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IN VIVO DEPOSITION AND INHALATION TOXICITY OF CEFDINIR LOADED FUNCTIONALIZED CARBON NANOTUBES AS NOVEL APPROACH OF FORMULATION OF RESPIRABLE PARTICLE.

Krishnat D. Dhekale[1] and Ravindra N. Kamble[*2]

[1,2]Department of Pharmaceutics, Poona College of Pharmacy, BharatiVidyapeeth University, Erandwane, Pune 411038, Maharashtra, India.

Abstract:

A dry-powder inhaler (DPI) is a device that delivers medication to the lungs in the form of a dry powder which are commonly used to treat respiratory diseases. Present work was attempted to investigate the potentialities of Multi-walled carbon nanotubes (MWCNT) as a pulmonary carrier for targeting Cefdinir tolung infection. Functionalization of MWCNTs by reported method and loaded with Cefdinir using the Incipient Wetness impregnation method to formulate Cefdinir loaded respirable particle(CLRP) which has efficient treatment against lung infections.This is evaluated by particle size, flow properties in vitro release kinetics, in vivo deposition study, acute inhalation toxicity study and Ex-vivo antimicrobial study. The results indicated that CLRP are nontoxic and 95% entrapment with better flow properties was achieved. The optimized formulation had particle size is6.482±0.09 µm. The release pattern of CLRP was shown to release in a controlled manner for 24 hours (i.e. after 24 hours it was 88 %). In vivo lung deposition study showed that maximum amount of drug is present in lung. Formulated respirable particle show better antimicrobial activity against gram (+ve) andgram (-ve). This work established a novel, easy to prepare with better drug loading efficiency.

Keywords: Respirable, Cefdinir, Deposition, Functionalization, Wetness Impregnation Method.

1. Introduction:

Respirable particle gaining much importance in the present day research field because it enables to target the drug delivery directly to lung both for local and systemic treatment, it has great potential to produce maximum therapeutic benefits it provides local action within the respiratory tract, gives rapid drug action, and allows for a reduction in systemic side-effects. Because of limitations associated with the conventional treatment of various chronic diseases a growing attention has been given to the development of targeted pulmonary drug delivery systems(1)(2). The conventional oral formulations have high dosing frequency and large dose often leads to local and systemic toxicity. Therefore, novel dry powder inhaler controlled release carrier systems may provide a possible solution to these problems by reducing dosing frequency and increasing drug bioavailability can lead to improved patient

compliance(3).The use of dry powder inhalers (DPIs) to administer treatments for respiratory diseases has increased significantly in recent years. There is now a wide range of DPIs available that vary considerably in design, operational techniques, output characteristics, and drug delivery across a range of inhalation patterns (4). Dry powder inhalers (DPIs) are devices through which a dry powder formulation of an active drug is delivered for local or systemic effect via the pulmonary route. DPIs have a number of advantages over other methods of pulmonary drug delivery, for example, direct delivery of drug into the deep lungs utilizing the patient's respiration and are increasingly being explored as a mechanism for the delivery of systemic drugs. Successful delivery of drugs into the deep lungs depends on the integration between powder formulations and the device performance (5). Carbon nanotubes (CNTs) are tubes made of carbon with diameters typically measured in nanometers, often refer to single-wall and multi wall carbon nano tube. The functionalization of multi-wall CNTs consists on the affirming of organic moieties to their tubular structure.
Through the functionalization of CNTs, it is possible to modulate their physicochemical properties, incrementing their ease of dispersion, manipulation, and process ability (6). Functionalization of CNTs has further opened new perspectives in the application of CNTs in drug delivery. Attachment of organic moiety to nanosized tubes has facilitated their use for diagnostic as well as targeting purposes, especially in infectious disease treatment. Modification of CNTs by introduction of a drug molecule onto the walls and/or sides of CNTs is referred to as CNT functionalization. This functionalization may be utilized for their enhanced biocompatibility, enhanced encapsulation tendency, and multimodal drug delivery (7). Several studies on the fate of nanotubes in the body have suggested that the functionalized CNTs loaded with drug molecules could easily pass into the cells and further into the cell nucleus, thus attaining targeted drug delivery both at cellular and nuclear levels (8). As above interesting properties of FMWCNTs may be a superior DPI carrier system.
The mechanism of action of cephalosporin's is similar to that of penicillin it interferes with bacterial cell wall synthesis. Bacterial cell walls contain peptidoglycans, which work is to keep external fluids and particles from entering those cells. By disrupting that synthesis, the antibiotic blocks the protein that links peptidoglycans, leaving bacterial cell walls open. An imbalance within the cell resulting complex process referred to as lytic cell death and bacteria dies.The "lung infections" category includes: influenza, pneumoniaand other acute lower respiratory infections, these infections are especially common and severe among the poor (9). The main mechanisms of lung defense against bacterial colonization are mucociliary clearance, polymorpho nuclear neutrophil phagocytosis, and local production of antibacterial cationic peptides. These systems of defense are poorly effective under conditions of increased viscosity and osmolarity, resulting in chronic lung infection (10).
Cefdinir is an expanded-spectrum, oral, third-generation cephem antimicrobial agent first synthesized in 1988 and approved by the USFDA in December 1997. (11).Structurally cefdinir is characterized by a vinyl group at C-3 position, and a 2-(2-aminothiazol-4-yl)-2-(hydroxyl-imino) acetyl moiety at C-7, which results in a marked increase in antimicrobial activity against both gram positive and negative bacteria, and also enhanced pharmacokinetic properties (12). The drug distributes very well in respiratory tract tissues and fluids; its pharmacokinetic profile allows once- or twice –daily administration. Cefdinir has tablet, capsule, and suspension formulations in the market but they need to be administered frequently and oral bioavailability is low (16 – 21 %). DPI of cefdinir may have effective results than oral and enhance the bioavailability(13).The conventional oral dosages of cefdinir for treatment of lung infectious diseases have multiple drawbacks such as orally given drugs cannot provide sufficient amount of drug to the lung site that requires increased amount of dose due to this there is need to dosage in respirable form. CLRPmay be effective result than orally and minimize the dose and enhance the bioavability.In this research, the

