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Effect of solvents on formation of disulphide bond in peptides: A comparative study to produce the peptide using solvents used for purification and therefore to reduce the introduction of chemicals or metal ions in the manufacturing process.

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Abstract: *A novel approach to the formation of disulphide bonds in peptides is developed by doing a series of experiments to evaluate effect of solvents on the disulphide bond formation in peptides in terms of time required, recovery, ease of processing during reaction working up and use of solvents. For evaluating the effect, experiments were setup using Water:Acetonitrile, Water:Methanol and Water:Ethanol for disulphide bond formation of Desmopressin and the results were compared with the aerial oxidation conducted in water. For each solvent, a set of oxidation reactions was studied at 0.5 mg/ml, 1 mg/ml and 5 mg/ml concentration. The acetonitrile water combination at concentration of either 0.5mg/ml or 1 mg/ml proved to deliver the best result in terms of purity, yield and time required to complete reaction.*

Keywords: *Acetonitrile, Desmopressin Bis-SH, Ethanol, Methanol Peptide*

I. Introduction:

Disulfide bridges play a crucial role in the folding and structural stabilization of many important extracellular peptides and proteins. In addition, the artificial introduction of extra disulfide bond into peptides or proteins allows the creation of conformational constraints that can improve biological activity or thermo stability. Due to that, disulfide bond containing peptides or proteins are very challenging to manufacture. Now a-days biologically active molecules that are natural in sources or mimic of natural source are playing an important role in curing/treating life threatening conditions. Scientists are working on peptide based formulations which can lead to less aggressive treatment for those conditions, and as peptides are the naturally occurring chemical entities in the body and therefore it is easier to control the diseases without too many side effects.

Methods of disulphide bridge formation (1, 3 to12)

Lot of efforts have been put towards developing cyclisation strategies of Cysteine containing peptide. A review of summarized work can be summarized as follows: **(1, 3 to12)**

1. **Cyclisation of SH containing precursor by Aerial oxidation**: In this method during the synthesis protecting groups are removed by side chain deprotection reagent and the resulting Bis-SH peptide is subjected to aerial oxidation in presence of molecular oxygen or oxygen bubbling. In this method the peptide is oxidized typically at low concentration (0.1 mg/ml to 1 mg/ml).
   That method is easy and straightforward to apply and also very mild for other amino acids in the molecule (i.e fewer chances of side reaction happening) but being in diluted state it introduces for use of additional concentration step such as reverse osmosis or adsorption on synthetic resins. Sometimes reactions take days to complete.
2. **Symmetrical Cyclisation from S-protected precursor:**
   Cyclisation of cysteine protected by Acm-group (i.e acetamidomethyl $–CH_2$-NH-CO-CH_3) is done by using iodine, thallium (III) Trifluoroacetate and a variety of alkyl trichlorosilanesulphoxide combinations. This approach is used for intramolecular or intermolecular disulfide bond formation and it has the drawback that side reaction are frequent specially on residues susceptible of entering redox reactions (e.g. Tryptophan).

Following are some protocols for oxidation of peptide containing cysteine:

1. Air oxidation
2. Oxidation by I_2, Tl (III) Trifluoroacetate
3. Oxidation by potassium ferricyanide
4. Oxidation mediated by Charcoal
5. DMSO oxidation both in acidic and basic condition.

Every protocol has its advantages and disadvantages. Air oxidation is very clean and doesn't contain any harsh chemical apart from the buffer, however some reaction take long time to complete (e.g. 40-72 days) even at highly alkaline pH which might hydrolyse the peptide. In other protocols metal ions and high boiling solvents are used as oxidizing agent are not easy to remove. So overall the cyclisation reaction of Cysteine containing peptide is a key step in the manufacturing process, the quality and the quantity of peptide is dependent on the outcome of this reaction. On industrial scale if we cannot get good yields with best quality then the resulting product becomes difficult to process therefore in cyclic peptides oxidation is the key reaction and can affect the quality and quantity of the product obtained.

II. Materials and Methods:

Cyclisation of desmopressin Bis-SH was studied at 3 different concentration i.e 0.5mg/ml, 1mg/ml, 5 mg/ml using Water, 50% ACN+50%WATER or 50% MEOH+50%WATER or 50% ETOH+50%WATER as follows:

Concentration: 0.5 mg/ml,

50 mg Desmopressin Bis-SH were weighed and dissolved separately in 100 ml of water, 50% ACN+WATER, 50% MEOH+WATER or 50% ETOH+WATER to perform reaction at 0.5 mg/ml concentration.

