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Preparation, Characterization and Evaluation of Some Metronidazole Formulations as Films and The study of Their Medicinal Activities Against Pathogenetic Microorganisms Causing Dermatitides

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Abstract

In the present study, a labor has been made to develop film type of transdermal therapeutic system comprising different concentrations of metronidazole with hydrophilic polymeric combinations using solvent casting (evaporation) technique. The DSC analysis confirmed that ester bonds were formed between the used polymers in preparing the films. The prepared films were considred semi-IPNs hydrogels and the mixture between the used polymers and metronidazole in preparing the films is considred physical mixing viz., chemical, physical and therapeutical properties of drug remained themselves. Two main parts including chemical and biochemical were discussed in this study. In the chemical part, the prepared films F_0 and F_3 were evaluated via the study of weight and thickness uniformities and swelling behaviour. *In vitro* drug release and kinetics of drug release were studied for all the prepared pharmaceutical formulations in this study. F_3 was higher average weight and thickness as compared to F_0 and in addition to their possession of the same swelling behaviour which was explained on the basis of the increase in swelling index up to period at 70 min and which was followed by the decrease in this index according to cleavage of the formed ester bonds in the crosslinked structure, erosion process with physical disintegration and mass loss. Also, the prepared formulation F_3 followed zero order, Korsmeyer-Peppas model and Fickian ($n<0.5$) transport mechanism (diffusion controlled release). In the

biochemical part, the biochemical activities of all the prepared formulations (as antimicrobials) were studied (*in vitro*) in four types of microbes including *Staphylococcus aureus* (Gram-positive), *Staphylococcus epidermidis* (Gram-positive), *Candida albicans* (yeast) and *Trichophyton* sp. (mold) by using agar diffusion method. These formulations showed increasing in their biochemical activities against these isolates and these activities associated with the increase in drug concentrations which were loaded with the selected polymers in this study and in addition to their possession of highest antimicrobial and biochemical activities to inhibit the growth of these microbial isolates as compared to pure drug and commercial gel at ($p<0.05$). Also, the prepared formulations including F_0 and F_3 were applied (*in vivo*) to skin of male rabbits for studying subacute toxicity test (LD_{50}

1. Introduction

Over the past thirty year greater attention has been focused on the development of controlled and sustained drug delivery systems. Amongst the extensive research has been carried out in designing of polymeric drug delivery systems. In the past few years, transdermal drug delivery is one of the most promising methods for drug application. Increasing numbers of drugs are being added to the list of therapeutic agents that can be delivered to the systemic circulation via skin[1]. Transdermal delivery of drugs through the skin to the systemic circulation provides a convenient route of administration for a variety of clinical indications[2]. Metronidazole was used as a model drug, which is an anti-microbial and anti-inflammatory agent with topical and systemic action that can be incorporated into several pharmaceutical forms[3-5]. It was used as a model drug in this study due to its very effectiveness for the therapy of mucous membrane diseases as vaginal infections[6, 7], skin irritation, inflammatory papules, pustules, and erythema of rosacea[8-22]. It is being used to treat anaerobic (B.fragilis) burn wound infections[23] and periodontal infections[24], when administered topically. As well as it has also proved to be very effective for the therapy of protozoal infections (trichomoniasis, , lambliasis (giardiasis), amoebiasis)[25], numerous anaerobic infections especially gram negative bacteria. It has been found to be mutagenic in bacterial assay[26] and murine spermatozoa[27] when administered orally. Many studies have been conducted concerning the efficacy of metronidazole as a local spermicide[28]. Another important rational of using metronidazole, is its unique, low molecular weight offering the greater permeation a benefit through vaginal epithelial membrane[6, 7]. Various dosage forms available in the market are solutions, suppositories, creams, ointments, gels, foams, sprays, tablets, capsules, films, etc[29]. Topical chemotherapeutic preparations meant for burns should not inhibit the reepithelization and cause injury to viable cells[23]. Not only the chemotherapeutic component but also the base used should not inhibit healing. In this study, the utility of gels were prepared from Carbopol 940 (C_{940}), Poly (vinyl alcohol) (PVA) and hydroxyl ethyl cellulose (HEC) for the controlled release of metronidazole, has been investigated.

2. Materials and Methods

2.1. Materials

Metronidazole was supplied by (NDI Co.- Iraq & Sigma–Aldrich Co. USA). Hydroxy ethyl cellulose (HEC) was supplied by (Aldrich Co., Germany). Polyvinyl alcohol (Degree of hydrolysis 98%, M_W =72000Da) was supplied by (Merck Co., Germany). Carbopol940 (C_{940}) was supplied by (Sigma-Aldrich Co., Germany). Nutrient agar (NA) and Sabouraud's dextrose agar (SDA) were supplied by (Hi Media Lab., India). Muller–Hinton Agar (MHA) was supplied by (Titan Biotech Co., Rajasthan, India). All other chemicals were of reagent grade.