functionalized MWCNTs were loaded with cefdinir drug CLRP which have efficient treatment against lung infections which is evaluated by particle size, flow properties, release kinetics in vivo deposition study and acute inhalation toxicity study.

2. Materials and Methods:

Multiwall carbon nanotubes were purchased from the Applied Science Innovations Pvt. Ltd, India. Cefdinir was received as a gift sample from Covalent Laboratories Pvt. Ltd, Hyderabad India. All other chemicals were of analytical grade purchased from local suppliers.

2.1. Functionalization of MWCNTs:

MWCNT were functionalized by the primary basic treatment followed utilization of hydrochloric acid for generation of functionalized MWCNTs (FMWCNT) covalently. By this process, the underlying vital treatment by hydrogen peroxide and hydroxide produced oxidized MWCNTs and later treating with HC lcreatdcarboxylated MWCNTs. 500 mg of MWCNT was scattered in 25 ml of the mixture of 25 % ammonium hydroxide and 30% hydrogen peroxide (V: V = 1:1) in a 100 ml round bottom flask, furnished with a condenser and dispersion was warmed to 80ºC and left for 5 h. Later on, the subsequent scattering was diluted in water and removed. The residue was then washed with water up to unbiased pH and the sample was dried in vacuum at 40ºC overnight. The treatment with hydrochloric acid produced carboxylated MWCNTs. In this method 500mg of MWNCTs was placed in round bottom flask and 200 ml of HCl was added. The resultant reaction mixture was stirred using magnetic stirrer for 2 h, then diluted, filtered and washed with ultrapure water dried in vacuum overnight.(14)

2.2. Drug loading:

Cefdinir was loaded using slight modification in Wetness Impregnation method. A concentrated solution of cefdinir in phosphate buffer PH 7.4 was prepared, this solution added to FMWCNT with continuous agitation using ultra-sonicator. This solution thus obtained was stirred for 2h and then the dispersion obtained was filtered by using vacuum filtration assembly with membrane filter (0.45µ). The obtained microparticles suspension was lyophilized using mannitol (2.5% w/v) as a cryoprotectant to get CLRP based DPI (16).

2.3. Freeze drying of CLRP with 2.5 % manitol (cryoprotectant):

To the CLRP and 2.5 % manitol (cryoprotectant)was added insufficient amount of phosphate buffer pH 7.4. This dispersion was frozen using ultra low freezer for 24 h at - 80°C followed by lyophilization for 48 h using single stage freeze dryer at chamber pressure of 0.120 mBar. The resultant CLRP carrier system was stored in desiccators until further use (19).

2.4. Characterization of CLRP:

CLRP characterized for different parameters determined as follows:

2.4.1. Drug content:

The drug content of CLRPwas calculated by dissolving the formulation in a suitable quantityof phosphate buffer Ph 7.4 by use of a cyclomixer and ultrasonicator. The drug content was determined at 286 nm using a spectrophotometer (V-530; JASCO, Japan) after suitable dilutions. The percentage drug content was reported as gram of drug in 1 g of formulation(18).

$$\text{Drug content} = \frac{\text{Amount of drug present}}{\text{Weight analyzed}} \text{X } 100 \ldots (2)$$

2.4.2. Flow properties:

DPIs evaluated for all important flow properties discussed below:

a. Angle of repose:
The fixed height cone method was used to check the flow property of the formulations. A glass funnel with 5mm internal diameter was fixed at 2.5 cm height over the flat surface. The gentle flowing of the powder through the funnel was carried out. The diameter of the powder cone formed was measured. The angle of repose was calculated by the following equation.
Tan θ = height / radius

b. Carr's index (Ci)
Carr's index (Ci) is calculated using the values of bulk and tapped density (20).

Ci = (Tapped density-bulk density)/ Tapped density X 100…. (3)

c. Tapped density:
The tapped density was evaluated using a small graduated tube with a defined volume size into which known weight of the powder was added. Tapped volume is calculated by using a tap density tester (Electrolab, tap density tester, USP) following 100 taps. Tapped density is determined by dividing the mass of powder by volume.
Tapped density = mass/volume after tapping

d. Bulk density:
Bulk density is determined by dividing the mass of the powder by the volume.
Bulk density = mass/volume

e. Hausner's ratio
Hausner ratio defines the flow ability of powder mixture. It is defined as per the formulae below:(21).

Hausner ratio = Bulk density/ Tapped density… (4)

2.4.3. Particle size determination:

The mean particle size of the CLRP was determined by laser diffraction technique using Malvern 2000 SM (Malvern Instruments, Malvern, UK); which works on the mechanism of photon correlation spectroscopy. The mean particle size analysis was carried out for 100 sec at room temperature by keeping an angle of detection at 90°.It allows sample measurement in the range of 0.05 -20,000 μm. The mean particle size was expressed in terms of D(0.9) i.e. size of the 90% of the particle (22)(23).

