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Stability and release of antiviral drugs from ethylene vinyl acetate (EVA) copolymer

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Abstract The use of polymer based drug delivery systems in dentistry is a relatively new area of research with the exception of the inhibition of secondary caries by the release of fluoride ions from polyalkenoate cements and their predecessors silicate cements. The present study was to test on orally biocompatible material, ethylene vinyl acetate copolymer (EVA), for release of antiviral drugs at oral therapeutic levels over extended periods of time. We also determined their stability during film casting and release. Materials studied include gancyclovir (GCY), acyclovir (ACY), dichloromethane (DCM), and ethylene vinyl acetate (EVA). The square films ($3 \times 3 \times 0.1$ cm) were prepared from the dry sheet obtained by solvent evaporation of polymer casting solutions. These solutions were made of EVA and the drug (40:1) in 70 ml of dichloromethane at 38°C. Then drug release characteristics from the drug loaded films were examined at 37°C for a minimum of 14 days in 10 ml medium (ddwater) replaced daily. Kinetics of drug release were followed by spectral measurements using previously determined λ_{max} values (GCY = 250 nm; ACY = 253 nm). A minimum of three samples was tested and reproducible results were obtained. Drug stability (ACY) during film casting and its release was determined using ^{1}H NMR spectrometer (Bruker DRX-500 and 400). Rate of drug release was determined from the part of the curve (rate vs. time) after the onset of the "burst." Although GCY has a larger molecular weight (255) than ACY (225), GCY exhibited about three times higher rate of release than ACY. This difference in rate values may be explained due to its relatively greater solubility in EVA, facilitating faster diffusion of the molecules through the channels present in EVA. This is consistent with the observation that the rate at which drug molecules diffuse through the channels of the polymer, can be increased by decreasing the molecular weight. In the case of ACY, the molecules may be undergoing molecular associations, perhaps dimerization or trimerization in addition to its lower solubility in EVA. The diffusion of ACY tends to be slower under these circumstances compared to GCY resulting in lower rate value than in the case of GCY. Biological studies revealed that ACY exhibited a remarkable decrease in a number of viral organisms present in virus infected cell culture system using real-time polymerase chain reaction (RT-PCR). NMR analysis indicates that the chemical structure of the drug remains stable during film casting process and release.

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Introduction

An increasing incidence of immunosuppressive-associated oral diseases, including those associated with HIV provided an opportunity to implement appropriate interventions. The observation has been made that oral viral infections and lesions may develop throughout HIV/AIDS progression, both by infection and reactivation. These oral viral infections and lesions often add to the morbidity of the primary illness, and the complex environment of the mouth presents a formidable challenge for the selective control of these pathogenic viruses.

These organisms are inhibited by ACY which competitively inhibits the viral DNA polymerase after phosphorylation by viral thymidine kinase and cellular kinases. In cell culture ACY has antiviral potency against HSV1 and 2, VZV,

EBV, HHV8 and CMV (listed in decreasing order of potency) [1]. Traditionally, these infections have been treated systemically not locally.

Although the dental literature contains numerous publications on the general topic of oral drug delivery, very few have discussed this with reference to the application of drug delivery systems. The use of polymer based drug delivery systems in dentistry is a relatively new area of research, with the exception of (a) the inhibition of secondary caries by the release of fluoride from polyalkenoate cements and their predecessors silicate cements and (b) promoting bone growth [2, 3]. Recently, certain types of composite filling materials, the so-called "compomers" [4], some orthodontic adhesive resins [5] and a few methacrylate based copolymer systems [2] were reported to release fluoride ions in order to reduce dental caries. The delivery systems have not been so far extended to include the incorporation of antiviral agents and are currently limited to subgingival delivery. The current study was undertaken to overcome some of the existing problems.

The main objective of this study was to determine whether a biocompatible ethylene vinyl acetate (EVA) copolymer could release drugs at therapeutic levels (effective concentrations) at a constant rate for prolonged period of time. In this study, using proton NMR technique, we also examined whether or not the released drug from the drug-loaded films remained physically and chemically unchanged during the film casting process. Finally, we investigated the effect of drug impregnated EVA on viral load present in a virus infected cell culture system using quantitative polymerase chain reaction (PCR).

Materials and methods

Preparation of polymer thin films

Materials used in the study include non-inflammatory and bio-compatible EVA copolymer were obtained from Du Pont, Wilmington, DE (Elvax; Grade 140 Wg), acyclovir (ACY) and ganciclovir (GCY) from Sigma Chemical Company, St. Louis, MO, and dichloromethane (Cl_2CH_2) from Mallinckrodt Baker Inc. Spctr AR, Paris, KY. Drug loaded EVA polymer films with 2.5% wt% drug impregnated in EVA copolymer were prepared according to the method used in our earlier studies [6, 7].

Measurement of drug release

A volume of 10 ml distilled water or water-ethanol system was used, depending on the solubility of the drug under examination, to collect the drug release daily. Fresh samples of 10 ml of the media were used daily for at least 14 days and the concentration of the drug concentrations were followed by measuring the optical density (OD) spectrophotometrically (Beckman Du® −70 Spectrophotometer). The value of λ_{max} for GCY (250 nm) and ACY (253 nm) were determined by spectral measurements from 220 nm to 400 nm. Using standard plots between drug concentration and optical density (OD), decreases in drug concentration were determined each day [6, 7]. A minimum of three drug loaded samples was used at each temperature including 37°C. Effect of temperature on the rate of drug release was studied in order to determine "energy of activation" association with diffusion of drug molecules through the channel present in the matrix. Drug loaded polymer square films (30 mm × 30 mm × 1 mm) were cut from dry films.

