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Recovery and Purification of Rapamycin from the Culture Broth of Mtcc 5681

[1]Polavarapu Baby Rani, [1]Battula Suneel Kumar, [1]A.K.S. Bhujanga Rao,
[1]S. Sreenivasrao and [2]Mangamoori Lakshmi Narasu
[1]Department of Biotechnology, Natco Research Center, Hyderabad-500018, India
[2]Institute of Post Graduate Studies and Research, Jawaharlal Nehru Technological University,
Mahaveer Marg, Hyderabad 500 028, India

Abstract: In this study of the recovery and purification of rapamycin from the culture broth of an actinomycetes strain MTCC 5681, we investigated various factors such as biomass separation, suitable solvents for extraction, normal phase and flash chromatographic conditions and selective precipitation to obtain rapamycin in substantially pure form of the product. Adsorption chromatography particularly with normal phase and flash chromatography, in combination with centrifugal decantation is found to be the most suitable for separation as well as purification of rapamycin. Centrifugal decantation technique is likely to emerge as an efficient, industrially scalable, high yielding and economical process for biomass separation. The purity of rapamycin obtained through the method described was 99.4% which has not been reported so far.

Key words: Rapamycin recovery, purification, centrifugal decantation, crystallization, mycelia, normal phase chromatography, silica

INTRODUCTION

Rapamycin is a macrocyclic polyketide produced during fermentation by the strain Streptomyces hygroscopicus which was isolated from a soil sample from Easter Island (Vezina *et al*., 1975; Sehgal *et al*., 1975). This fermentation based drug substance compound was originally discovered as an antifungal agent and has since been studied for its potent immunosuppressive and anti cancer properties (Ritacco *et al*., 2005; Hidalgo and Rowinsky, 2000; Marx and Marks, 2001; Yu *et al*., 2001; Sehgal, 1998).

Fermentation broth is a complex aqueous mixture of cells, soluble extra cellular products, intra cellular products, converted substrates or unconvertible components (Thykaer and Nielsen, 2003; Demain, 1999; Khaw *et al*., 1998). Separation techniques useful for any given industrial bioprocess depend not only on the location of the product (intra cellular and extracellular) and its size, charge and solubility (Skoog *et al*., 1998; Worthen *et al*., 2001). Purity of drugs is an important factor for the manufacture of safe and effective pharmaceuticals (Ahmad *et al*., 2009). Most of the drugs obtained by fermentation processes are purified by chromatographic procedures. Chromatographic purification can be achieved by high performance liquid chromatography, displacement and elution chromatography using silica gel columns. Each chromatographic process has its own advantages and disadvantages over other techniques but elution chromatography using silica gel columns is mostly used because of its ease of operation (Still *et al*., 1978; Maleki *et al*., 2000; Volosov *et al*., 2001).

Rapamycin recovery from fermentation broth poses additional difficulties and challenges because of the problems in isolation from very dilute solutions of the target molecule, its separation from byproducts and isomers with similar properties (Demain, 2004, 2006; Grima *et al*., 2003; Sanchez and Demain, 2002). Hence, the product recovery and purification from fermentation broth requires a multi-pronged approach to develop an efficient process with high overall yield. An important element in the design of a viable biotechnological process is the selection of economical and efficient separation techniques (Cragg *et al*., 1997; Berdy, 2005). Very little data are available on rapamycin recovery and purification.

The objective of the present study is to isolate and purify rapamycin from culture of Streptomyces hygroscopicus MTCC5681 by normal phase and flash chromatography using silica gel columns and this article describes a new separation procedure to isolate rapamycin with high yields.

MATERIALS AND METHODS

Separation of biomass containing rapamycin from culture broth: Approximately 1600 L of rapamycin fermentation broth obtained from a large scale fermentation experiment was acidified to pH 4.0 by

Corresponding Author: Polavarapu Baby Rani, Department of Biotechnology, Natco Research Center, Hyderabad-500018, India
Tel: 914023710575 Fax: 914023710758

