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PHYSICO-CHEMICAL AND ANTIMICROBIAL EVALUA TIONS OF CIPROFLOXACIN AND MOXIFLOXACINE CONJUGATES OF CHITOSAN AND POLYETHYLENE GLYCOL POLYMERS

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ABSTRACT

Objective: The objective of the project was to study the physico-chemical characteristics of the chitosan and polyethylene glycol polymer conjugates of ciprofloxacin and moxifloxacin. In spite of the strong activities of these drugs the experiment was to evaluate their activities in conjugated forms and to assess whether this will confer any special advantage. **Methodology:** The conjugates were prepared by esterification reactions. The drug-to carrier ratios were determined. The ester conjugates were characterised by melting point, UV, IR, solubility in different solvent systems after which they were subjected to stability tests (hydrolysis) in different buffers systems. Half-lives were also estimated. Microbiological evaluations of the conjugates were determined. **Results:** Evidence for ester formation was observed from the physicochemical changes indicated below. The UV absorption maxima for pure ciprofloxacin, moxifloxacin, chitosan and polyethylene glycol were 270 nm; 295 nm; 260 nm, 240 nm respectively while ciprofloxacin-chitosan and moxifloxacin-chitosan, ciprofloxacin- PEG and moxifloxacin-PEG were 300 nm, 270 nm, 280 nm and 300 nm respectively. The drug - to- carrier ratios were found to be: Cipro - Chitosan; 1.0 : 5.1; Moxi- Chitosan, 1.0 : 4.7; Cipro - PEG. 1.0 - 1.1 and Moxi - PEG, 1.0: 1.05. The infrared spectra showed characteristic absorption bands in the carbonyl region (1700cm^{-1} and 1800 cm^{-1}). The melting points for ciprofloxacin-chitosan, moxiflolxacin-chitosan, ciprofloxacin-PEG and moxifloxacin-PEG were 245-248^{o}C; 270 - 275^{o}C; 280-283^{o}C and 290-293^{o}C respectively. All the conjugates were insoluble in ethanol, diethyl ether, n-hexane and acetone but soluble or sparingly soluble in water, methanol, and glacial acetic acid, hydrochloric and sulphuric acids. Hydrolysis constants for all the conjugates showed linearity for between 0 and 8 hours and flexes into a plateau at 10 hours in phosphate buffered solutions of pH 6.1, pH 7.4 and pH 8.1. Corresponding regression equations were generated. The rates of hydrolysis of all the conjugates at pH 6.1 varied from 1.73 to 9.04 x 10^{-2} s^{-1}; at pH 7.4 the rate varied from 1.47 to 8.54 x 10^{-2} s^{-1} and at pH 8.1 the rate varied from 2.85 to 9.60 x 10^{-2}.s^{-1} The corresponding half-lives of hydrolysis varied from 5.1 to 40.8 hours; at pH 7.4 the half-life varied from 8.1 to 47.0 hours and at pH 8.1 the half-life varied from 7.2 to 24.3 hours. Antimicrobial activities of the conjugates against *Staph. aureus, Staph. pneumonia, E.coli, Salmonella typhi and Pseu. aeroginosa.* showed enhanced sensitivity of between 40 and 50%.

KEYWORDS: Polymers, Conjugates, Moxifloxacin and Ciprofloxacin.

INTRODUCTION

In the last two to three decades considerable interest in biodegradable biopolymers was the popular concept among Scientists and Researchers.[1] Since this development, various polymers have been adapted for drug delivery purposes whereby drugs target their sites of action at constant rate and with greater precision, minimizing side-effects.[2, 12, and 16] This therapeutically optimized dosage and rate of delivery represents great advancement in this field. Presently anticancer drugs have been the greatest beneficiary of this technique.[3, 13] In fact, polymeric nanoparticles have come into the fray.[4]

The first example of this biodegradable polymer was liposome, used as drug carriers instead of the previous dosage forms.[5] In addition to natural biodegradable polymers, synthetic polymers have been developed that have considerable advantages, most importantly, the options available for their use.[6] Great examples of synthetic polymers include polyvinylpyrolidone and polyethylene glycol or polyacrylate based on hydrogels. On the other hand natural polymers have greater advantage such as immunogenecity. Among the most used natural polymers are collagen, gelatin, chitosan, alginate, starch, pectin and casein derivatives.[7, 14]

Of these examples chitosan and polyethylene glycol (PEG) and derivatives have continually received a great deal of interest.[8,15]

In this investigation ciprofloxacin and moxifloxacin both of which are fluoroquinolone antibacterial agents with broad spectrum of activities were selected and conjugated with chitosan and polyethylene glycol. In spite of their strong antimicrobial activities and favorable pharmacokinetics, the experiment was to evaluate their activities in conjugated forms and whether this will confer any special advantages. These drugs act via inhibition of DNA gyrase enzyme.[9]

MATERIALS AND METHODS

1. Synthesis of chitosan- and polyethylene glycol (PEG) conjugates.

Pure ciprofloxacin powder was a kind gift from Prof. Ikoni. Ogaji (Department of Pharmaceutics & Pharmaceutical Technology, University of Jos, Nigeria) and chitosan from Mr Jabil Isah (Zolon Pharmaceuticals Nigeria, Plc).