Proton NMR

Proton NMR (^{1}H NMR) measurements were performed using 400 MHz Bruker Spectometer. Individual spectrum ACY alone and the corresponding spectrum for the drug released from EVA were measured in D_2O. The spectrum for EVA background has been subtracted from the latter. Similar measurements were made with reference to GCY analysis using 500 MHz spectrophotometer.

Cell lines used

B-958 cells, which are Epstein-Barr Virus (EBV) latently infected B cells, were maintained in RPMI media supplemented with 10% fetal bovine serum. 12-O-tetradecanoylphorbol-13-acetate (TPA) induction was used to mimic lytic infection as previously described [8, 9].

Sample collection and DNA extraction

Samples were collected, and cells were spun at 400 × g. Approximately 1 mL supernatant was removed and spun at 14,000 × g for 1 hr prior to DNA extraction from the viral pellet. The cells in the remainder of the sample were counted and spun at 4000 × g for 1 hr before DNA extraction from the cell pellet. To isolate DNA from samples, the DNeasy kit from Qiagen, Valencia, CA, was used per manufacturer's instruction.

Real-time polymerase chain reaction (RT PCR)

Real Time PCRs were run in 25 μL reactions for both EBV viral DNA and β-actin, a cellular housekeeping gene. For EBV, reactions included 12.5 μL Taqman Master Mix, 2.5 pmol EBV forward primer, 2.5 pmol EBV reverse primer, 20 pmol EBV probe, and 50 ng DNA template. For β-actin, reactions

included 12.5 μL Taqman Master Mix, 15 pmol forward and reverse primers, 5 pmol β-actin probe and 50 ng DNA template. All reactions were done in duplicate. Reactions were performed at 95°C for 10 min then 40 cycles of: 95°C for 15 sec, 60°C for 1 min using an ABI Prism 7000.

Statistical treatment

Two-way analysis of variance (ANOVA) was applied to rate data transformed to the log scale to achieve approximate normality and variance homogeneity. Temperature and medium were considered as one variable, and drug as the second variable. Since the interaction was not statistically significant ($p = 0.15$), main effects of drugs and temperature/medium combinations were evaluated at the 0.05 significance level. If a main effect achieved overall statistical significance, pairwise comparisons among the levels of the main effect were conducted with statistical significance defined as a p-value less than $0.05/3 = 0.016$ to make a Bonferroni adjustment for multiple comparisons.

Results

Kinetics of drug release

Figure 1 represents a typical time release profile for ACY showing reproducibility and the commonly observed initial "burst" at a short interval of time followed by a pattern of constant drug release at longer interval. Values of percent "initial burst" of drug to the total drug incorporated for both GCY and ACY were shown in Table 1. The "initial burst"

Table 1 Values of percent initial burst to the total drug incorporated and the rate of drug release with respect GCY and ACY at 37°C

Drug	Average of molecular Weight	Percent initial burst to the total drug Incorporated	Rate of release (μg/cm^2/Day)
GCY	255	5.4	2.1
ACY	255	1.5	0.75

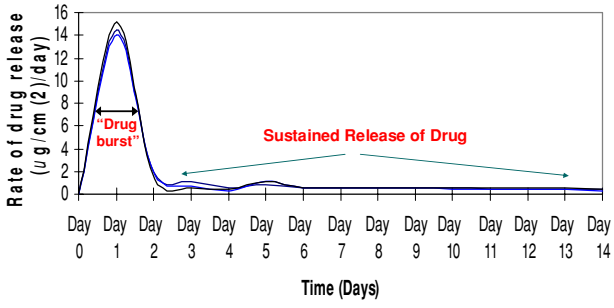


Fig. 1 Acyclovir Drug Release Profile at 37°C over time in ddH_2O

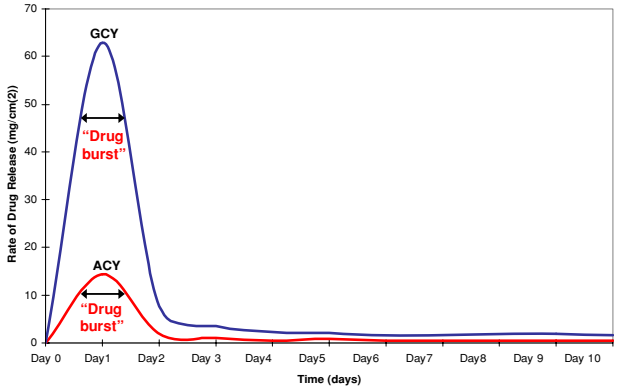


Fig. 2 A comparison between release profiles of GCY and ACY at 37°C with an initial burst of drug followed by a constant rate of drug release pattern

represents a sudden release of surface-bound drug molecules. The values of constant release rates at 37°C for a minimum of 14 days for GCY and ACY were determined and were also shown in Table 1.

Figure 2 represents a comparison between release profile of GCY and ACY at 37°C with an initial "burst" followed by a constant rate of drug release pattern. GCY exhibited about three times higher rate of release than ACY (Table 1).

Figure 3 Arrehius plot obtained for ACY between rate of release at the four different temperatures 32°C, 37°C, 42°C and 47°C versus $1/T$ in water as an extracting medium (Table 2).

