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Polymeric drug based on sulfanilamide: synthesis, antimicrobial and drug releasing studies

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Abstract

N-((4-amino sulfonyl)phenyl)acrylamide (APA) was synthesized using sulfanilamide and acryloyl chloride in the presence of triethyl amine at 0–5°C. Homo- and co-polymerization of 2-hydroxyethyl acrylate (HEA) and acrylic acid (AA) were done by adopting a solution polymerization technique using methyl ethyl ketone (MEK) as a solvent and benzoyl peroxide (BPO) as a free radical initiator at 70 ± 1°C. All the monomers and polymers were characterized by IR and NMR techniques. These monomers and polymers were tested for their antimicrobial activity against five different ATCC strain microorganisms (*Escherichia coli* (25922), *Pseudomonas aeruginosa* (27853), *Klebsiella* (70063), *Salmonella typhi* (6539) and *Staphylococcus aureus* (25923)). The effect of co-monomer, other than the active drug moiety present in the polymeric drug, is discussed. The antimicrobial activity of APA on Gram-positive bacteria was enhanced when copolymerized with AA and HEA. The polymer was made into a film form and that film was used for drug releasing study. The drug releasing rate was monitored by the absorption at 268 nm using a UV spectrophotometer. The effect of pH and the temperature on the drug releasing rate was monitored and found that the releasing rate was dependent on the co-monomer, pH and temperature of the medium.

Introduction

The design and application of polymeric drugs for human health is an interesting field that is in continuous expansion and development because of intrinsic advantages offered by specific macromolecular systems in new and low-risk therapies. The use of polymeric systems with pharmacological activity provides very good local activity, reducing the toxicological risks, and in addition could act as a release system of the pharmacological active residue, controlled by chemical reactions, mainly hydrolytic, under enzymatic and non-enzymatic processes. These chemically modified drug conjugates are usually prepared by esterification, acrylation or alkylation. By attaching bioactive substrates to the synthetic or naturally occurring macromolecules, it is expected to increase therapeutic efficiency while lowering toxicity. The basic idea behind this type of approach is that the substrate molecules would undergo hydrolysis or enzyme-catalysed cleavage when introduced into the system (Harris 1984). Drugs that contain reactive functional groups, such as hydroxyl or carboxyl groups, or amino groups, can be covalently linked to a wide variety of polymerizable derivatives (Ibrahim Erol 2004).

The activity of an antimicrobial drug initially starts from attacking the cell wall of a microorganism. The effect of the drug on the lipid bi-layers has been described by Mouritsen & Jorgensen (1994). According to Bolard et al (1991), the activity of antibiotic drugs is mainly due to the ability of the drug to bind to the membranes, which in turn is controlled by the lipid composition. The activity of the drug was enhanced and reported by several research workers under the heading of amphiphilic drugs (Attwood et al 1974). A classical review by Seeman (1972) described various reports on the membrane actions of the pharmacologically active compounds. Several studies have further reported a controlled-release system with hydrophilic character for curing chronic diseases by co-polymerising a drug moiety with hydrophilic components (Davaran & Entezami 1997, 1998).

Generally, the activity of the polymeric drug is related to the functional group and the nature of the polymeric substance. According to Vogl & Tirrell (1979), the introduction of a hydrophilic, hydrophobic or polyelectrolytic moiety will enhance the activity of the drug on

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microorganisms. Also, it was observed that poly(methacrylic acid) has no effect on microorganisms like *E. coli*. Incorporating hydroxyl or carboxylic groups in the polymer backbone could in fact increase the hydrophilic character of the polymeric drug. Most of the synthesized polymeric drugs having high hydrophilic character were produced by co-polymerizing the drug conjugates with acrylic acid as a hydrophilic part (Roman & Madruga 1989; Roman & Levenfeld 1990; Arshady 1993; Boundreaux et al 1996; Thamizharasi et al 2002; Arun et al 2003). Most of the acrylic type of synthesized drug monomers and their polymers are pharmaceutically active compounds (Arshady 1993). Significant pharmacological activity is, however, seen if high-molecular-weight acrylic polymers are incorporated with active drugs (Liso et al 1995). The advantage of acrylic acid as a co-monomer in a polymer chain bearing an active drug moiety is its ability to assist the cleavage of amide or ester linkage through neighbouring group participation mechanism (Savage et al 1978; Foolandi 1979). These properties make such systems quite interesting and provide longer delivery times with lower dosages.