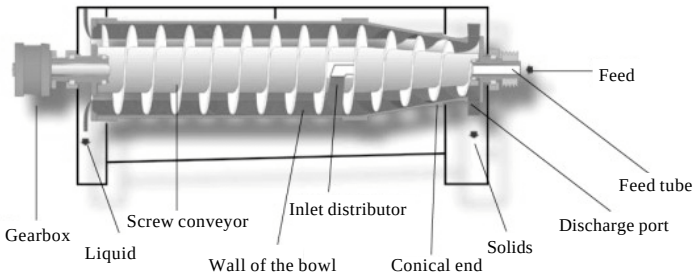


Fig. 1: Decanter centrifuge

addition of aqueous sulphuric acid. The acidified broth containing about 5% dried solids and 50% of suspended wet solids to be taken up for centrifugation. The decanter centrifuge (Fig. 1) consists of feed inlet, centrifuge bowl, screw conveyer assembly, bowl assembly and discharge port (ALDEC 20, Alfa laval, Sweden). The feed flow rate was set at $1m^3/h$ and the broth was pumped through a stationary feed inlet tube. The centrifuge was operated with a bowl speed of 4400 rpm at 3030 G centrifugal force. The dense solid particles get pressed outwards against the rotating bowl wall and then the rotating screw conveyer discharges the solids into the casing through discharge opening. Based on the operating volume, the dam plates were adjusted to receive the clarified liquid which was then discharged into the casing through dam plates. Approximately 1300 lt of clarified liquid was collected and analyzed by HPLC method as described. Very little rapamycin activity was detected in the decanted out liquid sample. About 350 kg of biomass cake thus obtained containing rapamycin was used for solvent extraction to isolate rapamycin.

Isolation of rapamycin from biomass: The biomass cake obtained above (350 kg) containing rapamycin was mixed with 1000lt of toluene and stirred for 4 h at 50°C. The extraction process was repeated with 1000lt of fresh toluene. The toluene extract was concentrated to 500 lt. The product rich concentrate was washed with 500 lt of 5% aqueous sodium bicarbonate solution, followed by washing with 2X500 Lt of water. The toluene extract was concentrated to obtain 10 kg of an oily residue which was mixed with 30 liters of acetone. Activated charcoal (5g) was added to this solution. The solution was stirred for 15 min at 40°C temperature, filtered and concentrated to obtain an amber colored oily residue of about 10 kg.

Chromatographic purification of rapamycin: The oily residue (3 kg) was loaded on to a column (length 1500 mm x dia 250 mm) packed with 30 kg of silica gel of 60-200 mesh size. 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 5.3 g of activated charcoal. The solution was stirred, filtered and concentrated under vacuum at 40°C. The residue was dried to obtain 400g of rapamycin powder (about 90% purity).

Crystallization of rapamycin: Rapamycin powder 5 g (purity 90% with tautomer content 6.5% at 1.1 RRT and 3.5% of other impurities) was dissolved in 30ml of isopropyl ether. The solution was stirred, filtered and concentrated at 25°C. The crystals were dried to obtain about 4.0g of rapamycin as a white powder (95-97% purity).

Purification of rapamycin by flash chromatography: Rapamycin powder 100mg (purity 95% with tautomer content of 2.5% at 1.1 RRT and other impurities amounting to 2.5%) was dissolved in 200ml of acetone. The solution was loaded on a column packed with flash specific media (cartridge from Grace Company, diameter 0.81 cm, particle size of silica 40µm, height 9.6cm). The product was eluted by gradient method.

Mobile phase: Buffer A: Hexane; Buffer B-Ethyl acetate

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 under reduced pressure concentrated at 15°C. The crystals were filtered and dried. About 50mg of rapamycin powder was thus obtained.

Estimation of rapamycin purity by HPLC: The purity of the product was determined by High Pressure Liquid Chromatographic (HPLC) analysis.

Table 1: Gradient elution of mobile phase for HPLC analysis

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

Column: UNISON UK C18, 3 μm, diameter 4.6 mm, length 250 mm
Flow rate: 1.0 mL/min
Detection wave length: 280 nm
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 elution schedule is as given in Table 1.

Statistics: Separation, extraction and purification techniques were conducted in triplicate using biomass produced by three fermentation batches. Statistical analysis of the purity measurement by the HPLC method at various stages and product yield starting from wet biomass to various stages of purification.

