Synthesis, Physico-Chemical Characterisation and Microbiological Evaluation of Moxifloxacin Conjugates of Peg and Chitosan

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ABSTRACT

Polyethylene glycol (PEG) and chitosan are biodegradable polymers which reportedly have good solubility characteristics and possess properties which make them suitable as drug carriers. The objective of the study was to synthesize and characterize the polymer conjugates of moxifloxacin in a bid to enhance drug delivery. Chitosan-moxifloxacin, and PEG-moxifloxacin were synthesized by reacting the carboxylic acid moiety of moxifloxacin with hydroxyl group of the polymer in an esterification reaction. The ester conjugates formed were characterized by analytical methods such as UV, IR and NMR. The Beer Lambert's plot showed good correlation between absorbance values and concentration with regression values, R^2 = 0.9543 for moxi-PEG and 0.9504 for moxi-chitosan respectively. The IR spectra showed absorption peaks at 3425.49 cm-1 for PEG and 3671.41 cm-1 for chitosan respectively. This indicated weak O-H groups which disappeared when the conjugates of the drugs (esters) were formed and this was further confirmed by ^{1}H and ^{13}C NMR. Solubility tests were also conducted on the conjugates and buffer hydrolysis was carried out to determine the rates of reaction and half-lives. The conjugates were observed to release their drugs better at physiological pH (7.4) than at pH 6.1 and 8.1. Antimicrobial inhibitory activities of the drug conjugates showed better activity compared to the parent antibiotic, moxifloxacin. It therefore, can be concluded that the conjugates were successfully synthesized and have potential for use in the management of some gram positive and negative bacteria where extended release is needed.

Keywords: Moxifloxacin, drug conjugates, biodegradable polymers, physicochemical evaluation, microbiological evaluation

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1. INTRODUCTION

1.1 Background To The Study

The use of polymers in the medical field is not a novelty - natural polymers have been used as components of herbal remedies for centuries.

Properties of chitosan

1. Biocompatibility
2. Analgesic property

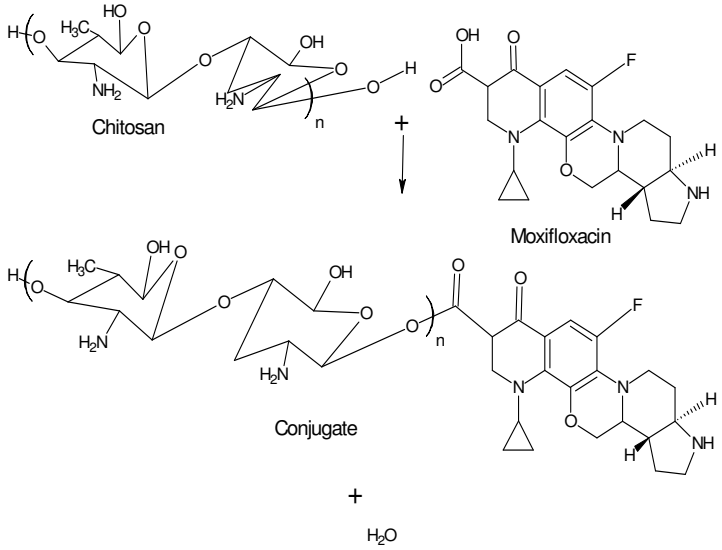
3. Haemostatic property
4. Antitumor Activity
5. Antimicrobial Activity
6. Antioxidative Activity
7. Biodegradability
   (Haug *et al.,* 2007)

2. METHODOLOGY

2.1.1 Synthesis of Conjugate

The starting process of the synthesis was esterification reaction.

The starting process of the synthesis was esterification reaction. An amount of 0.3M (120.42g) of pure moxifloxacin was added to 0.2mol of PEG in a round bottom flask and triturated for 2minutes to obtain a homogenous mixture then, 100ml of diluted sulphuric acid of 0.1 M was added into the mixture of pure moxifloxacin and polyethylene glycol then stirred vigorously for another two minutes. Also an amount of 0.3M (120.42g) of pure moxifloxacin was added to 0.125 mol of chitosan in a round bottom flask and triturated for 2minutes to obtain a homogenous mixture then, 100ml of diluted sulphuric acid of 0.1 M was added into the mixture of pure moxifloxacin and chitosan then stirred vigorously for another two minutes and both reflux for three (3hours) after which it was allowed to cool over night and the crystal filtered out as the product.



3. SEQUENCE IN THE CHARACTERIZATION AND PURIFICATION

1. Physical chemical determination and characterization
2. Recrystallization
3. Melting point determination
4. Preparative thin layer chromatography
5. Determination of calibration and validation curve
6. Infra-Red spectroscopy
7. NMR spectroscopy
8. Determination of Qualitative solubility test
9. Buffer Hydrolysis of the conjugate (stability)
10. Biological Assay

4. RESULT

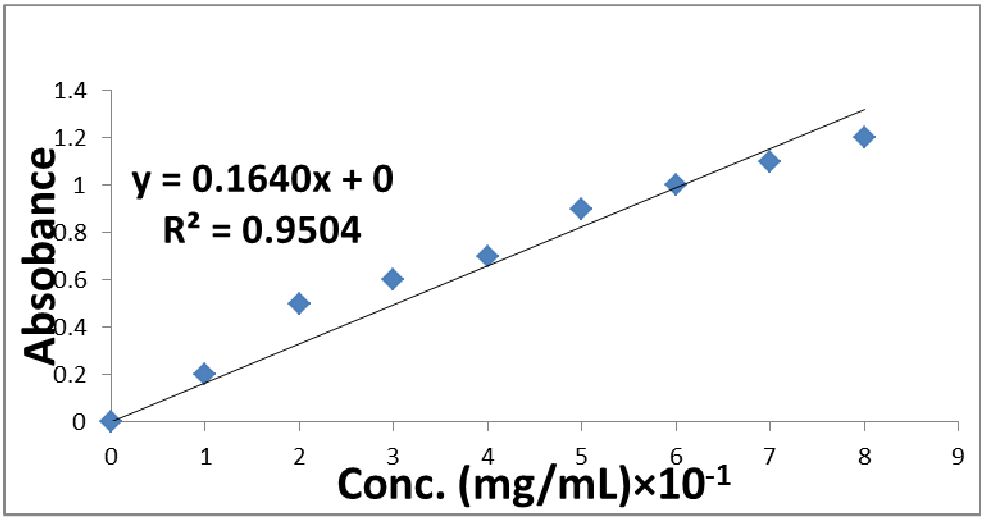


Figure I: Moxifloxacin-ChitosanConjugates

4.1 Moxifloxacin - PEG

3229.1862 cm^{-1} (weak and broad) N - H and C – H stretch
3052.785 cm^{-1} (medium and sharp) C – H stretch
2959.58 cm^{-1} (medium and broad) saturated C – H (CH_2 Stretch)
2512.267 cm^{-1} (weak and broad) secondary amine N – H
1884.83 cm^{-1} (very sharp and strong) ester functional group (COOR).
1521.472 cm^{-1} (Very sharp and strong) finger print region
1056.083 Cm^{-1} (medium, weak with shoulder) skeletal vibrations involving the bridge C-O

See Appendix A2.