Energy of activation ($\Delta E^{\neq}$) is characteristic of drug release process and determines the influence of temperature on the rate of drug release. $\Delta E^{\neq}$ for the diffusion process of ACY using water as extracting medium was determined from the slope of Arrehius plot [log(rate of drug release) verus $(1/T)$] using the Eq. (1).

$$\log k = \log A - \Delta E^{\neq}/(2.303 \times RT) \quad (1)$$

where k is the rate of chemical process, A is the frequency factor, $\Delta E^{\neq}$ is the energy of activation (cal. mole^{-1}), T is

Table 2 Constant release rates of acyclovir from EVA in water and percent initial burst to the total drug incorporated were determined at various temperatures (32°C, 37°C, 42°C and 47°C).

Temperature	Percent initial burst to the total drug incorporated*	Rate of drug release** (μg/cm^2/day)
32°C	0.96	0.48 ± 0.05
37°C	1.5	0.75 ± 0.03
42°C	3.2	1.14 ± 0.04
47°C	4.8	1.73 ± 0.04

*Drug in EVA (2.5%); Rate was expressed in term of mean ± SD;
** $\Delta E^{\neq}$: Energy of Activation = 16.45 k.cal/mole from the slope of Arrhenius Plot-Fig. 3).

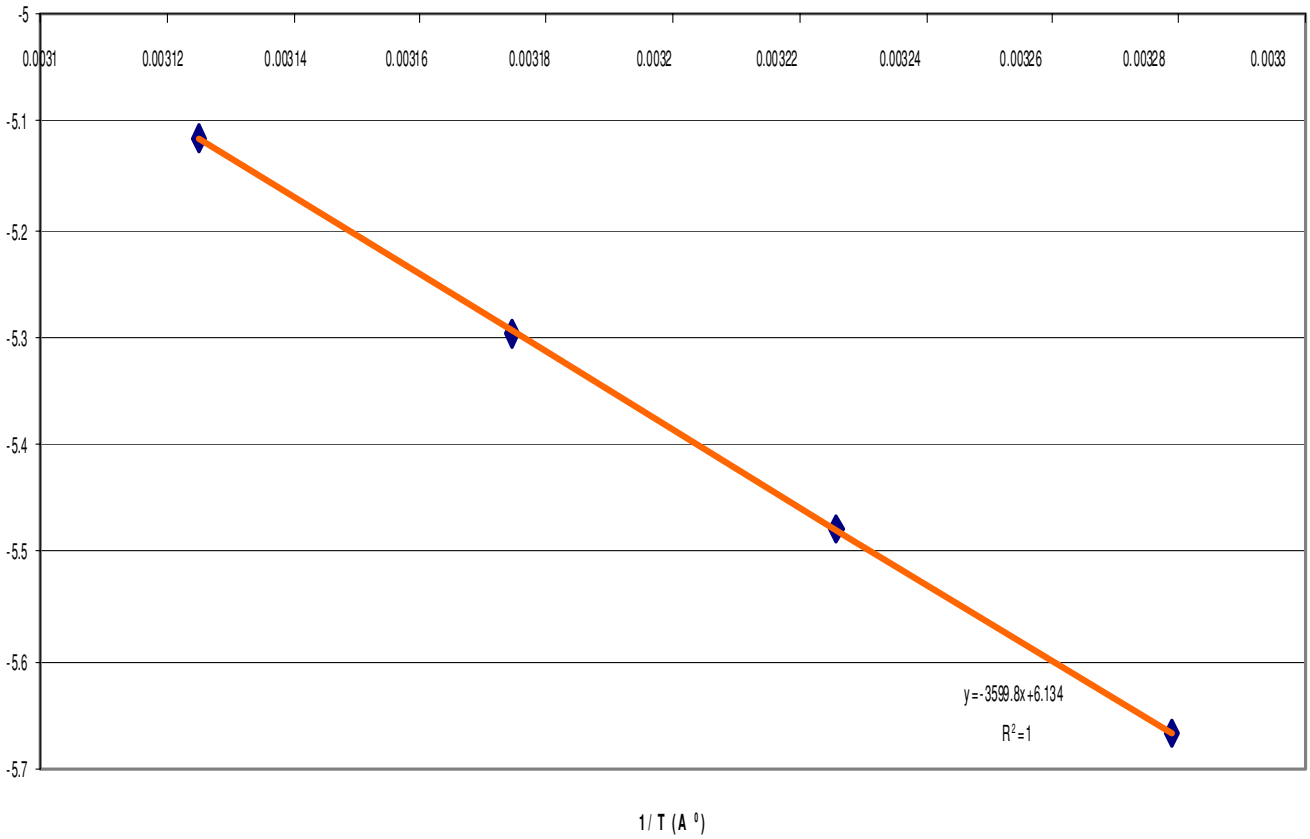


Fig. 3 An Arrhenius plot (log rate of drug release versus $1/T$) was obtained for acyclovir in order to determine energy of activation ($\Delta E^{\neq}$). The influence of temperature taken at 32°C, 37°C, 42°C and 47°C on the rate of drug release into water as an extracting medium