Nowadays, resistance to sulfa drugs is common in all parts of the world (Skold 2000, 2001). The antimicrobial activity of sulfanilamide is due to its structural analogy with *p*-aminobenzoic acid (PABA), which is involved in dihydrofolic acid synthesis. Sulfanilamide can competitively inhibit dihydropteroate synthesis by its structural analogy with PABA substrate. It can also function as an alternative substrate for dihydropteroate synthase (DHPS), forming a pterin adduct that cannot participate in the folate synthesis and ultimately diffuses from the cell. The idea behind our research work is to improve the antibacterial activity of sulfanilamide (without altering the basic structure and mode of action) by incorporating the hydrophilic co-monomer in the polymer chain, which already bears the pharmacologically active agent (sulfanilamide). Also, the co-monomer can be involved in the hydrolysis of the sulfanilamide from the polymer chain and thereby produces a sustained drug delivery system. The drug-releasing behaviour of the polymer sample was measured using UV visible spectroscopic technique.

Materials and Methods

Materials

2-Hydroxyethyl acrylate (HEA), triethylamine and acrylic acid (AA) were obtained from Fluka and used as such. Benzoyl peroxide (BPO) (Fluka) was purified by recrystallization using methanol–chloroform (1:1). Methyl ethyl ketone (MEK), methanol, dimethyl sulfoxide (DMSO) and acryloyl chloride were received from Aldrich Chemicals and purified according to standard procedure. Muller-Hinton broth and Muller-Hinton agar were obtained from Himedia and used as such. ATCC strains of Gram-negative bacteria (*Escherichia coli* (25922), *Pseudomonas aeruginosa* (27853), *Klebsiella* (70063), *Salmonella typhi* (6539)) and Gram-positive bacteria (*Staphylococcus aureus* (25923)) were obtained from the Christian Medical College (CMC; Vellore, India) and used for culturing the bacteria. FT-IR spectra of copolymer samples were recorded on Nicolet 20DXB (USA) using KBr pellets. ^{1}H and ^{13}C NMR spectra of the samples were run on Bruker CXP 320 MHZ in d_6-DMSO with TMS as an internal standard. UV-visible absorption measurement was conducted on a Schimadzu model 160A spectrophotometer. Molecular weights of the polymers were obtained using Shimadzu instrument (India) using an IR detector and THF as an eluent at a flow rate of 0.3 mL min^{-1}. Elemental analysis was done on a Coleman C-H-N analyser.

Synthesis of N-((4-amino sulfonyl)phenyl) acrylamide (APA)

Sulfanilamide (8.61 g, 0.05 mol) and 200 mL of MEK were taken in a 500-mL three-necked round-bottom flask fitted with a stirrer and thermometer. To this solution, 7.25 mL (0.05 mol) of triethylamine was added. The resulting solution was kept cool in an ice-bath for half an hour. Acryloyl chloride (4.22 mL, 0.05 mol) was dissolved in 25 mL of MEK and taken into a pressure equalizer. To this cooled solution, acryloyl chloride was added drop by drop at 0–5°C for about half an hour. After completion of the addition, the reaction mixture was kept at room temperature with constant stirring for 3 h. The mixture was then filtered and the quaternary ammonium salt was removed. The organic filtrate was washed three times with 50 mL distilled water to remove the unreacted reactants. The aqueous layer was removed and the organic layer was dried in a beaker containing anhydrous sodium sulfate for about 10 min. The resultant liquid was evaporated to get a crude acrylated product. The crude product was recrystallized using methanol. Yield = 10.26 g. Melting point = 135–137 °C. IR (cm^{-1}): 3476 (NH), 3050 (aromatic CH), 1674 (amide C = O), 1629 (CH = CH), 1595 (aromatic CH = CH), 1195 ($S(=O)_2$), 834 (1,4-substitution of benzene). ^{1}H NMR (δ, ppm): 10.5 (s, NH), 7.9–8.2 (m, Aromatic protons), 6.5 (t, vinylic CH), 6.0 (s, NH_2) and 5.8 (d, vinylic CH_2). ^{13}C NMR ((δ, ppm): 118 (vinylic CH_2), 126 (vinylic CH), 128, 131, 138, 141 (aromatic) and 163 (ester C = O). Elem. Anal. Calcd. for $C_9H_{10}N_2O_3S$: C, 47.78%; H, 4.45%; N, 12.38%. Found: C, 47.82%; H, 4.42%; N, 12.35%.