PEG Spectrum alone from the literature spectra (see appendix1):**At 3425.44 cm^{-1} is that of intermolecular and weakly bonded O-H,** 2739.682 cm^{-1} is assigned to CHO aldehydes and1657.296 cm^{-1} to ketones(C=O).When IR spectrum of moxifloxacin – PEG is compared with PEG, it showed purely esterification (reaction between carboxylic acid functional group from the moxifloxacin and the O-H group from the PEG) nature of the interaction between PEG non-Polar tail and moxifloxacin, (COO^-) which is the new signal at18848.830cm^{-1} (ester). 332.187 cm^{-1} is assigned to N-H primary amines and 3026.69202cm^{-1} and 2855.188cm^{-1} are assigned to C-H stretch. When MFX drug is added to the PEG solution in the presence of dilute sulphuric acid, the hydrogen ion from the acid protonate the NH_2 preventing it from interaction, **the loss of O-H in the product and a new signal at 18848.830cm^{-1}** show that the conjugate is formed.

4.2 IR Moxifloxacin – Chitosan

2892.45 cm^{-1} (Medium and strong) to C – H stretch
21022.92 cm^{-1} (broad and weak) assigned to COOR (Ester).
1625.185 cm^{-1} (sharp and strong) assigned to carbonyl carbon(C=O)
1591.89 cm^{-1} (sharp and strong) assigned to amide II band
1319.68 cm^{-1} (sharp and strong) assigned to asymmetric C – H band of CH_2 group
1056.35 cm^{-1} (strong, sharp with shoulder) assigned to skeletal vibrations involving the bridge C – O stretch.

4.3 IR Spectoscopy of Moxifloxacin – Chitosan Conjugate

FTIR Spectroscopy of moxifloxacin – chitosan between the selected polymer chitosan and the drug moxifloxacin. The spectra obtained from IR studies at wavelength from 4000cm–I to 400cm–I showed that there are loss of functional peaks between the spectra of drug and polymer.

Chitosan (CH) spectrum alone from literature spectrum(A4): 3347.194cm^{-1} (which is assigned to the N – H and hydrogen bonded O – H stretch vibrational frequencies while a sharp (shoulder) **peak at 3671.498cm^{-1} is that of free O – H bond stretch of glucopyranose units,** 2870.194cm^{-1} (C – H stretch) 2363.196 cm^{1} (C – N asymmetric band stretching) 1657.4395ccm $^{-1}$ (amide II band N – H stretch) 1319.594cm^{-1} (asymmetric C – H bending of CH_2 group) and 1056.0839cm $^{-1}$ (skeletal vibration involving the bridge C – O stretch) of glucosamine residue.

For chitosan – moxifloxcin complex, the spectra band appear at 3268.844cm^{-1} primary N-H stretch , absorption arising from C – H stretching in the alkanes occurs in the general region of 2892.485cm^{-1} symmetric or asymmetric CH_2 stretching vibration attributed to moxifloxacin and pyranose ring of chitosan). A band at 2102.29cm^{-1} refers ester functional group(COOR).While a weak absorption at around 1400cm^{-1}, 1400 – 1032cm^{-1} (1371.787cm^{-1}) (C – F band stretching of moxifloxacin 943 – 623cm^{-1} (mono and disubstituted benzene ring). When IR spectrum of moxifloxacin – chitosan compared with chitosan, it showed purely esterification (reaction between the carboxylic acid functional group from the moxifloxacin and the O-H group from the chitosan) nature of the interaction between chitosan non-Polar tail and moxifloxacin. (COO^-) which is the new signal at 2102.29 $cm.^{-1}$ (ester) When MFX drug added to the Chitosan solution in the presence of dilute sulphuric acid, the hydrogen ion from the acid protonate the NH_2 preventing it from interaction.

4.4 NMR

^{1}H Moxifloxacin– Chitosan

δ 0.9 ppm (s); sharp and weak ^{1}H; Methyl (CH_3)
δ 1.4 ppm (t) ;sharp; weak with shoulder ^{1}H; 2×CH_2 methylene
δ 2.0 ppm (s) ;sharp and strong ^{1}H; methylene (CH_2)
δ 2.2 ppm (s); Sharp and strong ^{1}H; methylene(CH_2)
δ 2.8 ppm (s); sharp and weak ^{1}H: primary amine (NH_2)
δ 3.7 – 4.5 ppm (m); sharp weak with shoulder ^{1}H :NCH_2 +CH Tertiary amine attached to methylene and methine
δ 9.0 ppm (s); sharp and weak ester (OCH_2 CH_2)
δ 11.5 ppm (s); sharp and strong ^{1}H: secondary amine (NH)

4.5 ^{13}C NMR Moxifloxacin– Chitosan

δ 19.0 ppm (s); sharp and strong methyl (CH_3)
δ 35.5 ppm (s); sharp and weak CH_2(methylene),NCH_2 Tertiary amine attached to methylene
δ 42.0 ppm (s); sharp and weak with shoulder OCH_3 methoxyl group
δ 62.0 ppm (s); sharp and weak C-F Fluoro carbon
δ 108.0 ppm (s); sharp and weak ester (OCH_2CH_2)
δ 152.0 ppm (s); sharp and strong ketone carbonyl carbon (C = O)

4.6 ^{13}C NMR Moxifloxacin-Chitosan

There is a methyl (CH_3) group at resonance δ19.0ppm(s) most shielded, mehtylene at δ 35.5ppm (Singlet) and at frequency δ 42 (dd) there is a methoxyl (OCH_3) functional group, at δ62ppm(s) there is fluorocarbon (C-F), and frequency δ 108ppm(s)corresponding to ester functional group ($COOCH_2CH_2$ –), and at frequency δ 152ppm (Singlet), there is a ketone group C=O most deshielded.

4.7 ^{1}H NMR Moxifloxacin-Chitosan

The proton NMR gives signals at frequency δ 0.9 ppm(s) corresponding to methyl group (CH_3) and at frequency δ1.4 there is 2×CH_2 methylene, there are methylene at δ 2.0ppm(s) and δ 2.2ppm(s), there isa primary amine (NH_2) at δ 2.8ppm(s) and tertiary amine (NCH_2 +CH) at frequency δ 3.7-4.5ppm(m).There is also OCH_2 group at δ 9.0 ppm(s), this is because its position of resonance is in order for an OCH_2 group. The position of OCH_2 resonance at δ 9.0pmm(s) suggests OCH_2CH_2- Groups, which are actually ester, ($COOCH_2CH_2$ –) and not ethers. The formation of ester as shown by the proton and ^{13}CNMR shows that a product (Conjugate) is formed from chitosan and moxifloxacin, signal at δ 11.5ppm (Singlet) correspond to carbonyl carbon (C=O) which is the most deshielded.

