Int J Pharma Res Health Sci. 2017; 5 (4): 1760-64
DOI:10.21276/ijprhs.2017.04.08
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CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

PHS Scientific House
PHARMAHEALTHSCIENCES

International Journal of Pharma Research and Health Sciences

Available online at www.pharmahealthsciences.net

Original Article

An Efficient Approach for Thio-Ether Bond Formation in Carbetocin

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ARTICLE INFO

Received: 27 May 2017
Accepted: 01 Jul 2017

ABSTRACT

An efficient approach for the formation of unnatural thio-ether side chain of carbetocin by performing the reaction using DMF and liquid Ammonia to produce Crude Carbetocin. Crude carbetocin SH was synthesized by solid phase synthesis. The cyclisation of Carbetocin-SH was done by variation in addition of carbetocin-SH and liquid ammonia in the reaction mixture. Cyclisation of carbetocin-SH was done using different pattern of input Raw-material and the dissolution of Carbetocin-SH. The approach was to avoid the dimer formation hindering the purification of Carbetocin and low yield.

Keywords: Peptide, DMF: Dimethyl Formamide, Carbetocin-SH, Ammonia, thioether, Monodisulfide bridge.

1. INTRODUCTION

Peptide with thioether linkage are effective mimics of cysteine linked structure, cyclisation of peptides is always challenging and special interest as there is always possibility of dimerisation between molecules of the carbetocin, during the reaction. The monosulfide bridge peptides provide greater stability to the peptide as compared to the disulfide bridge peptides.[8] A thioether linkage has been widely utilized as a stable option for Disulfide Bridge in the bioactive peptides, such as hormones, neurotransmitters and neuromodulators to prolong the biological activity. To increase the biological activity and stability against

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biodegradation thioether has been used to prepare cyclic analogs of normally acyclic polypeptides to restrict their conformational mobility. Thioether linked peptides have also found in nature specially in a family of polycyclic peptide antibiotics, lantibiotics, including nisin an important food preservative, epidermin, a therapeutic agent against acne, as well as enzyme inhibitor and immunological active peptides.[2] Carbetocin proved to be as effective as Oxytocin in prevention of postpartum hemorrhage in women with severe preeclampsia therefore Carbetocin is an appropriate alternative to Oxytocin.[3] The general procedure for thioether formation reactions is using microwave synthesizer with vessels of 4 ml volume using diisopropyl ethyl amine and dimethyl formamide as a solvent. In all irradiation experiments rotation of the rotor, irradiation time, temperature and power were monitored. Once the reaction reached to 50^0Cthe reaction mixture was held at this temperature for 10 min and the cooled rapidly to room temperature. The reaction content was washed with dimethyl formamide, dichloromethane. The final cyclisation step was performed after deprotection of 9-fluoroenylmethyloxy carbonyl group and HBTU/HOBt as activating group for 3 hours. The product was cleaved from resin and precipitated with ether. [1]

2. MATERIALS AND METHODS

Materials:

For doing the research work Carbetocin-SH, chemicals, Magnetic stirrer, glasswares, analytical HPLC were kindly provided by Hemmo Pharmaceuticals Pvt. Ltd.

Methods:

Details of Analytical Method used for Monitoring the Reaction:

1. Buffer A: In 800 ml Milli-Q water add 1 ml of Trifluoroacetic acid make up the volume to 1 lit and sonicate for 15 min
2. Buffer A: 100% Acetonitrile
3. Preparation of Working Standard of Carbetocin: 12.5 mg Carbetocin Working standard dissolved in 50% Acetonitrile:Milli-Q water to get a concentration of 0.5 mg/ml.
4. Preparation of Working Standard of Carbetocin-SH: 12.5 mg Carbetocin-SH Working standard dissolved in 50% Acetonitrile:Milli-Q water to get a concentration of 0.5 mg/ml.
5. Test solution: Sample 20 ml of solution from the reaction dilute it to 1/10 and inject

Table 1: Chromatographic conditions

Column:	C_{18}, 150 x 4.6 mm, 5 µm
Flow rate:	1.0 -1.5 ml/ min,
Detector Wavelength:	220 nm
Injection volume	20 µl
Run time:	40 min.
Carbetocin Retention Time:	About 9 min
Carbetocin-SH Retention Time:	About 11 min

Table 2: Gradient Program

Time (min)	Mob. Phase B Concentration
0.01	20
30.00	80
31.00	20
40.00	20
40.01	Stop

Experimental:

Experiment No.1: Cyclisation of Carbetocin-SH: Ammonia addition over the dissolved carbetocin-SH in Dimethyl formamide.

