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(54) **Title**: IMPROVED PROCESS FOR PREPARATION OF EPTIFIBATIDE BY FMOC SOLID PHASE SYNTHESIS

(57) **Abstract**: Eptifibatide is a cyclic heptapeptide containing six amino acids and one mercaptopropionyl (des-aminocysteine, Mpa) residue. An interchain disulphide linkage is formed between the cystiene amide and mercaptopropionyl moieties. It is a short acting parenteral antithrombotics drug used for treating Acute Coronary Syndrome (ACS). The present invention describes, simple, convenient, economical process for preparation of eptifibatide by Fmoc solid phase peptide synthesis. The linear peptide is assembled on Rink amide resin, in which thiol groups of cysteine and mercaptopropionic acid are protected by acm group and guanidine of homoarginine by pbf group. The assembled peptide is cleaved from the resin to give linear peptide in which thiols of cysteine and mercaptopopionic acid are protected by acm group. The linear peptide is oxidized by mild oxidation reagent iodine leading to afford eptifibatide. A three-stage HPLC purification afforded pharmaceutical grade eptifibatide with purity greater than 99%.

# Improved Process for Preparation of Eptifibatide by Fmoc Solid Phase Synthesis

## Field of the Invention

The present invention relates to a novel process for the preparation of Pharmaceutical grade eptifibatide **1** using solid-phase Fmoc-chemistry.

## Back Ground of the Invention

The eptifibatide, cyclic heptapeptide used for the treatment of cardio vascular disease. It is a short acting parenteral antithrombotics drug used for treating Acute Coronary Syndrome (ACS). It is also used for patients undergoing Percutaneous Coronary Interventions (PCI). Eptifibatide is believed to work by inhibiting platelet aggregation, specifically by blocking the platelet receptor glycoprotein IIb/IIIa.

Chemically eptifibatide is a cyclic heptapeptide containing six amino acids and one mercaptopropionyl (des-aminocysteine, Mpa) residue. An interchain disulphide linkage is formed between the cystiene amide and the mercaptopropionyl moieties. It is produced both by solution and solid phase peptide synthesis.

Map-Har-Gly-Asp-Trp-Pro-Cys-$NH_2$.

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**1**

In terms of peptide synthesis methodology, two major synthetic techniques dominate current practice. These are solution-phase, and solid-phase syntheses. Solution-phase route synthesis is laborious as compared to the solid-phase route as after each coupling the peptide formed has to be isolated. Whereas in the solid-phase synthesis, the excess reagents and by-products are washed off by simple filtration. In both the methods desired

peptide is prepared by the step-wise addition of amino acid moieties to a building peptide chain.

Reported synthetic approaches to eptifibatide have employed known techniques of solution-phase synthesis as described for example in US patents 2006/0036071A, 5506362.

US patents 5318899 and 5958732 claim recombinant techniques to produce peptides like eptifibatide. The peptide obtained by this recombinant process is modified by the conversion of lysine residue to homoarginine residue. These patent documents also claim solid-phase synthesis using Boc-chemistry

The patents US 5759999, US 576333, US 5770564, US 5807825, US 5807828, US 5843897, US 5968902, US 5935926, US 5344783, US 5780595 and US 5851839 describe the process for the preparation of eptifibatide using solid phase Boc-chemistry.

As compared to Boc-chemistry, Fmoc-chemistry based synthesis utilize procedures under milder process conditions and because of the base labiality of Fmoc-group, acid-labile side-chain protecting groups are employed providing orthogonal protection.

Fmoc based solid phase syntheses are descried in WO /05/121164 A, WO 03/093302, CN 1858060-A, CN 1500805, WO 2006045483, WO 2006 119388, WO 2006 0420 WO 2004 092202, WO 03/093302. These reports vary from each other in following aspects:

- Nature of solid support used for assemble of peptide
- Variance in the protected amino acids used for preparation of heptapeptide on the solid support
- Deprotection of thiol protecting groups of mercatopropionic acid and cysteine and subsequent oxidation of thiol group to form cyclic peptide

WO /05/121164 A and US 2007249806 A1, disclose the synthesis of the peptide eptifibatide using Rink amide resin as solid-support. These patents describe assembling a peptide chain comprising of six amino acids in required sequence on a solid support resin. The peptide obtained by this process is modified by solution phase synthesis for conversion of lysine residue to homoarginine residue. The conversion of lysine residue to homoarginine is through guanylation with the reagent 3,5-dimethyl pyrazole-1-carboxamid nitrite. These patents also claim the deprotection of the thiols with heavy metals and isolating the crude peptide as heavy metal salt.

The Chinese publication *Xiamen Daxue Xuebao, Ziran Kexueban* (2007), 46(1), 100-103 describes solid phase process that results eptifibatide of 98.5% purity employing TBTU/HOBt/DIEA activation system.

