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Synthesis, Characterisation and its Anti Bacterial Activity of Dalfampridine Genotoxic Impurity 4-Amino Pyridine N-Oxide

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Abstract: ***Objective: 4-Aminopyridines and its impurities are important intermediates for chemicals, pharmaceuticals and agrochemical industry, antineoplastic, anticoagulant, anti-inflammatory [1]. Dalfampridine used for the treatment of multiple sclerosis so, the drug availability in the finished dosage form is very important. In the present study we evaluated antibacterial activity of synthesized 4- amino pyridine n- oxide by using agar cup method. Methods: The serially diluted antibacterial test solution three doses of synthesized 4- amino pyridine n- oxide (50µg/ml, 75µg/ml and 100µg/ml) were added in the cups and all the plates stood incubated for 24 hours at 37^{0}C. The zone of inhibition was measured and compared with gentamycin as a positive control. Results: The synthesized 4-amino pyridine n- oxide (100µg/ml) was found to be effective and it exhibited substantial antibacterial activity against its direction of Escherichia-coli > Pseudomonas-aeruginosa > Bacillus-subtilis > Staphylococcus-aureus which activity is lesser than standard. Whereas100µg/ml dose showed higher anti bacterial activity with more zone of inhibition than 75µg/ml and 50µg/ml dose. Conclusions: The results proposed 100µg/ml of synthesized 4- amino pyridine n- oxide showed significant antibacterial activity but less activity than standard drug gentamycin.***

Keywords: Dalfampridine, Genotoxic impurities, N – Oxidation, Nitration, Reduction, LC-MS, ^{1}H NMR, Melting point, Anti bacterial activity

1. Introduction

Dalfampridine otherwise called as 4-Aminopyridine, 4-AP, fampridine. Dalfampridine is the first drug approved in the United States by USFDA to improve walking in patients with multiple sclerosis. Fampridine is also marketed as Ampyra in the United States by Acorda Therapeutics and as Fampyra in Europe.

Dalfampridine is a patent compound of 4- amino pyridine n-oxide. Dalfampridine and its impurities used to block K+ channel by non-selectively.

More than 90 % of patients with MS report difficulty in walking [2] Antibacterial drugs interfere chemically with the synthesis of function of vital components of bacteria. These differences provide us with selective toxicity of chemotherapeutic agents against bacteria.

2. Materials and Methods

Culture Media:
Nutrient agar(Hi-Media) and Mueller-Hinton agar(Hi-Media).

Materials

- The suitable mobile phases (solvent system for TLC) as applicable were developed using silica Gel-G ready plates. Pre-coated aluminium silica plates were used with suitable mobile phase.
- Progress of Reaction and monitoring for each stage is performing by LCMS.
- Mass of all synthesized compounds can be determined by using Thermo- DSQ-Trace and Advantage Max, Agilent 6130 LC/MS single quad mass spectrometer.
- Separation and purification of synthesized impurities carried out by using column chromatography and automated chromatography COMBI FLASH Rf device.
- The purity of all synthesized compounds identified by HPLC Agilent-1200 with PDA detector.

Methods

Scheme to synthesis 4- Amino pyridine N- Oxide [3]:
Step 1: Oxidation of Pyridine:

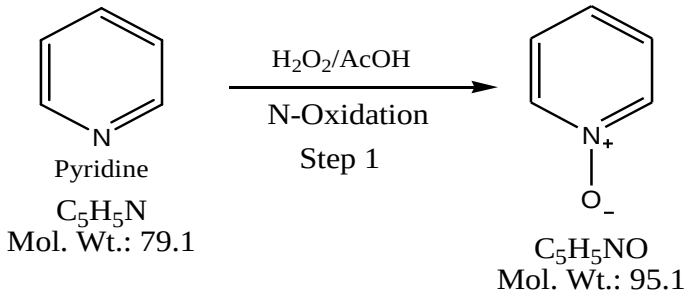


Figure 1: Step 1 Oxidation of Pyridine

Pyridine is oxidized into pyridine N oxide which on treatment with H_2O_2/ AcOH.10 grams of Pyridine was taken in the single neck RB flask, to this 30 ml of acetic acid was added and stirred for ten minutes. 50 ml of hydrogen peroxide was added and closed the flask with stopper. The reaction mixture was stirred over night.