500 ml Dimethyl formamide was taken in round bottom flask with two neck opening; 5.0 g of Carbetocin-SH provided by Hemmo Pharmaceuticals Pvt. Ltd was weighed and added slowly in Round bottom flask containing Dimethyl formamide. Stirring started with the help of magnetic stirrer. Allowed the Carbetocin-SH to get dissolved to clear solution. After complete dissolution of Carbetocin-SH the solution was cooled to -45±5°C by means of dry ice acetone bath. After attaining -45°C, addition of condensed ammonia solution at concentration of 0.2 lit/0.1 lit of DMF taken for dissolution of carbetocin-SH was started over the solution of Carbetocin-SH/Dimethyl formamide, Once the addition was over, dry ice acetone bath was replaced with hot water bath of 40°C. Sample was taken as a 0 hours and given for analysis as per the method mentioned in methods and analysis for monitoring the oxidation reaction. The reaction was monitored and continued till the percentage amount of Carbetocin-SH reduced to below 5.0 %. As per analytical results reaction progress was observed follows:

Table 3: Analytical results showing reaction progress at respective hours

Time of reaction monitoring	Carbetocin	Carbetocin-SH	Total area of carbetocin +carbetocin SH	%Carbetocin as per chromatogram	%Carbetocin-SH as per chromatogram	% of Carbetoc in SH as per total area	% of carbetocin as per total area
0	21909593	31428083	33619042	2.41%	34.65%	93.48 %	6.52%
1	25237180	27139249	52376429	24.12%	25.94%	51.82 %	48.18%
2	29493508	6960485	36453993	37.31%	8.80%	19.09 %	80.91%
3	30185821	3604024	33789845	40.87%	4.87%	10.67 %	89.33%
4	30191013	3317127	33508140	40.26%	4.42%	9.90%	90.10%

After completion of reaction the ammonia was evaporated by rising the temperature of the water bath to 60^0C and resulting solution containing dimethyl formamide was evaporated under vacuum and precipitate with ether to obtain powder. The Crude Carbetocin powder was analyzed as per the method prescribed, result are tabulated as follows

Table 4: Showing analytical result of crude Carbetocin powder

Weight of Crude Carbetocin	Purity of Product	peptide Content
3.9gm	60.04%	47.03%

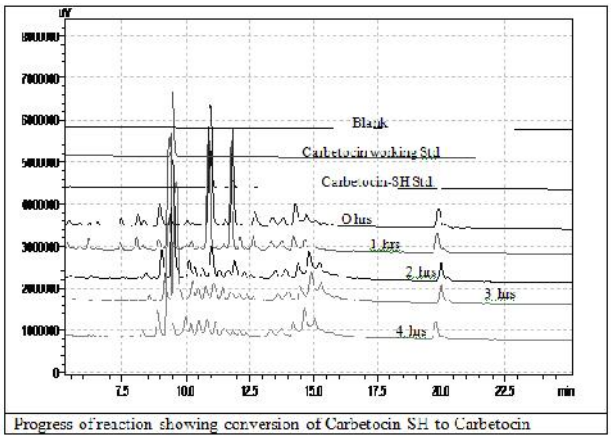

Fig 1: Overlapping of Chromatograms of the reactions monitoring

Observation:

1. Reaction has taken 3 hours to complete, but continued till 4th hour to see the progress of the reaction but no changed observed.
2. Starting area of Carbetocin-SH was 31428083 and after completion of reaction the area of Carbetocin was 30191013 that means 95.8 % conversion was there.
3. Purity at start was 34.65% and after end of reaction it was 40.26% which means 16% increase in purity observed
4. The purity of Carbetocin after precipitation was 60.04% and peptide content 47.03%.

Experiment No.2: Cyclisation of Carbetocin-SH with Carbetocin-SH powder addition over the Ammonia solution in Dimethyl formamide.