Synthesis of poly(APA)

A free radical solution polymerization technique was used for preparing the homopolymers. APA (1.13 g, 0.005 mol), 10 mL of MEK and 0.023 g (2 wt%) of BPO were taken in a polymerization tube. The mixture was flushed with a slow stream of oxygen-free nitrogen for about 15 min. The tube was tightly sealed and immersed in a thermostatic water bath for 24 h at 70 ± 1°C. The homopolymer was precipitated using excess methanol. The precipitate was filtered off and dried in vacuum at 30°C for 12 h. Yield = 1.02 g. IR (cm^{-1}): 3328 (NH), 3082 (aromatic CH), 2939 and 2912 (aliphatic CH), 1594 (aromatic CH = CH), 1332 and 1181 (SO_2), 833 (1, 4-substitution of benzene). ^{1}H NMR (δ, ppm) 5.9 (NH-SO_2), 7.9–8.2 (m, Ar-H), 10.5 (Ar-NH), 1.2–1.5 (m, CH and CH_2 of polymeric backbone).

Synthesis of poly(APA-co-AA)

APA (0.905 g, 0.004 mol) and 0.29 g (0.004 mol) of AA, 10 mL of MEK and 0.024 g (2 wt%) of BPO were used for the

synthesis of poly(APA-co-AA). Yield = 0.88 g. IR (cm^{-1}): 3231 (NH and OH), 2921 (aliphatic CH stretch), 1684 (amide C = O), 1643 (acid C = O), 1410 (OH bending of acid), 1320 (SO_2), 940 and 980 (acid stretching), 840 (1,4 substituted benzene) and 700 (aromatic CH, out of plane bending). ^{1}H NMR (δ, ppm): 5.9 (s, NH-SO_2), 7.9–8.2 (m, Ar-H), 10.5 (s, Ar-NH), 11.5 (s, OH) 1.2–1.5 (m, CH and CH_2 of polymeric backbone).

Synthesis of poly(APA-co-HEA)

APA (0.905 g, 0.004 mol) and 0.464 g (0.004 mol) of HEA, 15 mL of MEK and 0.027 g (2 wt%) of BPO were used for the synthesis of poly(APA-co-HEA). Yield = 0.94 g. IR (cm^{-1}): 3349 (NH and OH), 1732 (amide C = O), 1693 (ester C = O), 1598 (Ar-C = C), 1361 (SO_2), 1148 (C-O stretching) and 796 (substituted aromatic CH, out of plane bending). ^{1}H NMR (δ, ppm): 5.9 (s, NH-SO_2), 7.2–8.2 (m, Ar-H), 4.3 (s, OH), 3.3–3.6 (m, O-CH_2) and 1–1.5 (m, CH and CH_2 of polymer backbone).

Drug susceptibility test

The drugs were tested by a disc-diffusion method. Diluted bacterial cultures (100 μL) were spread on sterile Mueller-Hinton agar plates, after which 8-mm-diameter discs (sterile blank) impregnated with drug to be tested (100 μg) were placed on the plates. The plates were incubated for 24 h at 37°C under aerobic conditions and the diameter of the inhibition zone around each disc was then measured and recorded. If the drugs were found to be active in the disc-diffusion test (inhibition zone > 10 mm), they were further evaluated for determining minimum inhibitory concentration (MIC) values.

Minimum inhibitory concentration (MIC)

The drugs were screened for their antibacterial activity against *E. coli, P. aeruginosa, Kelbsiella, S. typhi* and *S. aureus*. MIC was evaluated by turbidity method. A loopful of bacteria was inoculated in 100 mL of nutrient broth at 37°C for 20 h in a test-tube shaker at 150 rev min^{-1}. The test compounds were prepared by dissolving in a minimal volume of DMSO and were serially diluted in Mueller-Hinton broth at concentrations in the range of 1–100 μg mL^{-1}. The 24-h bacterial cultures were then transferred into 10 mL of Muller-Hinton broth (control and test compounds) and incubated at 37°C for 24 h. The growth of the bacteria was determined by measuring the turbidity after 24 h. Thus, the MIC was generally read as the smallest concentration of drug in the series that prevents the development of visible growth of test organism. All the experiments were done in triplicate.