4.8 Solubility

The solubility tests carried out was a qualitative test to show if the drug conjugates can dissolve in the different solvent media. The solubility profiles are shown in Table 3. Moxifloxacin-PEG and moxifloxacin-chitosan dissolves in polar solvents because of the polarity polymers and its ability to form salts easily. As a result of this, it was not surprising that the conjugates are insoluble in non-polar solvents. The fact that the conjugates of PEG are soluble in aqueous medium suggests that these conjugates can be administered through the systemic route where plasma esterases can easily release the drugs into the system, thus enhancing the rapid increases in plasma concentration of these drugs. As regards chitosan derivatives, systemic administration might lead to sustained release of the drugs since the release will be equilibrium dependent. These results further confirm the justification for the use of these polymers as biocompatible polymers in drug delivery and sustained release vehicles see Table 3 below.

Table 3: Solubility of Drugs and its Conjugate

Moxifloxacin-PEG	Solvent	Moxifloxacin-Chitosan
Soluble	Water	Sparingly soluble
Soluble	Methanol	Sparingly soluble
Insoluble	Ethanol	Insoluble
Soluble	Glacial acetic acid	Soluble
Soluble	Hydrochloric acid	Soluble
Soluble	Sulphuric acid	Sparingly soluble
Insoluble	Diethyl ether	Insoluble
Insoluble	N- hexane	Insoluble
Insoluble	Acetone	Insoluble

Table 3: Solubility of Drugs and its Conjugate

4.9 Buffer Hydrolysis of Drug Conjugate at different pH over 12HRS
Rates and Half-life for First Order Reaction

$C=Coe^{-kt}$------1
Log= log Co-kt/2.303------2
Relating equation (2) to regression equation
Y=mx+C
Rate=dc/dt=kc
Rate of kinetics (k) =slope(s), x=s=k from the equation
(T ½ = 0.693/K

Table 4: Rate of Reaction and Half-lives

	P^H 6.1	P^H 6.1	P^H 7.4	P^H 7.4	P^H 8.1	P^H 8.1
Drug conjugates	Rate of reaction(mg/hr)	Half live	Rate of reaction	Half live	Rate of reaction	Half live
Mox-PEG	1.36×10^{-2}	5.1	7.7×10^{-2}	8.9	9.6×10^{-2}	7.2
Mox-chit	4.57×10^{-2}	15.2	8.54×10^{-2}	8.1	4.14×10^{-2}	16.7

The buffer (pH 6.1 to 8.1) hydrolysis of the polymer conjugates are shown in figure Ia, Ib, IIa and IIb. It was observed that over the 12hrs duration of the experiment, the rate of hydrolysis precede uniformly between 0 and 8hrs producing a linear plots (see fig Ia and IIa respectively). Hydrolysis of PEG-conjugates overall proceed most slowly at P^H 7.4 while it was fastest at P^H 6.1.The hydrolysis P^H 8.1 was intermediate. Even at that, the hydrolysis of PEG-moxifloxacin, generally proceed more smoothly. In the first 8hrs of the hydrolysis kinetics produced linear plots, in which the rates were calculated to be: Moxifloxacin-PEG at P^H 6.1; 1.36×10^{-1} mg/h, Moxifloxacin-PEG at P^H 7.4; 7.78×10^{-2} mg/h; Mox-PEG at P^H 8.1; 9.6×10^{-2} mg/h, Moxifloxacin-chitosan at P^H 6.1; 4.57×10^{-2} mg/h; Moxifloxacin-chitosan at P^H 7.4; 8.54×10^{-2} mg/h; Moxifloxacin-chitosan at P^H 8.1; 4.14×10^{-2} mg/h; respectively. Similarly, the calculated half-lives were P^H 6.1 Moxifloxacin-PEG 5.1hrs, Moxifloxacin-chitosan 15.2h, P^H 7.4 Mox-PEG 8.9h, Moxifloxacin-chitosan 8.1h, P^H 8.1 Moxifloxacin-chitosan 16.7h respectively. The overall hydrolysis profiles of the chitosan conjugates follow the same pattern as that of PEG polymers, with hydrolysis proceeding at slowest rate at P^H 7.4 and fastest at P^H 6.1. The chitosan polymer conjugates hydrolyzed much more smoothly when compared to PEG polymer.

The kinetics of hydrolysis of the chitosan conjugates produced rates. Moxifloxacin-chitosan at P^H 6.1; 4.57×10^{-2} mg/h; Moxifloxacin-chitosan at P^H 7.4; 8.54×10^{-2} mg/h; Mox-chit at P^H 8.1; 4.14×10^{-2} mg/h; for the different P^H values. Again the calculated rates of hydrolysisMoxifloxacin-PEG at P^H 6.1; 1.36×10^{-1} mg/h, Moxifloxacin-PEG at P^H 7.4; 7.78×10^{-2} mg/h; Moxifloxacin-PEG at P^H 8.1; 9.6×10^{-2} mg/h, Moxifloxacin-chitosan at P^H 6.1; 4.57×10^{-2} mg/h; Moxifloxacin-chitosan at P^H 7.4; 8.54×10^{-2} mg/h; Moxifloxacin-chitosan at P^H 8.1; 4.14×10^{-2}mg/h; respectively. Similarly, the calculated half-lives were P^H 6.1 Ciprofloxacin-PEG 40.8h, Moxifloxacin-PEG 5.1h, Moxifloxacin-chitosan 15.2h, P^H 7.4 Ciprofloxacin-PEG 9.12h, Moxifloxacin-PEG 8.9h, Moxifloxacin-chitosan 8.1h, Moxifloxacin-PEG, 7.2h, Moxifloxacin-chit 16.7h respectively with half-lives of P^H 6.1 Moxifloxacin-PEG 5.1hrs, Ciprofloxacin-chitosan 7.7h, Moxifloxacin-chitosan 15.2h, P^H 7.4 Moxifloxacin -PEG 8.9h, Moxifloxacin-chit 8.1h, P^H 8.1 Moxifloxacin-PEG 7.2h, Moxifloxacin -chitosan 16.7h respectively.