1. pH of the reaction was checked at the start and samples injected on HPLC as Before pH.
2. pH of the reaction was adjusted to 9.5±0.5 using ammonia solution.
3. Progress of reaction was checked by HPLC and pH was checked at regular interval.
4. After achieving a consume of the % of Desmopressin Bis-SH higher than 98.5% the reaction was stopped by addition of acetic acid.

Concentration: 1 mg/ml,

100 mg Desmopressin Bis-SH weighed and dissolved separately in 100 ml of water, 50% ACN+WATER, 50% MEOH+WATER or 50% ETOH+WATER to perform oxidation at 1 mg/ml concentration.

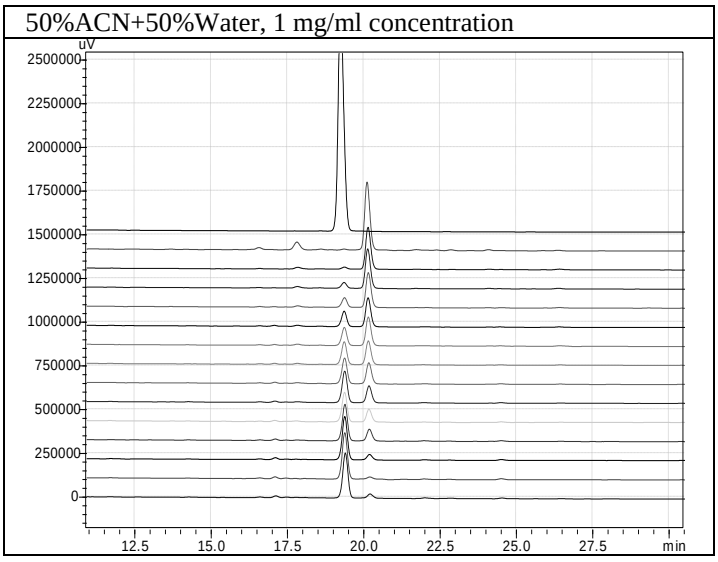
1. pH of the reaction was checked at the start and samples injected on HPLC as Before pH.
2. pH of the reaction was adjusted to 9.5±0.5 using ammonia solution.
3. Progress of reaction was checked by HPLC and pH was checked at regular interval.
4. After achieving a consume of the % of Desmopressin Bis-SH higher than 98.5% the reaction was stopped by addition of acetic acid.

Concentration: 5 mg/ml,

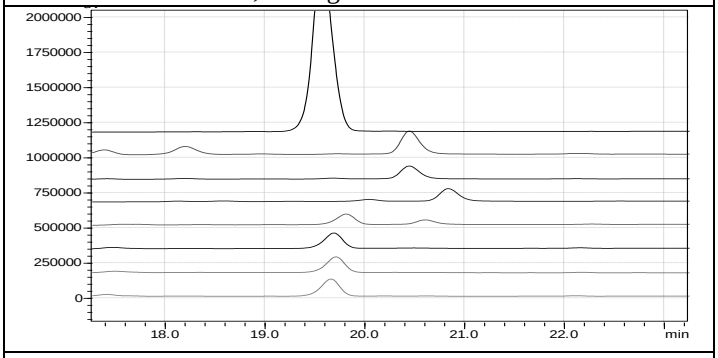
500 mg Desmopressin Bis-SH weighed and dissolved separately in 100 ml of water, 50% ACN+WATER, 50% MEOH+WATER or 50% ETOH+WATER to perform reaction at 5 mg/ml concentration.

1. pH of the reaction was checked at the start and samples injected on HPLC as Before pH.
2. pH of the reaction was adjusted to 9.5±0.5 using ammonia solution.
3. Progress of reaction was checked by HPLC and pH was checked at regular interval.
4. After achieving a consume of the % of Desmopressin Bis-SH higher than 98.5% the reaction was stopped by addition of acetic acid.