Patent CN 1858060 discloses the solid -phase synthesis of eptifibatide, which comprises attaching Fmoc- α-protected amino acid to the solid support Rink amide MBHA / AM. Thiol moieties of mercaptopropionic acid and cysteine are protected by benzyl ether. Cleavage of peptide from the resin is affected by TFA/HBr/AcOH system. These cleavage conditions create lot of impurities, as the peptide bonds are also susceptible for HBr/AcOH mixture, and thus form small peptide impurities in this process. This process claims oxidation of crude peptide in air at pH 7.5- 10. These conditions also initiate the formation of impurities.

Patent WO 2006045483 describes a general concept of on resin cyclization of cysteines with t-butylsulfenyl, dithiopropionyl thiol protecting groups using Siber resin. In this patent eptifibatide is chosen as model compound. There are no details regarding the purity.

Patents WO 2006 119388, WO 2006 0420 WO 2004 092202 from Novetide/Teva describe the preparation using trityl protected mercaptopropionic acid and on trityl resin.

CN 1500805 describes the preparation of eptifibatide on rink amide resin. In this patent thiol of mercaptopropionic acid is protected by triphenylmethyl and guanidine of homoargaine by N,Ndi-tert butoxycarbinyl protecting groups respectively.

Patent WO 03/093302 discloses the process for the solid-phase synthesis of eptifibatide comprising of attaching an α- nitrogen protected α-carboxamide amino acid to the solid support 4-methoxy trityl polystyrene resin through its thiol side chain, followed by removing the α-nitrogen protecting group and assembling the peptide chain on the α-nitrogen. Mercaptopropionic acid is protected by trityl or by disulphide of mercaptopropionic acid. The process consists of number steps and uses expensive raw materials.

The processes descried in the prior art have disadvantages such as;
(i) Protecting thiols with expensive and difficult to deblock thiol protecting groups, (ii) isolating free thiol peptide, which is susceptible for aerial oxidation leading to impurities, (iii) forming homoarginine from lysine using expensive guanylating reagents. (iv) product purity is not mentioned in most of the prior art procedures.
Keeping above points in mind an improved process has been invented to eptifibatide with the following advantages.

1. The present process does not involve the isolation of thiol peptide, susceptible to aerial oxidation leading to the formation of impurities, which hampers the purification of the final product and yield.
2. Homoarginine moiety is obtained from Fmoc-Har (Pbf)-OH. Protection of guanidine of homoargaine by Pbf group affords hydrophobicity for highly hydrophilic homoargnine, which facilitates smooth coupling.
3. Disulfide loop in the peptide amide is introduced through an efficient process by the deprotection and oxidation of thiol moieties in the single step without isolation of –SH peptide.
4. The present process yields pharmaceutical grade eptifibatide with >99% purity of isolated product.

**Summary of the Invention**

The present invention relates to a novel process for the preparation of Pharmaceutical grade eptifibatide **1**, which involves, (i) assembling amino acid residues and mercaptopropionic acid with an appropriate thiol protecting groups on a solid phase resin to yield resin bound peptide **2**, (ii) cleaving the peptide thus obtained from the resin with concomitant removal of side chain protecting groups except acm protecting group of thiol moiety to obtain crude peptide **3**. (iii) crude peptide **3** having protected thiol groups, is deprotected and oxidized with iodine in water/acetic acid to obtain crude eptifibatide. (iv) and finally subjecting to chromatographic purification.

The procedure described in the present invention is simple, efficient and cost effective and adoptable for large-scale production.

Map-Har-Gly-Asp-Trp-Pro-Cys-$NH_2$.

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**1**

Mpa(Acm)-Har-Gly-Asp-Trp-Pro-Cys (Acm)-$NH_2$

**3**

**Brief Description of Drawings:**

Figure1: HPLC Chromatogram of peptide **3**

Figure 2: HPLC Chromatogram of crude eptifibatide **1**

Figure 3: HPLC chromatogram of pooled fractions of primary purification

Figure 4: HPLC chromatogram of pooled fractions of secondary purification

Figure 5: HPLC chromatogram of lyophilized eptifibatide

**Detailed Description of the Invention**

A considerable number of known, naturally occurring small and medium-sized cyclic peptides as well as some of their synthetic derivatives and analogs possessing desirable pharmacological properties have been synthesized. However, wider medical use is often

hampered due to complexity of their synthesis and purification of the peptides to pharmaceutical grade. Therefore improved methods for making these compounds in simple, minimum steps and in a cost effective manner is desirable and it is the need of the industry.

The purity and yield of the peptides are important aspects of any route of synthesis. Purity is represented by the degree of presence of pharmacologically active related impurities. The trace amounts of impurities present in the peptide may disturb and adversely affect the beneficial action of the peptide when used as a therapeutic agent. In solution phase synthesis repeated purifications at each step invariable lead to low yield of the final peptide. The present invention is a novel process to achieve high purity with enhanced yield of the target peptide eptifibatide through solid phase methodology.

The step-wise introduction of N α-protected amino acids in solid phase peptide syntesis normally involves the carboxyl group activation of the incoming amino acid or the use of pre-formed activated amino acid derivatives. The reagents HOBt/ DIC, HBTU, TBTU, Py Boc and HATU are the preferred tools for in situ carboxyl activation. However HBTU, TBTU, Py Boc and HATU are expensive hence inexpensive HOBt/DIC is used for coupling.