RESULTS AND DISCUSSION

Separation of biomass containing rapamycin from culture broth: Fermented broth was obtained by a microbial process using a rapamycin producing microorganism MTCC 5681. Employing a pilot-plant scale industrial fermenter, the culture medium and fermentation conditions such as strain of organism, type of inoculum, time of fermentation, fermentation temperature etc were optimized to produce maximum yield of the desired product. At the cessation of the fermentation, the pH of the fermentation broth was adjusted to about 4.0 with aqueous sulphuric acid before separating mycelia. The pH adjustment was useful to remove residual soluble contaminants during separation of mycelia. After acidification of the fermentation broth, the mycelia were separated by centrifugal decantation technique. Centrifugal decantation refers to the process of passing fermentation broth for separating solids from liquids using special equipment known as a Decanter Centrifuge in one single continuous process (Fig. 1). When the fermentation broth passes through the unit, the centrifugal force compact the solids and expels surplus liquid. The dried solids are discharged from the bowl. The clarified aqueous liquid phase was then separated by flow path. The water insoluble compound of interest remains in the dried solids. Removal of mycelial solid and separation of liquid are a crucial function in this fermentation process. The decanter centrifuge of the type used here is most useful and preferred when the solid content in the fermentation broth exceeds more than 65% (w/v).

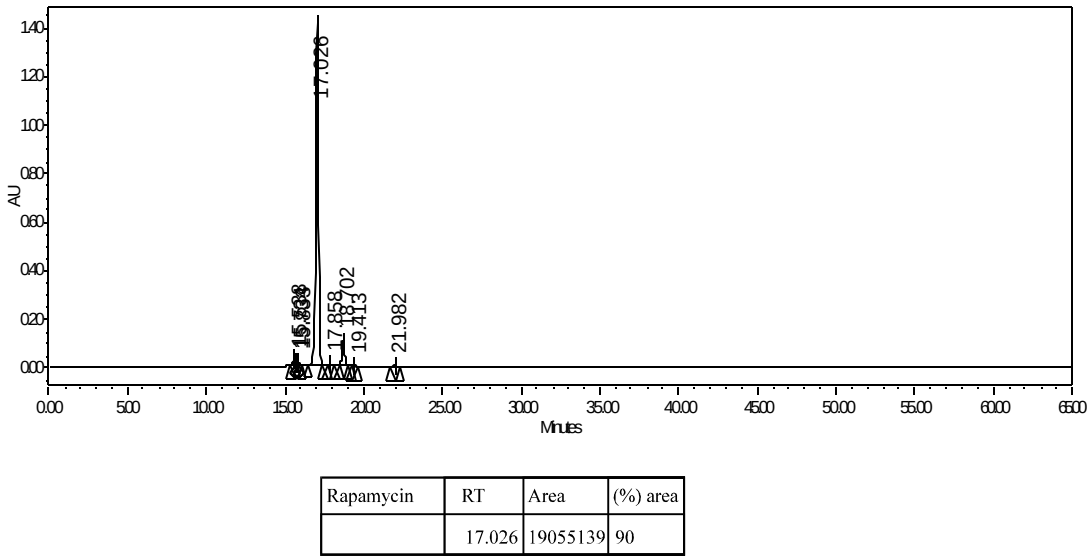
Isolation of rapamycin from biomass: It has been found that the separation of mycelia and isolation of rapamycin into solvent as described here leads to near quantitative recovery from the fermentation broth. The solid mass after centrifugal decantation was mixed with a solvent capable of solubilising the compound of interest to form liquid slurry. Toluene and ethyl acetate are particularly preferred hydrophobic extraction solvents and these are excellent for acidic metabolites. Extraction time optimization depending on the macrolide containing biomass, hydrophobic extraction solvent, equipment used and temperature. At the end of the operation, the extraction mixture contains rapamycin in the solvent as well as residual biomass. The amount of solvent used was generally atleast twice the amount of solid biomass after the centrifugal decantation. Typically four to six equivalent volumes were used. The impurities can be separated by filtration, phase separation or both. The organic layer rich in rapamycin was washed with aqueous sodium bicarbonate and water. Then activated charcoal was added and filtered. Following the extraction, rapamycin in hydrophobic solvent is concentrated to an oily residue. The concentration was carried out by using atmospheric pressure, attained with the aid of vacuum pump. The concentration was preferably carried out at a temperature of 25°C until the initial volume was reduced to about 0.5 to 1% to provide concentrated rapamycin as an oily residue. Crude rapamycin was isolated from the oily residue.

