THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN 2319-2119

RESEARCH ARTICLE

CH.RAMAMOHANA RAO et al, The *Experiment, November, .2012 Vol. 4(3), 243-252*

DEVELOPMENT OF NEW HPLC METHOD AND IDENTIFICATION AND QUANTIFICATION OF NALIDIXIC ACID, CIPROFLOXACIN IN FISH TISSUE.

1. INTRODUCTION

Nalidixic acid is the first of the synthetic quinolone antibiotics. In the technical sense, it is a naphthyridone, not a quinolone: its ring structure is a 1,8-naphthyridines nucleus that contains two nitrogen atoms, unlike quinoline, which has a single nitrogen atom.[1]Synthetic quinolone antibiotics were discovered by George Lesher and coworkers as a byproduct of chloroquine manufacture in the 1960s.[1] Nalidixic acid is effective against both gram-positive and gram-negative bacteria. In lower concentrations, it acts in a bacteriostatic manner; that is, it inhibits growth and reproduction. In higher concentrations, it is bactericidal, meaning that it kills bacteria instead of merely inhibiting their growth. It is especially used in treating urinary tract infections, caused, for example, by Escherichia coli, Proteus, Shigella, Enterobacter, and Klebsiella. It is also a tool in studies as a regulation of bacterial division. It selectively and reversibly blocks DNA replication in susceptible bacteria. Nalidixic acid and related antibiotics inhibit a subunit of DNA gyrase and induce formation of relaxation complex analogue. It also inhibits the nicking dosing activity on the subunit of DNA gyrase that releases the positive binding stress on the supercoiled DNA. It is the only FDA approved quinolone for treating UTI infections in children.[2] Aeromonas hydrophila, Clostridium and Haemophilus influenza species are generally susceptible to Nalidixic acid, while other species such as Bifidobacteria, Lactobacillus, Pseudomonas and Staphylococcus are resistant to Nalidixic acid. [4]

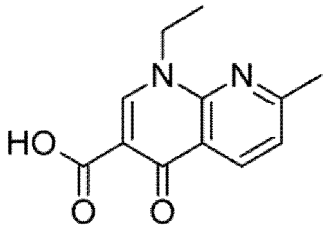


Figure.4.A Structure of Nalidixic acid

Ciprofloxacin is a synthetic antibiotic of the fluoroquinolone drug class[(5)(6)] It is a second-generation fluoroquinolone antibacterial. It kills bacteria by interfering with the enzymes that cause DNA to rewind after being copied, which stops synthesis of DNA and of protein. Ciprofloxacin was first patented in 1983 by Bayer A.G. and subsequently approved by the U.S. Food and Drug Administration (FDA) in 1987. Ciprofloxacin has 12 FDA-approved human uses and other veterinary uses, but it is often used for unapproved uses (off-label).. Ciprofloxacin is 1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid[(8)]. Its empirical formula is $C_{17}H_{18}FN_3O_3$ and its molecular weight is 331.4 g/mol. It is a faintly yellowish to light yellow crystalline substance. Ciprofloxacin hydrochloride is the monohydrochloride monohydrate salt of ciprofloxacin. It is a faintly yellowish to light yellow crystalline substance with a molecular weight of 385.8 g/mol. Its empirical formula is $C_{17}H_{18}FN_3O_3HCl{\bullet}H_2O$. Medical uses are [(13)] Chronic bacterial prostatitis (recommended as a first-line antibiotic choice)[(10)(11)], Lower respiratory tract infections,Skin and skin structure infections, Bone and joint infections, Infectious diarrhea[(13)]

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN 2319-2119

RESEARCH ARTICLE

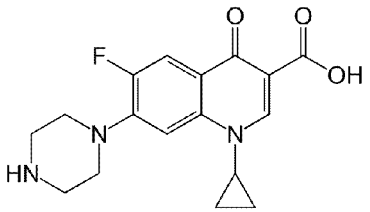


Figure.4.B Structure of Ciprofloxacin

2. Materials and Methods

2.1. Instrumentation

For quantitative estimation of Nalidixic acid, Ciprofloxacin in Fish an isocratic peak hplc instrument with chromosil c18, c8 column (250 mm x 4.6 mm, 5µ), (150 mm x 4.6 mm, 5µ) was used. The instrument is equipped with a LC 20AT pump for solvent delivery and variable wavelength programmable UV-Visible detector, SPD-10AVP. A 20µL Hamilton syringe was used for injecting the samples. Data was analyzed by using PEAK software. techcomp UV 2301UV-Visible spectrophotometer (Hitach software) was used for spectral studies. Degassing of the mobile phase was done by using a Loba ultrasonic bath sonicator. A Denver balance was used for weighing of the materials.