In case of homoarginine, simple alpha amino protected Fmoc-homoargaine without side chain guanidine protection is used initially. In this case coupling did not take place efficiently, hence Fmoc-Har (Pbf)-OH is used.

Oxidative cyclization of protected or non-protected sulfhydryl groups with formation of disulfide structures is usually carried out at the final synthetic step. In few cases it is also carried out before cleavage of the peptide molecule from the solid support. The oxidation of open-chain peptide containing free and / or certain types of protected sulfhydryl groups with iodine in methanol or acetic acid, acetic acid/water is a convenient and safe process. In the present invention the deprotection and oxidation of acm protecting groups of cysteine and mercaptopropionic acid is effected by iodine.

There has been a long awaited requirement for obtaining eptifibatide, which will circumvent the limitations associated with the processes of the prior art. An industrial process of peptide synthesis containing homoarginine, tryptophan, disulfide loops, etc. demands appropriate choice of protecting groups and reaction conditions to build up the peptide chain. This objective has been successfully achieved in the present invention.

**Example 1: Synthesis of Mpa (Acm)-Har (Pbf)-Gly-Asp(OtBu)-Trp-Pro-Cys(Acm)-Resin (2)**

Synthesis of the peptide was carried out by a regular stepwise Fmoc SPPS (solid phase peptide synthesis) procedure starting from Rink amide resin. The resin was transferred to the reaction vessel of the peptide synthesizer (Endeavor 90, AAPPTEC peptide synthesizer) and the linear peptide was assembled on it using 6.0 times mole excess amino acid derivatives, on the peptide synthesizer. The first amino acid Fmoc-Cys (Acm)-OH was coupled to the resin by deprotection the Fmoc-group on the resin followed by activation of Fmoc-Cys (Acm)-OH. Fmoc-Cys (Acm)-OH (14 mmole) and HOBt (14 mmole) were dissolved in DMF (150ml), DIC (14 mmole) was added to the reaction mixture and agitated for 5 minutes before being charged to the damp resin in the reaction vessel. The coupling reaction took place for about 6 (coupling monitored by Kaiser test) hours.

For coupling of the next amino acid proline, the α-nitrogen of the first amino acid Fmoc-Cys (Acm)-OH was deprotected. This was followed by activation of Fmoc-Pro-OH by DIC/HOBt as described above and then transferred the mixture to the reaction vessel. This process was repeated with all the amino acids till the entire linear peptide chain was assembled on the solid support. The Mpa (Acm)-OH was assembled at the end. Each coupling was carried out for a time range of 3-10 hours. After the couplings were completed, the resin was washed with DMF followed by methanol.

**Example 2: Cleavage of the peptide from the resin to yield Mpa(Acm)-Har-Gly-Asp-Trp-Pro-Cys (Acm)-$NH_2$ (3)**

The assembled peptide resin was treated with 300ml of cleavage cocktail consisting of TFA (95%), TIS (2.5%), EDT (2.5%) for 2 hours at room temperature. The reaction mixture was filtered and precipitation of the peptide was carried out by addition of 3 L of cold ether. The crude peptide precipitate in the solvent was let to stand at -20°C for 15 hours. The peptide was isolated by filtering through sintered funnel followed by cold solvent wash (200ml x 3) to remove the scavengers used in the cleavage cocktail. The crude peptide precipitate was dried under vacuum and characterized by RP-HPLC (**Figure1**, HPLC Chromatogram of peptide **3**)

Yield: 11.5grms.

**Example 3: Deprotection and oxidation of peptide 3 to yield eptifibatide 1**

Crude protected peptide **3** (11g) was dissolved in 5.5 liters of water /acetic acid (9/1), iodine (1.5g) dissolved in 100 ml methanol was added during the course of 1-2h. The reaction mixture was kept at 4°C for 24 hours. Excess iodine was neutralized with ascorbic acid and then taken up for purification (**Figure 2**, HPLC Chromatogram of crude eptifibatide **1**)

**Example 4: Purification of crude eptifibatide**

*Primary Purification*

The above crude eptifibatide solution was loaded on to prep C-18 column (50x 250mm, 100A). Fractions with >95%purity was pooled and taken up for secondary purification (**Figure 3**, HPLC chromatogram of pooled fractions of primary purification).