2.4.4. In vitro release study:

CLRP equivalent to 1 mg of CEF was dispersed in 2 ml phosphate buffer pH 7.4 solution in the dialysis membrane bag (MembracelMD 34-14, cut–off 14kD). Dialysis tubing consisted of regenerated cellulose, a material chemically and physically treated to increase its resistance. The dialysis bag was suspended in 100 mL PBS at pH 7.4 and maintained at 37 ± 0.5 °C. The dispersion was rotated at 100 rpm/min in a magnetic stirrer. At predetermined

time intervals 1-mL aliquots were sampled and replaced with 1 mL fresh pH 7.4 PBS, which was maintained at 37 ± 0.5 ℃. The sample was analyzed by UV spectrophotometrically at 286 nm (24).

2.4.5. Acute inhalation toxicity study:

Healthy male wistar albino rats (n = 6) (National Institute of Biosciences, Pune, India) weighing between 180 and 250 g were used for control group, FMWCNT, CLRP, toxicity study. They were maintained in cages with a 12:12 h dark/light cycle and humidity (45–55 %) controlled environment and provided free access to standard food and tap water. The studies were performed according to Organization for Economic Cooperation and Development (OECD 2001) guidelines. The study protocols were approved by the Institutional Animal Ethics Committee of Poona college of Pharmacy, Pune, India. The animals were fasted but provided free access to water overnight before the study. The rats were randomly divided into Three groups (n = 6). The first group (control group) received distilled water orally. The second groups were received formulation equivalent to 10 mg of Cefdinir as a single exposure by fabricated inhalation apparatus, respectively (27). In 3rd group 5 mg FMWCNT intra-trachialy. After 28 days, the blood samples collected with the anticoagulant were used immediately for the determination of hematological parameters such as red blood corpuscles (RBC) count, white blood corpuscles (WBC) count, hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelet count were performed using a veterinary blood cell counter (ERMA Inc., PCE 210 Vet). The differential leukocyte count (DLC) was performed with an optical microscopy after staining.At the end of the protocol, the animals were anaesthetized by ether inhalation and necropsy was performed on randomly selected two animals of each group to analyze the macroscopic external features of trachea and lungs tissue. These tissues were carefully removed and fixed in 10 % buffered formalin and embedded in paraffin. Histology sections (5 μm thick) were stained with hematoxylin and examined under a light microscope. The parameters observed, evaluated and interpreted for the toxicity study are presented below:

Hematology:	RBC, WBC, Hb, Platelet, PCV, MCV, MCH, MCHC.
Liver function tests:	GGTP, Total protein, albumin, globulin, SGOT, SGPT, ALP, Total Bilirubin, conjugated bilirubin, unconjugated bilirubin.
Kidney function tests:	Creatinine, BUN (Blood urea nitrogen), Sodium, potassium, urea, chloride, calcium, bicarbonate, phosphorus, uric acid.
Histopathology:	Sacrificed the animals at end of study, organs were removed, weighed and finally processed for histopathology of kidney, heart, liver, and lung.(28)(29)

2.4.6. In vivo deposition study:

The research experiment was performed on healthy Wistar albino rats weighing between 180 and 250 g (National Institute of Biosciences, Pune, India).The studies were performed as per the Organization for Economic Cooperation and Development (OECD 2001) guidelines. All studies were approved by the Institutional Animal Ethics Committee (IAEC) of Poona College of Pharmacy (1703/PO/C/13/CPCSEA) Pune, India bearing protocol number CPCSEA, PCP/PCT 07/2017-2018. The study was performed using a randomized design with (n=6) one group rats were exposed to CLRPformulations equivalent to 10 mg of cefdinir as a single exposure by inhalation apparatus. The serial blood samples (0.5-2 mL) were collected using retro-orbital puncture technique at predetermined time intervals (5 min, 9, and

15 h). Serum was separated from whole blood by centrifugation at 10,000 rpm after clotting for 1 h at 4ºC (Cryocentrifuge 2810R, Eppendorf, USA). The collected serum samples were analyzed to quantify Cefdinir using reported validated HPLC method. Cefdinir was extracted from serum by liquid-liquid extraction method. The mixture was extracted with 1 mL of chloroform by vortex mixing for 15 min which was then subjected to centrifugation at 10,000 rpm for 10 minutes. The separated organic layer was collected and evaporated to dryness at 40ºC under a gentle stream of nitrogen gas. The obtained residues were reconstituted in 100 µLPBS 7.4 pH with vortex mixing from which 20 µL was injected to the HPLC system. At last, same group of animals were euthanized by intraperitoneal administration of 3383 henobarbital and thoracic cavity was opened. Each time point, different group of animals was used. The lungs along with tracheal segments were removed. The sterile saline solution (1 mL of 0.9%) was used to rinse the lungs to get broncho-alveolar lavage fluid (BALF). The collected BALF solution was centrifuged, cell debris was removed and collected supernatant was used to quantify the CEF. Moreover, trachea and lungs were harvested in order to quantify the CEF. Tissue samples were blotted with paper towel to remove excess fluid, rinsed in ice-cold saline, weighed and stored at -20°C until to analyze. The obtained tissues were cut and minced in to small pieces for the efficient extraction of cefdinir. Further homogenization and centrifugation of tissues was carried out using PBS 7.4 and extract was evaporated to dryness. The residue was reconstituted in mobile phase and cefdinir concentration was quantified. The HPLC system specifications were as follows: Pump, PU-1580 (JASCO, Japan); Injector, auto sampler (AS-1555; JASCO); Column, Hypersil C18, 250 × 4.6 mm, 5 µm (Thermo Electron Corporation, USA); Detector, UV/visible (UV-1575; JASCO). Data acquisition and analysis were carried out using Borwin/HSS 2000 software (LG 1580-04; JASCO). The mobile phase was a mixture of acetonitrile:methanol:water (13:5:2 v/v). The column temperature and flow rate were 40°C and 1 mL/min. The wavelength was 286 nm. The HPLC analytical method and process of extraction were well validated. Limit of detection and quantification for CEF were 5 to 25 ng/mL and retention time is 7.5 min. The CEF calibration curve was linear ($y = 263873$, $r^2 = 0.9987$ in serum, $y = 263398$, $r^2 = 0.9977$ in BALF and $y = 264111^*$ (-28887), $r^2 = 0.9996$ in lung tissue) at a concentration range of 5 to 25 ng/mL. The peak area of CEF was used for quantification. The deposition fraction of cefdinir in BALF, lung tissue, and plasma was calculated(18).