Workup:
After completion of the reaction, removed the excess acetic acid and peroxide by concentraring in the rotavapor and dried completely. Three times added a toluene and removed the solvent in rotavapor to expel the traces of water. Product Yield is 10 gm.

Step 2: Nitration of Pyridine N – Oxide:

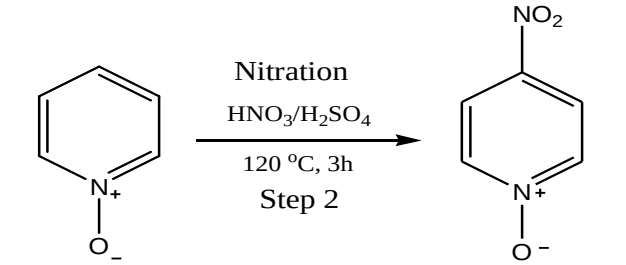


Figure 2: Step 2 Nitration

10gms of pyridine-N-oxide are filled in the 250ml reaction flask and heated to 60°C. The nitrating mixture is added into an addition funnel and added dropwise within 30 minutes without further heating. Thereby the internal temperature drops to about 40°C. Afterwards the reaction mixture is refluxed for 3 hours to 125-130°C internal temperature.

Work up

After cooling the reaction mixture is poured in a 1 Litre beaker containing 150g finely crunched ice. Then about 170 mL of a saturated sodium bicarbonate solution are added carefully until a pH-value of 7 - 8 is reached. Finaly yellow crystalline solid precipitates formed and its consisting a product and sodium sulfate. Crude yield: 5.5 g

Acetone is added to that yellow crude product and the insoluble white salt is separated over a Buechner funnel. The solvent is evaporated by rotary evaporator, the remaining yellow product is dried in a desiccators which is nitrated compound. Product Yield is 3.5 g.

Step 3: Reduction:

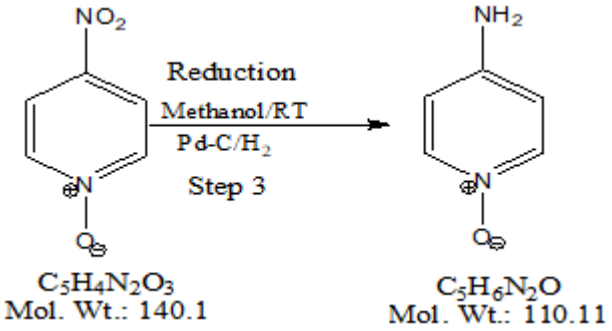


Figure 3: Step 3 Reduction

Nitro group rapidly reduced at room temperature to amino group in methanol in the presence of 10% palladium on carbon. 0.5gm of 4-nitro pyridine -N- oxide was dissolved in 30 ml of methanol .100 mg of 10% palladium added in that reaction mass. After added palladium the reaction mass carried under hydrogen pressure condition for overnight. The reaction is monitored by TLC and LC-MS.

Workup

The reaction mass is filtered off by using high vacuum to remove palladium. The filterate is concentrated to remove methanol by using rotary evaporator.Yield:190 mg

3. Evaluation of Anti Bacterial Activity

For our study, we used the agar cup method, to assess the minimum inhibitory concentration (MIC) [4].