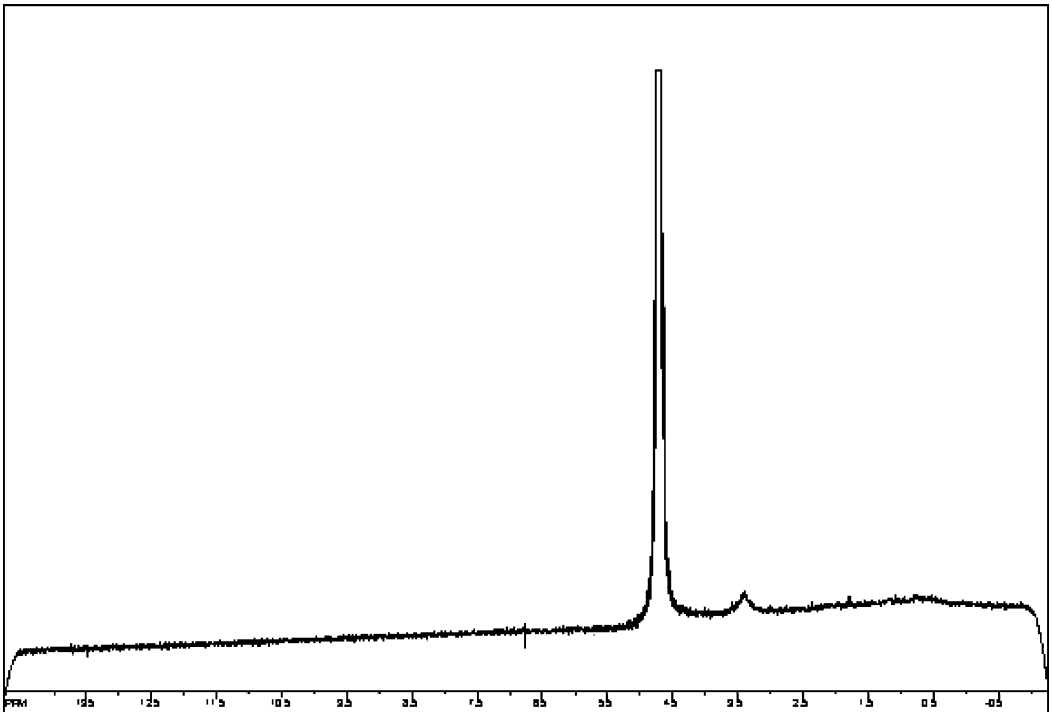
the absolute temperature and R is the gas constant (1.987 cal. Degree^{-1} mole^{-1}). Slope here refers to $-\Delta E^{\neq}/(2.303 \times 1.987)$.

Proton NMR analysis

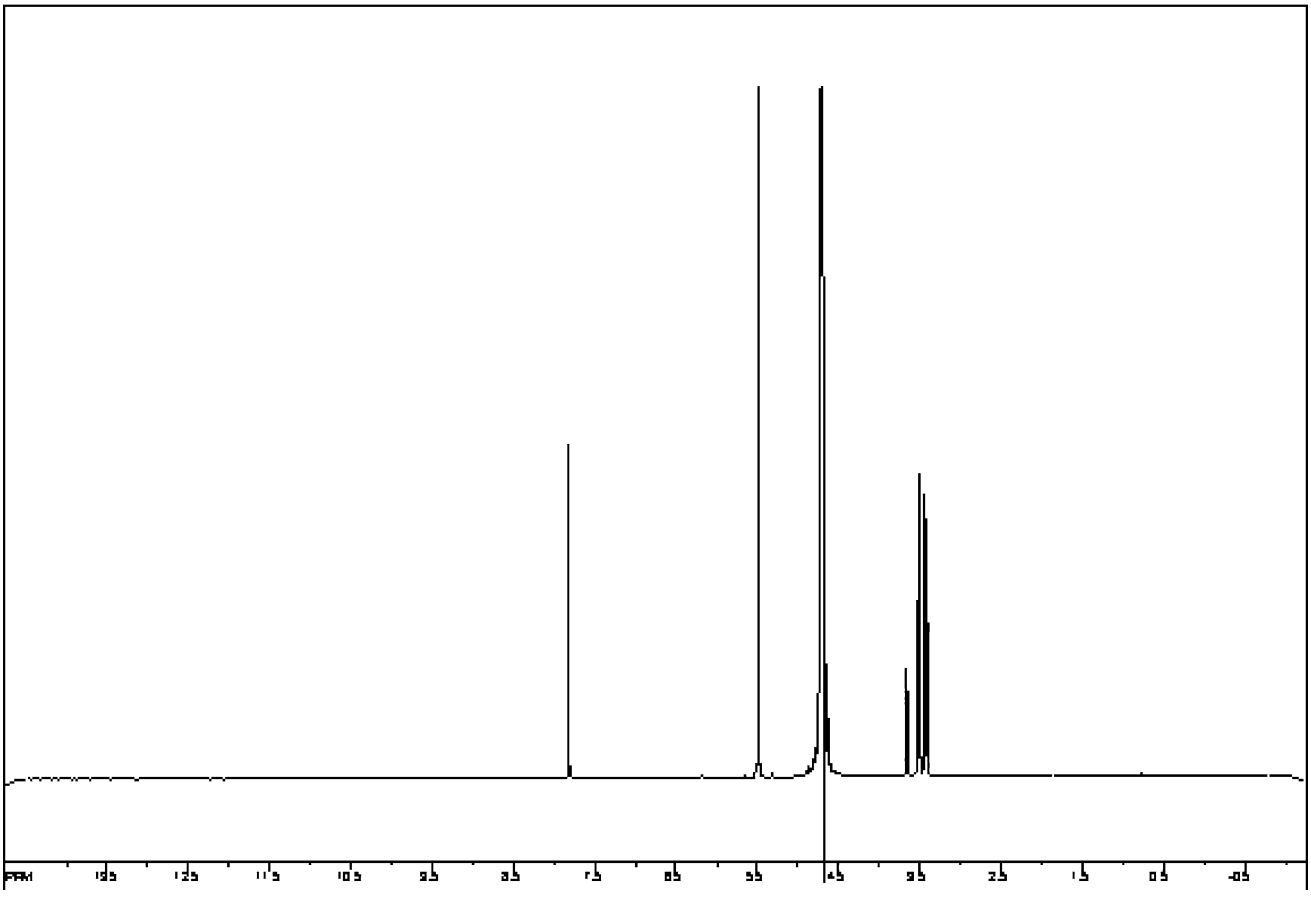
Proton nuclear magnetic resonance spectral studies also were performed on EVA alone (Fig. 4(a)), GCY alone (Fig. 4(b)), or GCY released from EVA (Fig. 4(c)). The spectral results showed that chemical shifts were identical between the two conditions, GCY alone (Fig. 4(b)) and GCY extracted from EVA (Fig. 4(c)), indicating that the chemical structure of the drug remains unchanged during the film casting process. The solvent peak appears at approximately 4.8 ppm on both spectra. The spectral signals noted in the EVA were distinct from those of GCY alone or extracted. Similar measurements were made with reference to EVA alone (Fig. 5(a)) ACY alone (Fig. 5(b)), and ACY released from EVA (Fig. 5(c)). NMR spectral measurements showed that chemical shifts for ACY (Fig. 5(b)) and ACY from EVA (Fig. 5(c)) were identical, suggesting that ACY remained unchanged during film casting and release processes.