Reagents include: Sulphuric acid (Farm Italia, Conloerba; Italy), sodium hydroxide and sodium hydrogen carbonate (BDH, UK); Ammonia solution (Wardle Chemicals, USA); Glacial acetic acid (Wardle chemicals, USA); Ethanol (BDH, UK); Chloroform (Merck, USA); Methanol (BDH, UK), Ethyl acetate (Farm, Italy; Toluene (BDH, UK). All reagents and solvents were of analytical grade and therefore not reprocessed.

In the synthesis of Conjugate, approximately 165.68 g (0.50 M) of ciprofloxacin was weighed and transferred to a solution of 190.80g chitosan (0.125 M) in a round bottom flask and stirred to mix properly; this was followed by 100.0 ml sulphuric acid (1.0 M) after which it was stirred again vigorously.

The mixture was refluxed for three hours. The product was an ester and was separated and washed with sodium hydrogen carbonate to remove any excess sulphuric acid; the ester was dried and recrystallized. The same procedure was adapted for ciprofloxacin-PEG, moxifloxacin-chitosan and moxifloxacin-PEG with appropriate adjustments to the weights and volumes. During the refluxing process, the progress of reaction was followed by Thin-Layer Chromatography (TLC). The products were finally purified by preparative TLC (methanol: chloroform: Glacial acetic acid; 1: 16: 3).

Recrystallization of the esters

The esters were soluble in methanol but insoluble in diethyl ether, therefore the compounds were recrystallized from diethyl ether - methanol solution. The crystals were washed and dried in a desiccator for later use.

Melting Point Determination

The melting point of the individual conjugates were packed in capillary tubes and placed in a melting point apparatus (Gallenkamp, UK) previously set at ambient temperature. The transition temperatures were recorded.

Infrared and Ultraviolet absorptions

Infra-Red spectrophotometer was used to detect the presence of ester bonds in the conjugates. Pellets of KBr in the conjugates were prepared and scanned between 600 cm^{-1} and 4000 cm^{-1}.

Determination of solubility profiles

Qualitative solubility tests on the conjugates were carried out in the following solvents: water, methanol, ethanol, glacial acetic acid, hydrochloric acid, sulphuric acid, acetone, diethyl ether, and n-hexane. A mass of 200 mg of each conjugate was transferred into beakers on a water bath maintained at 37 ± 1^{o}C. Approximately 20 mL of water was added to each beaker and stirred for more than 10 minutes. The conjugate was considered soluble if no crystals were left undissolved, i.e. a neat solution was formed. This was repeated for other solvents.

Development of UV Absorption maxima and calibration curve

A mass of 100 mg of each pure drug and their conjugates were dissolved in aqueous glacial acetic acid. The solutions were each transferred into 10 mL volumetric flasks (10 mg / mL). Each solution was scanned in the UV/ visible regions (200 nm to 800 nm) and the plots recorded. Beer-Lambert plots were generated by carrying out graded dilutions and absorbances at absorption maxima read against the concentrations; their correlation coefficients were calculated. The calibration curves were validated by standard protocols.

Determination of Drug-to-Carrier Ratio

The molar drug-to-carrier ratio for the various conjugates was determined by the method of Dai et al. (2014; DOI: 10.1038/srep05871). Essentially, the pure drugs (10 mg / mL) were individually dissolved in aqueous glacial acetic acid in 10 mL volumetric flasks and absorptions read at the individual UV absorption maximum, i.e. ciprofloxacin (270 nm); Moxifloxacin (295 nm); Ciprofloxacin-PEG (280 nm); Moxifloxacin- PEG (300 nm); Ciprofloxacin - chitosan (300 nm) and moxifloxacin - chitosan (270 nm). The UV absorbances of ciproloxacin-PEG, ciprofloxacin- chitosan; moxifloxacine- PEG and moxifloxacin- chitosan were read respectively at 280 nm; 300 nm; 300, and 270 nm. A mass of ciprofloxacin-PEG; moxifloxacin-PEG; ciprofloxacin-chitosan moxifloxacin-chitosan were individually diluted to known concentrations followed by hydrolysis for 60 minutes at 60^{o}C and absorbances recorded at individual absorption maximum. The mass of each drug in the conjugate was calculated relative to the mass of polymer, i.e. mDrug / mConjugate.