Buffer A: 0.1% TFA in water + acetonitrile (95+5)

Buffer B: 0.1% TFA in water + acetonitrile (50+50)

Gradient program

| Time (min) | A (%) | B (%) |
|---|---|---|
| 0 | 100 | 0 |
| 10 | 85 | 15 |
| 70 | 55 | 45 |
| 75 | 0 | 100 |
| 85 | 0 | 100 |
| 86 | 100 | 0 |
| 95 | 100 | 0 |

*Secondary purification*

Fraction with purity greater than 95% are pooled and loaded again onto to prep C-18 column (50x 250mm, $100A^0$). The fraction with more than 99% purity were pooled and taken up for salt exchange (**Figure 4**, HPLC chromatogram of pooled fractions of secondary purification)

Buffer A: 0.5% AcOH in water + acetonitrile (95+5)

Buffer B: 0.5% AcOH in water + acetonitrile (50+50)

Gradient program

| Time (min) | A (%) | B (%) |
|---|---|---|
| 0 | 100 | 0 |
| 10 | 100 | 0 |
| 15 | 85 | 15 |
| 60 | 65 | 35 |
| 70 | 0 | 100 |
| 80 | 0 | 100 |
| 81 | 100 | 0 |
| 90 | 100 | 0 |

*Salt exchange*

Fraction with purity greater than 99% are pooled and loaded again onto to prep C-18 column (50x 250mm, $100A^0$). The fraction with more than 99% purity were pooled and lyophilized (**Figure 5**, HPLC chromatogram of lyophilized eptifibatide).

Buffer A: 0.5% AcOH in water + acetonitrile (95+5)

Buffer B: 0.5% AcOH in water + acetonitrile (50+50)

Buffer C: 0.1 M Ammonium acetate in water + acetonitrile (95+5), pH 6.5

Gradient program

| Time (min) | A (%) | B (%) | C(%) |
|---|---|---|---|
| 0 | 100 | 0 | 0 |
| 10 | 100 | 0 | 0 |
| 10.1 | 0 | 0 | 100 |
| 20 | 0 | 0 | 100 |
| 20.1 | 100 | 0 | 0 |
| 30 | 100 | 0 | 0 |
| 30.1 | 0 | 0 | 100 |
| 40 | 0 | 0 | 100 |
| 40.1 | 100 | 0 | 0 |
| 50 | 100 | 0 | 0 |
| 65 | 50 | 50 | 0 |
| 80 | 50 | 50 | 0 |
| 81 | 0 | 100 | 0 |
| 89 | 0 | 100 | 0 |
| 90 | 100 | 0 | 0 |

**We claim:**

1. A process for the preparation of eptifibatide **1** by Fmoc solid phase synthesis technique,

Map-Har-Gly-Asp-Trp-Pro-Cys-$NH_2$.
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**1**

The said process comprising.

a) Assembling a peptide chain consisting of six amino acids and a mercaptopropionic acid in a required sequence on a solid support resin by coupling to one another by peptide bonds to obtain a peptide bound resin **2** as given below.

Mpa(Acm)-Har (Pbf)-Gly-Asp (OtBu)-Trp-Pro-Cys (Acm)-Resin (**2**)

**2**

b) Cleaving and deprotection of all groups except acm group, the peptide of step (a) from the resin to obtain peptide **3** as given below.

Mpa (Acm)-Har-Gly-Asp-Trp-Pro-Cys (Acm)-$CONH_2$ (**3**)

c) Treating the peptide **3** with iodine in mixture of water/acetic acid for deprotection of acm groups and instantly oxidation of sulfhydryl groups to obtain eptifibatide **1**, and

d) Purifying the crude eptifibatide of step (c) by RP-HPLC in isolation or in combination with IEC chromatographic techniques.

2. A process of claim 1, where in the solid support used is Rink amide resin.
3. A process of claim 1, where in the cleavage of the resin with the linker leads to the release of assembled peptide amide **2**.

4. A process as claimed in claim 1, wherein the assembly of the amino acids gives a peptide bound resin of **2**

Mpa(Acm)-Har (Pbf)-Gly-Asp (OtBu)-Trp-Pro-Cys (Acm)-Resin (**2**)

5. A process of claim 1,where in the protecting for gaunidino group of homoargine is pbf.
6. A process of claim 1,where in the protecting group for thiol functional group of cysteine and mercaptopropionic acid is acm group.
7. A process of claim 1,where in the peptide of compound **1** obtained has purity greater than 99%.
8. A process for preparation of eptifibatide **1,** essentially as described in the examples 1-4 above.