Statistical analysis

The MIC value and the drug releasing rate were measured in triplicate. Statistical analysis of the MIC value was performed using the unpaired Student's *t*-test. Differences were considered significant when $P < 0.01$.

Preparation of film

The drug sample (200 mg) was dissolved in a minimum quantity of the DMSO. The resulting solution was placed into a film forming glass plates. This glass plate was dried in vacuum at 30°C for 24 h. The film was peeled from the glass plate and the thickness of the film was measured using an electronic screw gauge. The thickness of the film was 100–200 μm. This film was used for the controlled drug delivery studies.

In-vitro drug release study

The in-vitro drugs release pattern was studied at different temperature (37 and 40°C) and at different medium pH (7.4 and 9.2). Pieces (1–1.5 cm^2) of co-polymer films (100–200 μm thickness) were taken and soaked in 10 mL of phosphate-buffered solutions of pH 7.4 and 9.2. Five millilitres of the solution were periodically collected for analysis and replaced by the same volume of fresh medium. The amount of APA in the medium was determined using UV spectroscopy absorption at $\lambda_{max} = 268$ nm. These measurements were performed by a UV/visible detector systronic system. The cumulative drug released was plotted against the time intervals. All the experiments were done in triplicate.

Results and Discussion

A novel APA monomer carrying an active drug moiety was synthesized, at high purity, using sulfanilamide and acryloyl chloride (Figure 1). Co-polymerization was done using AA and HEA with a view to increasing the interaction between the polymer chain to that of the cell wall of microorganism (Figure 2). All the monomers and polymers were characterized by IR and NMR techniques. The average molecular weight of polymers was around 6000 g mol^{-1} (Table 1). The polydispersity values of the polymers were < 2, quantitatively suggesting that the polymer was terminated by disproportion method, which was typical of acrylates.

Antimicrobial activity

The MIC values (average of triplicates) of the sulfanilamide, APA monomer, poly(APA), poly(APA-co-AA) and poly(APA-co-HEA) are shown in Table 1. The effect of the co-monomer in the polymer chain on the antimicrobial activity was investigated by comparing the MIC values of poly(APA), poly(APA-co-AA) and poly(APA-co-HEA). The results showed that the presence of hydrophilic co-monomer in the polymer chain enhanced the antimicrobial activity on the Gram-positive bacteria. The activity of poly(APA-co-AA) against *S. aureus* was found to be very high. The MIC value (3 μg mL^{-1}) was higher than, or equal to, the MIC value of linezolid (Ford et al 1999) (a new oxazolidinone class of compound), which is in early phase III of human clinical trials. Besides having a similar MIC value, the synthesis of poly(APA-co-AA) was very simple, making it a good competitor for the oxazolidinone class of compound. But, in the case of Gram-negative bacteria, the antimicrobial activity was

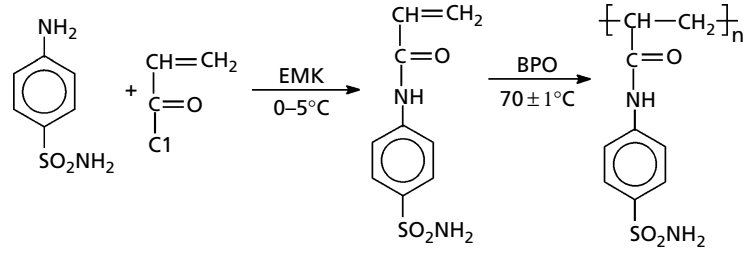
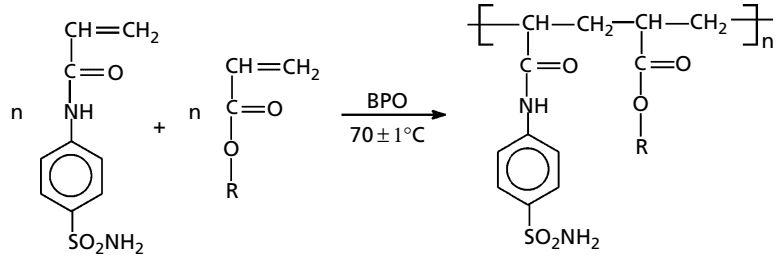


Figure 1 Synthesis of APA and poly(APA).