2.4.7. Ex-vivo antimicrobial study:

The antimicrobial action of the CLRP was measured utilizing the disc diffusion technique. For inoculums planning and test of antibacterial activity, the Muller Hinton agar was utilized. Standard cefdinir (a stock solution of the medication in methanol was set up at a concentration of 120 mg/ml) was utilized as positive control and the negative control was FMWCNT.

3. Results:

3.1. Entrapment efficiency:

Encapsulation efficiencyfor CLRP was 95%, which means that it was effective in maintaining the drug inside during the process of drug loading.

3.2. Flow properties:

The flow properties of CLRP formulation were characterized. The powder has shown better angle of response, carr's index, tapped density, bulk density, and hausner ratio of 23±4.20°C (excellent), 20.38±3.15 (below 15 % is considered as poor stream), 0.201±0.06 (g/cm3),

0.204±0.05 (g/cm^3), and 1.13 ± 3.15 (Below 1.25 Hausner's ratio shows the best flow characteristics than the greater one)which proved its acceptability as a carrier. The formulation exhibiting good flow properties displayed better aerosolization characteristics. The better angle of repose, Carr's index and hausner ratio would be one of the contributing factors.

3.3. Drug content:

The drug content of CLRPwas 98.67 ± 0.32 % w/w. The higher drug content was observed due to tight encapsulation of drug with FMWCNT due to higher acceptance ratio of it during drug loading, and also during freeze drying there was no drug loss observed.

3.4. Particle size determination:

Particle size in the range of 1-6 µm has a vital role to get more deposition in the lung. The particle size of CLRP formulation was 6,48µm as depicted in figure 1. Particles less than 1 µm are always exhaled resulting less deposition efficiency. The particles more than 6 µm are deposited in the throat (18). The formulation was within the limits of inhalable range. The less span value of the formulation was the indication of uniformity and narrow size distribution of the particles.

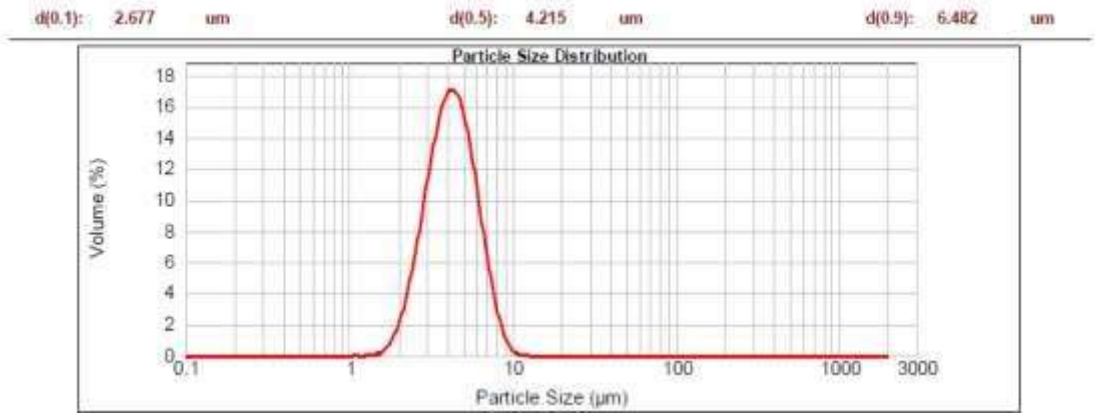


Figure 1: Particle size determination of CLRP

3.5. In vitro release study:

The in vitro dissolution of CLRP and pure CEF equivalent to 1 mg of CEF was checked by dialysis technique. Dissolution was conducted in PBS (pH 7.4) for 24 h at a temperature of 37 ± 0.5 ºC. The pure CEF resulted44.26 ± 0.71 % of release in 4 h and then decline the release. But the CLRP form showed initial burst release of 34.30 ± 0.61 % and then showed drug release in a controlled manner and after 24 hours it showed 88.56 ± 0.78 % as shown in figure 2.