## HPLC Chromatogram of Peptide 3

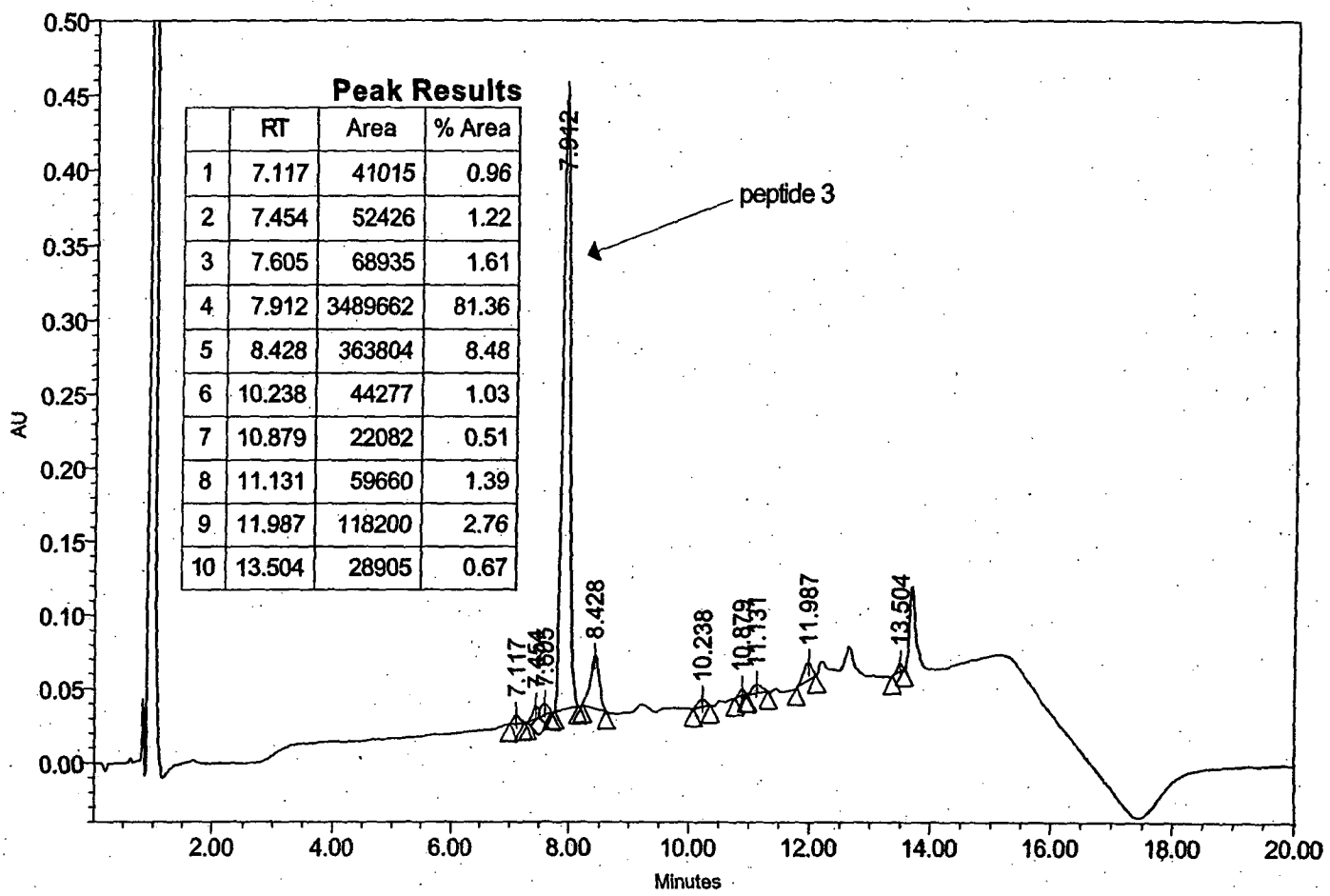



Figure 1

## HPLC Chromatogram