Chromatographic purification of rapamycin: The purity of rapamycin as obtained above was 90% with tautomer content 6.5% at 1.1 RRT and 3.5% of other impurities. The HPLC chromatogram of crude rapamycin is shown in Fig. 2. The details of chromatogram are given in Table 2.

The choice of appropriate stationary phases as well as the mobile phase is crucial for obtaining optimum separation of components, maximizing the recovery of solutes and avoiding irreversible adsorption of solutes onto the packing material. The retention of a specific compound also depends on the polarity of the mobile phase. The mixture of rapamycin and analogous compounds were dissolved in acetone and subjected to column chromatography using silica gel having a mesh size of 60-200. The mixture was adsorbed on the column

Table 2: The HPLC chromatogram details for crude rapamycin

Peak No.	Retention time (min)	Area (µV*sec)	(%) Area	SD	RT Ratio	Identification
1	15.538	231885	1.1		1.095	
2	15.734	105818	0.326		1.082	
3	15.833	72809	0.517		1.075	
4	17.026	19055139	90.01	0.1	1	Rapamycin
5	17.858	108947	0.701		1.048	
6	18.702	1337704	6.501	0.04	1.098	Tautomer
7	19.413	64489	0.326		1.14	
8	21.982	92334	0.519		1.291	



Rapamycin	RT	Area	(%) area
	17.026	19055139	90

Fig. 2: HPLC chromatogram-Rapamycin (crude)

and is successively eluted with organic solvents. The fractions rich in rapamycin were pooled to recover rapamycin. Preferred eluants are mixtures of acetone and hexane. The chromatographic fractions were treated with activated charcoal to remove colored impurities. After the concentration of the fractions containing crude rapamycin, the product was processed further by crystallization and chromatography. Rapamycin powder thus obtained was about 90% pure.

Crystallization of rapamycin: A solvent is chosen such that the compound of interest is neither excessively soluble nor insoluble. Input was 5gm of rapamycin containing purity 90% and output was 4.0 g containing rapamycin purity of about 95% (2.5% of tautomer at 1.1 RRT and other impurities of 2.5%) using isopropyl ether (IPE) crystallization. The HPLC chromatogram of rapamycin purified as above using isopropyl ether is shown in Fig. 3. The details of chromatogram are given in Table 3.

Input was 5 g of rapamycin containing rapamycin powder purity 90% and output was 3.5 g containing rapamycin purity about 97% (tautomer content of 1.5% at 1.1 RRT and other impurities of 1.5%) using diethyl ether crystallization. The HPLC chromatogram for rapamycin purified as above using diethyl ether is shown in Fig. 4. The details of chromatogram are given in Table 4.

Purification by flash chromatography: Prystallized rapamycin of 95-97% purity was purified by flash chromatography to get rapamycin of pharmaceutical grade with >99% purity.

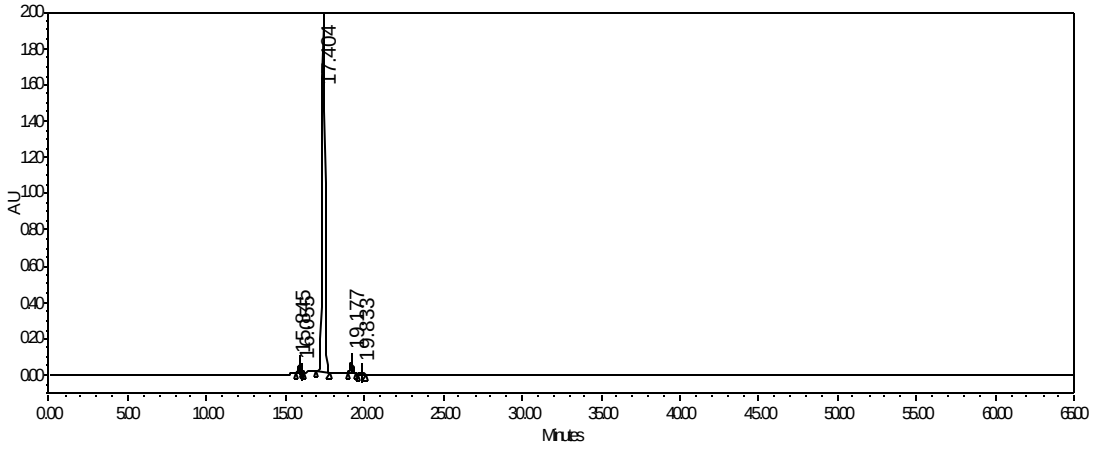
In flash chromatography, silica flash cartridges particle size was 40 µm. A smaller particle size increases resolution. These cartridges increase yield, resolve more and reduce run time. The purity of rapamycin powder was 99% with tautomer content 0.4% at RRT 1.1 and 0.6% of other impurities after chromatographic purification. The HPLC chromatograms for rapamycin (reference standard) and that of rapamycin produced in substantially pure form using flash chromatography are shown in Fig. 5 and 6, respectively. The details of chromatogram as in Fig. 5 are given in Table 5 and that of Fig. 6 is given in Table 6.

