PROCESS FOR ISOLATION AND PURIFICATION OF RAPAMYCIN FROM FERMENTATION BROTH

FIELD OF THE INVENTION

The present invention relates to isolation and recovery of rapamycin using decanter centrifuge technology and normal phase chromatography. The invention also relates to purification of rapamycin in a substantially pure form with a tautomer impurity of less than 0.5%.

BACKGROUND OF THE INVENTION

Rapamycin is a macrocyclic triene antibiotic produced naturally by Streptomyces hygroscopicus. It has been found useful in an array of applications based on its antitumoral and immunosuppressive effects. Rapamycin and its derivatives continue to be studied for treatment of these disorders.

Rapamycin, its preparation and its antibiotic activity were described in U.S. patent no 3929992.

Rapamycin (US patent 4885171 has been shown to have antitumor activity.

US 5616595 discloses a process for recovering water insoluble compounds (including FK506, FK520 and rapamycin) from a fermentation broth which includes sequential steps of concentrating, solubilising and diafiltrating the compound of interest, all through a single closed recirculation system to isolate the compound for further downstream purification.

US5508398 discloses a process for separating a neutral non-polypeptide macrolide from acidic, basic and non polar impurities present in a concentrate of fermentation broth extracts containing neutral macrolide by selective solubility.

Use of silver for separating cis-trans isomers of an unsaturated aliphatic acid having same number of carbon atoms is known through J.Chromatography, 149, 417-419(1978).

PCT application WO 05/019226 discloses a process for recovery of a macrolide by treating with water immiscible solvent, followed by mixing with water miscible solvent, performing hydrophobic interaction chromatography, extracting the fraction containing macrolide with water-immiscible solvent and then performing silica gel chromatography to obtain the macrolide..

US patent application No 2004/0226501 discloses a method of crystallizing a macrolide from a concentrate of whole broth extraction containing macrolide biomatter, by combining the macrolide, a polar solvent like ethyl acetate, hydrocarbons solvent like n-hexane and sodium hydroxide to attain a pH of 7 or above.

The methods known in the prior art do not disclose isolation of rapamycin by decanter centrifuge technique and the process literature methods known for purification of rapamycin do not result in pure form of the product.

The present invention provides decanter centrifuge technique which is less tedious, industrially scalable efficient process, involving less solvent consumption and substantially improved yields.

The HPLC analysis of Rapamune$^{(R)}$ (Wyeth - brand name for the drug product) revealed the existence of three isomeric forms isomer A (cis), isomer B (rapamycin), Isomer C (trans) and other unidentified impurities.

The present invention provides rapamycin in substantially pure form and a process to obtain the same. The invention discloses a process of obtaining rapamycin with an impurity content of less than 0.8% excluding Isomer C (tautomer). The Isomer C

(tautomer) which is a major hurdle in conversion of rapamycin derivatives like temsirolimus and everolimus etc obtained less than 0.5% at 1.1 RRT.

BRIEF DISCLOSURE OF THE INVENTION

Briefly the invention relates to a process for isolation and purification of rapamycin from fermentation broth, comprising the steps of;

1. Adjusting the pH of the fermentation broth to 4.0 and concentrating by centrifugal decanter.
2. To the concentrated biomatter containing rapamycin, addition of hydrophobic extraction solvent where extraction temperature is between 35 to 45°C. The Solvent extract is evaporated under reduced pressure to yield oily residue containing crude rapamycin.
3. Isolation of crude rapamycin by adsorbing on silica gel from a solution in a non polar solvent, followed by elution therefrom with a second solvent more polar than the first solvent.
4. The purification process includes treatment of rapamycin with isopropyl ether and/or diethyl ether to yield a purified rapamycin product. This is precipitated, collected by filtration, washed and then dried under vacuum in to yield a product of about 97% purity.
5. The product from step (d) is loaded on a flash chromatographic column and the final product with total impurity content of less than 0.8% and tautomer content less than 0.5% is isolated.

Other aspects and advantages of the invention will be apparent from the following detailed description.

BRIEF DESCRIPTION OF DRAWINGS

FIG 1: Diagrammatic representation of a decanter centrifuge.

FIG 2: Standard chromatogram of Rapamycin

FIG 3: Chromatogram of crude rapamycin

FIG 4: Chromatogram of purified rapamycin with isopropyl ether crystallization

FIG 5: Chromatogram of purified rapamycin with diethyl ether crystallization

FIG 6: Chromatogram of substantially pure form of rapamycin.