of Crude Eptifibatide 1

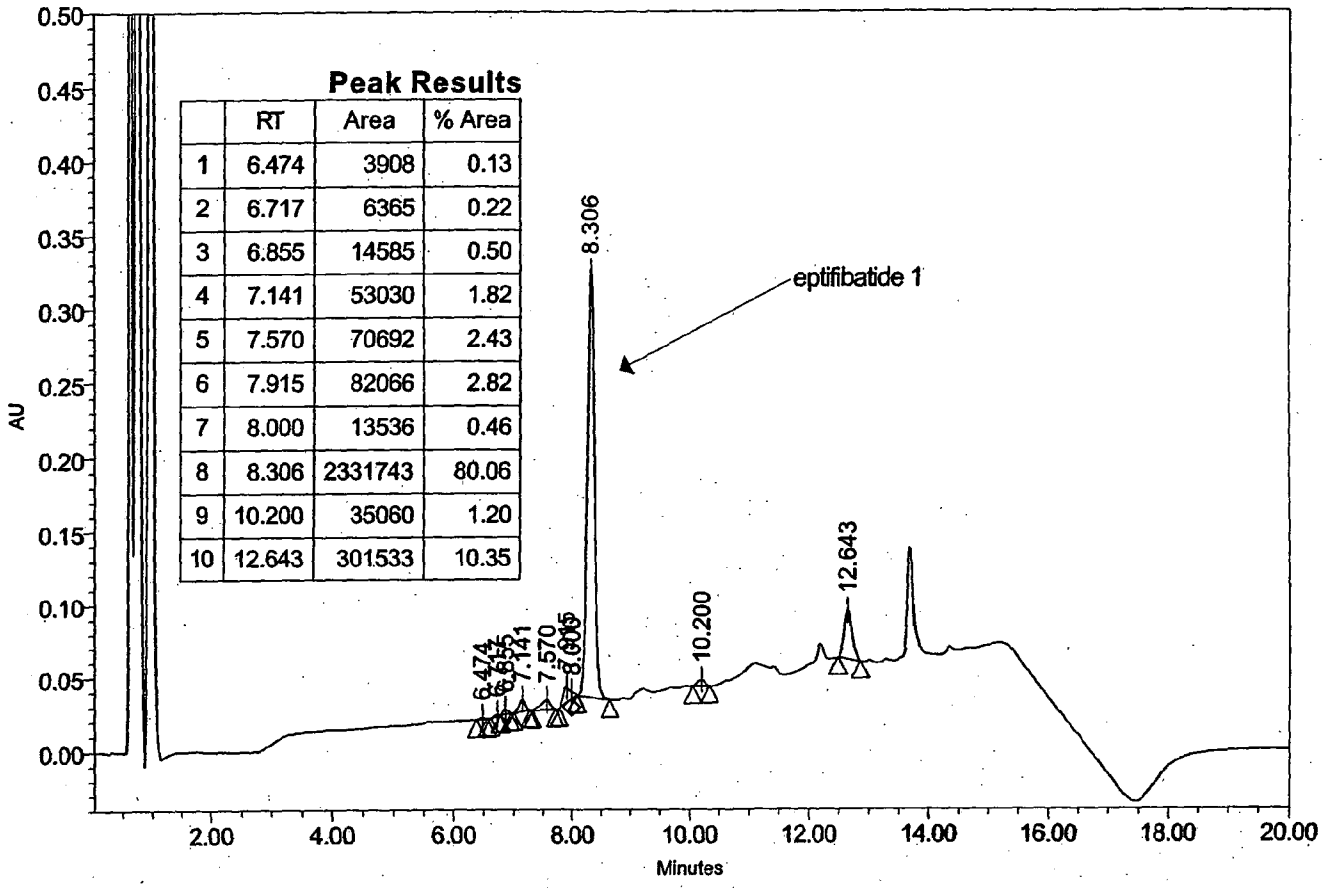
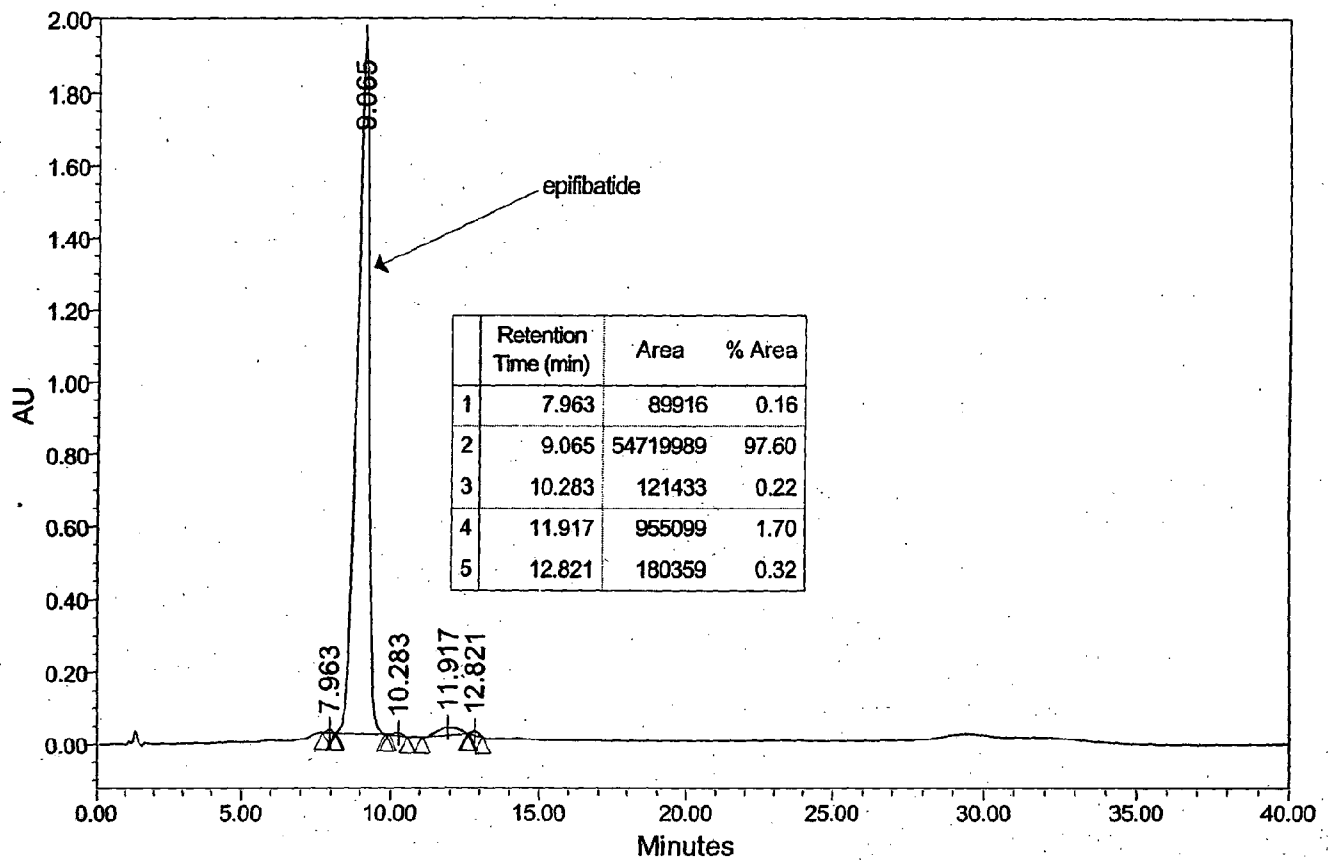