2.2. Chemicals and Solvents

The reference standard sample was obtained from ZEN Pharma, Gujarat. The fish samples were collected from the local markets, Aqua fields, fishing companies. Acetonitrile, Methanol, Water used is HPLC grade are purchased from Merck Specialties Private Limited, Mumbai, India. M Phosphate Buffer, T.E.A of AR grade purchased from local market.

2.3 Sample collection

Fish samples were collected from aqua fields (where antibiotics are using to prevent bacterial infection), i.e Bhimavaram, West Godavari district, Andrapradesh, Pittalavanipalem, Guntur Distric, Andrapradesh, few fish samples from local fish markets in vijayawada and machilipatnam (body weight; 250 grms ±10.5 g). Then the fish were stocked into the 500 L capacity circular plastic tanks

2.4. The Mobile Phase

Three different suitable mobile phases are prepared individually for analysis of target antibiotics in fish. The prepared mobile phases are sonicated up to 30 min, and filtered through 0.45 µ nylon filter paper.

CIPROFLOXACIN: Acetonitrile, 0.25 M H3PO4 (80:20 v/v).

NALIDIXIC ACID: 30.0 ml of acetonitrile with 70.0 ml of 0.5 M Phosphate buffer.

2.5. Standard Solution of the Drug

For analysis of Ciprofloxacin, Nalidixic acid 1000 ppm stock solutions are prepared with reference standards of Ciprofloxacin, Nalidixic acid anti biotic drugs with their mobile phases. From the stock solution calibration curves prepared to estimate target anti biotic drugs.

2.6. Preparation of FISH Samples:

Extraction of antibiotics from fish protein. [14-16]

The methanol extract of fish protein was prepared with optimized method using a method as described by Hellioet al, 50 grms of fish protein was mixed with 50 ml of Methanol and homogenized using apolytron homogenizer. The mixture was then centrifuged at 10,000 rpm for 30 minutes . Supernatant was then collected and filtered with Whatman no.1 filter paper. Then, the supernatant was collected and purified using a syringe with 0.22 µm filter. Elutes were then collected and stored in refrigerator at 4°C. This sample was used for quantitative and qualitative analysis of target antibiotics in fish protein. The samples were then sonicated for 5 minutes and analyzed using HPLC system.

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN 2319-2119

RESEARCH ARTICLE

3. Optimization of HPLC methods from Standard Methods

During HPLC method optimization, a systematic study on effect of various factors was performed by varying one parameter at a time and keeping all other conditions constant. Method development consists of selecting the appropriate wavelength and chromatographic conditions like stationary and mobile phase. The following studies were conducted for this purpose.

3.1. Detection Wavelength: The proper wavelength was needed to determine maximum detector response. The first step was to run a UV-VIS spectrum (from 190-320 nm) using an HPLC system equipped with the Photo Diode Array Detector.

3.2. Choice of Stationary Phase: In general, develop all methods with HPLC columns from the same vendor. The preferred brand of HPLC column should be selected primarily based on the long term stability and lot-to-lot reproducibility. Preliminary development trials have performed with octadecyl columns form different manufacturers with different configurations.

3.3. Selection of the Mobile Phase: Liquid chromatography method development began with the optimizing mobile phase composition and column type. The feasibility of several mixtures of solvent such as acetonitrile, water and methanol using different buffers such as ammonium acetate, ammonium formate, acetic acid and formic acid with variable pH range 3–6 was tested for complete chromatographic resolution.

In order to get sharp peak and base line separation of the components, a number of experiments were carried out by varying the composition of various solvents and its flow rate. Under isocratic conditions, mixtures of solvents like methanol, water and Acetonitrile with and without different buffers indifferent combinations were tested as mobile phase on a C18 stationary phase.

3.4. Flow Rate

Flow rate of the mobile phase was changed from 0.5 – 1.5 mL/min for optimum separation. A minimum flow rate as well as minimum run time gives the maximum saving on the usage of solvents.