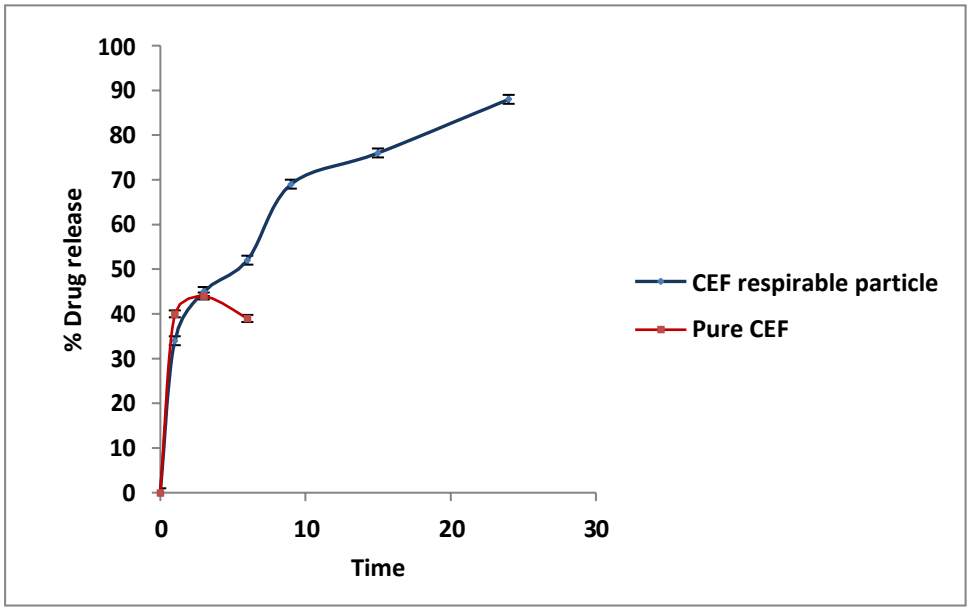


Figure 2: In vitro release study

3.6. Acute inhalation toxicity study:

All the important hematological parameters, liver function tests, and kidney function tests of all the groups (three) and compared with control group were examined and the results obtained are depicted in table 1, 2, and 3respectively. From table 1, 2, and 3 which shows hematological parameters (Hb (g/dl), WBCs (10^3/mm^3), RBCs (10^3/mm^3), Platelets (10^3/mm^3), PCV, MCV (µm^3), MCH (mmg), MCHC (mg/dl)), the liver function tests (GGTP, Total Protein, Albumin (g/dl), Globulin, Alkaline phosphatase, SGPT, SGOT, Total Bilirubin, Conjugated Bilirubin, Unconjugated bilirubin), and the kidney function tests (Creatinine, Sodium, Potassium, Chloride, Urea, BUN (blood urea nitrogen) (mg/dl), Bicarbonate, Calcium, Phosphorus, Uric acid)respectively it can be stated that all the parameters were within the ideal ranges for CLRP group, and for remaining Pure Cefdinir and FMWCNT group showed some negligible variations for particular parameters which are not considered as harmful.

Table 1: Hematology study

Sr. No.	Parameter	Control group		Pure Cefdinir		FMWCNT		CLRP	
		Male	Female	Male	Male	Female	Female	Male	Female
1.	Hb (g/dl)	13.10±0.29	13.001±0.78	11.07±0.92	13.10±0.29	13.001±0.78	11.31±0.59	12.11±0.47	13.01±0.28
2.	WBCs (10^3/mm^3)	9.50 ± 0.15	8.15 ± 0.12	9.03± 0.10	10.50 ± 0.15	9.15 ± 0.12	10.40 ± 0.40	9.51 ± 0.21	9.51 ± 0.21
3.	RBCs (10^3/mm^3)	6.4 ± 0.2	6.5 ± 0.3	6.04 ± 0.47	6.40 ± 0.2	7.01 ± 0.3	6.90 ± 0.33	6.60 ± 0.15	6.97 ± 0.18
4.	Platelets (10^3/mm^3)	480±2.31	474±2.31	397±2.11	470±2.31	494±2.31	401±2.01	422±1.31	484±1.24
5.	PCV	39±1.3	46 ±1.2	45±2.6	49±1.3	46 ±1.2	44 ±1.5	49 ± 1.4	46±1.1
6.	MCV (µm^3)	61 ± 1.70	71 ±1.50	69±0.79	61 ± 1.70	61 ±1.50	68±1.30	60±0.94	62±0.52

7.	MCH (mmg)	20±0.34	20±0.41	18±0.31	20±0.34	20±0.41	18±0.40	20±0.26	21±0.26
8.	MCHC (mg/dl)	34±0.50	28±2.50	35±0.23	34±0.50	33±2.50	35± 0.19	35±1.10	36±0.24