Determination of Rate of Hydrolysis

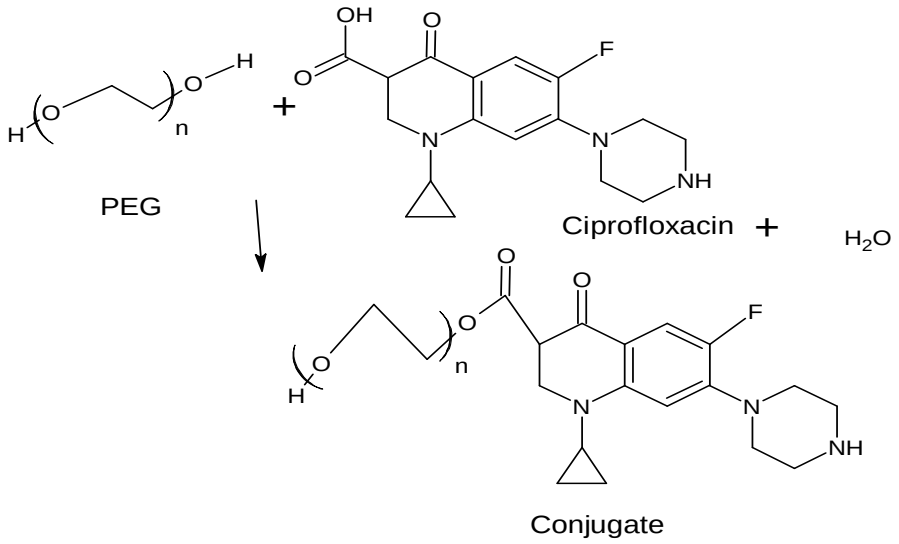
Hydrolysis or stability studies was carried out in phosphate buffers at pH 6.1; 7.4; pH 8.1 maintained at 37^{o} ± 1^{o}C. A mass of 100 mg of each conjugate, (i.e. ciprofloxacin-chitosan moxifloxacin - chitosan; ciprolfoxacin - PEG and moxifloxacin- PEG) were individually dissolved in sufficient methanol and made up to 10 mL in volumetric flasks (10 mg/mL final concentration). The content of each volumetric flask was turned over to 20 mL beakers on a water bath (37^{o} ± 1^{o}C). For example, aliquots of 1.0 mL of ciprofloxacin - chitosan were withdrawn at regular intervals of 1 hour for 12 hours. Each time 1.0 mL of the aliquot was removed, it was replaced by a fresh 1.0 mL solvent, thus maintaining a predictable concentration. Hydrolysis of each conjugate was investigated by determining how much of the drug had been released. This was repeated for other conjugates.

Antimicrobial studies

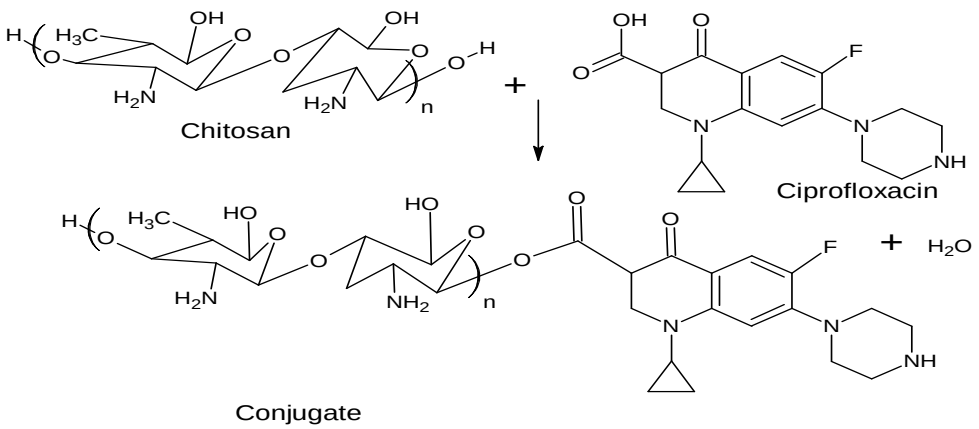
Nutrient agar was prepared and innoculated with the microorganisms according to established protocols.[10] The agar plate for each microorganism was incubated, first with pure drugs as positive controls, then with their conjugates; they were transferred to the incubator maintained at 37^{o}C for 24 hours. Zones of inhibitions were measured.

RESULTS AND DISCUSSION

Evidence for the successful synthesis of the conjugates was found in their melting points: Cipro - Chitosan 245^{o}C - 248^{o}C; Moxi - Chitosan, 270^{o}C - 275^{o}C; Cipro - PEG, 280^{o}C - 283^{o}C and Moxi - PEG, 290^{o}C - 293^{o}C: (See Table 1 below). UV absorptions were: Cipro - Chitosan, 300 nm; Moxi - Chitosan, 270nm, Cipro - PEG 280 nm and Moxi - PEG, 300 nm. FT-IR absorptions for all the conjugates ranged between 1700^{-1}cm and 1800 cm^{-1} all of which were different from their parent compounds. A typical reaction between ciprofloxacin, moxifloxacin with PEG and chitosan are illustrated in scheme 1a and b below.



(a)



(b)

Scheme I (a and b): Synthesis of Ciprofloxacin-, moxifloxacin with Chitosan and PEG conjugates respectively.