The kinetics order of reaction in both polymers was determined to be first order reaction for the first 8h and zero order from 8h to 12h. Hydrolysis of mox-chit over 12h at P^H 6.1, shows much difference in the pattern of release of the drug from the polymer conjugates as the concentration in mass release into blood system is higher compared to that of P^H 7.4 and P^H 8.1from the hydrolysis profile, there is a constant release of the drug from the conjugate from 0h to 10h and a slight decrease at 10h and rises from 10h to 12h, showing a steady release of the drug see figure Ia. At P^H 7.4, there is a gradual rise from 0h to 8h and begins to decrease from 8h to 12h suggesting a decrease in concentration as the drug is being release from the polymer conjugate upon the action of esterase enzyme. Then at P^H8.1, the drug hydrolyses steadily from 0h to 6h then decreases slightly from 6h to 8h and rises sharply from 8h to 10h and became constant from 10h to 12h,suggesting a saturation point at 12h. See figure Ia. The observation that the rate of hydrolysis at P^H 6.1 is faster than the rate at P^H 7.4 suggests that the hydrolysis is based catalyzed. The buffer hydrolysis is based on enzymatic action of esterase enzyme on the ester (polymer conjugates) where by releasing the drug and polymer for therapeutical activity. The activity of the esterase enzyme varies in different buffer P^H, but it was far better in buffer P^H of 8.1

Buffer Hydrolysis Curve for Moxifloxacin-Chitosan

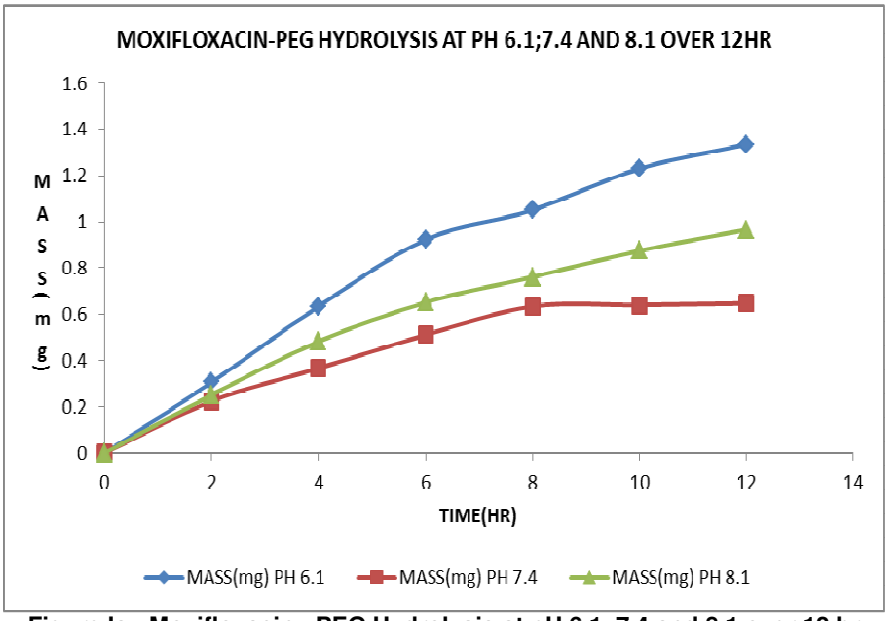
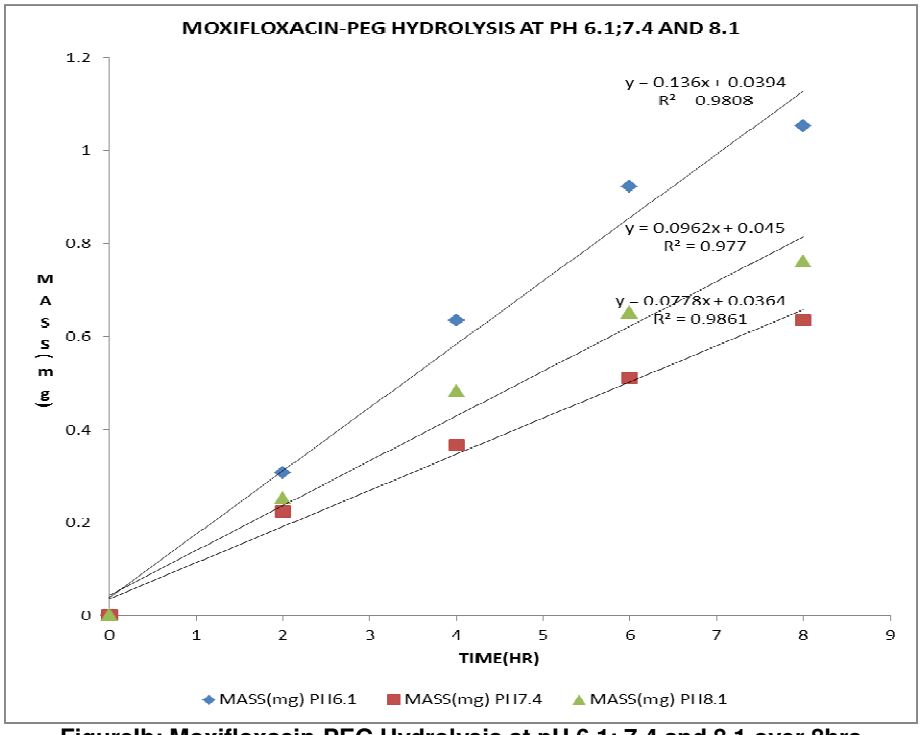