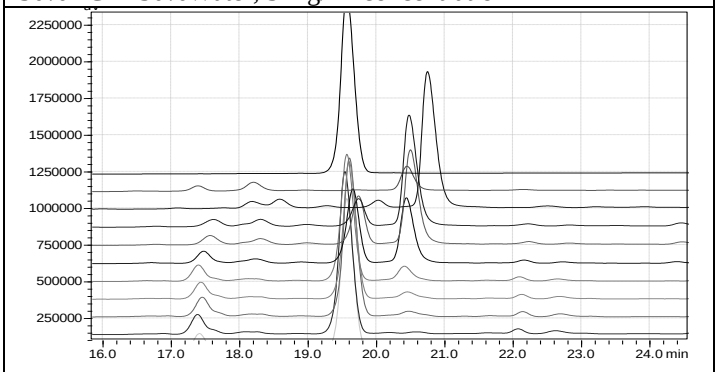
Progress of reactions as follows:



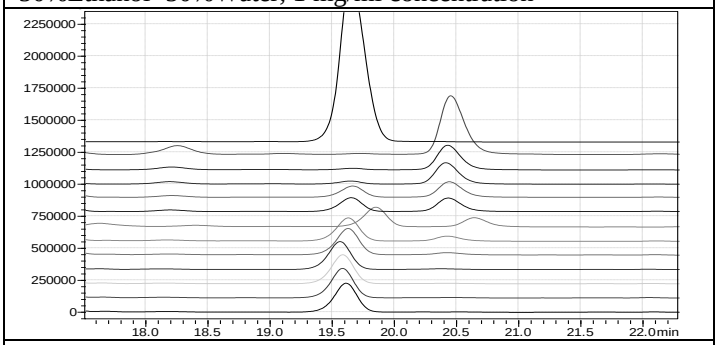
50%ACN+50%Water, 0.5 mg/ml concentration



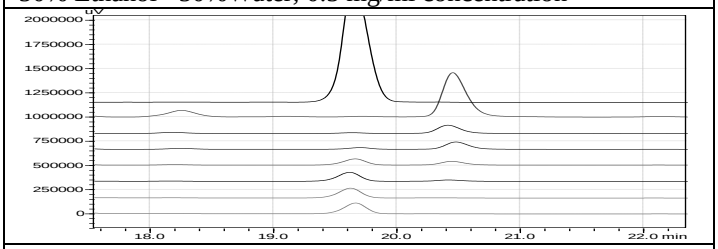
50%ACN+50%Water, 5 mg/ml concentration



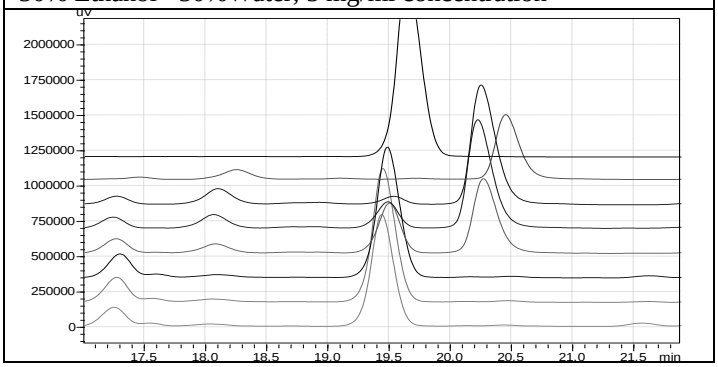
50%Ethanol+50%Water, 1 mg/ml concentration



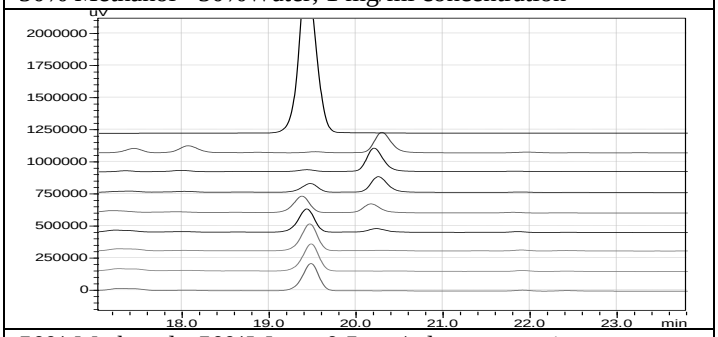
50% Ethanol +50%Water, 0.5 mg/ml concentration