Biological studies

Viral replication in EBV infected B958 cells was induced by the addition of TPA. Induced and uninduced cultures were treated with ACY alone or EVA impregnated ACY. Viral DNA was quantitated by amplification of the EBV BNRF1 gene. β-actin served as a control. It was necessary to determine whether ACY released from the drug impregnated polymer would be biologically effective. It was then found that reduced quantities of viral DNA were detected upon the release of ACY from the drug impregnated polymer indicating the efficacy of released drug in an *in vitro* system, suggesting biological effectiveness. The assay detects EBV viral load present in EBV infected lymphocytes, B958 cells, that are latently infected or in which virus is induced to replicate by the addition of phorbol esters such as TPA. In this study, virus was induced to replicate with the addition of TPA to the cultures and cells were then treated with ACY alone or ACY released from polymer. The resulting raw data was standardized using the cellular housekeeping gene β-actin to obtain the Δ (ΔCt), where Ct stands for the cycle number at which the sample reaches the threshold. The addition of ACY to the TPA induced and uninduced B958 cells reduced viral



(a)



(b)

Fig. 4 A proton NMR spectral analysis of GCY impregnated polymer. (a) EVA alone; (b) GCY alone; (c) GCY released from the EVA

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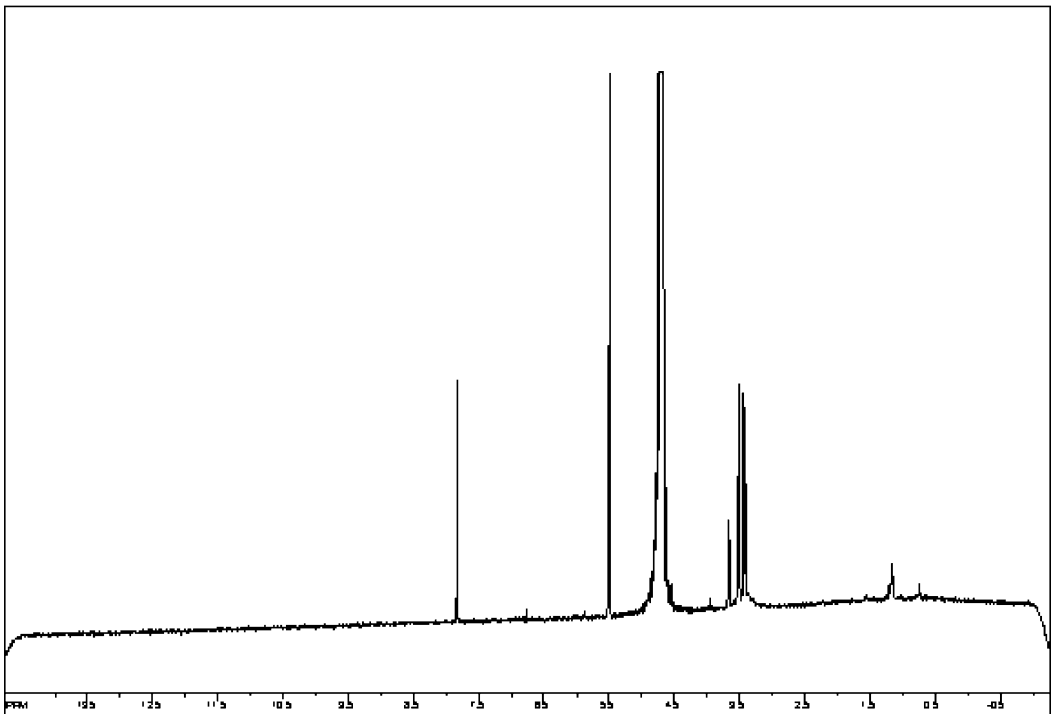


(c)

Fig. 4 (*Continued*)

load significantly (Fig. 6(a)). The ACY impregnated polymer was equally effective in reducing the EBV viral load in culture. Upon addition of EVA + ACY (Fig. 6(b)), the amount of viral DNA was reduced drastically in both the induced and uninduced B958 cells while addition of EVA alone did not affect the viral load significantly. In these assays, it was determined that treatment with both free ACY and with drug impregnated polymer significantly reduced the amount of viral DNA present as determined by a 7–8 cycle difference in each case (Fig. 6(a) and (b)).