Figure Ia: Moxifloxacin - PEG Hydrolysis at pH 6.1, 7.4 and 8.1 over 12 hr



Figurelb: Moxifloxacin-PEG Hydrolysis at pH 6.1; 7.4 and 8.1 over 8hrs

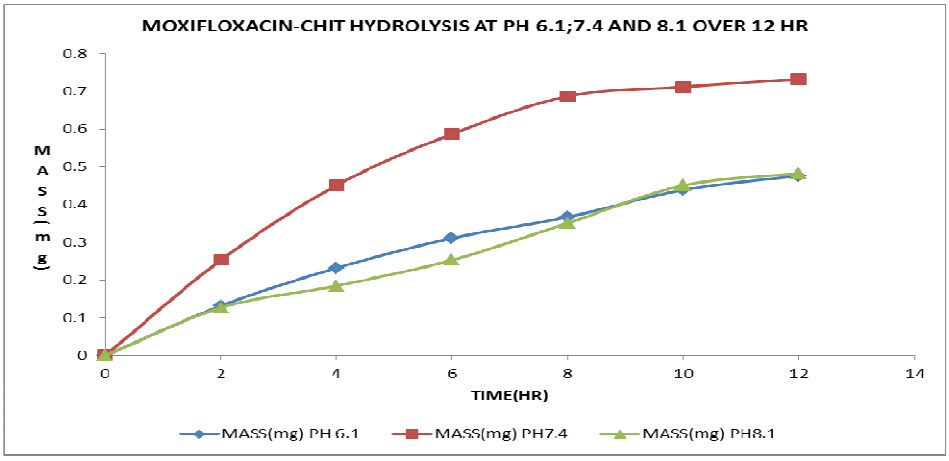


Figure IIa: Moxifloxacin-Chitosan Hydrolysis at pH 6.1, 7.4 and 8.1 over 12hr

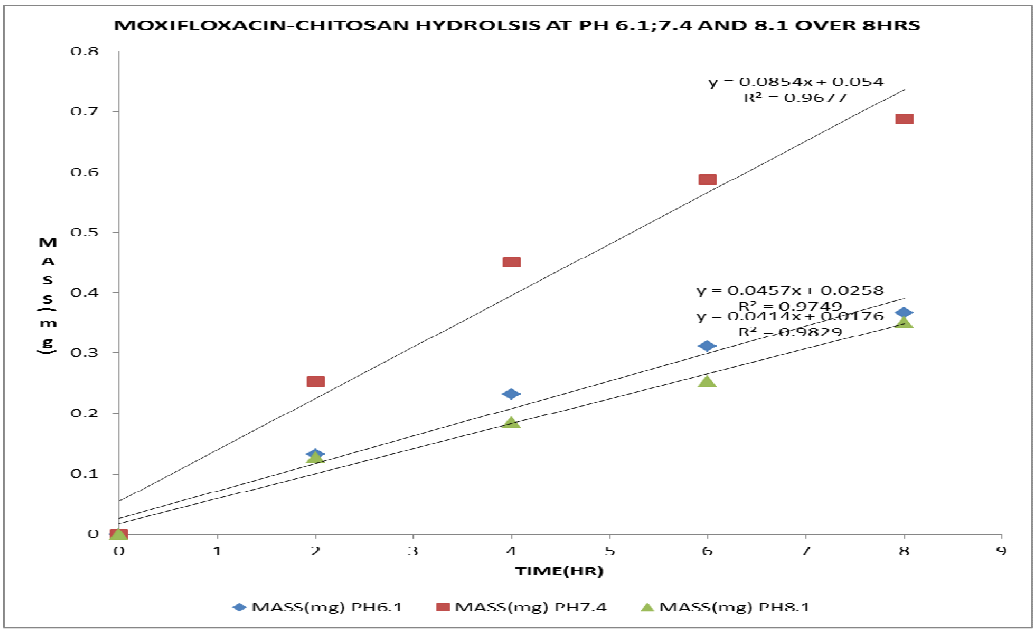


Figure IIb: Moxifloxacin-Chitosan Hydrolysis at pH 6.1, 7.4 and 8.1 over 8hr

Antimicrobial activity of PEG-moxifloxacin and chitosan-moxifloxacin complex and its solution against *staphylococcus aureus, streptococcus pneumonia, e.coli, salmonella typhi* were measured by agar diffusion method. The same procedure applied to all the antibiotics and conjugates. After 24hrs incubation at 37^0C, the antibiotics and conjugates showed antibacterial effect on the micro organisms cultured.The antimicrobials inhibited by the polymer conjugates have better inhibition than the parent drugs (antibiotics) with significant inhibition difference of 20.0mm, 7.8mm, 12.0mm for, Mox-chit, and Mox-PEG respectively. This increase in inhibition of antimicrobial activity is because the polymer alone has mild antimicrobial activity and this tends to potentiate the antimicrobial activity of the parent drug. When the polymer conjugates gets to the blood system, it is hydrolyzed by the enzyme esterase in the blood thereby releasing the parent drug and the polymer which, both has antimicrobial activity hence producing a better therapeutic effect against the micro organism. The inhibitory activity was measured based on the diameter of the clear inhibition zone.

When compared with the control (acetic acid 0.1 percent v/v). Both polymers showed clear inhibitory effect against gram positive and gram negative bacteria. For the solvent (control) no antimicrobial activity was observed against all the tested micro organisms used. From the comparative study between drug and complex it is clearly evident that chitosan-moxifloxacin, PEG- moxifloxacin complex is much more effective than moxifloxacin alone, see figure V, showing the result of inhibition in bar chat. The inhibitory additives showed figure that the complexes were effective for inactivating bacteria with the enhancement in the total activity.This is possible due to the sustained release effect of polymers conjugate and also due to the synergistic effect of both the chitosan and moxifloxacin, PEG and moxifloxacin, in the composite when esterase enzyme act on it releasing the drugs. The result suggests that the antimicrobial activity of moxifloxacin can be enhanced by its conjugation with chitosan and PEG in the form of sustained release.

Table II: Antimicrobial inhibition

Drug conjugates	S. Aureus	S. Pneumonia	E. coli	S. Typhi	P. Aeroginosa
Growth control (glacial acetic acid)	-	-	-	-	-
Moxifloxacin(mox) antimicrobial inhibition	6.0mm	12.0mm	13.0mm	9.0mm	7.0mm
Chitosan(chit) antimicrobial inhibition	21.0mm	25.0mm	21.3mm	21.0mm	5.0mm
PEG antimicrobial inhibition	5.0mm	24.4mm	20.0mm	4.0mm	26.0mm
Mox-PEG antimicrobial inhition	5.0mm	27.0mm	27.8mm	16.0mm	27.5mm
Mox-PEG inhibition difference	0.0mm	2.6mm	7.8mm	12.0mm	1.5mm
Mox-chit antimicrobial inhibition	26.0mm	27.2mm	27.4mm	17.0mm	25.0mm
Mox-chit inhibition difference	4.0mm	2.2mm	6.1mm	-4.0mm	20.0mm

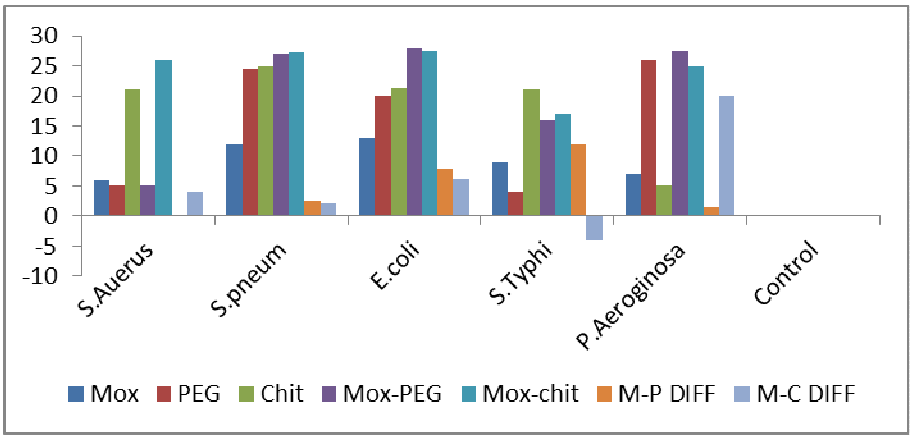


Figure IV: Bar chart showing antimicrobial inhibition and inhibition difference

5. CONCLUSION

From the characterization process using UV,IR,NMR, it was proven that the polymers can actually be conjugated with fluoroquinolones (moxifloxacin) and from the buffer hydrolysis and antimicrobial screening of the drug conjugate, it can be deduced that the drug conjugate actually have better antimicrobial activity than the parent drugs. Therefore it can be concluded that the conjugates were successfully characterized and have potential for use in the management of some gram positive and negative bacteria where extended release is useful.