FTIR spectrosphotometry was used to confirm the success of the synthesis because of the absorption at 1720 cm^{-1} which was strong and sharp. The FTIR spectra of a typical ester is shown in Fig. 1

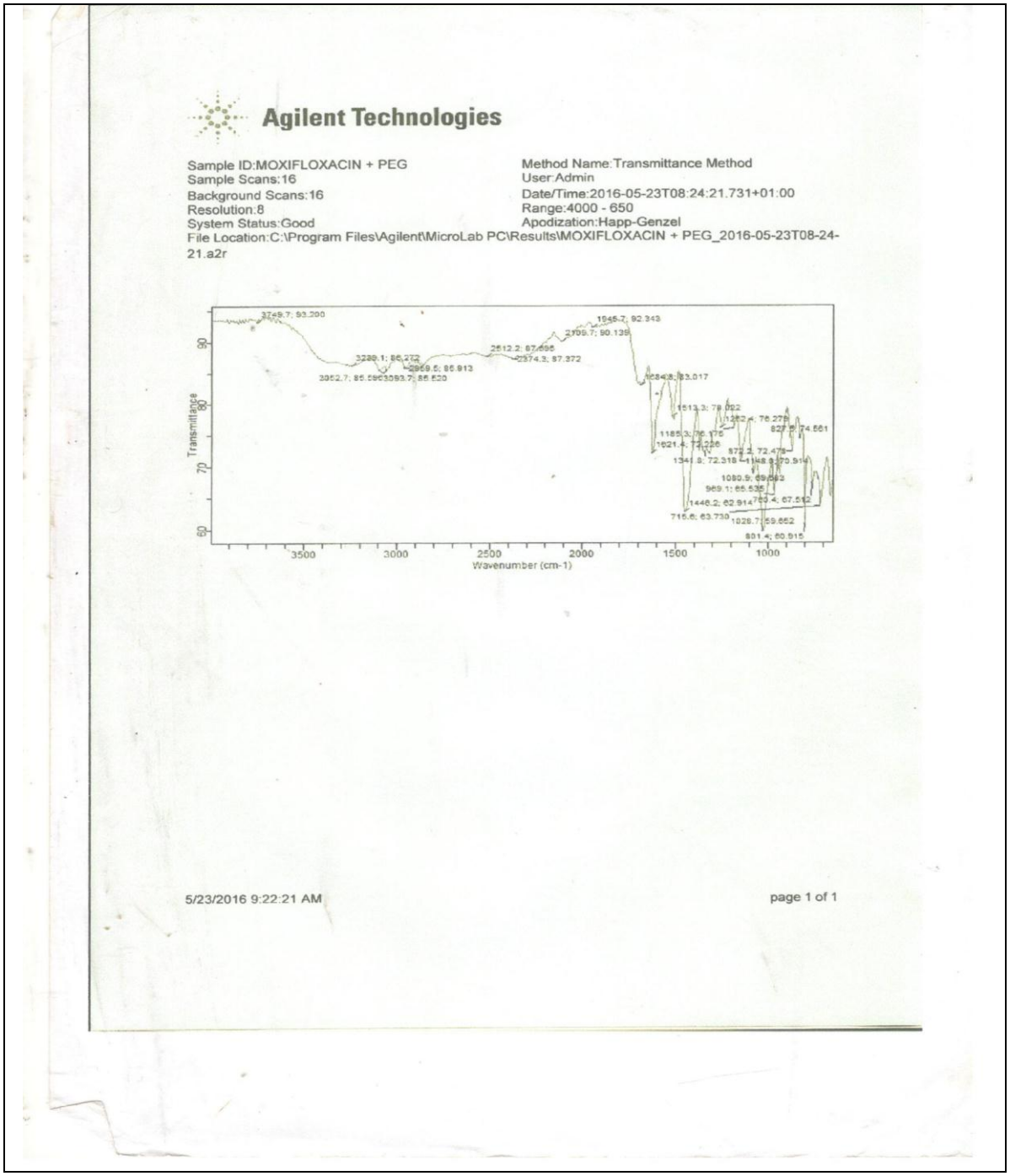


Fig. 1 : The typical FT-IR spectrum of a conjugate.

The differences in the melting points between the pure drugs and their conjugates further suggest that the synthesis was achieved (see Table 1). The lower melting points for the conjugate molecules might be due to increase molecular size and reduced electrical charges on the conjugate resulting from the reactions.

Table 1: Melting points of the drugs and their conjugates.

Drug/polymer Conjugates	Molecular weight (g/Mol)	Melting point ^{o}C
Pure Cipro	331.35	254-256
Pure moxi	401.43	240-242
Pure chitosan	1526.46	220-223
Pure PEG	4000.00	55-57
Cipro-chitosan	1839.81	245-248
Cipro-PEG	4313.34	280-283
Moxi-chitosan	1909.90	270-274
Moxi-PEG	4383.43	290-293

The UV absorption profiles of ciprofloxacin and moxifloxacin and their conjugates were as follows: pure ciprofloxacin and moxifloxacin absorbed maximally at 295 nm and 270 nm respectively. Similarly, PEG and chitosan respectively absorbed maximally at 240 nm and 260 nm. Their conjugates with ciprofloxacin and moxifloxacin absorbed maximally at 280 nm and 300 nm respectively. These absorption data were used to determine the rates of hydrolysis and the drug-polymer ratios.

From the absorption maxima, calibration curves were generated according to the following generalized equation:
$y = mx + c$
Cipro; $y= 0.2631x + 0$, $R^2= 0.9932$
Moxi, $y=0.2781x + 0$; $R^2 = 0.9871$
Their conjugates have the following regression equations:
Cipro-PEG: $y = 0.2762x + 0$, $R^2 = 0.9791$.
Moxi- PEG: $y = 0.2172x + 0$, $R^2 = 0.9843$
Cipro- chitosan: $y = 0.2301x + 0$, $R^2 = 0.9793$
Moxi-chitosan: $y = 0.1641x + 0$, $R^2 = 0.9804$.