Discussion

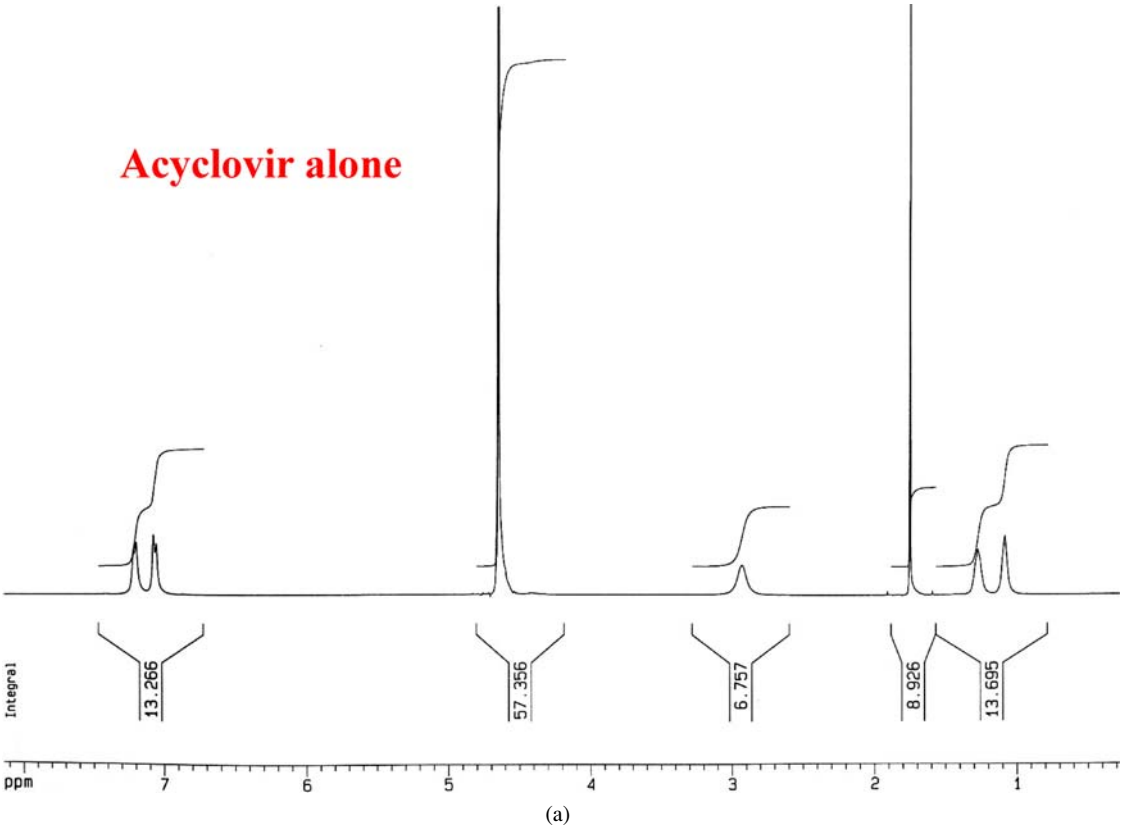
This study demonstrates that GCY and ACY, after an initial burst, show a sustained rate of drug release from EVA over extended periods of time. This study also shows that the rate of drug release increases, as expected, with increasing temperature in double-distilled water extracting medium. Finally proton NMR analysis of GCY and ACY strongly suggested that chemical structure of these two drugs remained unchanged during film casting process.

Rate data presented in Table 1 are based on the part of the curve after the onset of initial burst (Fig. 1). Release profiles usually reveal an initial burst of drug at short intervals followed by a longer period of continuous release. The initial drug "burst" is due to the porosity present in the matrix or liberation of surface-bound drug (Fig. 1) [10–14].

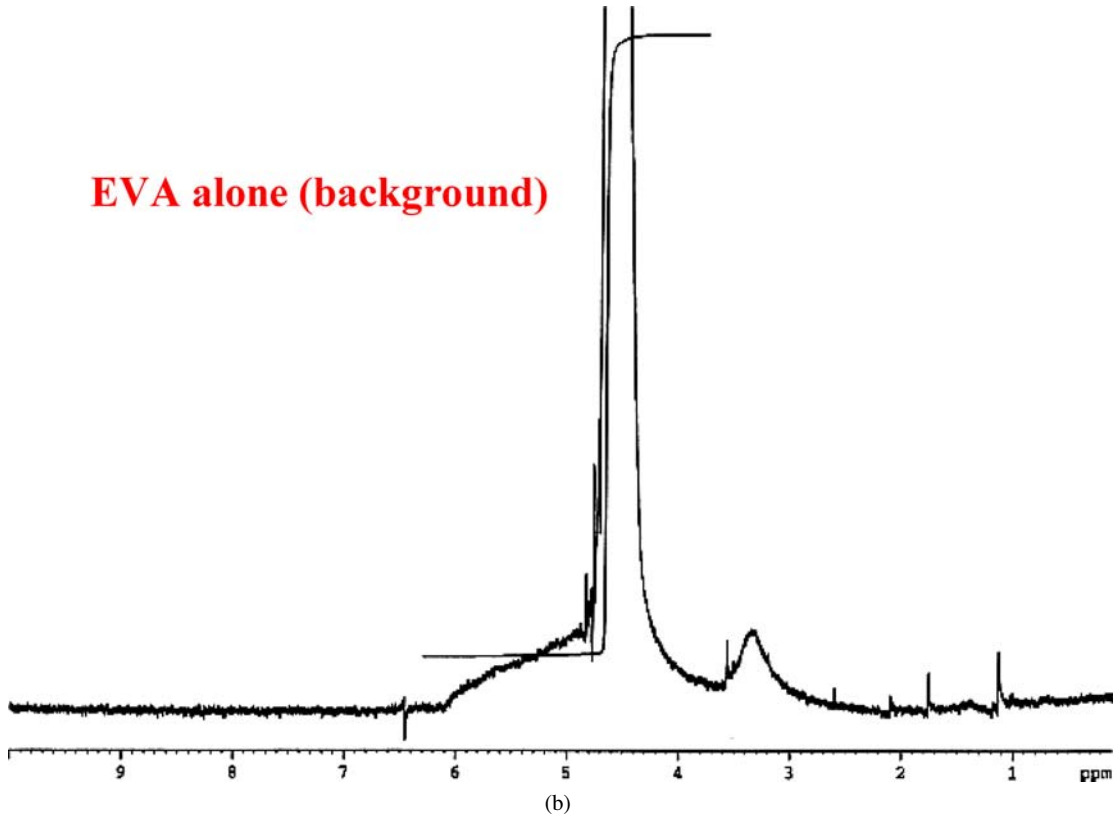
It seems clear that among several factors, the rate of dissolution of drug in the polymer matrix and the rate at which the drug molecules diffuse, influence drug release process. On the basis of molecular weight of ACY (225) and GCY (255), we expect to see that ACY should diffuse at a rate faster than that of GCY. Contrary to expectation, ACY exhibited a slower rate than GCY. The observed lower rate of ACY may be interpreted as due to molecular association such as dimerization and trimerization resulting in slower diffusion through the channels and hence the observed lower rate (about three times lower than GCY) (Table 1 and Fig. 2).