DETAILED DESCRIPTION OF THE INVENTION

The process of the present invention is directed towards recovering water insoluble compound rapamycin that is produced by large scale fermentation. "Recovering" as used here in refers to the process of removing related impurities from the compound of interest and encompasses removing excess fluid and dissolved soluble impurities. Rapamycin containing biomatter can be the starting material for practice of the present invention. Fermented broth is obtained by a microbial process using a rapamycin producing microorganism MTCC 5681. In most circumstances and particularly large scale industrial fermentations, the culture medium and fermentation conditions (strain of organism, type of inoculums, time of fermentation, fermentation temperature etc) are optimized to produce maximum yield of the desired product.

At the cessation of the fermentation, the pH of the fermentation broth is adjusted to below 7 preferably 4.0 to 7.0 before separating mycelia. The acid can be organic or inorganic including hydrochloric acid, sulphuric acid, phosphoric acid, acetic acid and formic acid etc. The pH adjustment is useful to remove residual soluble contaminants during separation of mycelia.

In the process after acidification of the fermentation broth, the mycelia are separated by centrifugal decantation technique. Centrifugal decantation refers to the process of passing fermentation broth for separating solids from liquids using a special equipment known as a Decanter Centrifuge in one single continuous process (Fig 1). This is done by centrifugal force. When the fermentation broth passes through the unit, the centrifugal force compact the solids and expels surplus liquids. The dried solids are discharged from the bowl. The clarified aqueous liquid phase is then separated by flow path. The water insoluble compound of interest remains in the dried solids. Removal of mycelial solids and separation of liquids are crucial function in a fermentation process. This type of decanter centrifuge is most useful and preferred when the solid content in the fermentation broth exceeds more than 65% (w/v).

It has been found that the separation of mycelia and isolation of rapamycin into solvent leads to near quantitative recovery from the fermentation broth.

In the second step of the present invention, the solid mass after centrifugal decantation is mixed with a solvent capable of solubilising the compound of interest to form liquid slurry. Solvents useful in the present invention include alcohols (C_4 to C_6), esters of lower alcohols (C_1 to C_6), chlorinated hydrocarbons and lower ketones (C_3 to C_6). Preferred extraction solvents are isobutyl acetate, n-butyl acetate, t-butyl acetate, ethyl acetate, propyl acetate, ethyl formate, dichloromethane, chloroform, carbon tetra chloride and toluene. Toluene and ethyl acetate are particularly preferred hydrophobic extraction solvents. Those skilled in the art can easily select a suitable solvent knowing the physic chemical properties of the rapamycin.

Extraction can be carried out at any convenient temperature between 2°C about 70°C. Preferably the extraction is carried out at a temperature about 25°C and about 50°C. The skilled artisan will know to optimize the extraction time depending on the macrolide containing biomass, hydrophobic extraction solvent, equipment used and temperature. At

the end of the extraction, the extraction mixture contains rapamycin in the extraction solvent as well as residual extracted biomass.

The amount of solvent used is generally atleast twice the amount of solid biomass after the centrifugal decantation. Typically two to six equivalent volumes are used. The impurities may be present in the solid or liquid. The impurities can be separated by filtration, phase separation or both. The organic layer rich in rapamycin is washed with base and water. Then activated charcoal is added and filtered. The base can be selected from inorganic or organic bases. Preferably, the base can be aqueous sodium bicarbonate. Following the extraction, rapamycin in hydrophobic solvent is concentrated to an oily residue. The concentration can be at atmospheric pressure or it can be at reduced pressure, attained with the aid of vacuum pump or water aspirator. The concentration is preferably carried out at a temperature above 25°C. The concentration is carried out until the volume of the rapamycin containing solvent is reduced to about 0.5 to 1% of its initial volume, or less to provide concentrated rapamycin as an oily residue. Crude rapamycin can be isolated from the oily residue.