Table 2: Liver function test

Sr. No.	Parameters	Control group		Pure Cefdinir		FMWCNT		CLRP	
		Male	Female	Male	Male	Female	Female	Male	Female
1.	GGTP	9±0.71	7±0.58	10±1.06	10±0.71	10±0.58	11±0.2	13.5±0.95	12.8±0.53
2.	Total Protein	7.9 ± 0.24	8.3 ± 0.08	7.12±0.08	8.9 ± 0.24	7.3 ± 0.08	8.7±0.07	8.7±0.11	9±0.05
3.	Albumin (g/dl)	0.99±0.06	1.5±0.07	5.8±0.11	3.8±0.06	4.2±0.07	5.2±0.06	4.8±0.06	4.9±0.05
4.	Globulin	6.9±0.2	6.8±0.04	7.6±0.14	7.9±0.2	7.8±0.04	7.2±0.12	7.6±0.1	7.1±0.06
5.	Alkaline phosphotase	182±19	154±44	173±24	182±19	184±44	163±22	162±56	168±9.2
6.	SGPT	89 ± 6.6	101 ± 8.8	90±6.8	89±6.6	90±8.8	90±1.9	101±3.4	100±0.48
7.	SGOT	270±8.5	235±1.5	273±3.9	270±8.5	265±1.5	286±2.5	263±5.1	283±8.7
8.	Total Bilirubin	0.18±0.039	0.22±0.035	0.24±0.039	0.18±0.039	0.22±0.035	0.19±0.031	0.17±0.035	0.20±0.0087
9.	Conjugated Bilirubin	0.045±0.023	0.03±0.01	0.031±0.02	0.045±0.003	0.03±0.001	0.02±0.001	0.043±0.003	0.035±0.005
10.	Unconjugated bilirubin	0.14±0.056	0.19±0.026	0.17±0.049	0.14±0.056	0.15±0.026	0.17±0.034	0.15±0.039	0.16±0.041

Table 3: Kidney function test

Sr. No.	Parameters	Control group		Pure Cefdinir		FMWCNT		CLRP	
		Male	Female	Male	Male	Female	Female	Male	Female
1.	Creatinine	0.47±0.022	0.59 ± 0.018	0.83±0.51	0.77±0.022	0.89 ± 0.018	0.79±0.46	0.86±0.022	0.88±0.064
2.	Sodium	146±7.5	102±4.4	135±0.96	146±7.5	140±4.4	142±0.71	147±0.65	144±0.48
3.	Potassium	4.6±0.14	4.1±0.024	4.5±0.36	4.6±0.14	4.1±0.024	4.1±0.05	6.07±0.17	5.3±0.20
4.	Chloride	99±0.48	99±0.33	95±1.2	99±0.48	99±0.33	98±0.85	100±0.71	100±0.65
5.	Urea	43±3.1	50±2.5	46±25	43±3.1	50±2.5	55±1.7	41±0.41	49±1.6
6.	BUN (blood urea nitrogen) (mg/dl)	20±1.4	23±1.2	23±0.77	19±1.4	13±1.2	22±0.78	19±0.19	14±0.73
7.	Bicarbonate	24±1.9	21±0.48	23±0.61	24±1.91	21±0.48	21±0.52	24±0.89	21±0.21
8.	Calcium	9.3±0.18	10±0.1	9.1±0.065	9.3±0.1	10±0.15	9.8±3.1	8.3±2.	9.9±0.1

			5		8			2	1
9.	Phosphorus	7.5±0.36	6.1±0.3	7.7±0.49	7.5±0.36	6.1±0.30	5.9±0.35	7.6±0.23	5.9±0.23
10.	Uric acid	0.97±0.30	0.99±0.054	1.5±0.34	0.97±0.30	0.99±0.054	2.04±0.06	1.0±0.15	1.0±0.01

Histopathology:

Acute inhalation toxicity study from the obtained results it can be stated that all the parameters were within the ideal ranges for CLRPgroup, and for remaining Pure Cefdinirand FMWCNT group showed some negligible variations for particular parameters which are not considered as harmful.

Histopathology study was conducted in the Wistar rats to prove the safety of formulated cefdinir loaded DPI formulation. Histopathological observations of the control group, Pure Cefdinir, FMWCNT, CLRPformulation were performed with an equivalent dose of 10 mg of cefdinir. Kidney, heart, liver, and lung sections of all formulation treated groups were observed histopathologically after 28 days of dosing. The control group treated has not depicted any signs of toxicity as shown in figure 3 (A, B, C, and D). This proved the safety of cefdinir as a suitable candidate for the pulmonary route of administration. Histopathology of Pure Cefdinirdepicted in figure 4, histopathology of the kidney showed, one of the kidneys has mild focal mononuclear inflammatory infiltrate which was determined by mild bleeds (figure 4A). No signs of inflammation ($P < 0.05$) were observed in the sections of the heart from which it can be concluded that formulation was safe for the pulmonary administration. Section of liver tissue showed mild focal mononuclear periportal inflammation along with few eosinophils which are determined by mild tissue damage (figure 4C). In histopathology of lungs, there were some blood clots and some tissue damages which determine bronchioles with necrosed mucosa and marked mild acute inflammatory infiltrate with abscess formation (figure 4D). Figure 5 and 6 depicts the histopathology of all the above organs treated with FMWCNT and CLRP respectively. Histopathology of the kidney showed, one of the kidneys has mild blood clots from which it can be concluded that there is a focal mononuclear inflammatory infiltrate. No signs of inflammation ($P < 0.05$) were observed in the sections of the heart. Big tissue damage was observed from histopathology of the liver which is meaning there is a focal mild mononuclear periportal inflammation along with few eosinophils. From the histopathology of the lung section, it can be observed that there are some mild tissue damages and blood clots in the lung sections.