1) Necessary controls were used. Like,
   - Drug (Standard) control
   - Organism control
   - Gentamycin (Known antibacterial agent) as reference was used in the present study.
   - Muller Hinton Agar (MHA) (Hi Media) was used as nutrient medium for growth of microorganisms.
   - The cultures used for testing were obtained from NCL which were equivalent to ATCC cultures.
   - All microorganisms were inoculated in Tryptic Soya Bean Broth (Hi Media).
   - All the drugs were dissolved in sterile Methanol and DMSO (AR Grade).
   - The dissolved drugs were serially diluted according to NCCLS guidelines.
2) **Microbial strain**:

Name of Microorganism Used	Type	Strain Used
A. Staphylococcus-aureus.	(Gram Positive)	ATCC 25923
B. Bacillus-subtilis	(Gram Positive)	ATCC 21332
C. Pseudomonas-aeruginosa.	(Gram Negative)	ATCC 27853
D. Escherichia-coli.	(Gram Negative)	ATCC 25922

3) Sterile molten MHA was poured aseptically under laminar air flow unit into sterile Petri plates containing the test microorganism and was allowed to solidify. After solidification of the media cups/wells were bored using 'T' borer [5].
4) The serially diluted antibacterial test solution (synthesized compounds) concentrations are 50µg/ml, 75µg/ml and 100 µg/ml were added in the cups and allowed to diffuse in the agar by placing the Petri plates under refrigeration for 10 minutes.
5) At 37^0C all the plates stood incubated for 24 hours.
6) The inhibition zone of the test antibacterial solution and the control drug was measured using Vernier callipers and the MIC was determined.

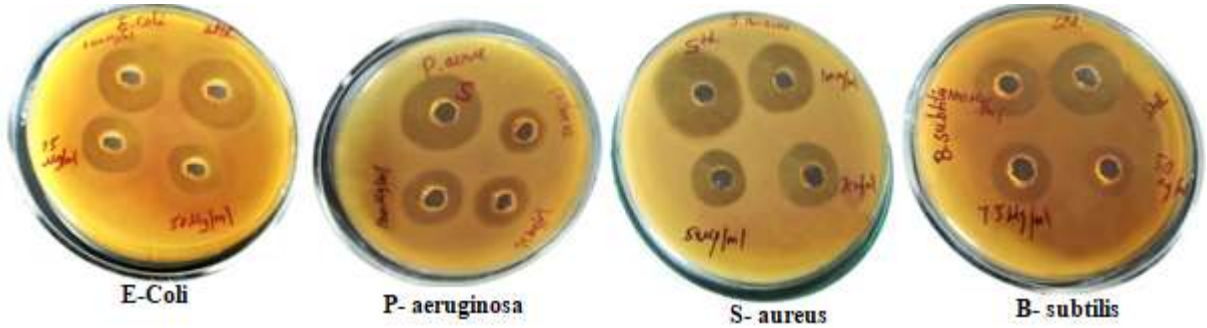


Figure 4: Photographs showing zone of inhibition of synthesized 4- Amino pyridine N- Oxide

4. Results

Characterisation results:

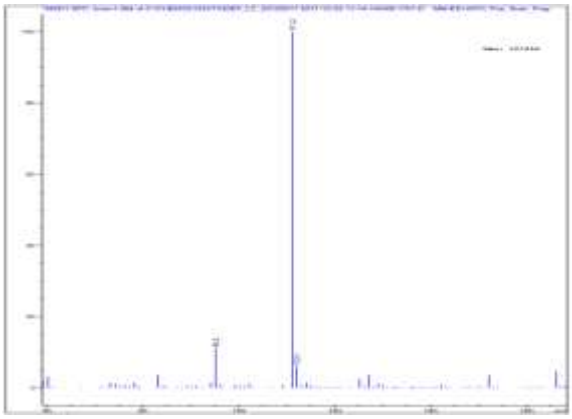
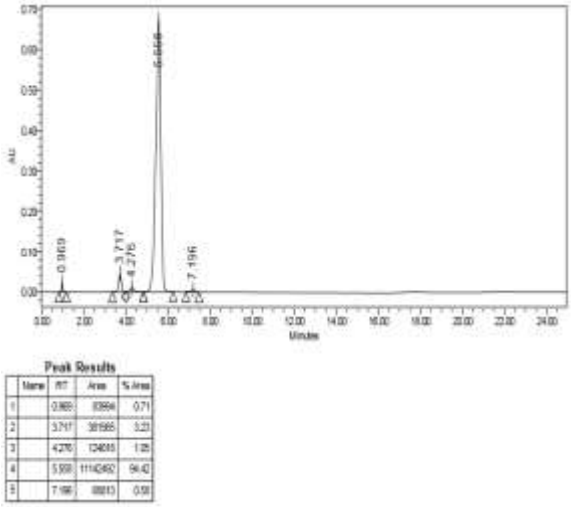