Drug-to-polymer ratio

From the standard calibration curves for ciprofloxacin- and moxifloxacin- conjugates, the coefficients for ciprofloxacin and moxifloxacin were calculated. Further dilutions of the conjugates were prepared in series, i.e. 2.5 mg/mL; 5.0 mg/mL, 7.5 mg/mL and 10 mg/mL in volumetric flasks and their absorbances read-off. Combining this information with coefficients, the amount of each drug in the conjugate was calculated to be in the ratio of 1.0: 5.1 for between ciproflocacin and chitosan and 1.0: 4.7 between moxifloxacin and chitosan. Similarly, the ratios between ciprofloxacin and PEG and moxifloxacin and PEG were respectively 1:1.1 and 1: 1.05. The result for ciprofloxacin- and moxifloxacin-chitosan suggest that not all of the hydroxyl groups of chitosan were possibly esterified because of probable hindered positions of some hydroxyl groups in the polymer chain.

STABILITY STUDIES

The buffer hydrolysis (pH 6.1 to 8.1) of the polymer conjugates are shown in the figure (2 – 5). It was observed that over the 12 hours duration of experiment, the rate of hydrolysis proceed uniformly between 0 hours and 8 hours producing linear plots (See figures 2- 4 below). Further hydrolysis for the next four hours produced a plateau or stable hydrolysis. Similarly, hydrolysis of PEG-Conjugates in overall proceeded smoothly for the first 8 hours, but proceeded more slowly for the next four (4) hours, these figures, therefore illustrate the linear parts of the plots after regression. From the plots it could be observed that hydrolysis was fastest at pH 6.1 followed by pH 7.4. At pH 8.1 the rate was fairly constant over the next 4 hour period. Since physiologic pH is around 7.4, it could reasonably be assumed that administration of these conjugates could have beneficial effect when applied for delivery purposes. The advantages of this kind of drug delivery might be to circumvent the first-pass metabolic process and other transport processes. These reaction rates follow first order kinetics. The regression equations at different pH values are as follows: (pH 6.1) $y = 0.0904x + 0.624$ and $R = 0.9610$; (pH 7.4) $y = 0.0147x + 0.0072$; $R =0.9750$; (pH8.1) $y= 0.0648x- 0.0238$; $R = 0.9916$. The overall hydrolysis profiles of the chitosan conjugates follow the same pattern as that of PEG polymers, with hydrolysis proceeding at reduced rate at pH 7.4 and fastest at pH 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 of reaction as seen in table 3. The principle behind the buffer hydrolysis is the assumption that drugs in the systemic circulation employs esterases that act on the ester bonds of conjugates thereby releasing the drug from polymer for therapeutic activity. The activity of the esterase enzyme varies in different buffer pH, but it was much better at pH 7.4 which is about the normal physiological pH. These rates of reactions were used to calculate the half-lives, (Table 3), and the fact that the conjugate are freely soluble in dilute hydrochloric acid, suggest that pH 7.4 gave best hydrolysis medium. It could be inferred that since the physiologic pH hovers around 7.4, this drug conjugate could do well both as drug delivery and as a sustained release vehicle.