In the third step of the present invention, oily residue containing rapamycin is purified by column chromatography on a silica gel column. The mixture of rapamycin and analogous compounds are dissolved in a suitable solvent and subjected to column chromatography using silica gel having mesh size of 60 – 200. The mixture is adsorbed on the column and is successively eluted with organic solvents. The fractions rich in rapamycin can be pooled to recover rapamycin. The organic solvent for elution can be for example an aliphatic hydrocarbon, a C_{2-10} ester, a chlorinated hydrocarbon, ketone, C_{4-8} ethers or mixtures thereof. The C_{2-10} esters can be for example ethyl acetate, iso butyl acetate and n-butyl acetate etc. Aliphatic hydrocarbons can be for example n-pentane, n-hexane, n-heptane and cyclo hexane etc. Aliphatic ketone can be for example acetone, 2-butanone etc. The eluant can be isocratic, that is of constant composition or the composition of the

eluant can be varied during elution. Preferred eluants include mixtures of acetone and hexane.

After the concentration of the fractions containing crude rapamycin, the product may optionally be processed further by crystallization and/or chromatography. The term crude rapamycin as used here in refers to a rapamycin powder that contains less than about 10% impurities including the tautomer.

Another aspect of the present invention relates to a method for purifying rapamycin by crystallization (precipitation) and includes i) Dissolving crude rapamycin in isopropyl ether ii) Mixing over a period of 2-3hrs . iii) Filtering the product to obtain rapamycin powder. iv) Washing the rapamycin powder with chilled isopropyl ether and v) drying the product. The obtained rapamycin powder contains less than about 5% impurities including tautomer.

In a further aspect, a method for purifying rapamycin is provided by recrystallization and includes;

i) Dissolving rapamycin powder with a purity of about 90% in diethyl ether. ii) Mixing over a period of 2-3hrs. iii) Filtering the product to obtain rapamycin powder. iv) Washing the rapamycin powder with chilled diethyl ether and v) Drying the product. The obtained rapamycin powder contains less than 1.5% impurities excluding tautomer and tautomer content is less than 2%.

As a further aspect of the invention, the purified product by crystallization from isopropyl ether and/or diethyl ether may be further purified with a suitable chromatographic medium. The present invention also relates to a method for purification of rapamycin in substantially purified form using flash chromatographic technique. The stationary phase can be silica with particle size of about 40μm with a narrow range of size distribution.

Using this silica, resolution of closely eluting compounds dramatically increased and also yields fractions of high purity with improved loading capacity. Thereby reduction in solvent consumption is achieved. The elution is carried out using an organic solvent selected from a group comprising acetone, ethyl acetate, hexane, diethyl ether. Preferably the elution is carried out with ethyl acetate and hexane. The pure rapamycin powder thus obtained contains total impurity content less than 0.8% excluding tautomer. The tautomer content obtained is less than 0.5% at 1.1 relative retention time (RRT) with rapamycin purity of about 99% (HPLC).

The present invention relates to rapamycin with total impurity content less than 0.8%.by HPLC excluding tautomer. The present invention also relates to rapamycin with tautomer content less than 0.5% at 1.1 RRT. All RRTs are here with respect to rapamycin retention time (R.T). The purity of rapamycin thus obtained is more than 99%.

The details of HPLC method for analysis of crude and purified forms of rapamycin in the present invention are as follows:

Column: UNISON UK C18, 3µm, diameter – 4.6mm, length – 250mm
Flow rate: 1.0ml/min
Detection wave length: 280nm
Injection volume: 20µl
Diluent: Methanol
Temperature: 55°C
Approximate retention time (RT) of rapamycin: 17 min
Mobile phase: Buffer A: Water; Buffer B – 80% methanol and 20% Acetonitrile. The gradient is as given in Table 1.

TABLE 1: GRADIENT ELUTION OF MOBILE PHASE

Time (min)	Buffer A (%)	Buffer B (%)
0	30	70
3	30	70
10	20	80
40	20	80
42	30	70
65	30	70

The identity of the product isolated and purified was confirmed as rapamycin through comparison of physic-chemical, spectral and chromatographic properties with those of a sample of authentic rapamycin. The purity of the product was determined by high pressure liquid chromatographic analysis.

The invention will now be described further by way of examples. The examples are merely illustrative of the invention and are not intended to limit the invention in any way.