Figure 2

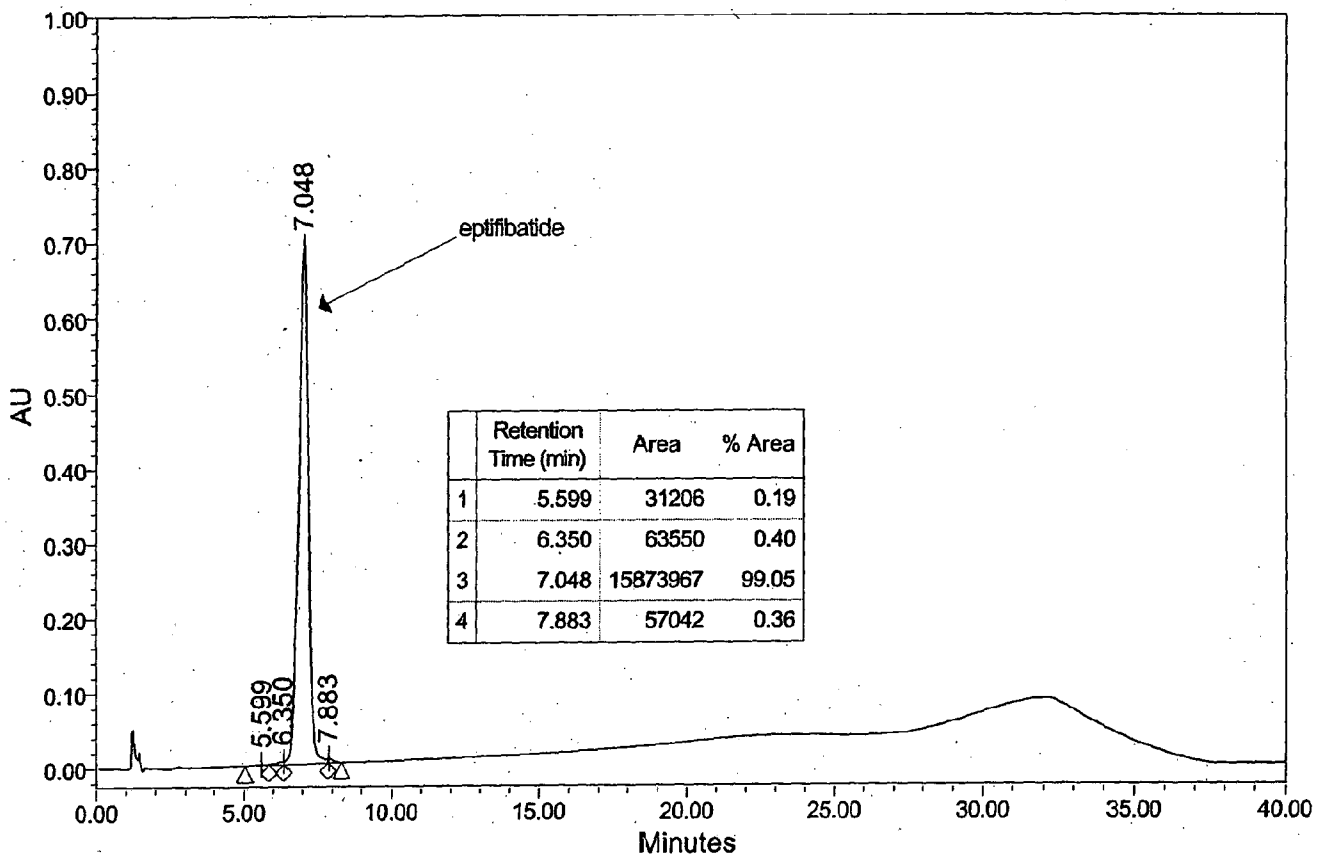
## HPLC Chromatogram of Pooled Fractions of Primary Purification




| | Retention Time (min) | Area | % Area |
|---|---|---|---|
| 1 | 7.963 | 89916 | 0.16 |
| 2 | 9.065 | 54719989 | 97.60 |
| 3 | 10.283 | 121433 | 0.22 |
| 4 | 11.917 | 955099 | 1.70 |
| 5 | 12.821 | 180359 | 0.32 |