3.5 HPLC Conditions Optimization for Analysis of NALIDIXIC ACID [17-19]

For analysis of NALIDIXIC ACID in tissue samples, HPLC with UV-detector set at 266 nm was used, with low sensitivity and specificity. So, HPLC with PDA detector is used to analysis of NALIDIXIC ACID. In this study C18 reversed phase thermo column was employed at Ambient temperature Acetonitrile-0.05KH_2PO_4 Phosphate buffer (30%:70% v/v) pH (5.1) as the mobile phase. The isocratic elution under the condition employed allows the separation of NALIDIXIC ACID. Good separation and peak shape was obtained at flow rate of 1 ml/min.

TABLE. 4.1 Chromatographic conditions of NALIDIXIC ACID

S.NO	Parameter	condition
1	Mobile phase	30.0 ml of acetonitrile with 70.0 ml of 0.05 M KH_2PO_4buffer
2	Column	C18, 150 mm×4.6 mm
3	Wavelength	266 nm
4	Flow rate	1 ml/min
5	Sample volume	20 µL
6	Mobile phase pH	5.1
7	Run time	10 min
8	Column Temperature	Ambient

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN 2319-2119

RESEARCH ARTICLE

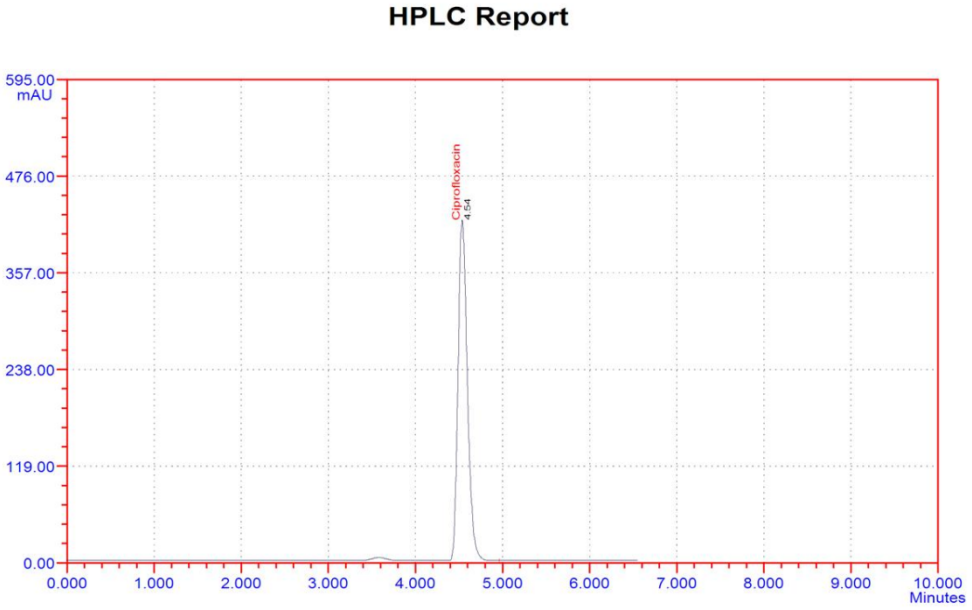
3.6 HPLC Conditions Optimization for Analysis of CIPROFLOXACIN [20-22]

For analysis of CIPROFLOXACIN in tissue samples, HPLC with UV-detector set at 282 nm was used, but has low sensitivity and specificity. So, HPLC with U.V detector is used to analysis of CIPROFLOXACIN. In this study C18 reversed phase thermo column was employed at Ambient temperature, Methanol, 0.25M H3PO4 (80%:20% v/v) P^H (6.8)as the mobile phase. The isocratic elution under the condition employed allows the separation of CIPROFLOXACIN. Good separation and peak shape was obtained at flow rate of 1ml/ min.