Where,

R = H or CH_2CH_2OH

Figure 2 Monomeric units present in the co-polymer chain.

Table 1 Molecular weight and MIC (μg mL^{-1}) value of APA, poly(APA), poly(APA-co-AA) and poly(APA-co-HEA)

Compound	*E. coli*	*S. aureus*	*P. aeruginosa*	*Klebsiella*	*S. typhi*	MW × 10^3	$M_n \times 10^3$	MW/M_n
Sulfanilamide	12.7 ± 0.27	24.1 ± 1.33	12.5 ± 0.86	12.9 ± 0.40	25.4 ± 1.08	—	—	—
APA	13.4 ± 0.16	13.1 ± 0.34	6.3 ± 0.42	11.7 ± 0.66	12.0 ± 0.44	—	—	—
Poly (APA)	12.9 ± 0.22	12.2 ± 0.48	6.3 ± 0.66	6.7 ± 0.14	5.7 ± 0.37	4.86	3.04	1.60
Poly(APA-co-AA)	13.0 ± 0.25	3.1 ± 0.17	6.7 ± 0.52	13.1 ± 0.98	6.0 ± 0.50	6.39	3.51	1.82
Poly (APA-co-HEA)	11.9 ± 0.56	6.3 ± 0.59	6.9 ± 0.18	11.5 ± 0.27	12.8 ± 0.14	6.08	3.67	1.66

MIC was calculated using three values.

found to be unaltered or decreased (Table 1) when the active drug moiety was co-polymerized with AA or HEA. Differences in the cell wall constituents of microbes might have brought about such a variation in the co-monomer effects between the two types of strains (Prescott et al 2002). In the case of Gram-negative bacteria, the remarkable observation lies in the MIC value of *P. aeruginosa. P. aeruginosa* is considered to be a highly resistant species among the Gram-negative bacteria (Normark & Normark 2002). Several studies have demonstrated the low susceptibility of *P. aeruginosa* towards both hydrophobic and hydrophilic antibiotics (Nikaido 2000, 2001). The MIC value of poly(APA-co-AA) over *P. aeruginos*a, although found to be high (6.7 μg mL^{-1}) considering the intrinsic resistance of the bacterium, represented a considerable advantage. Besides, the high hydrophilic nature of the poly(APA-co-AA) chain bearing the active drug moiety provided a considerable advantage for effective penetration through the outer membrane of *P. aeruginos*a, thereby efficiently inhibiting the folic acid synthesis.

The antimicrobial property of co-monomer AA in poly(APA-co-AA) is not apparent and can be ruled out as poly(AA) showed absence of any antimicrobial activity in similar systems (Vogl & Tirrell 1979). This provides a clear picture that the enhancement of antimicrobial activity against *S. aureus* of poly(APA-co-AA) over that of poly(APA) was due to the availability of active drug moiety (sulfanilamide) adjacent to the cell wall of the microorganism or the opening up of the cell wall to allow the active drug moiety to penetrate through and interfere with the folic acid synthesis. With regards to the first condition, we feel that the availability of the drug adjacent to the cell

wall was increased due to the polymeric chain forming a cage-like structure around the microorganism.

Such structures could be stabilized by both ionic and hydrogen bonding between the polymeric chain and the cell wall of the microorganism (Bolard et al 1991). As for as the second possibility, cell wall opening can be triggered by the ionic strength and the hydrophilic nature of the medium adjacent to the cell wall of the microorganism (Arshady 1993; Roman & Madruga 1989). In poly(APA-co-AA), the co-monomer AA was involved in increasing the ionic strength of the medium adjacent to the cell wall of the microorganism. It appears from the results that these two factors operate simultaneously to increase the activity of poly(APA-co-AA) over that of poly(APA) and poly(APA-co-HEA). The co-monomer AA acts as an anchor to deliver the active drug moiety through the above-said two mechanisms.