Effect of temperature

Increases of temperature invariably result in increases of rate of diffusion of molecules either in liquids or in solids [15, 16]. The same observation can be extended to include drug delivery process involving the diffusion (translocation) of the drugs through the channels in thin polymer films when immersed in extracting medium. Consistent with this, it was seen in Table 2 and Fig. 3 that the drug studied exhibited a significant increase in rate values as the temperature was increased from 32°C to 47°C, and is attributed to the diffusion of drug molecules through the channels or interconnecting porous network present in EVA thin films surrounded by water as extracting medium [3, 17, 18]. A rise in temperature



(a)



(b)

Fig. 5 Proton NMR spectral analysis of ACY impregnated polymer. (a) ^{1}H NMR spectrum of acyclovir alone; (b) EVA alone as background; and (c) Acyclovir extracted from EVA with background spectrum of EVA subtracted. For all spectra, D_2O was used as a solvent

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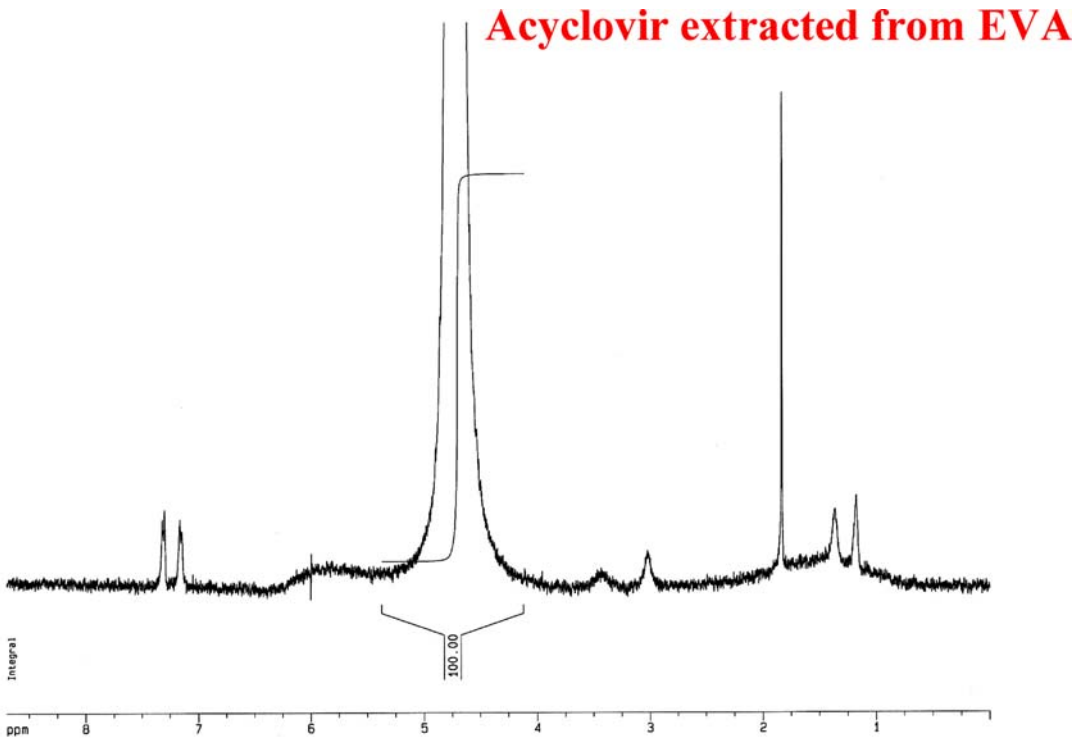


(c)

Fig. 5 (*Continued*)

of 10°C from 37° to 47°C doubled the release rate consistent with general expectation [15] (see Table 2).

Among the critical factors which are responsible for the observed increase in the rate values, drug particle size, geometry, drug-drug interactions, drug-polymer matrix interactions, length and size of the channel play a major role in the translocation of these drug molecules.

Real time polymerase chain reaction amplifying the BNLF1 region of the EBV viral genome, detected reduced quantities of viral DNA upon release of ACY from the polymer. This indicates the efficacy of released drug in an *in vitro* system. How assay works, controls all results were normalized to the cellular housekeeping gene beta actin.