Table 3: HPLC chromatogram details of purified rapamycin using isopropyl ether

Peak No.	Retention Time (min)	Area (µV*sec)	% Area	SD	RT Ratio	Identification
1	15.845	374736	1.328		0.91	
2	16.055	67800	0.24		0.923	
3	17.404	26951553	95.519	0.15	1	Rapamycin
4	19.177	729637	2.586		1.102	Tautomer
5	19.833	92167	0.327	0.06	1.14	

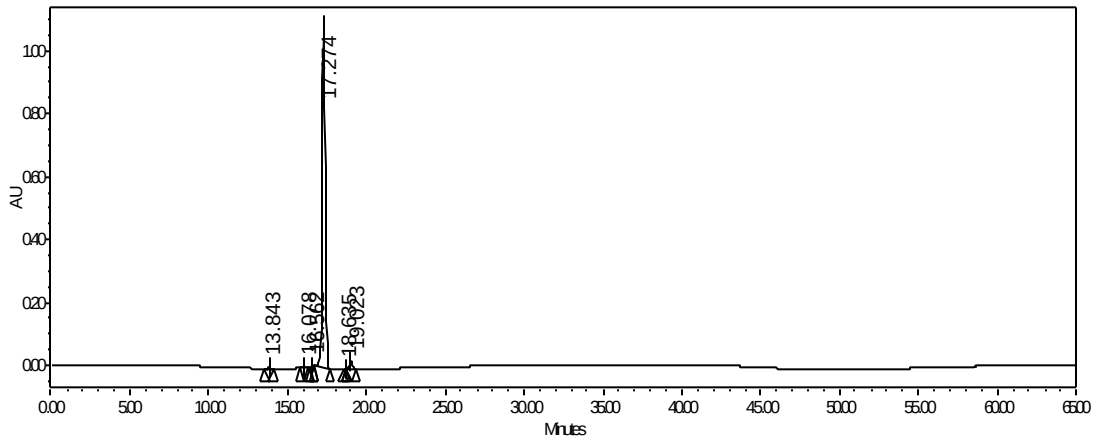
Table 4: HPLC chromatogram details for purified rapamycin using diethyl ether

Peak No.	Retention time (min)	Area (µV*sec)	% Area	SD	RT Ratio	Identification
1	13.843	23786	0.488		0.801	
2	16.078	26799	0.364		0.93	
3	16.562	10828	0.203		0.958	
4	17.274	15853156	97.266	0.08	1	Rapamycin
5	18.635	3174	0.264		1.078	
6	19.023	381054	1.415	0.01	1.101	Tautomer



Rapamycin	RT	Area	(%) area
	17.404	26951553	95.519

Fig. 3: HPLC chromatogram-Rapamycin (crystallized from IPE)