Effect of co-monomer on *S. aureus*

When we compared the activity of the poly(APA-co-AA) with that of poly(APA-co-HEA) on Gram-positive bacteria, the former was twice as active. This might be attributed to the greater ionic strength of AA than HEA, which enhances the antimicrobial activity considerably. Moreover, the AA present in the poly(APA-co-AA) can engage in neighbouring group participation mechanism for the detachment of active drug moiety from that of the polymeric chain (McCormick 1985). This also will add to the evidence that poly(APA-co-AA) is a very active polymeric drug when compared with poly(APA) and poly(APA-co-HEA) towards Gram-positive bacteria.

Controlled release studies of copolymers

The drug release pattern for polymer carrying AA and HEA as a co-monomer is shown in Figures 3 and 4. This showed that the rate of release of active drug moiety from the polymer backbone was enhanced when the pH of the medium was changed from 7.4 to 9.2 (Arun & Reddy 2005). This might be due to the fact

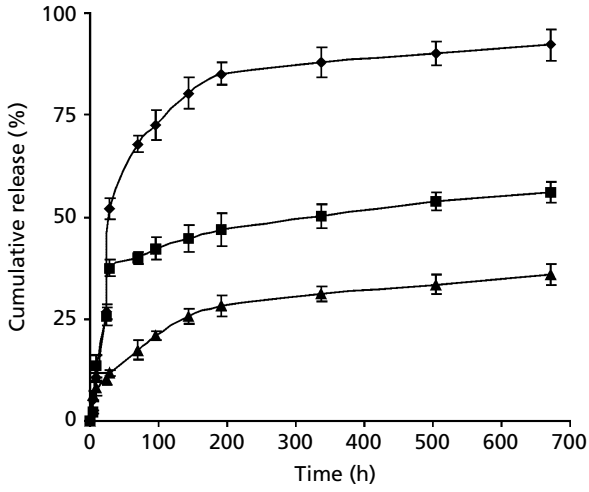


Figure 3 Effect of pH and temperature (diamonds = pH 9.2 at 37°C, squares = pH 7.4 at 40°C and triangles = pH 7.4 at 37°C) on the drug releasing rate of copoly(APA-AA) film (average ± s.d., n = 3).

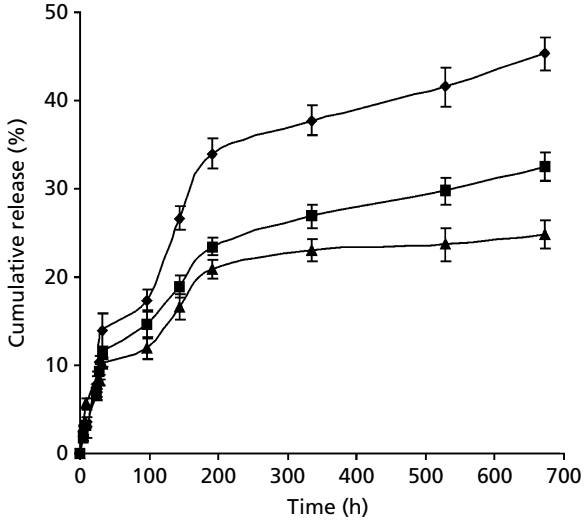


Figure 4 Effect of pH and temperature (diamonds = pH 9.2 at 37°C, squares = pH 7.4 at 40°C and triangles = pH 7.4 at 37°C) on the drug releasing rate of copoly(APA-HEA) film (average ± s.d., n = 3).

that in slightly alkaline pH (pH 7.4), the amide linkage that connects the drug to the polymer backbone is resistant to hydrolysis. The drug releasing graph clearly indicates that the releasing pattern was not uniform in nature. This may be due to the irregularity in the drug releasing pattern shown by the polymeric film. Earlier studies have reported a similar type of irregularity in the release of the drug and this can be overcome by changing the type of testing sample from the film type to granular type (Sharkawi et al 2005). Since we were studying a releasing pattern at different temperatures, we intended to go for the film type rather than granular type of testing sample. The drug releasing pattern in the acidic medium was not studied here since the amide linkage showed more stability towards hydrolysis (Gallardo et al 2001) in acidic conditions. As the basicity of the medium increased, the amide linkage was more prone to hydrolysis and released the active drug moiety to the surroundings (Elvira et al 2001) at a faster rate. The other factor that was responsible for the higher release rate of the drug was the presence of the aromatic system of the drug over an aliphatic system. This sort of aromatic system, exhibiting a faster release rate than an aliphatic system, has been observed by many previous research workers (Gallardo et al 2001). The hydrolysis of the amide bond in the basic medium was reported earlier (Mikes et al 1974; Gallardo & Sanroman 1993; Elvira et al 2001). We observed a slight increase in the release rate of the drug when the temperature was changed from 37 °C to 40 °C at pH 7.4. This is due to the fact that the temperature increases the chain mobility, which in turn allows the water molecules to penetrate through the polymer matrix to enhance the hydrolysis rate of the system. This sort of chain mobility, responsible for the enhancement of releasing rate and the effect, may be more pronounced when the polymer sample was in film form. This type of pH responsiveness has major advantages — one of the most important being sustained oral drug delivery provided by the pH difference between the stomach and the intestine (Davaran et al 1999).