500 ml Dimethyl formamide was taken in Round bottom flask with two neck opening, chilled to -45°C using dry ice acetone bath. After attaining the temperature, 1 lit Ammonia solution collected using condensed ammonia gas through the cold trap using dry ice acetone ice to obtain concentration of 0.2L ammonia /0.1L of Dimethyl formamide over the chilled Dimethyl formamide. Once the solution of ammonia and Dimethyl formamide was ready, 5.0 g of Carbetocin -SH provided by Hemmo Pharmaceuticals Pvt. Ltd. was added slowly with constant stirring using magnetic stirrer. Carbetocin –SH was allowed to dissolution to get clear solution. After complete dissolution of Carbetocin -SH, dry ice acetone bath was replaced with hot water bath of 40°C.Sample was taken as a 0 hours and given for analysis as per the method mentioned in methods and analysis for monitoring the oxidation reaction. The reaction was monitored and continued till the percentage amount of Carbetocin-SH reduced to below 5.0 %. As per analytical results reaction progress was observed follows:

Table 5: Analytical results showing reaction progress at respective hours and the impurity profile

Time of reaction monitoring	Carbetocin	Carbetocin-SH	Total area of carbetocin +carbeto	%Carbetocin as per chromatogram	%Carbetocin-SH as per chromatogram	% of Carbetocin SH as per total	% of carbetocin as per total area
0	25936261	22870636	48806897	26.53%	23.39%	46.86%	53.14%
1	28485666	7216977	35702643	35.30%	8.94%	20.21%	79.79%
2	28496269	4484262	32980531	37.23%	5.86%	13.60%	86.40%
3	28528434	3777721	32306155	38.12%	5.04%	11.69%	88.31%
4	28476231	3694249	32170480	37.40%	4.80%	11.48%	88.52%

After completion of reaction the ammonia was evaporated by raising the temperature of the water bath to 60^0C and resulting solution containing dimethyl formamide was evaporated under vacuum and precipitate with ether to obtain powder. The Crude Carbetocin powder was analyzed as per the method prescribed, result are tabulated as follows

Table 6: Showing analytical result of crude Carbetocin powder

wt. of Crude Carbetocin	Purity of Product	peptide Content
4.2gm	53.8%	40.17%

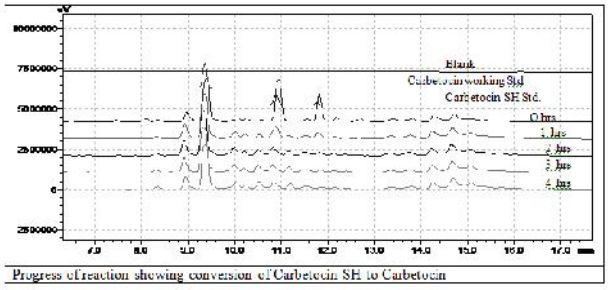

Fig 2: Overlapping of Chromatograms of the reactions monitoring

Observation:-

1 Reaction has taken 4 hours to complete.

2 Starting area of Carbetocin-SH was 22870636 and after completion of reaction the area of Carbetocin was 28476231 that means 125 % conversion was there.

3 Purity at start and end was maintained

4 Purity of Carbetocin after precipitation was 53.8% and peptide content was 40.17%.

Experiment No.3: Cyclisation of Carbetocin-SH with addition of solution of carbetocin-SH in DMF over the prechilled Ammonia solution.

500 ml Dimethyl formamide was taken in Round bottom flask with two neck opening, 5.0 g of Carbetocin -SH provided by Hemmo Pharmaceuticals Pvt. Ltd was weighed and added slowly in Round bottom flask containing Dimethyl formamide. Stirring started with the help of magnetic stirrer. Carbetocin -SH was allowed to dissolve to get clear solution. After complete dissolution of Carbetocin -SH temperature was adjusted to -25±5°C by using dry ice acetone bath. At same time collection of Ammonia solution was done separately in prechilled round bottom flask at -45°C by dry ice acetone bath equipped with cold trap for ammonia gas chilled at -45°C chilled by dry ice acetone. After collection of 1 lit ammonia solution, the solution of Carbetocin-SH/Dimethyl formamide was added onto the ammonia solution containing Round bottom flask under

constant stirring. Once the addition complete, dry ice acetone bath was removed and was replaced with hot water bath of 40°C. Sample was taken at 0 hours and given for analysis. The reaction was monitored and continued till the percentage amount of Carbetocin-SH reduced to below 5.0 %. As per analytical results reaction progress was observed follows:

Table 7: Analytical results showing reaction progress at respective hours and the impurity profile