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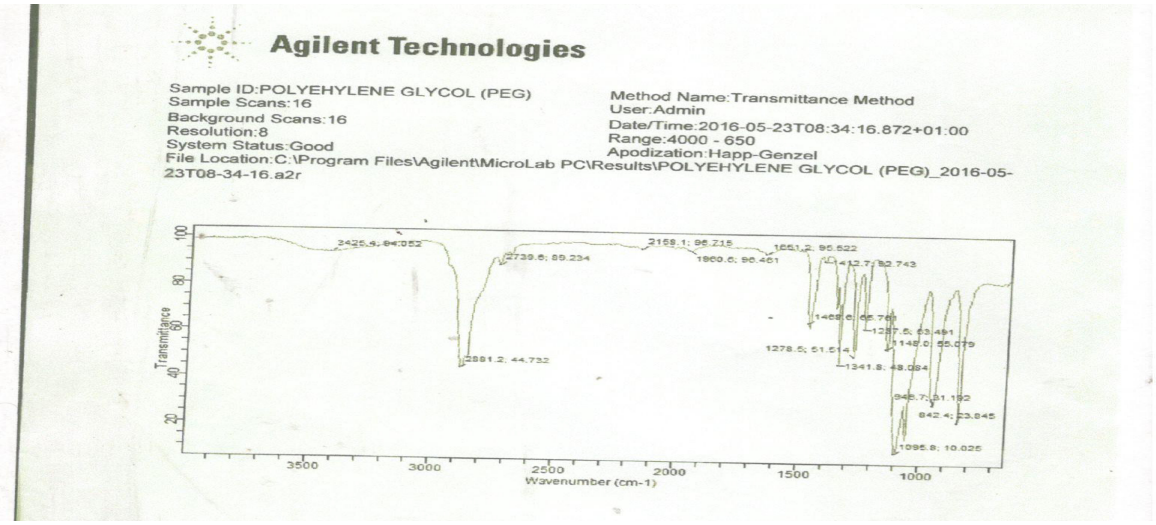
APPENDIX

Appendix A1: IR Spectra of PEG

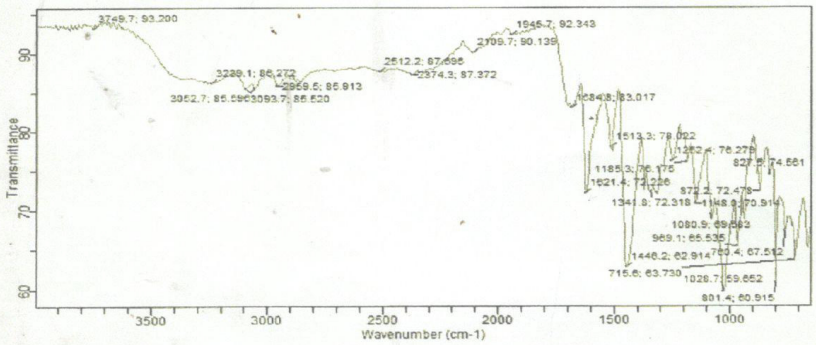


AppendixA3: IR Spectra of Moxifloxacin-PEG

Agilent Technologies

Sample ID:MOXIFLOXACIN + PEG
Sample Scans:16
Background Scans:16
Resolution:8
System Status:Good
File Location:C:\Program Files\Agilent\MicroLab PC\Results\MOXIFLOXACIN + PEG_2016-05-23T08-24-21.a2r

Method Name:Transmittance Method
User:Admin
Date/Time:2016-05-23T08:24:21.731+01:00
Range:4000 - 650
Apodization:Happ-Genzel



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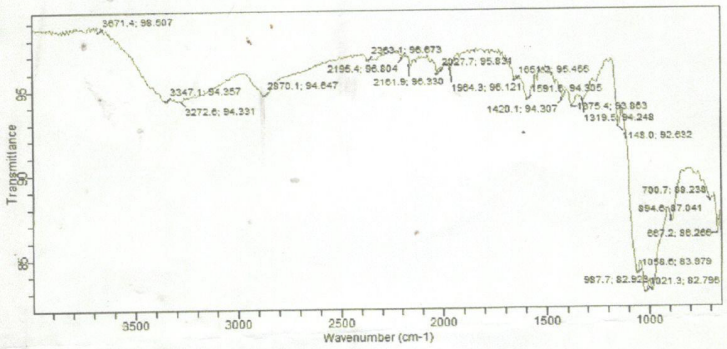
AppendixA3: IR Spectra of Chitosan

Agilent Technologies

Sample ID:CHITOSAN
Sample Scans:16
Background Scans:16
Resolution:8
System Status:Good
File Location:C:\Program Files\Agilent\MicroLab PC\Results\CHITOSAN_2016-05-23T08-29-51.a2r

Method Name:Transmittance Method
User:Admin
Date/Time:2016-05-23T08:29:51.656+01:00
Range:4000 - 650
Apodization:Happ-Genzel



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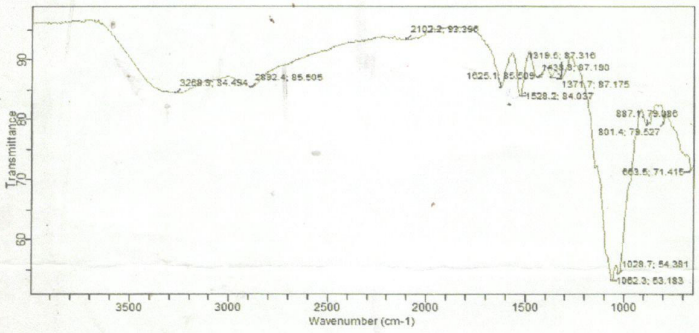
AppendixA4: IR Spectra of Moxifloxacin-Chitosan

Agilent Technologies

Sample ID:MOXIFLOXACIN + CHITOSAN
Sample Scans:16
Background Scans:16
Resolution:8
System Status:Good
Method Name:Transmittance Method
User:Admin
Date/Time:2016-05-23T08:26:52.832+01:00
Range:4000 - 650
Apodization:Happ-Genzel
File Location:C:\Program Files\Agilent\MicroLab PC\Results\MOXIFLOXACIN + CHITOSAN_2016-05-23T08-26-52.a2r