EXAMPLE 1

BIOMASS SEPARATION

Approximately 1600 liters of rapamycin fermentation broth obtained a large scale fermentation experiment was acidified by addition of dil.sulphuric acid to pH 4.0. The acidified broth containing 5.1% dried solids and 50% of suspended wet solids were fed into a receiver tank. The decanter centrifuge (Fig-1) consists of centrifuge, conveyer assembly, bowl assembly and frame and casing assembly (ALDEC 20, Alfa laval, Sweden). The feed flow rate was set at $1m^3/hr$ and the broth was pumped through a stationary feed inlet tube. The centrifuge was operated with bowl speed of 4400 rpm at 3030 G (centrifugal force). The dense solid particles were pressed outwards against the rotating bowl wall, then the screw conveyer was rotated and solids were discharged into

casing through solid discharge opening. Based on volume, the dam plates were adjusted to receive clarified liquid. Then the clarified liquid was discharged into casing through dam plates. Approximately 1300 liters of clarified liquid was collected and analyzed by HPLC; very little rapamycin activity was detected in the liquid sample. About 350 kilograms of biomass cake thus obtained containing rapamycin was used for solvent extraction to isolate rapamycin.

EXAMPLE 2

RECOVERY OF RAPAMYCIN

350kilogram of biomass cake from example 1 was mixed with 1000liters of toluene and stirred for 4hrs at 50°C. The extraction process was carried out twice with 1000liters of toluene. The toluene extract was concentrated to 500 liters. The rich concentrate was washed with 500 liters of 5% sodium bicarbonate solution, followed by wash with 2X500 L of water. The toluene extract was concentrated to obtain 10 kilogram of oily residue. The oily residue was mixed with 30 liters of acetone. Activated charcoal (5.25grams) was added to this solution. The solution was stirred for 15 minutes at 40°C temperature, filtered and concentrated to obtain oily residue of about 10 kilograms.

EXAMPLE 3

RECOVERY OF RAPAMYCIN

The oily residue (3kg) was applied to a column packed with 30kg of silica gel of 60 – 200 mesh size. The column diameter was 250mm and length was 1.5m. The elution was carried out with gradient mobile phase of acetone and hexane (90% hexane, 10% acetone – 60 lt, 80% hexane, 20% acetone – 60 lts, 70% hexane, 30% acetone – 60 lt, 60% hexane, 40% acetone – 150 lt). The drug rich fractions were eluted at 60% hexane, 40% acetone ratio. The collected fractions were mixed with 5.25g of activated charcoal. The

solution was stirred, filtered and concentrated under vacuum at 40°C. The residue was dried to obtain 400g of crude powder .The rapamycin purity was 90% with tautomer content 6.5% at 1.1 RRT and other impurities 3.5%.

The HPLC chromatogram for crude rapamycin recovered as above is shown in Fig 3. The details of chromatogram are given in Table 2.

TABLE 2

Peak No	Retention Time (mins)	Area (µV*sec)	% Area	RT Ratio	Identification
1	15.538	231885	1.100	1.095	
2	15.734	105818	0.326	1.082	
3	15.833	72809	0.517	1.075	
4	17.026	19055139	90.010	1.000	Rapamycin
5	17.858	108947	0.701	1.048	
6	18.702	1337704	6.501	1.098	Tautomer
7	19.413	64489	0.326	1.140	
8	21.982	92334	0.519	1.291	

EXAMPLE 4

PURIFICATION OF RAPAMYCIN

Rapamycin powder (10g) obtained from example 3 was dissolved in 30ml of isopropyl ether. The solution was stirred, filtered and concentrated at 25°C. The crystals were dried to obtain 9g of white rapamycin powder. The purity of rapamycin powder was 95% with tautomer content of 2.5% at 1.1 RRT and other impurities amounting to 2.5%.

The HPLC chromatogram of rapamycin purified as above using isopropyl ether is shown in Fig 4. The details of chromatogram are given in Table 3.

TABLE 3

Peak No	Retention Time (mins)	Area (μV*sec)	% Area	RT Ratio	Identification
1	15.845	374736	1.328	0.910	
2	16.055	67800	0.240	0.923	
3	17.404	26951553	95.519	1.000	Rapamycin
4	19.177	729637	2.586	1.102	Tautomer
5	19.833	92167	0.327	1.140	

EXAMPLE 5

PURIFICATION OF RAPAMYCIN

Rapamycin powder (5g) obtained from example 3 was dissolved in 10ml of diethyl ether. The solution was stirred, filtered and concentrated at 25°C. The crystals were dried to obtain 4.0g of white rapamycin powder. The purity of rapamycin powder was 97% with tautomer content of 1.5% at 1.1 RRT, and other impurities amounting to 1.5%.