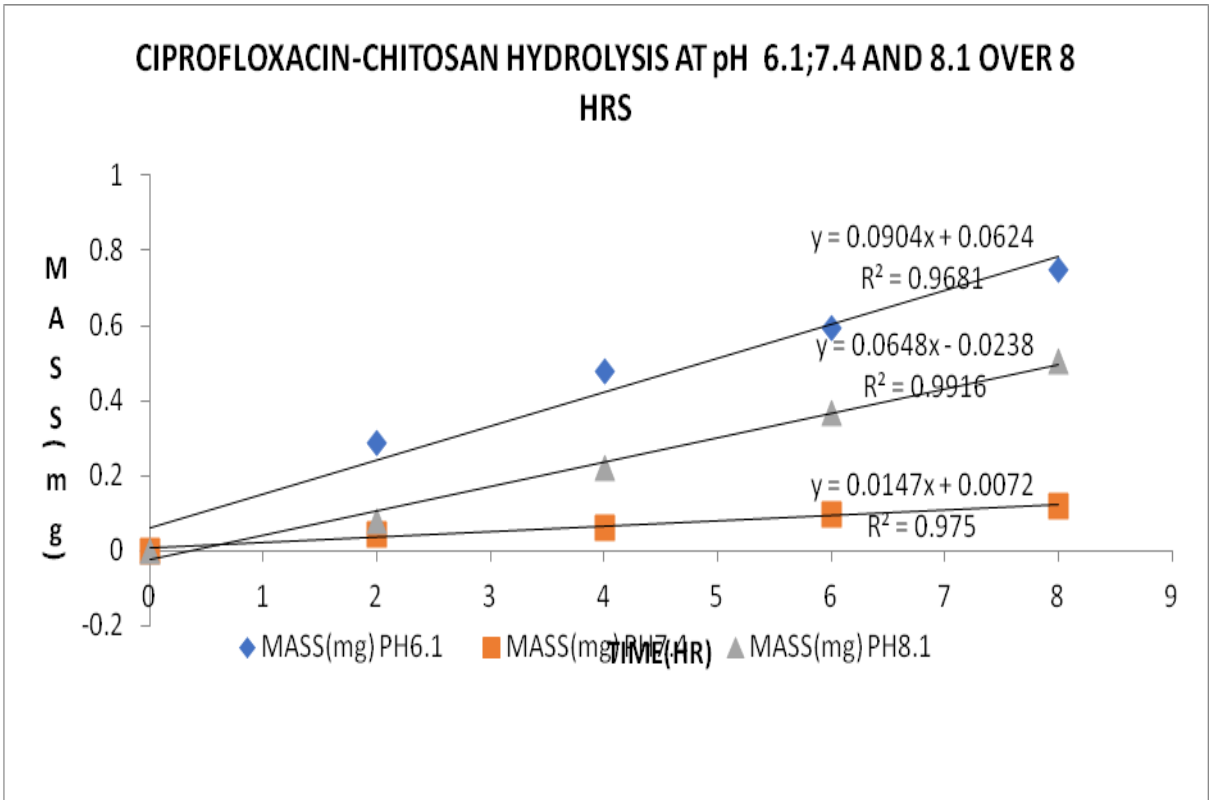


Figure 2: Ciprofloxacin-Chitosan Hydrolysis at pH 6.1, 7.4 and 8.1 over 8hrs.

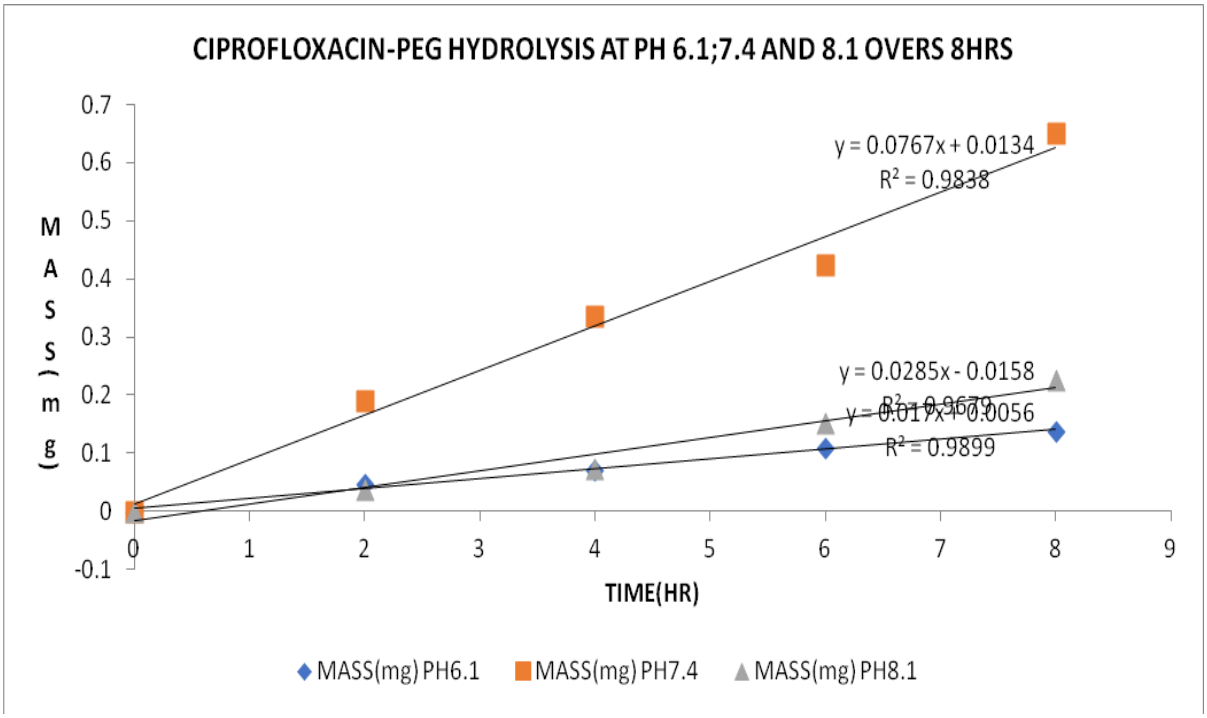


Figure 3: Ciprofloxacin-PEG Hydrolysis at pH 6.1, 7.4 and 8.1 over 8hr.