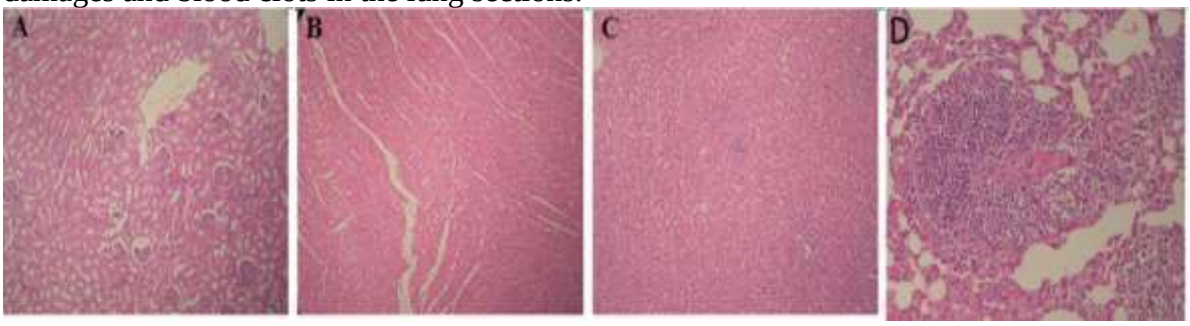


Figure 3: Histopathology of A: kidney, B: heart, C: liver, D: lung (control group)

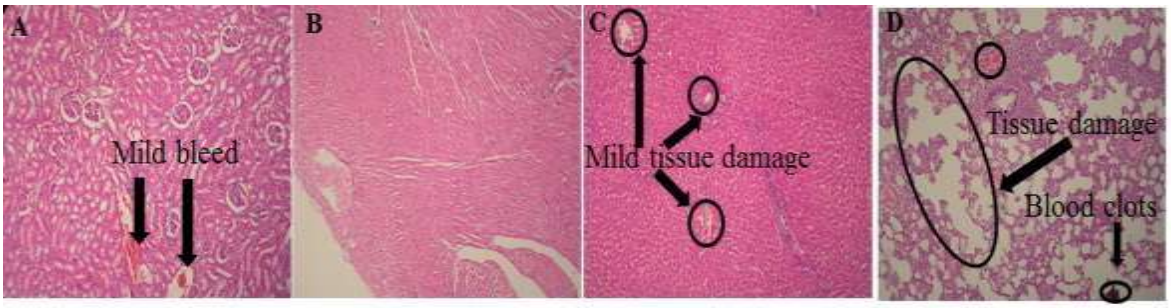


Figure 4: Histopathology of A: kidney, B: heart, C: liver, D: lung (Pure Cefdinir)

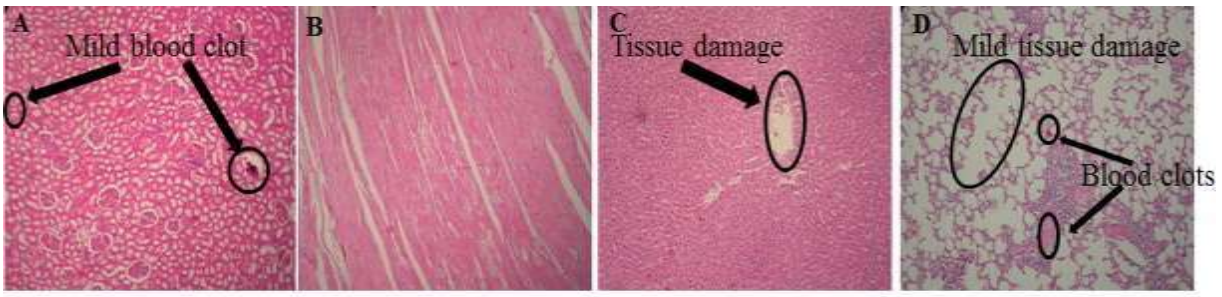


Figure 5: Histopathology of A: kidney, B: heart, C: liver, D: lung (FMWCNT)

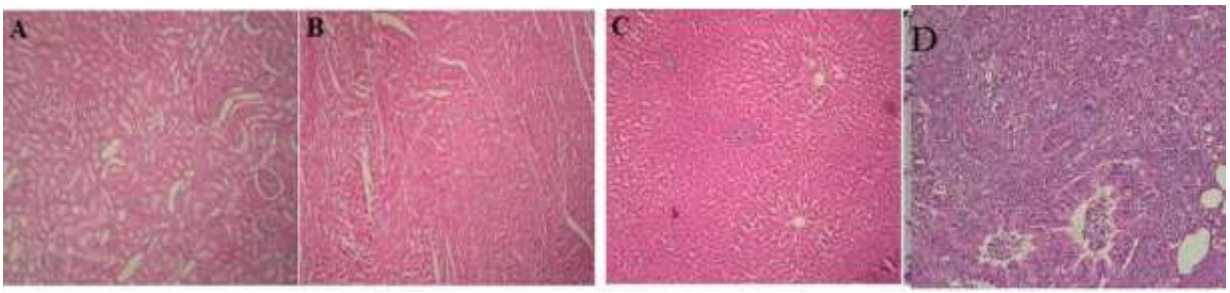
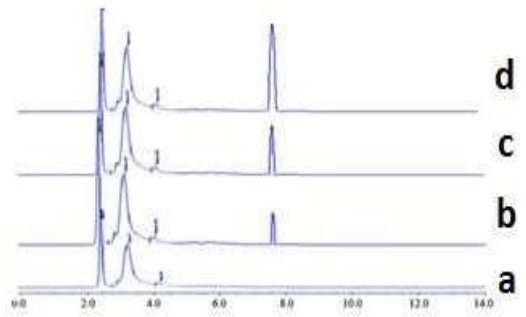