TABLE. 4.2 Chromatographic conditions of CIPROFLOXACIN

S.NO	Parameter	Condition
1	Mobile phase	Methanol and 0.25 M H3PO4 (80:20 v/v)
2	Column	C18, 150 mm×4.6 mm
3	Wavelength	282 nm
4	Flow rate	1 ml/min
5	Sample volume	20 µL
6	Mobile phase pH	6.4
7	Run time	10 min
8	Column Temperature	Ambient

Figure: 4.C HPLC CHROMATOGRAM FOR CIPROFLOXACIN

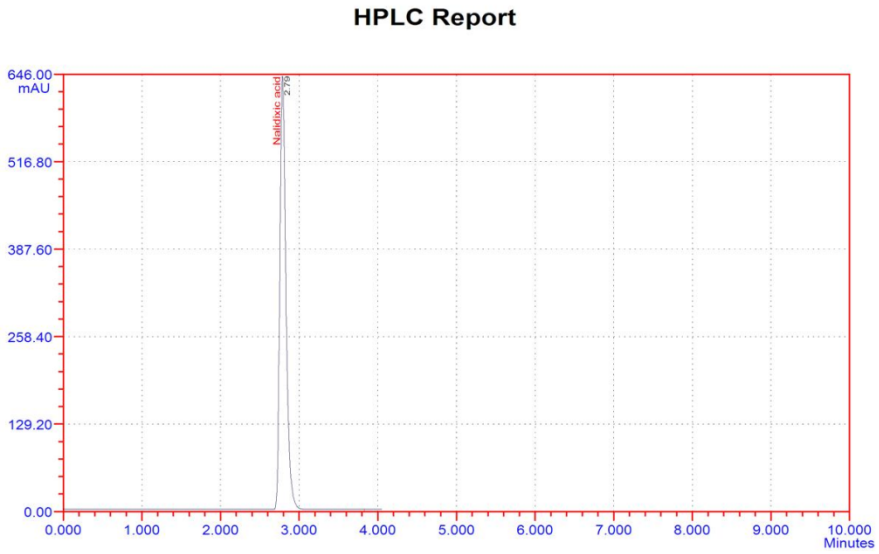


THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN 2319-2119

RESEARCH ARTICLE

Figure: 4.D HPLC CHROMATOGRAM FOR NALIDIXIC ACID



4. RESULTS

1. Calibration curve with standard

from the stock solutions different concentrations Nalidixic acid (0.5-3ppm), Ciprofloxacin of standard drug solutions are injected in to hplc at system suitable condition what are optimized from standard procedures. the calibration curves are plotted between area of peak and drug concentrations.

Table.4.3 Calibration Table for Nalidixic acid

S.NO	Standard concentration (ppm)	Peak Area
1	0.5	1008
2	1	2216
3	1.5	3148
4	2	4325
5	2.5	5364
6	3	6587
7	Slope = 2184.429	Intercept = -41.2143

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN 2319-2119

RESEARCH ARTICLE

Fig 4.E Calibration curve Nalidixic acid

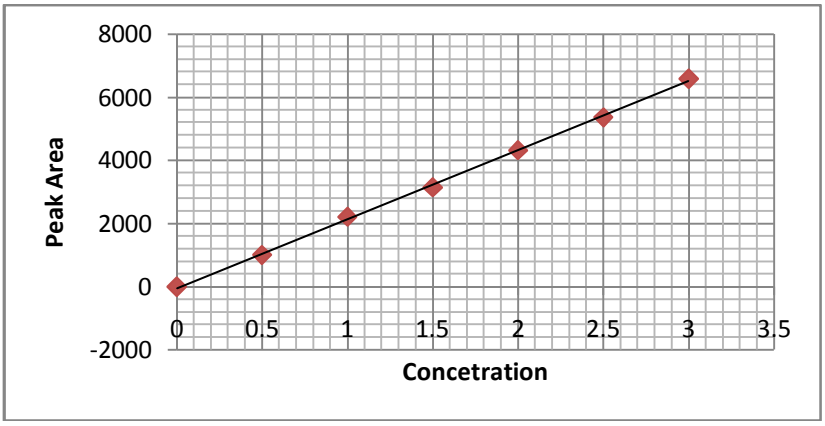


Table 4.4 Recovery studies of NALIDIXIC ACID

S.NO	% OF RECOVERY	Fixed conc in ppm1	Spiked conc in ppm1	Total sample concetration	Amount of recovery	% of recovery	% of Average recovery
1	50%	1.0	0.5	1.5	1.492	99.46	99.503
2	100%	1.0	1.0	2.0	1.989	99.45	
3	150%	1.0	1.5	2.5	2.49	99.6	