The HPLC chromatogram for rapamycin purified as above using diethyl ether is shown in Fig 5. The details of chromatogram are given in Table 4.

TABLE 4

Peak No	Retention Time (mins)	Area (μV*sec)	% Area	RT Ratio	Identification
1	13.843	23786	0.488	0.801	
2	16.078	26799	0.364	0.930	
3	16.562	10828	0.203	0.958	
4	17.274	15853156	97.266	1.000	Rapamycin
5	18.635	3174	0.264	1.078	
6	19.023	381054	1.415	1.101	Tautomer

EXAMPLE 6

CHROMATOGRAPHIC PURIFICATION OF RAPAMYCIN

Rapamycin powder (100mg) obtained from example 4 was dissolved in 200ml of acetone. The solution was loaded on a column packed with flash specific media (cartridge from Grace Company, diameter 0.81cm, particle size 40μm, height 9.6cm). The product was eluted by gradient method.

Mobile phase: Buffer A: Hexane; Buffer B –Ethyl acetate. The gradient is as given in Table 5.

TABLE 5

Time (min)	Buffer A (%)	Buffer B (%)
0-	70	30
10	65	35
14	65	35
19	60	40
29	58	42
39	57	43
64	52	48
85	20	80
95	20	80

The product was eluted at a ratio of 60% hexane, 40% acetone and 58% hexane, 42% acetone. The fractions containing pure rapamycin were pooled and concentrated at 15°C. The crystals were filtered and dried. 50mg of rapamycin powder was obtained. . The purity of rapamycin powder was 99% with tautomer content 0.4% at RRT 1.1 and 0.6% of other impurities.

The HPLC chromatograms for rapamycin (reference standard) and that of rapamycin produced in substantially pure form as above using flash chromatography are shown in Fig 2 and Fig 6 respectively. The details of chromatogram as in fig 2 are given in Table 6 and that of fig6 is given in Table 7.

TABLE 6

Peak No	Retention Time (mins)	Area (μV*sec)	% Area	RT Ratio	Identification
1	15.687	551902	0.7764	0.912	
2	15.900	247455	1.0021	0.924	
3	17.195	27225638	95.2694	1.000	Rapamycin
4	18.913	843756	2.6261	1.099	Tautomer
5	19.548	94542	0.326	1.136	

TABLE 7

Peak No	Retention Time (mins)	Area (μV*sec)	% Area	RT Ratio	Identification
1	15.907	165308	0.400	0.933	
2	16.365	38976	0.100	0.960	
3	17.033	27042609	99.00	1.000	Rapamycin
4	18.373	32869	0.100	1.078	
5	18.732	129663	0.400	1.099	Tautomer

We claim

1. Rapamycin having a purity of greater than 99% and tautomer content less than 0.5%.

2. Rapamycin as claimed in claim 1 is obtained by a process of isolation and purification.

3. The process of isolation as claimed in claim 2 comprises:

    a) adjusting pH of fermentation broth to pH 4 with 20% aqueous sulphuric acid
    b) separation of biomass and acidified broth using decanter centrifuge
    c) extracting the biomass with suitable solvent mixture and washing the extracted rapamycin with sodium bicarbonate and water
    d) concentrating the rapamycin rich extract
    e) dissolving the concentrate in a suitable solvent
    f) mixing the dissolved product with activated charcoal and filtering
    g) concentrating the filtrate
    h) chromatographic separation of oily residue using silica gel having a mesh size of about 60-200µm
    i) eluting with a mixture of suitable organic solvent using gradient method.

4. The process according to claim 3, where in step c) the solvent mixture is toluene and ethyl acetate.

5. The process according to claim 3, where in step e) the solvent is acetone.

6. A process according to claim 3, where in step i) the organic solvent is selected from aliphatic hydrocarbons and ketones.

7. A process according to claim 6, where in aliphatic hydrocarbon is selected from n-pentane, n-hexane, n-heptane, cyclo hexane or mixtures thereof.

8. A process according to claim 6, where in ketone is acetone.

9. The process according to claim 3, where in the rapamycin produced thereby has a purity of about 90% with tautomer content of less than 6.5%.