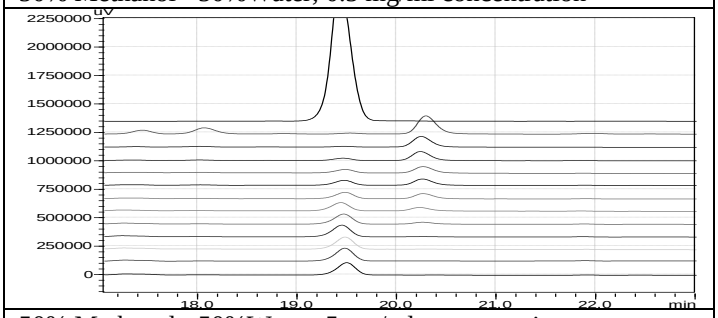
50% Ethanol +50%Water, 5 mg/ml concentration



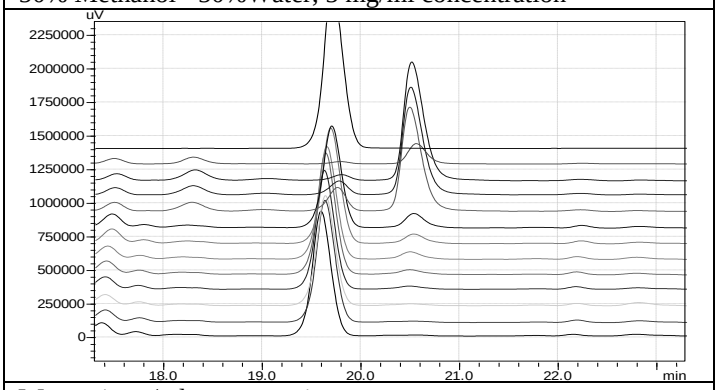
50% Methanol +50%Water, 1 mg/ml concentration



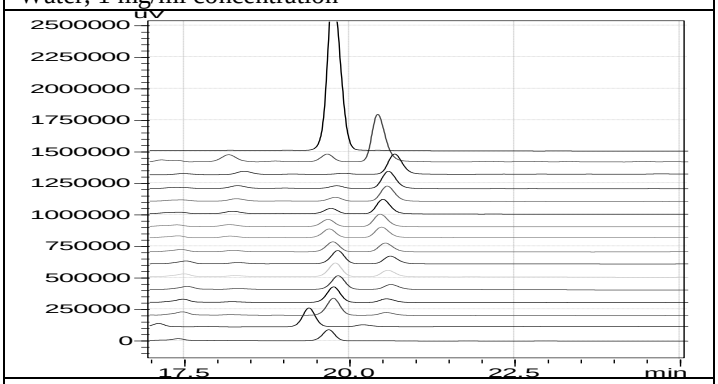
50% Methanol +50%Water, 0.5 mg/ml concentration



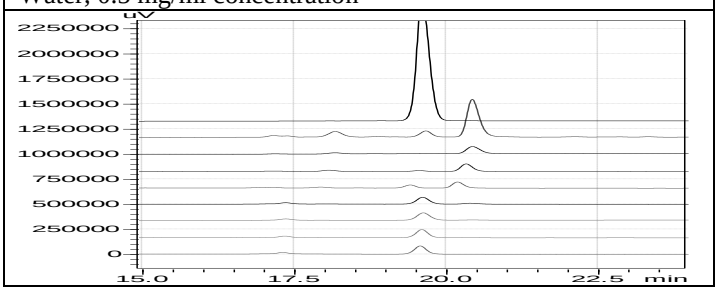
50% Methanol +50%Water, 5 mg/ml concentration

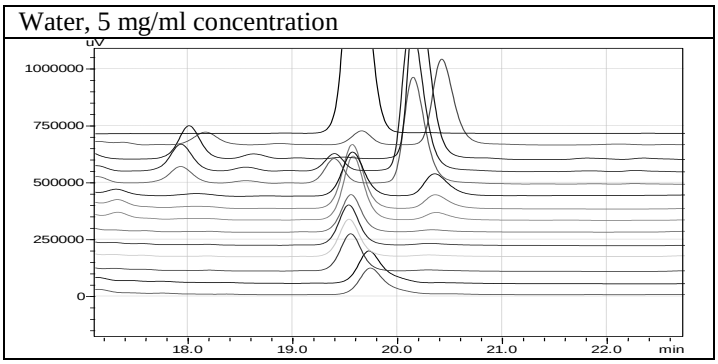


Water, 1 mg/ml concentration



Water, 0.5 mg/ml concentration





The overlapped chromatograms above are showing the progress of the oxidation in each reaction condition. The overlap is followed in the sequence from top to bottom as desmopressin std, desmopressin Bis-SH standard, reaction at 0 hours to completion of reaction. The first graph in each is overlap is of desmopressin std, second is of desmopressin bis-SH std, third is of 0 hours and then subsequent hours, the last graph is after acetic acid addition.