In summary, the matrix material composed of EVA copolymer appears to be a useful vehicle for the sustained release of drugs after the initial burst. This polymer system has uniform drug distribution and the drug release kinetics was reproducible. Based on the statistical analysis, it has been demonstrated that differences in log rates across temperature did not depend on the nature of the drugs.

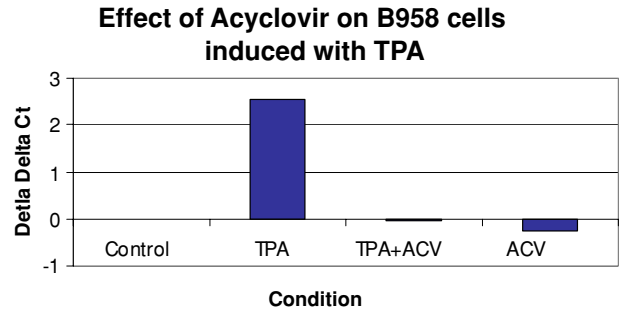
Results of ^1H NMR spectral analysis

A comparison of relative intensities and the ^{1}H chemical shifts of spectra obtained for solution of EVA (Fig. 4(a)), GCY alone (Fig. 4(b)) and GCY + EVA (Fig. 4(c)) suggest strongly that the chemical structure remained unaffected during the film casting process. In other word, this spectrum (Fig. 4(c)) is so nearly a simple addition of the two previous spectra (Figs. 4(a) and (b)) that no further analysis is necessary to conclude that the GCY comes out of the film with the same structure as the one that was incorporated into EVA.

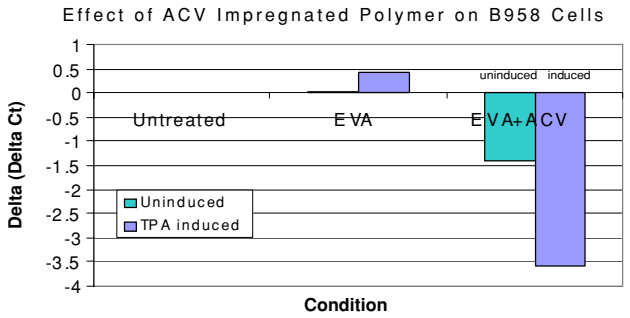
In the ^{1}H chemical shifts of spectra of EVA: Fig. 5(a); ACY: Fig. 5(b); ACY + EVA (Fig. 5(c)) there is similar correspondence between the spectra. Judging from the similarity between the lines (both shape and position) in the ACY powder and in the mixture, it is strongly suggested that ACY comes out of the EVA film with the same structure as the one that was incorporated into EVA. A further confirmation that the extractable drug is unchanged comes from studies showing that the drug is effective as antiviral agent.

Biological studies

Our *in vitro* results support the biological effectiveness of the ACY impregnated device. Quantitative PCR was used as a means to monitor the biological effectiveness of the ACY impregnated polymer by measuring the ability of the polymer to reduce the EBV viral load in cell culture. Phorbol esters such as TPA were used for chemical induction of EBV production in B958 cells to mimic the condition of the virus under permissive conditions in its host. Induction



(a)



(b)

Fig. 6 The Biological Effect of ACV Impregnated Polymer on EBV in infected B958 cells as determined by the Real Time PCR assay. Viral replication in EBV infected B958 cells was induced with the addition of TPA. Induced and uninduced cultures were treated with ACY alone or EVA impregnated ACY. Viral DNA was quantitated by amplification of EBV BNRF1 region. β-actin served as a control. Representative graphs show EBV viral load was assayed under three conditions as a function of cycle number and delta ct. Bar graphs in Fig. 6(a) and (b) depict viral DNA load as a function of change in cycle count normalized to beta actin and to untreated cells (delta-delta Ct). (a) Viral DNA load in untreated Control cells, TPA treated cells, cells treated with acyclovir and TPA or cells treated with acyclovir alone. (b) EBV viral DNA load in Untreated control cells, cells treated with (induced) or without TPA (uninduced) in the presence of EVA alone, cells treated with (induced) or without (uninduced) TPA in the presence of acyclovir impregnated EVA

results in increased viral DNA replication. Reduced viral load was detected after exposure to the ACY impregnated polymer in virus infected induced and uninduced B958 cells. The untreated and EVA alone controls were comparable, demonstrating that EVA has no effect upon the amount of virus present.

These studies demonstrate that not only is the impregnated device stable, it is effective in reduction of viral replication. Additionally, others have shown that ACY is capable of transbuccal permeation in a porcine model [19]. Thus the released drug should be effective in the oral cavity. The device is feasible and will be useful clinically. Implementation of such an impregnated biocompatible polymer may change the way that local oral mono and polymicrobial infections are treated clinically. Direct local delivery of drugs to the infected sites will be efficacious and should omit the need for systemic treatment of these conditions. Additionally, the time-release nature of the product will avoid past problems of creams, ointments and troches that only provide transient short-term drug administration. The application of this technology is far reaching and can potentially be applied to other populations that encounter significant morbidity secondary to oral viral infectious agents. Taken together, the information gained from this study will provide the basis for clinical studies in the near future.

Conclusions

^{1}H NMR spectroscopy indicates that incorporation of GCY, or ACY, into EVA does not change the structure of these drugs. This indicates the anti-viral properties/activity of ACY and GCY were not affected during film casting process. ACY was shown, by RT-PCR, to decrease the number of viral organisms present in a virus infected cell culture system. The information gained from this study will allow us to develop prosthetic devices that can be used for clinical studies in the future.

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