Time of reaction monitoring	Carbetocin	Carbetocin-SH	Total area of carbetocin +carbetocin SH	%Carbetocin as per chromatogram	%Carbetocin-SH as per chromatogram	% of Carbetocin SH as per total area	% of carbetocin as per total area
1	7791290	30216306	38007596	26.53%	23.39%	79.50%	20.50%
2	29041920	18506867	47548787	35.30%	8.94%	38.92%	61.08%
3	31026559	5257820	36284379	37.23%	5.86%	14.49%	85.51%
4	31042867	2850215	33893082	38.12%	5.04%	8.41%	91.59%
5	31030696	2561127	33591823	43.39%	3.50%	7.62%	92.38%

After completion of reaction the ammonia was evaporated by raising the temperature of the water bath to 60^0C and resulting solution containing dimethyl formamide was evaporated under vacuum and precipitate with ether to obtain powder. The Crude Carbetocin powder was analyzed as per the method prescribed, result are tabulated as follows

Table 8: Showing analytical result of crude Carbetocin powder

wt. of Crude Carbetocin	Purity of Product	peptide Content
4.3gm	67.32%	52.75%

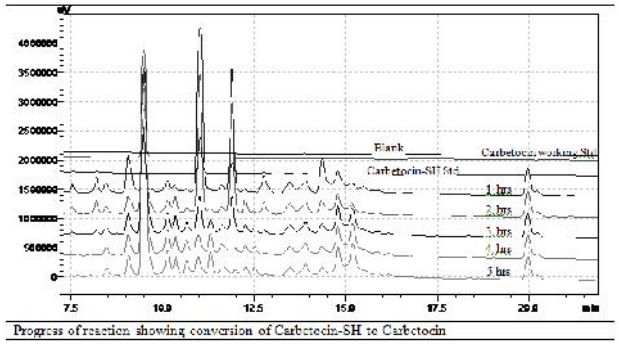

Fig 3: Overlapping of Chromatograms of the reactions monitoring

Observation:

1. Reaction has taken 5 hours to complete.
2. Starting area of Carbetocin -SH was 30216306 and after completion of reaction the area of Carbetocin was 31042867 that mean the conversion was more than 102.6%.
3. The purity of Carbetocin after precipitation was 67.32% and peptide content was 52.75%.

3. RESULT AND DISCUSSION

The results of the above experiments are tabulated in following Table-9:

Table 9: Crude Carbetocin recovery

Expt. No.	Crude Carbetocin obtained in gm	Purity	Peptide Content	Pure Carbetocin as per Peptide Content (gm)
1	3.9	60.04%	47.03%	1.83
2	4.2	53.80%	40.17%	1.69
3	4.3	67.32%	52.75%	2.27

In terms of purity: The cyclized Carbetocin obtained by alkaline oxidation and at three different experiments showed comparative purity of crude carbetocin. Crude carbetocin from Experiment no 3 is having highest purity of 67.32%, while the least is from experiment no 2 that is 53.80%. Crude carbetocin obtained from experiment no 1 shows 60.04% purity.

In terms of Time: Time required for cyclisation of Carbetocin-SH during three experiments was 3hrs, 4hrs and 5 hrs respectively. Even though the time taken by experiment no 3 was more than the successor but the product obtained from the process was of far better quality than that of first two experiments.

Yield in terms of peptide content: Yield of experiments determined in terms of peptide content of the crude carbetocin powder. Peptide content of crude carbetocin of experiment no 3 was 52.75% that means 4.3 gm crude carbetocin powder contains 2.27 gm of pure carbetocin. Similarly amount of pure carbetocin in crude carbetocin from first two experiments is 1.83 and 1.69 gm with peptide content of 47.03% and 40.17% respectively.

4. CONCLUSION

From above observation it is clear for cyclisation of carbetocin like peptides not only ammonia is the key reagent but the pattern of addition of Carbetocin-SH precursor dissolved in dimethyl formamide is also important for formation of cyclized entity. Among the experiments done the pattern of dissolution of carbetocin-SH in DMF with simultaneous collection of ammonia, followed by addition of carbetocin-SH in DMF solution over ammonia proven to be better route for formation of Carbetocin.

5. ACKNOWLEDGEMENT

We are thankful to Hemmo Pharmaceuticals Pvt. Ltd. for the support provided for research.

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Conflict of Interest: None

Source of Funding: Nil