Figure 5: LC-MS Spectrum for 4- Amino pyridine N- Oxide



Peak Results

	Name	RT	Area	% Area
1		0.969	83866	0.71
2		3.717	381995	3.23
3		4.276	124015	1.05
4		5.938	11142462	94.42
5		7.196	68813	0.58

Figure 6: HPLC Spectrum For4- Amino pyridine N- Oxide

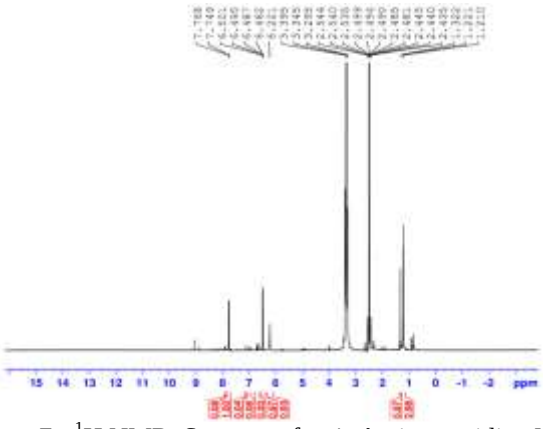


Figure 7: ^{1}H NMR Spectrum for 4- Amino pyridine N- Oxide

Antibacterial Activity Results of 4- Amino Pyridine N- Oxide

Table 1: Zone of Inhibition of 4- Amino pyridine N- Oxide

S. No.	Concentration (µg/ml)	Bacterial Culture Zone of Inhibition in mm			
		E.coli	*P.aeruginosa*	*S. aureus*	*B.subtilis*
1	Std (Gentamicin)	25	25	26	26
2	50	10	08	08	09
3	75	13	13	12	12
4	100	15	15	13	14

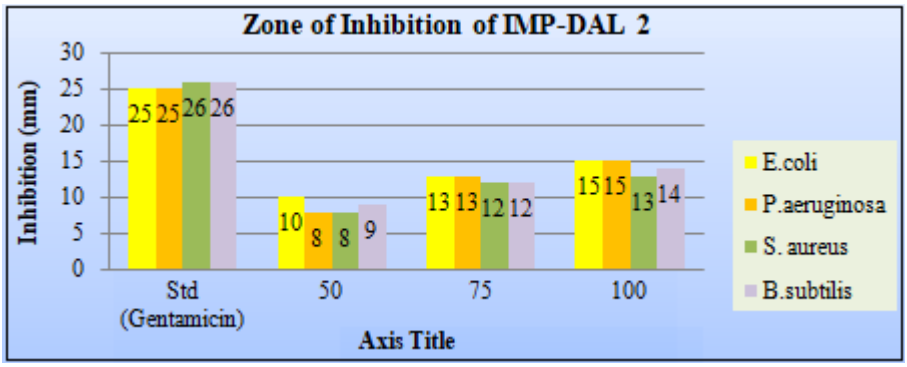


Figure 8: Zone of Inhibition of 4- Amino pyridine N- Oxide.

5. Conculsions

The targeted molecule synthesized with good yield and high purity. To achieve high purity attention was drawn forwards automated combi flash and it was performed by developing different analytical methods for separation of synthesized genotoxic impurity. 4- Amino pyridine N- Oxide assessed for its in-vitro anti-bacterial activity compared to both gram-positive and gram-negative bacteria using the standard drug Gentamycin. We are concluded as molecule exhibits substantial antibacterial activity against its direction *Escherichia-coli* > *Pseudomonas-aeruginosa* > *Bacillus-subtilis* > *Staphylococcus-aureus*. 100 µg/ml of synthesized molecules showed more activity compared to other concentrations.

6. Acknowledgement

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