Rapamycin	RT	Area	(%) area
	17.274	15853156	97.266

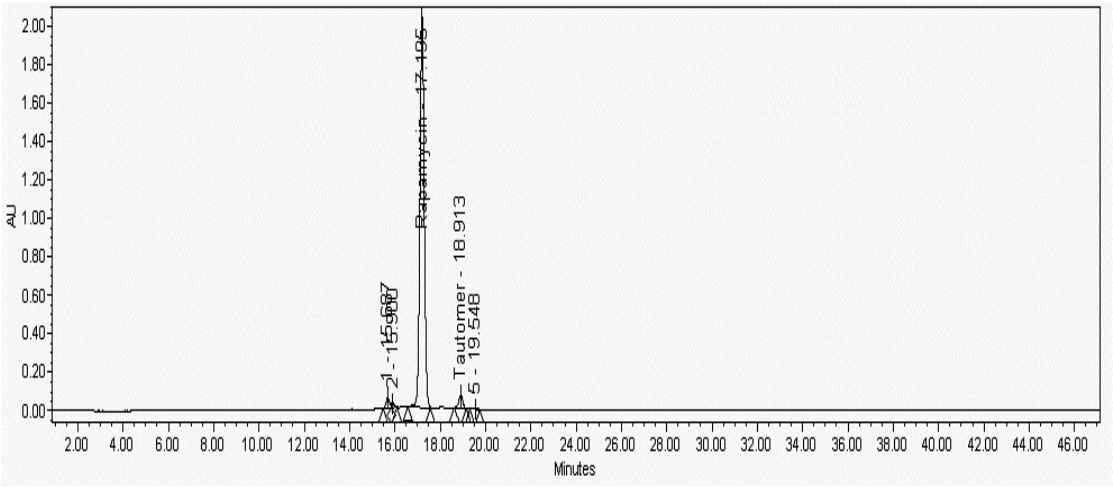
Fig. 4: HPLC chromatogram-Rapamycin (crystallized from Diethyl ether)

Table 5: The HPLC chromatogram details for rapamycin reference standard

Peak No.	Retention time (min)	Area (µV*sec)	% Area	SD	RT Ratio	Identification
1	15.687	551902	0.7764		0.912	
2	15.9	247455	1.0021		0.924	
3	17.195	27225638	95.2694	18	1	Rapamycin
4	18.913	843756	2.6261		1.099	Tautomer
5	19.548	94542	0.326	0.01	1.136	

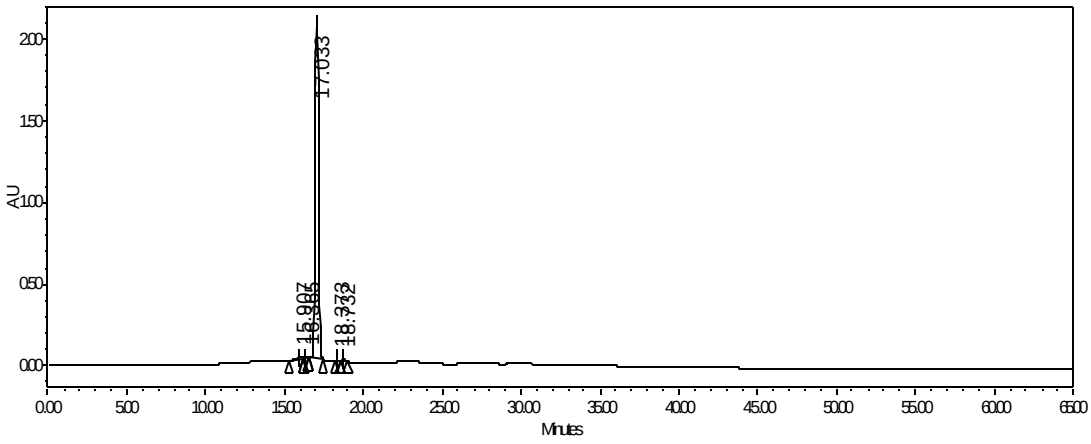
Table 6: HPLC chromatogram details for rapamycin purified using flash chromatography

Peak No.	Retention time (min)	Area (µV*sec)	% Area	SD	RT Ratio	Identification
1	15.907	165308	0.4		0.933	
2	16.365	38976	0.1		0.96	
3	17.033	27042609	99	0.03	1	Rapamycin
4	18.373	32869	0.1		1.078	
5	18.732	129663	0.4	0.01	1.099	Tautomer



Rapamycin	RT	Area	(%) area
	17.195	27225638	95.2694

Fig. 5: HPLC chromatogram-Rapamycin reference sample



Rapamycin	RT	Area	(%) area
	17.033	27042609	99

Fig. 6: HPLC chromatogram-Rapamycin (Purified by flash chromatography)

CONCLUSION

The need to upgrade the purity of rapamycin to pharmaceutical grade (> 99%) leads us to develop a multi step commercially viable purification process. Centrifugal decantation is the preferred method for recovering the biomass from the fermentation broth. Adsorption and flash chromatographic methods have been developed for good separation to achieve pharmaceutical grade purity of product. Drastic reduction in tautomer content (0.4%) and isolation of high purity rapamycin (>99%) have been achieved by a combination of separation and purification techniques.

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