Details of Analytical Method used for Monitoring the Reaction:

1. Buffer A: Dissolve 9.08 gm of Potasium dihydrogen phosphate in 1 L water and add 5 ml of Trifluoroacetic acid and 5 ml Triethyl amine mix well and adjust pH to 5.01 with 10N NaOH.
2. Buffer B: 500 ml Buffer A+425 ml Acetonitrile+75ml Tetrahydrofuran.
3. Preparation of Desmopressin Standard Solution: Desmopressin Working Standard Vial dissolved in 25 ml of Milli-Q water.
4. Preparation of Desmopressin Bis-SH Standard Solution: 12.5 mg of desmopressin Bis-SH were dissolved in 25 ml of water.
5. Resolution Solution: Oxytocin Desmopressin validation mixture dissolved in water to obtain a concentration of 0.25mg/ml
6. Test Solution: 1 ml of sample diluted to 10 ml Milli-Q water.

Chromatographic condition:

Column	C18, 250X4.6mm, 5 µ
Flow rate	1.5ml/min
Injection volume	50 µL for Blank, Std solution (Desmopressin and Desmopressin Bis-SH)
	50 µL for Test solution
Run time	50 min

System Suitability: Details of Analysis

Sample Solution	**Retention Time of Principal Peak**
Desmopressin Std Solution	Around 19 min
Desmopressin Bis-SH Std Solution	Around 20 min
Resolution between main peak due to Desmopressin and main peak due to Oxytocin	NLT 1.5

III. Result and Discussion:

Cyclisation reaction in water:

In terms of purity: The cyclized desmopressin obtained by aerial oxidation at three different concentration of 0.5mg/ml, 1 mg/ml, 5 mg/ml showed purities of 62.71%, 56.06% and 61.51% respectively which is lower as compared to other experiments done with other solvents.

In terms of Time: The cyclisation reaction of desmopressin by aerial oxidation, at three different concentration of 0.5mg/ml, 1 mg/ml, 5 mg/ml took 17 hours, 33 hours and 35 hours respectively. The time at 1 mg/ml and 5 mg/ml is the longest time taken by reaction for completion as compared to other reaction conditions.

In terms of Yield: Yield of the cyclisation reaction of desmopressin by aerial oxidation, at three different concentrations of 0.5mg/ml, 1 mg/ml, 5 mg/ml was 107.68%, 113.30% and 18.42% respectively.

From the above observation it is clear that even though water is the cleanest way to do the cyclisation of peptides, due to solubility issues of peptides in water there are chance of low reaction yield. As the reaction takes longer time for completion therefore there are more chances of disulphide scrambling and thus more unwanted dimers or polymers can form during cyclisation reaction, which leads to low yields and poor purity levels, purity. Therefore it is clear that the water alone is not a suitable medium for cyclisation reaction of peptides.

Cyclisation reaction in Water: Acetonitrile:

In terms of purity: The cyclized desmopressin obtained by Water:Acetonitrile oxidation and at three different concentrations of 0.5mg/ml, 1 mg/ml and 5 mg/ml showed purity by HPLC of 76.61%, 74.76% and 72.92% respectively which is comparably higher when as compared to other experiments done with other solvents.

In terms of Time: The cyclisation reaction of desmopressin by Water: Acetonitrile oxidation, at three different concentrations (0.5mg/ml, 1 mg/ml, 5 mg/ml) took 8 hours, 11 hours and 18 hours respectively. Even though the reaction took comparably similar time with respect to reaction in Water: Methanol, Water: Ethanol in terms of purity and yield it was higher in acetonitrile: water mixtures than in any of the other experimental conditions.

In terms of Yield: Yield of the cyclisation reaction of desmopressin by Water: Acetonitrile oxidation, at three different concentrations (0.5mg/ml, 1 mg/ml, 5 mg/ml) was 133.81%, 106.07% and 133.81% respectively.