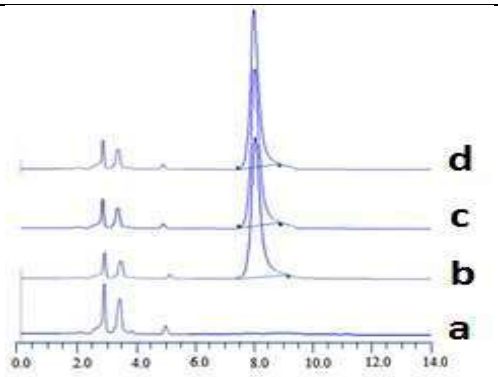
Figure 6: Histopathology of A: kidney, B: heart, C: liver, D: lung (CLRP**)**

3.7. In vivo lung deposition study:

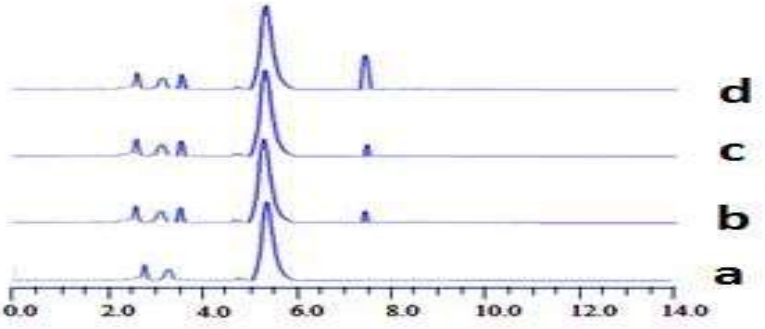
In pulmonary absorption studies, lung deposition of CLRPusing simple, rapid and validated reported HPLC method. Cefdinir was eluted at 7.5 min in BALF, lung tissue and plasma. The CLRPhas shown maximum lung deposition. After 5 min initial dosing and gradually increased 9hr and 15hr deposition for CLRPin BALF lung tissue and serum was observed. This was observed due to favorable physicochemical properties which helped to achieve required MMAD and lung deposition. Moreover, CLRP having less tapped density with spherical nature and rod shaped surface was responsible to reduce surface contact leading to higher fluidization and efficient deposition. The better aerodynamic behavior of formulation was result the enhanced lung deposition fraction. The CLRP indicated less cefdinir absorption for initial in lung. After which controlled Cefdinir absorption was observed up to 15 h as shown in the Fig. (7). Thein vivo absorption profile of CLRPshowed 1.73 folds increased cefdinir concentration in the lung tissue. The controlled site specific release of cefdinir from CLRP attributed to maintain higher concentration of cefdinir in the lung tissues as compared to BALF and serum.



A – Balf (a – Balf blank, b – Cefdinir in balf (5 min), c – Cefdinir in balf (9 hr), d – Cefdinir in balf (15 hr))



B – Lung tissue (a – Lung tissue blank, b – Cefdinir in lung tissue (5 min), c – Cefdinir in lung tissue (9 hr), d – Cefdinir in lung tissue (15 hr))

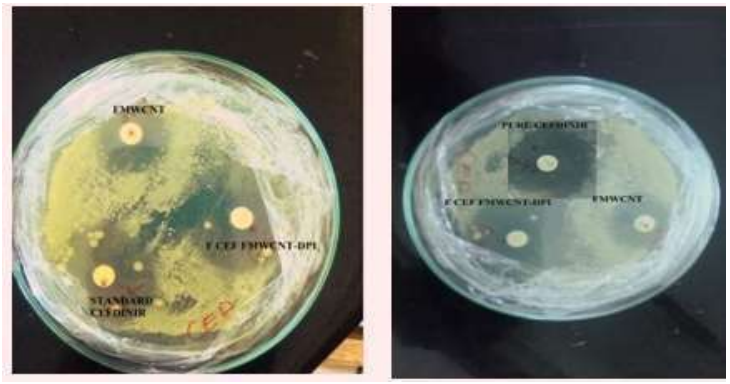


C – Plasma (a – Plasma blank, b – Cefdinir in plasma (5 min), c – Cefdinir in plasma (9 hr), d - Cefdinir in plasma (15 hr)).

Figure 7: Concentration of Cefdinir In vivoin vitro lung deposition study by HPLC method

3.8. Ex-vivo antimicrobial study:

The antibacterial activity of the CLRP was assayed using the disc diffusion method. In figure (8A) Formulated CLRP was shows a clear zone around a disc was evidence of antimicrobial activity against Gram-positive (Staphylococcus aureus) and in figure (8B) Formulated CEF loaded FMWCNT-DPI was shows a clear zone around a disc was observed that is evidence of antimicrobiall activity against Gram-negative (e.coli). Formulated CLRP shows better activity against gram positive and gram negative dut synergistically enhance activity FMWCNT.Standard cefdinir were used as positive control and the negative control was FMWCNT.



A **B**

Figure 8: A.Ex-vivo antimicrobial study against Gram +ve (Staphylococcus aureus) B.Ex-vivo antimicrobial study against Gram -ve (e.coli)

4. Conclusion:

Conclusively it can be stated that the system avoided the hepatic first pass metabolism and non-invasive route for drug administration. The formulation of CLRP for drug delivery is useful to reduce dose of drug and reduced toxicity. Formulation can show better activity due to site specific delivery with increasing Bioavability and Formulated CLRP shows better activity against gram positive and gram negative bacteria.

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