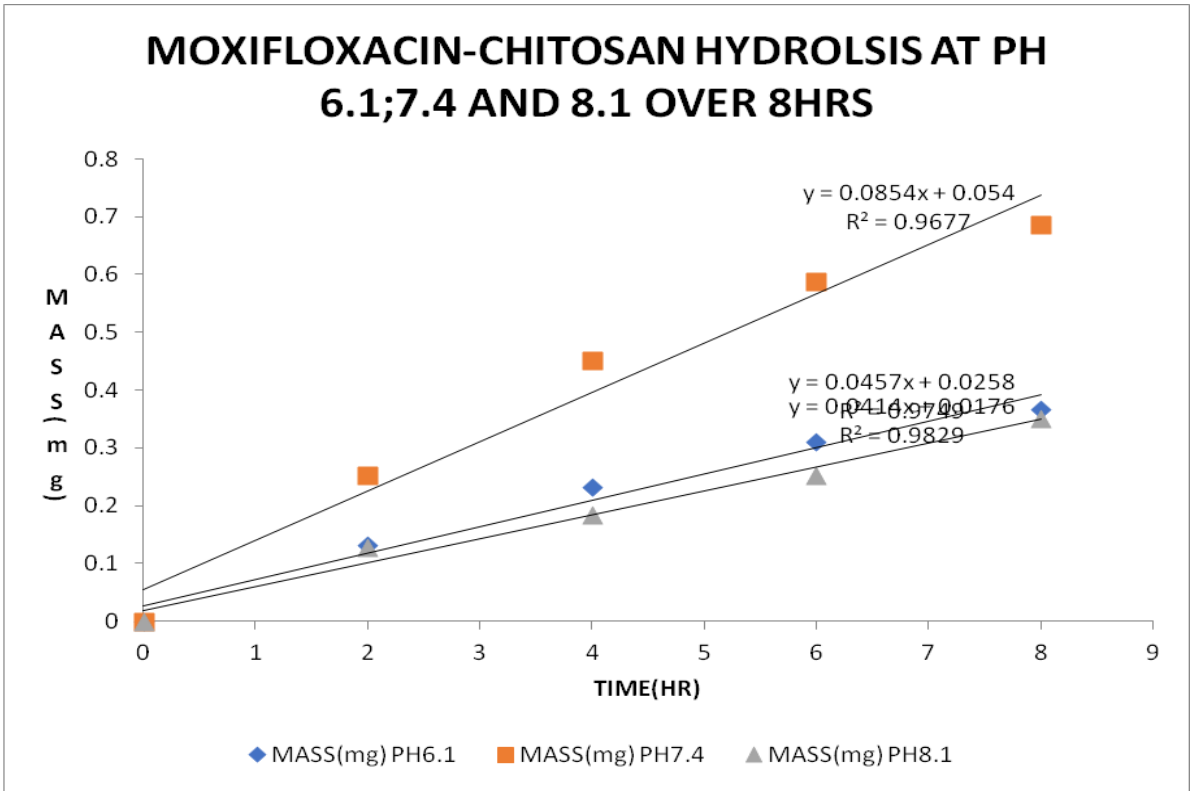


Figure 4: Moxifloxacin-Chitosan Hydrolysis at pH 6.1, 7.4 and 8.1 over 8 hr.