Considering overall Yield, time taken for completion of reaction and purity then the Water:Acetonitrile reaction condition is comparatively cleaner and therefore preferred over Water:Methanol and Water:Ethanol solvent conditions

Cyclisation reaction in Water: Methanol:

In terms of purity: The cyclized desmopressin obtained by Water:Methanol oxidation and at three different concentrations (0.5mg/ml, 1 mg/ml, 5 mg/ml) was having purity of 76.82%, 77.68% and 76.25% respectively which is comparably high at all three concentration conditions when compared with results for other solvents

In terms of Time: The cyclisation reaction of desmopressin by Water: Methanol oxidation, at three different concentrations (0.5mg/ml, 1 mg/ml, 5 mg/ml) took 18 hours, 10 hours and 22 hours respectively. Even though the purity of desmopressin using this condition is better than the previous, The time required for reaction is higher as compared to other conditions and that can lead to lower yield.

In terms of Yield: Yield of the cyclisation reaction of desmopressin by Water: Methanol oxidation, at three different concentrations (0.5mg/ml, 1 mg/ml, 5 mg/ml) was 136.89%, 114.74% and 102.29% respectively.

Even though Reaction at 0.5mg/ml was having comparable yield and purity with cyclisation reaction using Water: Acetonitrile at 0.5 mg/ml and 1mg/ml, Still time taken to complete the reaction in Water: Methanol media at 0.5mg/ml was 18 hours which longer than the Water: Acetonitrile (8 hours). Reaction at 1 mg/ml took only 10 hours to complete. The yield was only 114.74% which is 18.07 % lower than Water: Acetonitrile reaction at 0.5mg/ml. Reaction at 5 mg/ml took 22 hours to complete and had lower yields due to longer reaction times.

Cyclisation reaction in Water: Ethanol:

In terms of purity: The cyclized desmopressin obtained by Water:Ethanol aerial oxidation and at three different concentrations (0.5mg/ml, 1 mg/ml, 5 mg/ml) was having purity of 72.82%, 73.23% and 76.25% respectively which was comparably lower as compared to experiments done in Water:Acetonitrile and Water:Methanol.

In terms of Time: The cyclisation reaction of desmopressin by Water:Ethanol aerial oxidation, at three different concentrations (0.5mg/ml, 1 mg/ml, 5 mg/ml) took 7 hours, 17 hours and 7 hours respectively. The reaction time in Water: Ethanol is comparable to that of Water: Acetonitrile mixtures. Yields are also comparable but purities are slightly lower (76.61% in Water: Acetonitrile and 72.82% in Water: Ethanol).

In terms of Yield: Yield of the cyclisation reaction of desmopressin by Water: Ethanol oxidation, at three different concentrations (0.5mg/ml, 1 mg/ml, 5 mg/ml) was 134.94%, 124.26% and 88.93% respectively.

Conclusion:

The results of the above experiments are tabulated in following table in order of decreasing yield.

Medium of reaction	**concentration of reaction in mg/ml**	**Time required for completion in Hours**	**% yield as compared to area of desmopressin Bis-SH at start and Desmopressin at end of reaction**	**Purity of desmopressin**
50%MEOH +50% Water	0.5	18	136.89%	76.82%
50%ETOH + 50%Water	0.5	7	134.94%	72.82%
50%Acetonitrile+50%Water	0.5	8	133.81%	76.61%
50%Acetonitrile+50%Water	5	18	132.01%	72.92%
50%ETOH + 50%Water	1	17	124.26%	73.23%
50%MEOH +50% Water	1	10	114.74%	77.68%
Water	1	33	113.30%	56.06%
Water	0.5	17	107.68%	62.71%
50%Acetonitrile+50%Water	1	11	106.07%	74.76%
50%MEOH +50% Water	5	22	102.29%	76.25%
50%ETOH + 50%Water	5	7	88.93%	66.25%
Water	5	35	18.42%	61.51%

From above observation it is clear that reaction in acetonitrile and water as a medium is one of the best combination for disulphide bond formation in peptides. Higher yields of the reaction are indicating towards an improved solubility of peptides in the Water: solvent mixtures. The time required for reactions is comparably less as compared to reactions in water. Due to lower reaction times and higher yields the concentration condition of 0.5 mg/ml and 1 mg/ml in Water: Acetonitrile are selected as suitable for cyclisation of desmopressin at industrial scale. Similarly this can be applied to other peptides to improve the yields and lowering the time of processing by using organic solvents for oxidation compatible with those used during the purification step.

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