10. A process according to claim 2 wherein the purification process comprises:
    j) crystallizing the rapamycin using isopropyl ether or diethyl ether
    k) subjecting the crystallized rapamycin to flash chromatography using specialized silica with particle size distribution of about 25 to 45μm
    l) eluting with a mixture of suitable organic solvent using gradient method

11. The process according to claim 10, where in step j) the crystallized rapamycin produced thereby, has a purity of greater than 96% with tautomer content less than 2%.

12. The process according to claim 10, where in step l) the organic solvents are selected from aliphatic hydrocarbons and C_2-C_{10} esters.

13. The process according to claim 11, where in the aliphatic hydrocarbon is n-hexane.

14. The process according to claim 11, where in C_2-C_{10} ester is ethyl acetate.

15. The process according to claim 10 , where in step k) the rapamycin obtained has a purity of greater than 99%, where in rapamycin content is about 99% with the tautomer content less than 0.5% and purity of rapamycin including isomer is about 99.4%.

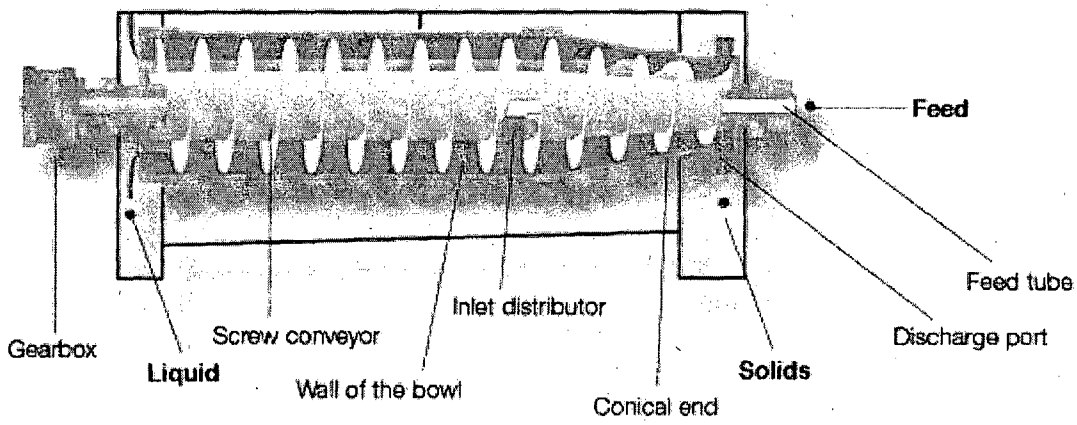

FIG 1: DECANTER CENTRIFUGE

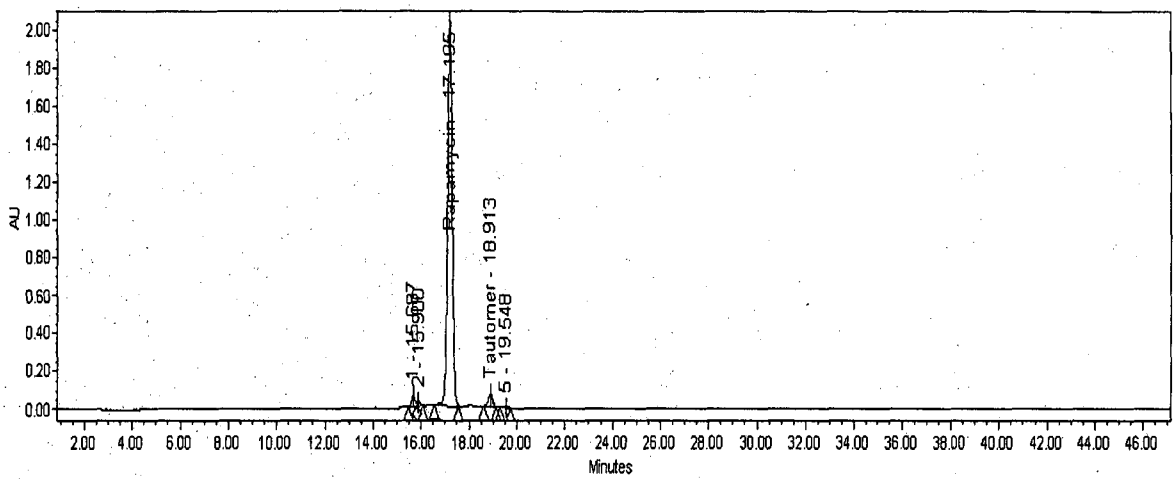

FIG 2: RAPAMYCIN REFERENCE STANDARD CHROMATOGRAM

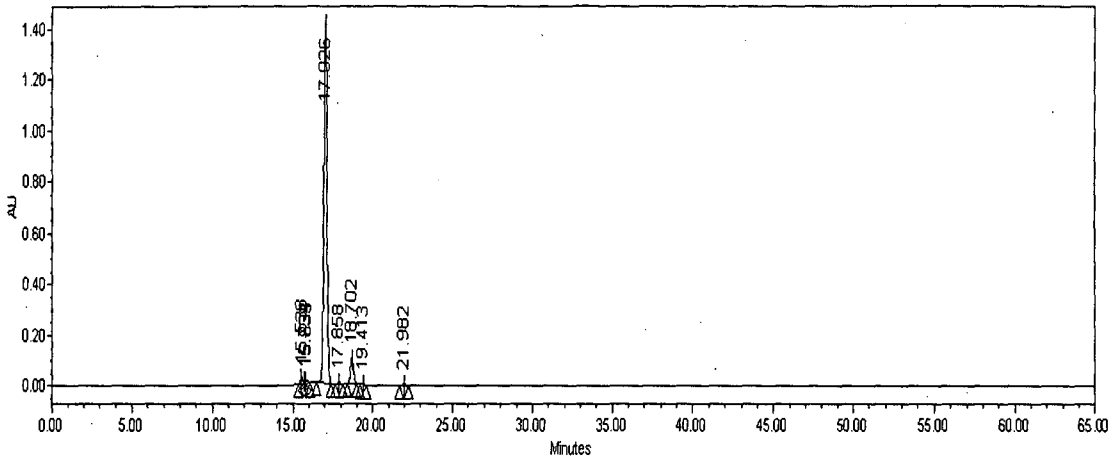

FIG 3: RAPAMYCIN CRUDE HPLC CHROMATOGRAM

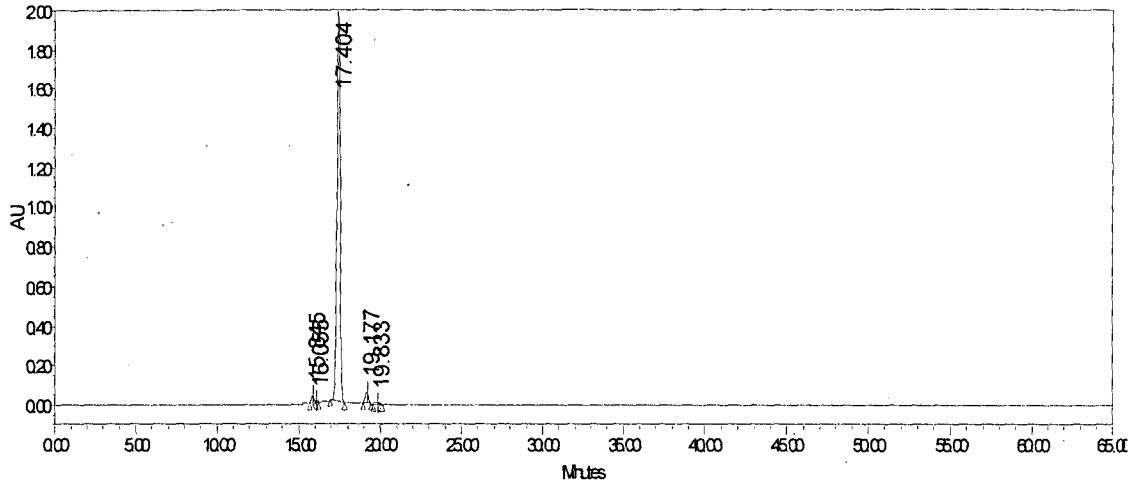

FIG 4: CHROMATOGRAM OF PURIFIED RAPAMYCIN WITH ISOPROPYL ETHER CRYSTALLIZATION

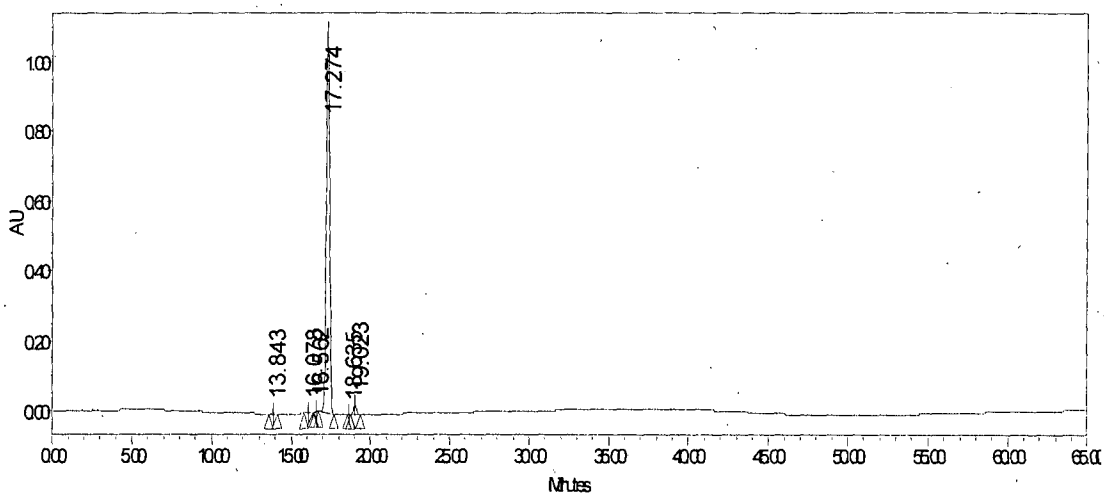

FIG 5: CHROMATOGRAM OF PURIFIED RAPAMYCIN WITH DIETHYL ETHER CRYSTALLIZATION

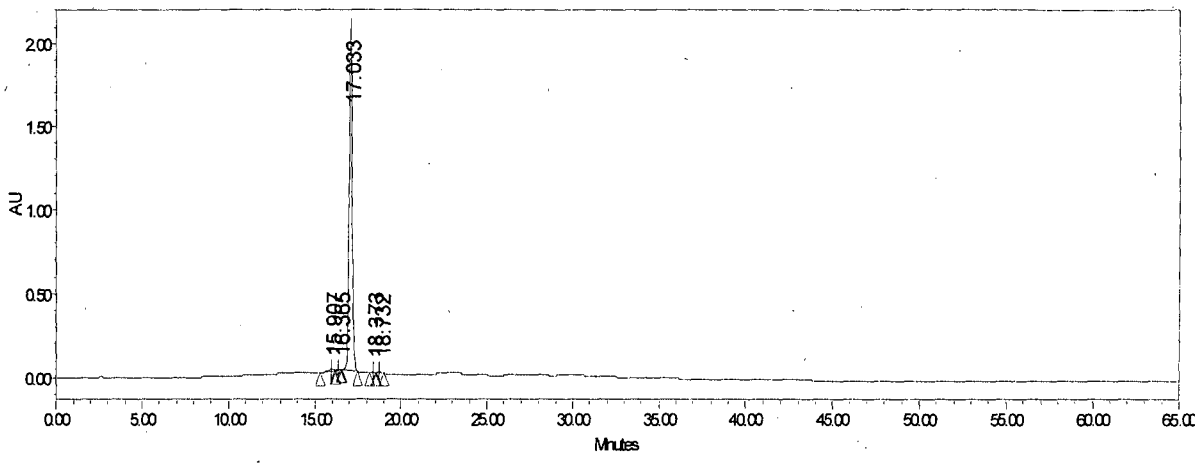

FIG 6: CHROMATOGRAM OF SUBSTANTIALLY PURE FORM OF RAPAMYCIN

INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2012/000728

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07D498/18
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2005/019226 A1 (BIOCON LTD [IN]; PATIL NITIN SOPANRAO [IN]; KHEDKAR ANAND PRAKASH [IN]) 3 March 2005 (2005-03-03) cited in the application example 1	1-15
Y	WO 2008/056372 A1 (BIOCON LTD [IN]; PATIL NITIN SOPANRAO [IN]; HUSSAINI SYED IDRIS [IN];) 15 May 2008 (2008-05-15) example 3	1-15
A	US 5 508 398 A (GLETSOS CONSTANTINE [US]) 16 April 1996 (1996-04-16) cited in the application examples	1-15

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
23 July 2013	30/07/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Menegaki, Fotini

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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