Table.4.5 Calibration Table for Ciprofloxacin

S.NO	Standard concentration (ppm)	Peak Area
1	1	1316
2	2	2357
3	3	3267
4	4	4231
5	5	5349
6	6	6257
7	7	7356
8	Slope = 1025.44	Intercept = 177.5833

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN 2319-2119

RESEARCH ARTICLE

Figure 4.F Calibration curve Ciprofloxacin

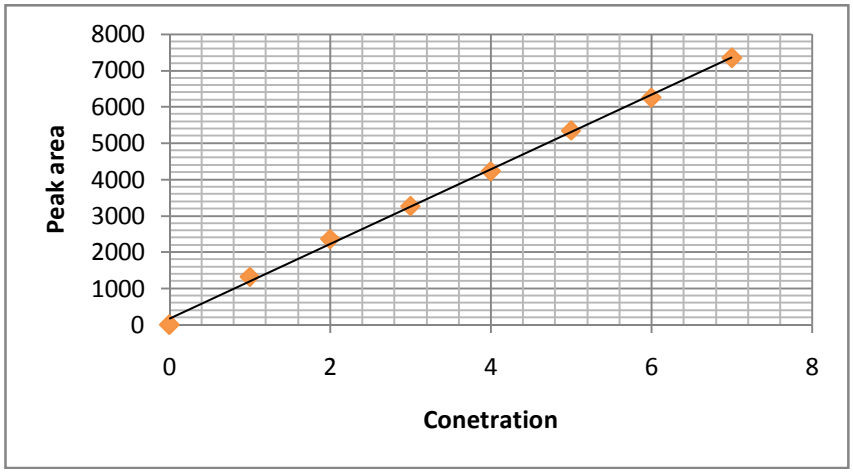


Table 4.6 Recovery studies of Ciprofloxacin

S.NO	% OF RECOVERY	Fixed conc in ppm1	Spiked conc in ppm1	Total sample concetration	Amount of recovery	% of recovery	% of Average recovery
1	50%	2.0	1.0	3.0	2.991	99.7	99.67
2	100%	2.0	2.0	4.0	3.983	99.57	
3	150%	2.0	3.0	5.0	4.987	99.74	

Table.4.7 Concentration of Nalidixic acid, Ciprofloxacin in fish protein (Bhimavaram samples)

S.NO	SAMPLE LOCATION	Nalidixic acid concentration in protein sample (ppm)	Ciprofloxacin concentration in protein sample (in ppm)
1	Bhimavaram	5.63	9.52
2	Bhimavaram	7.59	8.996
3	Bhimavaram	8.32	15.28
4	Bhimavaram	7.305	16.34
5	Bhimavaram	9.056	18.27
6	Bhimavaram	11.250	19.45

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN 2319-2119

RESEARCH ARTICLE

Table.4.8 Concentration of Nalidixic acid, Ciprofloxacin in fish protein (Pittalavanipalem samples)

S.NO	SAMPLE LOCATION	Nalidixic acid concentration in protein sample (ppm)	Ciprofloxacin concentration in protein sample (in ppm)
1	Pittalavanipalem	18.6	15.525
2	Pittalavanipalem	15.9	16.495
3	Pittalavanipalem	14.35	17.364
4	Pittalavanipalem	18.65	15.236
5	Pittalavanipalem	17.12	18.135
6	Pittalavanipalem	18.35	16.471

Table.4.9 Concentration of Nalidixic acid, Ciprofloxacin in fish protein (Hyderabad samples)NDL = below detection limit

S.NO	SAMPLE LOCATION	Nalidixic acid concentration in protein sample (ppm)	Ciprofloxacin concentration in protein sample (in ppm)
1	Hyderabad	3.25	BDL
2	Hyderabad	BDL	3.274
3	Hyderabad	2.48	1.247
4	Hyderabad	BDL	1.435
5	Hyderabad	4.286	2.493
6	Hyderabad	2.347	1.36

Table.5.0 Concentration of Nalidixic acid, Ciprofloxacin in fish protein (Vijayawadasamples)

S.NO	SAMPLE LOCATION	Nalidixic acid concentration in protein sample (ppm)	Ciprofloxacin concentration in protein sample (in ppm)
1	Vijayawada	1.523	0.684
2	Vijayawada	0.569	0.623
3	Vijayawada	0.846	0.476
4	Vijayawada	0.357	0.264
5	Vijayawada	1.235	0.179
6	Vijayawada	1.761	0.43