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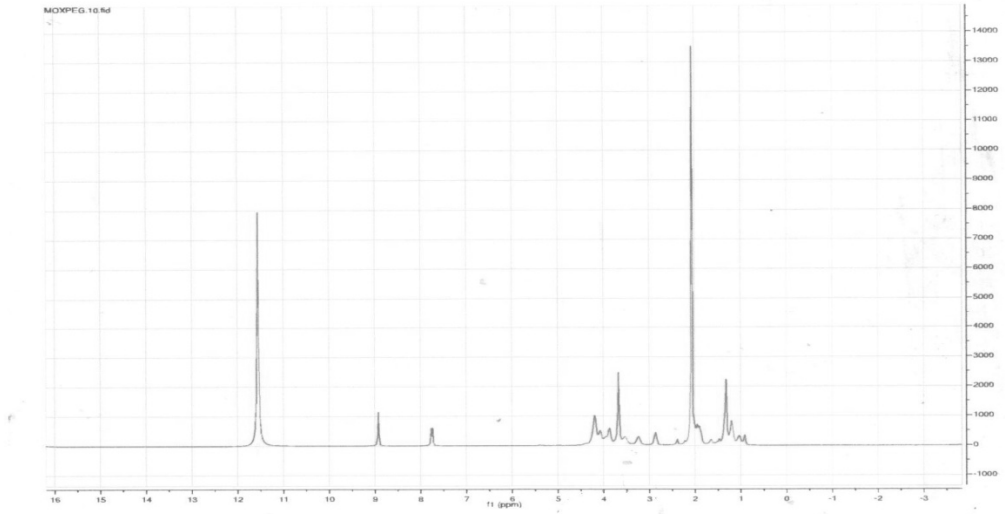
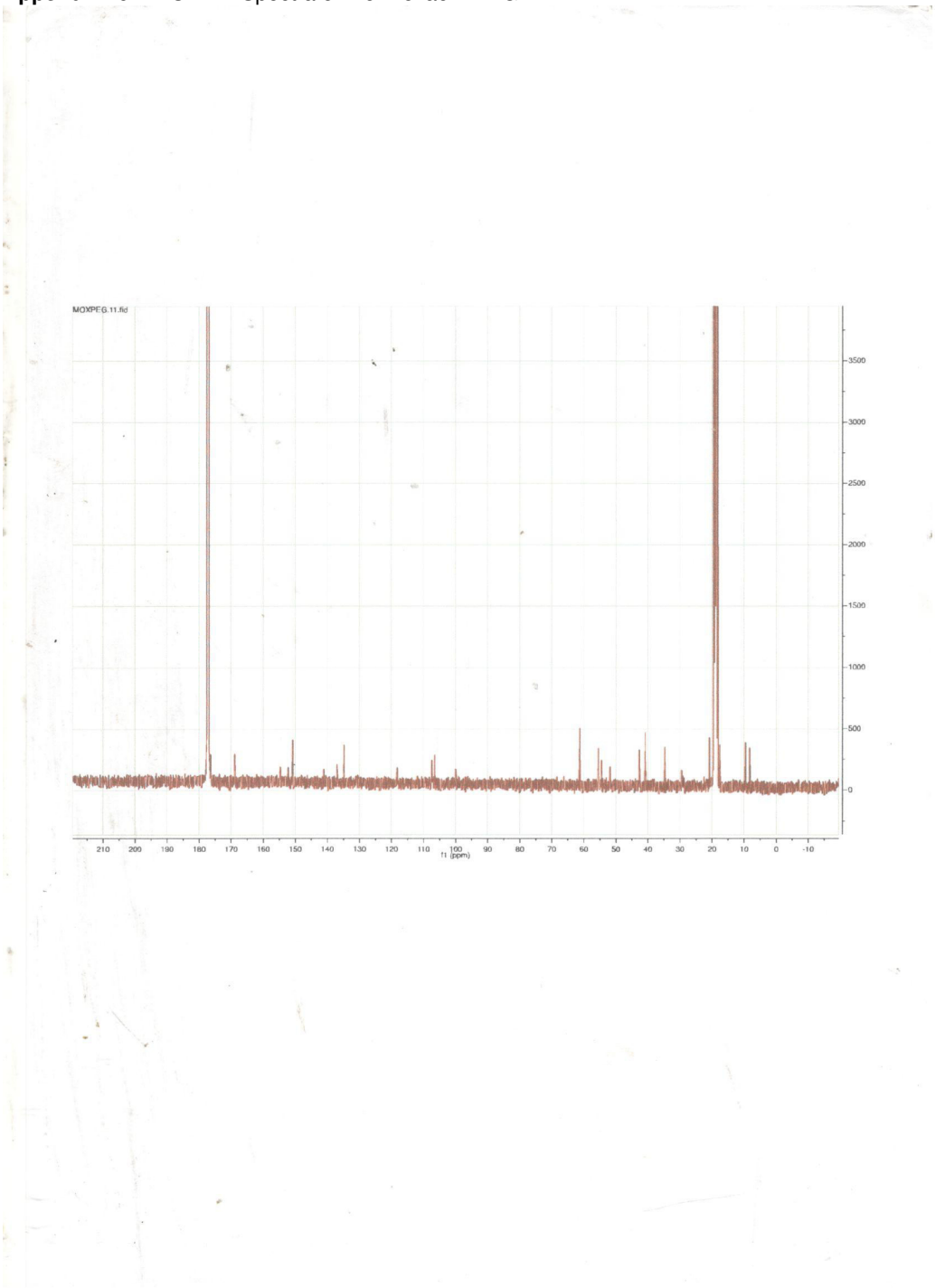
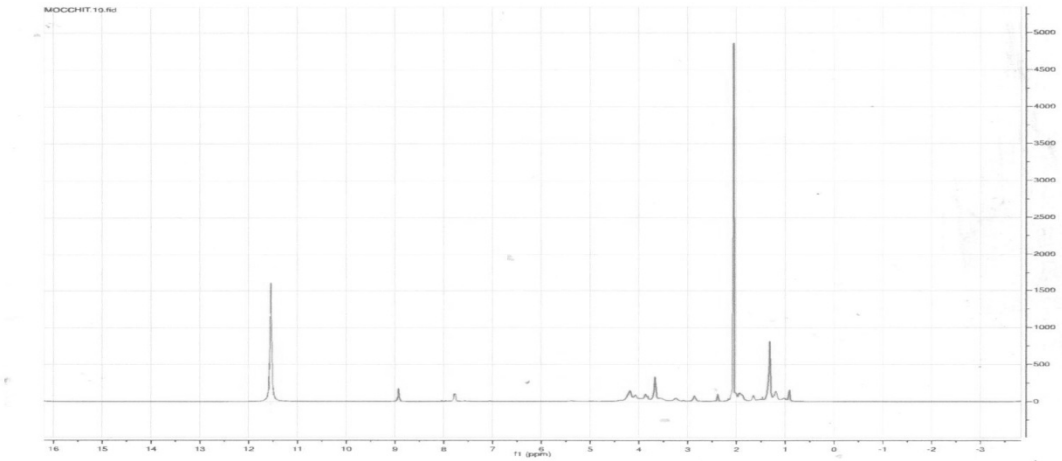
AppendixA5: ProtonNMR Spectra of Moxifloxacin-PEG