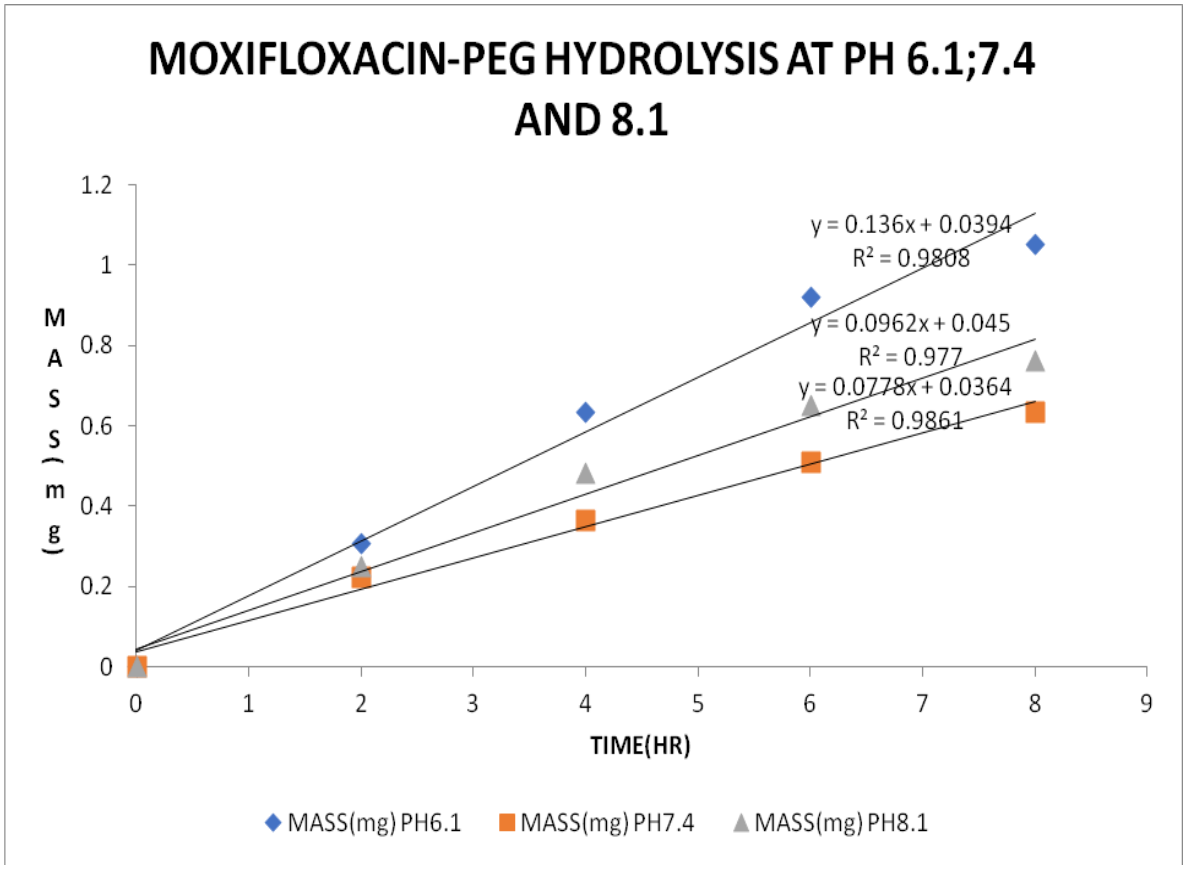


Figure 5: Moxifloxacin -PEG Hydrolysis at pH 6.1, 7.4 and 8.1 over 8 hr.

Table 2: Rates and Half-life for First Order Reactions.

	pH 6.1	pH 6.1	pH 7.4	pH 7.4	pH 8.1	pH 8.1
Drug conjugates	**Rate of reaction (mg/hr)**	**Half live (hrs)**	**Rate of reaction (mg/hr)**	**Half live (hrs)**	**Rate of reaction**	**Half live (hrs)**
Cipro-PEG	1.7×10^{-2}	40.8	7.6×10^{-2}	9.12	2.85×10^{-2}	24.3
Cipro-chit	1.36×10^{-2}	7.7	1.47×10^{-2}	47	6.48×10^{-2}	10.7
Mox-PEG	9.04×10^{-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

Antimicrobial studies

The antimicrobial activities of the conjugated compounds showed moderate increases relative to the unconjugated and pure drugs (Fig.6). From the bar chart it could be observed that moxifloxacin- conjugates show lower activity than the corresponding ciprofloxacin conjugates towards all the microorganisms studied. Over all, there are no significant advantages in conjugating these drugs, except for probable steady state release that might occur if administered.

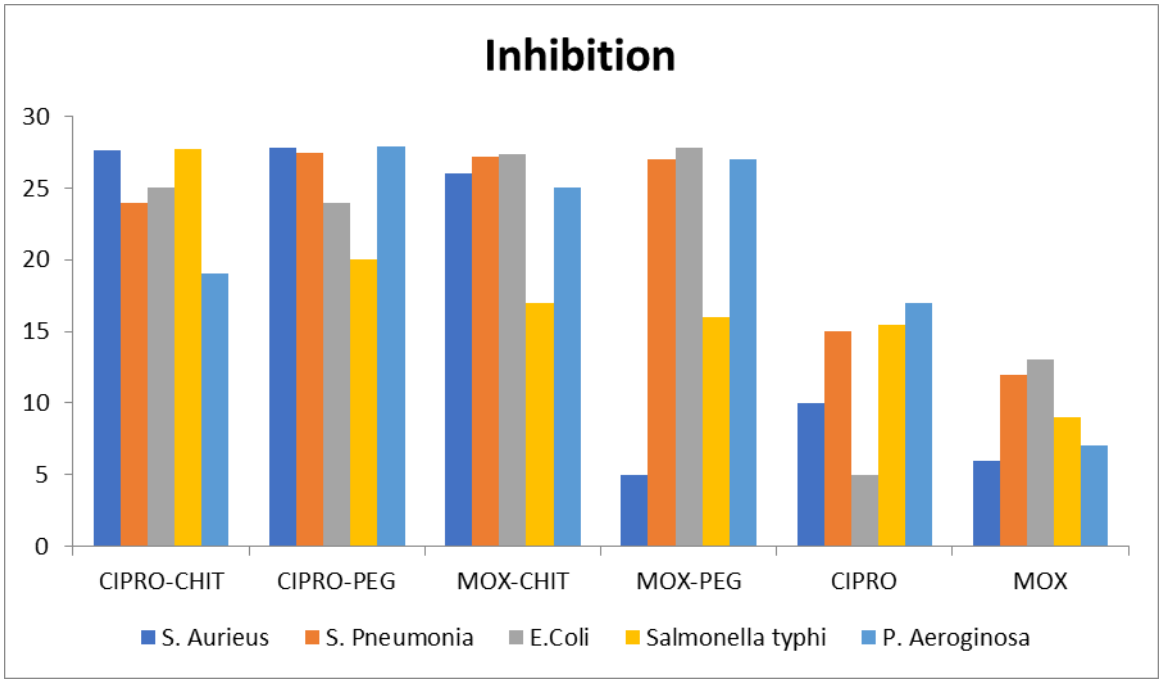


Figure 6: Bar chart showing drug and drug conjugates inhibitions.

CONCLUSION

The conjugates were successfully synthesized and characterized by the usual protocols. From the result of the buffer hydrolysis it was possible to calculate the drug-polymer conjugate ratios as a prerequisite for adaptation for use as delivery vehicle. The order and rates of reactions, half-live of the conjugates and other physico-chemicals studies reported here will serve as a pointer to the potential use for clinical applications.

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