From the comparative graph (Figure 5), it is evident that the polymer sample made up of AA released the drug at a

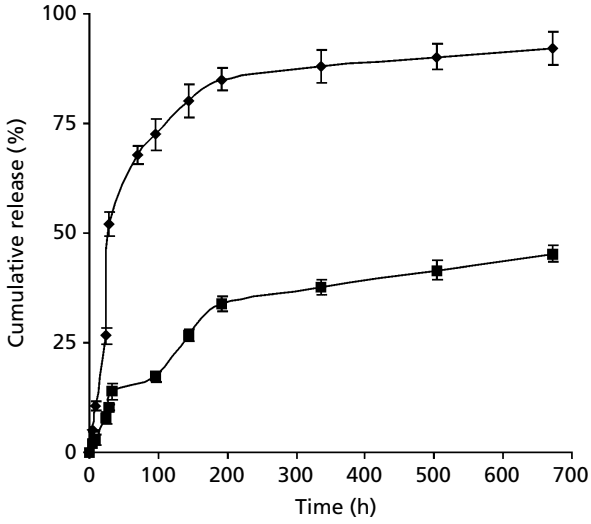


Figure 5 Effect of co-monomer type (diamonds =AA, squares = HEA) on the drug releasing rate from the polymer film (pH 9.2 at 37°C) (average ± s.d., n = 3).

faster rate than the HEA. This is due to the fact that the AA in the polymer back bone assisted the hydrolysis through the neighbouring group participating mechanism (McCormick 1985) and this type of assistance was absent in the case of polymer bearing HEA as a co-monomer. No doubt the co-monomer HEA present in the polymer chain was involved in increasing the swelling of the polymer matrix, thereby allowing the water particles to participate in the hydrolysis of the amide bond, but the effect was comparatively lower than that of AA, which effectively participated in the neighbouring group participation mechanism.

Conclusions

An attempt was made to increase the antimicrobial activity of the conventional drug sulfanilamide without altering either its basic structure or its mode of action by the polymerization process. The APA monomer was synthesized using sulfanilamide and acryloyl chloride. Co-polymerization of APA was done using two different monomers, AA and HEA. All the monomers and polymers were characterized by IR and NMR techniques. All the monomers, APA homopolymer and poly(APA-co-AA) and poly(APA-co-HEA) co-polymers were tested for their antimicrobial activity with five different microorganisms (*E. coli, P. aeruginosa, Klebsiella, S. typhi* and *S. aureus*). We found that the difference in the MIC values of five different bacteria was due to the difference in their cell-wall constituents.

Also, we observed that the polymer carrying the co-monomer AA and HEA apart from the active drug moiety (sulfanilamide) showed enhanced activity towards *S. aureus*. Remarkably, *P. aeruginosa* also showed less resistance towards the polymeric drug. This was due to a cage-like structure formation and increase in ionic strength of the medium that acted between the polymeric chain and the cell wall of the microorganism. Even though several other authors previously reported these two types of mechanisms, this is the first time that we have reported this methodology to increase the antimicrobial activity of sulfanilamide. The drug releasing pattern of the polymer film was monitored for 4 weeks using UV/visible spectroscopic technique. The drug releasing graph suggests that the co-monomer, pH and the temperature of the medium affect the releasing rate of drug from the polymer film. We also found that the rate of release increased upon increasing the pH and temperature of the medium. Also, an enormous difference in releasing behaviour was observed between poly(APA-co-AA) and poly(APA-co-HEA).

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