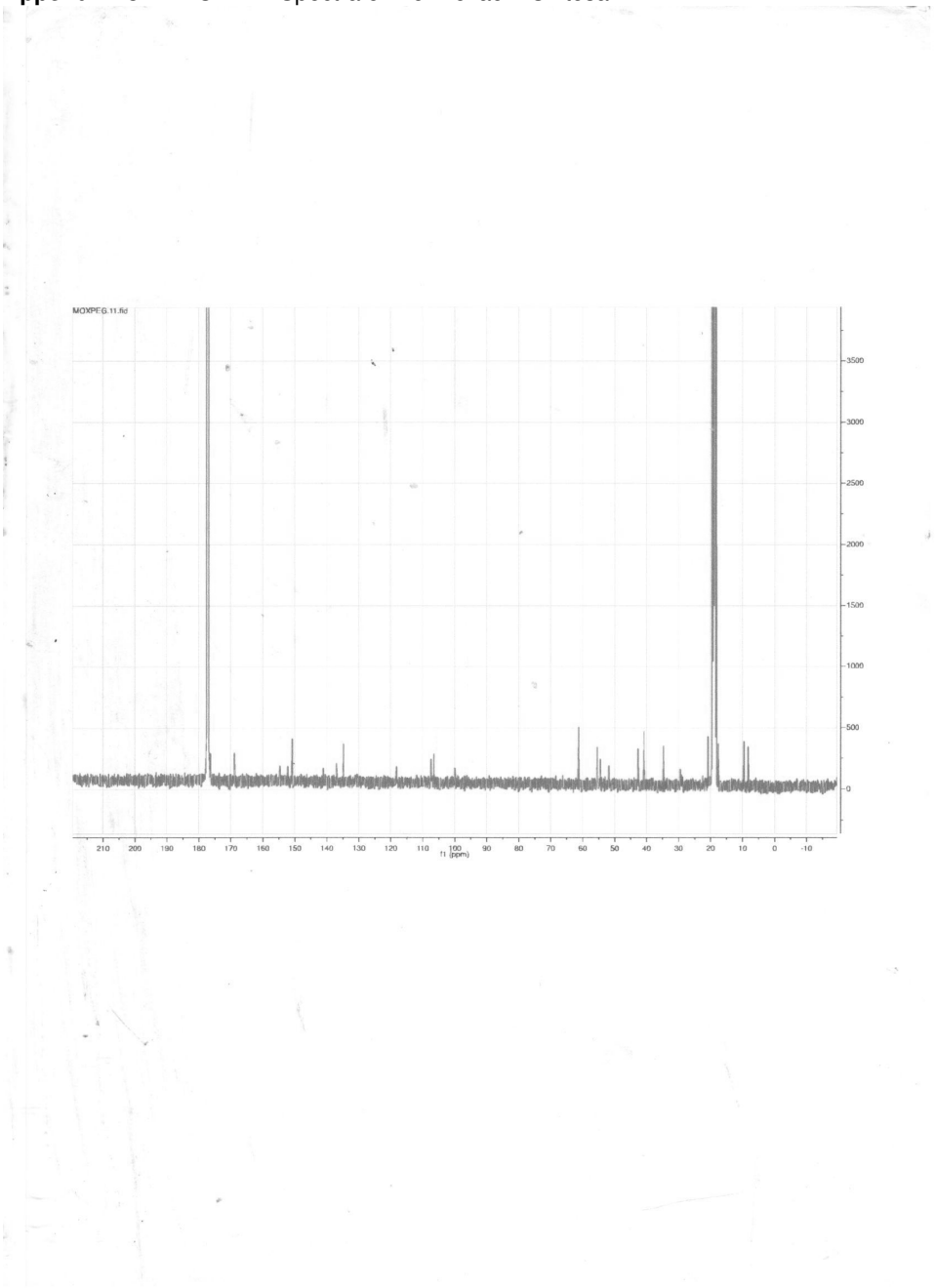
AppendixA6: ^{13}CNMR Spectra of Moxifloxacin-PEG



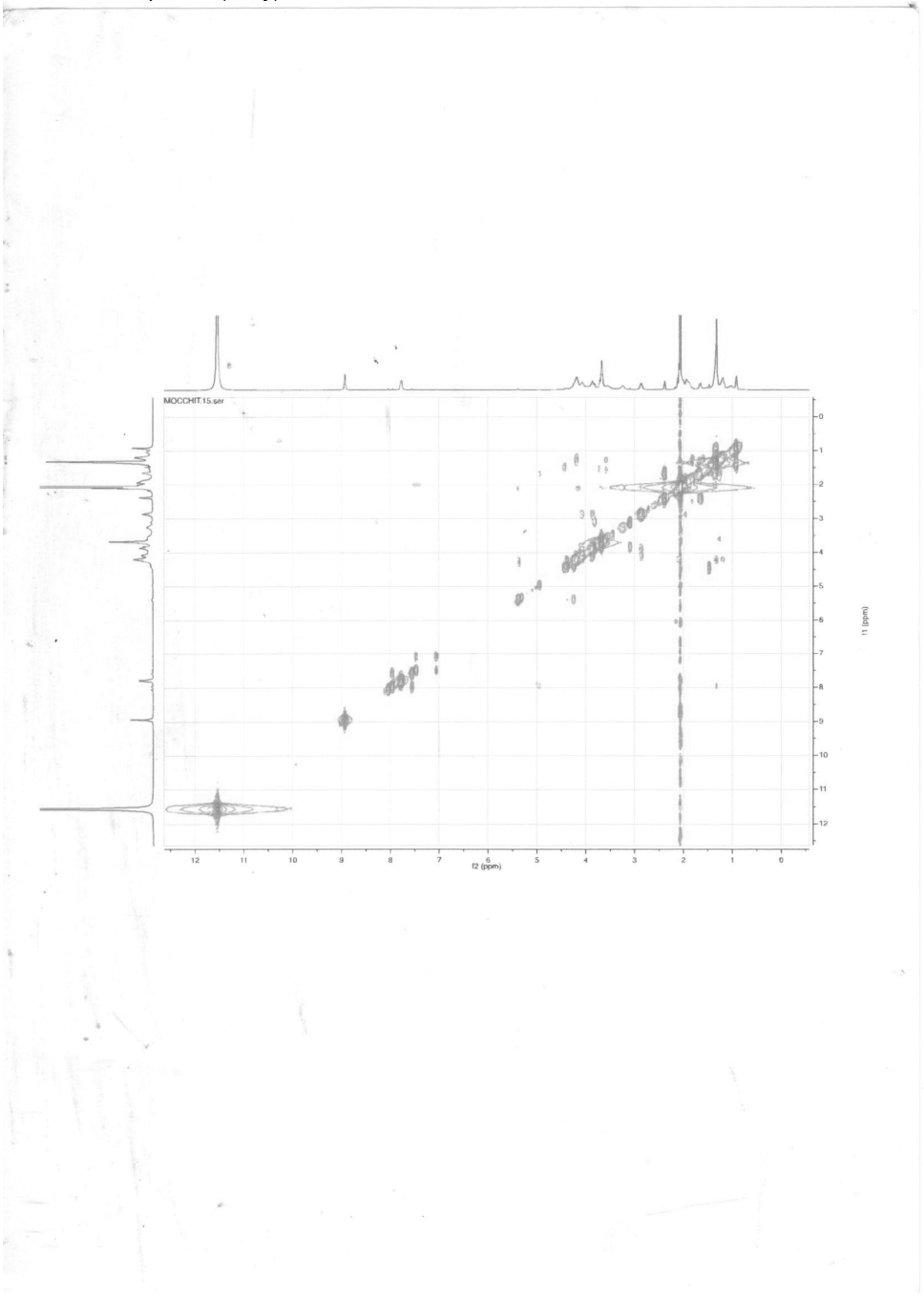
AppendixA7: Proton Spectra of Moxifloxacin-Chitosan



AppendixA8: ^{13}C NMR Spectra of Moxifloxacin-Chitosan



AppendixA9: NMR Spectra (cosy) of Moxifloxacin-PEG



AppendixA10: NMR Spectra (cosy) of Moxifloxacin-Chitosan