THE EXPERIMENT
INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

ISSN 2319-2119

RESEARCH ARTICLE

Table.5.1 Concentration of Nalidixic acid, Ciprofloxacin in fish protein (Machilipatanam samples)

S.NO	SAMPLE LOCATION	Nalidixic acid concentration in protein sample (ppm)	Ciprofloxacin concentration in protein sample (in ppm)
1	Machilipatanam	4.235	3.214
2	Machilipatanam	0.412	2.159
3	Machilipatanam	0.652	2.657
4	Machilipatanam	0.357	4.601
5	Machilipatanam	1.294	8.054
6	Machilipatanam	1.356	6.327

5. DISCUSSION

In aqua fields, aqua formers are using antibiotics to prevent diseases in fishes. At the time of catching fish formers adding antibiotic drugs to the water to skip unexpected loss due to diseases. From this condition fishes are directly entering in to homes through different places like shops, sellers, fish markets, Hypermarkets. Those antibiotics are entering unnecessary in to humans through this route.
We are developed a new HPLC method for analysis of Nalidixic acid, Ciprofloxacin. The developed method for NALIDIXIC ACID is, wavelength 266 nm, at Ambient temperature, mobile phase is Acetonitrile 0.05 M Phosphate buffer (30%:70% v/v) pH (5.1), at flow rate of 1 ml/min. The HPLC method for CIPROFLOXACIN is UV-detector at 282 nm, C18 reversed phase thermo column, with Ambient temperature, mobile phase as Methanol, 0.25M H3PO4 (80%:20% v/v) P^H (4.6)as the mobile phase, at flow rate of 1ml/ min. Our method showing 99.503 % and 99.67% recovery for Nalidixic acid, Ciprofloxacin respectively.

For research analysis we are selected few places i.e Bhimavaram, Machilipatnam, Vijayawada, Hyderabad, Pittalavanipalem. In this list Bhimavaram, Pittalavanipalem are aqua culture places in Andrapradesh, so we are collected fish samples directly from aqua fileds. From Machilipatnam, Vijayawada, Hyderabad, we are collected samples from fish markets.

In our survey we observed usage of Nalidixic acid, Ciprofloxacin vigorously. So we analyzed the fish samples for these two antibiotics. Nalidixic acid estimated in 5.63 ppm – 11.25 ppm in Bhimavaram, 14.35 ppm- 18.65ppm in pittalavanipalem. These two areas are aqua culture places. We concluded that the aqua formers are using anti bitotics in huge.0.357 ppm – 1.761 ppm in vijayawada, 0.357 ppm to 4.235 in Machilipatnam, 2.48 ppm -4.286 ppm in Hyderabad.

Ciprofloxacin estimated in 9.52 ppm – 19.45 ppm in Bhimavaram, 15.236 ppm- 18.135ppm in pittalavanipalem. These two areas are aqua culture places. We concluded that the aqua formers are using anti bitotics in huge.0.264 ppm – 0.684 ppm in vijayawada, 2.159 ppm to 8.054 in Machilipatnam, 1.247 ppm -3.274 ppm in Hyderabad

7. REFERENCE

1. Emmerson, A. M.; Jones, A. M. (2003). "The Quinolones: Decades of Development and Use" . The Journal of Antimicrobial Chemotherapy 51 13–20. doi:10.1093/jac/dkg208. PMID 12702699. edit
2. Barclay, L. (Sep 2011). "AAP Reviews Use of Systemic and Topical Fluoroquinolones". MedScape.
3. Fraser, A. G.; Harrower, A. D. (December 1977). "Convulsions and Hyperglycaemia Associated with Nalidixic Acid". British Medical Journal 2 (6101): 1518.
4. "Nalidixic acid spectrum of bacterial susceptibility and Resistance", Toku-E. 2011-09-14. Retrieved 2012-05-14.
5. Drusano GL, Standiford HC, Plaisance K, Forrest A, Leslie J, Caldwell J, GL (September 1986). "Absolute oral bioavailability of ciprofloxacin". Antimicrob Agents Chemother. 30 (3): 444–6.