**Figure 3**

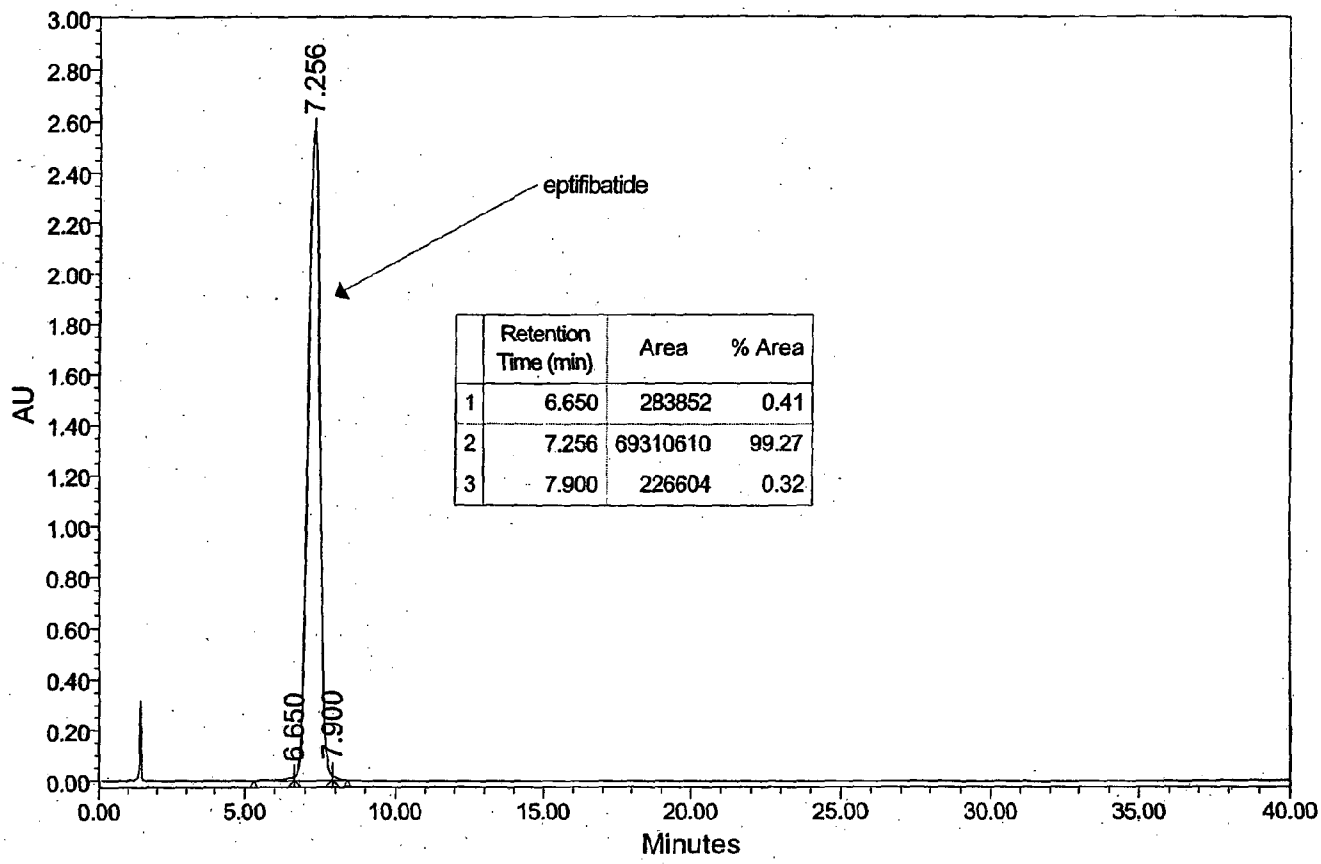
## HPLC Chromatogram of Pooled Fractions of Secondry Purification




| | Retention Time (min) | Area | % Area |
|---|---|---|---|
| 1 | 5.599 | 31206 | 0.19 |
| 2 | 6.350 | 63550 | 0.40 |
| 3 | 7.048 | 15873967 | 99.05 |
| 4 | 7.883 | 57042 | 0.36 |

Figure 4

## HPLC Chromatogram of lyophilized eptifibatide




| | Retention Time (min) | Area | % Area |
|---|---|---|---|
| 1 | 6.650 | 283852 | 0.41 |
| 2 | 7.256 | 69310610 | 99.27 |
| 3 | 7.900 | 226604 | 0.32 |

Figure 5

# INTERNATIONAL SEARCH REPORT

International application No
PCT/IN2008/000360

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K7/06

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)
C07K

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 practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
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| X | WO 2006/041945 A (NOVETIDE LTD [IL]; TEVA PHARMA [US]; TOVI AVI [IL]; EIDELMAN CHAIM [IL) 20 April 2006 (2006-04-20) examples 23,24 | 1-8 |
| X | WO 03/093302 A (AVECIA LTD [GB]; EVANS DAVID JOHN [GB]; SIMPKIN DEAN STEVEN ANTHONY [G) 13 November 2003 (2003-11-13) cited in the application example 1 | 8 |

-/--

[X] Further documents are listed in the continuation of Box C.

[X] 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 |
|---|---|
| 8 January 2009 | 19/01/2009 |

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

Form PCT/ISA/210 (second sheet) (April 2005)

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International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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