6. Nelson, JM.; Chiller, TM.; Powers, JH.; Angulo, FJ. (Apr 2007). "Fluoroquinolone-resistant Campylobacter species and the withdrawal of fluoroquinolones from use in poultry: a public health success story.". Clin Infect Dis 44 (7): 977–80.
7. Kawahara, S. (Dec 1998). "[Chemotherapeutic agents under study]". Nippon Rinsho 56 (12): 3096–9.
8. "Ciprofloxacin-Hydrochloride". The American Society of Health-System Pharmacists.
9. "Fluroquinolone Drug Class Review". Oregon State University College of Pharmacy. 2002. Retrieved 9 September 2011.
10. Schaeffer AJ (September 2004). "NIDDK-sponsored chronic prostatitis collaborative research network (CPCRN) 5-year data and treatment guidelines for bacterial prostatitis". Int. J. Antimicrob. Agents 24 Suppl 1: S49–52.doi:10.1016/j.ijantimicag.2004.02.009.
11. Zuger A. "Ciprofloxacin for Acute Exacerbations of Chronic Bronchitis". Journal Watch (General)1998 (1103): 4. "
12. Vardakas, KZ; Siempos, II; Grammatikos, A; Athanassa, Z; Korbila, IP; Falagas, ME (December 2008). "Respiratory fluoroquinolones for the treatment of community-acquired pneumonia: a meta-analysis of randomized controlled trials".CMAJ 179 (12): 1269–77.
13. Donaldson, PM; Pallett, AP; Carroll, MP (May 1994)."Ciprofloxacin in general practice". BMJ (Clinical Research Ed.)308 (6941): 1437.
14. Screening of antibacterial activity of mucus extract of Snakehead fish, Channa striatus (Bloch), ONG YEONG WEI, R. XAVIER, K. MARIMUTHU, European Review for Medical and Pharmacological Sciences, 2010; 14: 675-681
15. SUBRAMANIAN S, ROSS NW MACKINNON SL. Comparison of antimicrobial activity in the epidermal mucus extracts of fish. Comp Biochem Physiol B 2008; 150: 85-92.
16. MAT JAIS AM, MATORI MF, KITTAKOOP P, SOWANBORIRUX K. Fatty acid composition in mucus and roe of Haruan, Channa striatus, for wound healing. Gen Pharmacol 1998; 30: 561-563.
17. Screening of antibacterial activity of mucus extract of Snakehead fish, Channa striatus (Bloch), ONG YEONG WEI, R. XAVIER, K. MARIMUTHU, European Review for Medical and Pharmacological Sciences, 2010; 14: 675-681
18. SUBRAMANIAN S, ROSS NW MACKINNON SL. Comparison of antimicrobial activity in the epidermal mucus extracts of fish. Comp Biochem Physiol B 2008; 150: 85-92.
19. 25) MAT JAIS AM, MATORI MF, KITTAKOOP P, SOWANBORIRUX K. Fatty acid composition in mucus and roe of Haruan, Channa striatus, for wound healing. Gen Pharmacol 1998; 30: 561-563.
20. Determination of ciprofloxacin in plasma and urine by HPLC with ultraviolet detection Marika Kamberi[a], , Kimiko Tsutsumi, Tsutomu Kotegawa, Koichi Nakamura and Shigeyuki Nakano, Clinical ChemistryJune 1998 vol. 44 no. 6 1251-1255
21. Simple High-Performance Liquid Chromatographic Method for Determination of Ciprofloxacin in Human Plasma, Mohsen Amini, Mahnaz Khanavi, Abbas Shafiee, Iranian Journal of Pharmaceutical Research (2004) 2: 99-101
22. High Performance Liquid Chromatography (HPLC) Method Development and Validation Indicating Assay for Ciprofloxacin Hydrochloride, Sani A. Ali, Chijioke C. Mmuo, Rafat O. Abdulraheem, Sikirat S. Abdulkareem, Emmanuel T. Alemika, Musa A. Sani and Mohammed Ilyas, Journal of Applied Pharmaceutical Science 01 (08); 2011: 239-243

CH.RAMAMOHANA RAO, L.CYRIL ARUN KUMAR*

Dept of Bio-Technology, Acharya Nagarjuna University, Nagrjunanagar, Guntur,A.P, India.

Email id: ramohan.phd@gmail.com