2.2. Methods

2.2.1. Chemical Part

2.2.1.1 Preparation of Cast Films Containing Metronidazole

Mucoadhesive films were prepared by solvent casting (evaporation) technique[30, 31]. Glass moulds were used for casting in films. For preparing 10% PVA (w/w), 500mg PVA was dissolved in 5mL distilled water at 90°c with stirring to produce a clear solution and was kept for 1hr to remove all the air bubbles [32]. 100mg C_{940} was placed in 4mL of distilled water and was stirred for 60 minutes (min). 100mg HEC was dissolved in 3mL distilled water. The three polymeric solutions were mixed and was stirred for 1hour (hr). Then one drop of (0.0294g) glycerin (glyceraldehyde) was added to the polymers solution as plasticizer. Determined amount of metronidazole was weighed accurately and was dissolved in 1mL of methanol in another beaker. The drug solution (in different concentrations) was added to 1g of the polymers solution and was mixed thoroughly with the help of a magnetic stirrer. The glass mould with a size of 2.54x7.62cm^2 was placed on a flat surface. The casting solution (3mL) was poured into glass mould and was dried for 24hrs at the room temperature for evaporation. The films were removed by peeling and were cut into square dimensions of 1x1cm^2. These films were kept for 2days in a desiccators for further drying and they were shown in table (1).

Table(1) Solid formulations of various metronidazole films

Metronidazole (mg)/1g of polymers solution	Formulation code
0.0	F_0
2.5	F_1
5.0	F_2
10.0	F_3
15.0	F_4
20.0	F_5
25.0	F_6
33.0	F_7

It is worth mentioning that all of the determined amounts of metronidazole in table (1) were dissolved in 1mL of the solvent (methanol).

2.2.1.2 Evaluation of the Films

Formulated films were subject to the preliminary evaluation tests. Films with any imperfections, entrapped air, or differing in thickness, weight or content uniformity were excluded from further studies.

2.2.1.2.1 Weight Uniformity of the Films[33]

For the evaluation of film weight, three films from F_0 and F_3 (1cm^2) were cut by using sharp blade from different areas and weighed individually on a digital balance.

The results were analyzed for mean and standard deviation(SD).

2.2.1.2.2 Thickness Uniformity of the Film

The thickness of F_0 and F_3 was measured using digital screw guage (thickness tester) at different positions of the films.The results were analyzed for mean and SD.

2.2.1.2.3 Swelling Study[34]

The most important property of hydrogel is its ability to imbibe water. Thus the permeability of the membranes, their mechanical properties, their surface properties, and the resultant behavior at biological interfaces are all a direct consequence of the amount and nature of water held in this way. The amount of water absorbed at a given temperature is usually expressed as the equilibrium water content[35, 36]. Water content of hydrogel can also be expressed as swelling index (S.I). For the determination of S.I the preweighed two patches (1×1cm²) from each formulation were placed in a beaker (containing 10 ml of distilled water at 37°C). After particular interval of time patches were removed and wiped (blotted) with filter paper and weighed.

$$S.I=(Wt-W0/\ W0)\times 100$$

Where W_0 is initial weight of bioadhesive film at zero time (before dipping into beaker) and Wt is the weight of swollen bioadhesive film after time t (after dipping in beaker) & wiped. The S.I is governed by several factors. These include the nature of hydrophilic group present in the gel, the nature and density of crosslink and the external factors such as temperature, the tonicity (together with the nature of the constituent ions), and the pH of hydrating medium. It is, therefore, a fact that the nature of hydrogels is dominated by the effect of water within the matrices that gives them their unique role in the field of biomedical applications.

2.2.1.3 Standard Calibration Curve of Metronidazole

For representation of standard calibration curve of metronidazole, the standard ($stock_1$) solution of metronidazole was prepared by weighing accurately 100mg of pure metronidazole dissolving in minimum quantity of methanol and diluting to 100.0mL with phosphate buffer pH7.0. 10.0mL of the $stock_1$ solution was taken and was diluted further to 100.0mL with phosphate buffer pH7.0 as $stock_2$ solution. 0.03, 0.06, 0.125, 0.25, 0.5, 1.0, 1.5, 2.0, 4.0 and 6.0mL were pipetted out of the $stock_2$ solution and were diluted to 10.0mL with phosphate buffer pH7.0 to obtain a concentrations range of 0.3 to 60.0µg/ml. The absorbance of all drug solutions was measured at 325nm using UV-spectrophotometer in triplicate readings (n=3) for avoiding any error case.

2.2.1.4 *In Vitro* Release (Dissolution) Studies

Dissolution testing is an essential requirement for the development and establishment of the release of drug model. In this study, skin permeation studies were performed using dialysis tube method[37]. 25mg with area $1cm^2$ of each prepared film was placed in the synthetic dialysis bags and were immersed into 125mL phosphate buffer solution pH7.0 after it was fixed in sterilized beakers. Each beaker was accurately covered with glass watch and was fixed on a magnetic stirrer at 100rpm and 37±1°C. 4mL aliquot of the dissolution fluid was withdrawn at regular time interval

and was replaced with fresh quantity dissolution fluid. The samples were analyzed spectrophotometrically at 325nm to determine the dissolved drug concentration (drug content) using UV-spectrophotometer. All the experimental units were analyzed in triplicate (n=3).

2.2.1.5 *In Vitro* Release Kinetic Studies

In order to study the exact mechanism of drug release from the prepared formulations, the drug release data was analyzed according to zero order[38], first order[39], Higuchi square root[40] and Korsmeyer-Peppas model[41]. The criterion for selecting the most appropriate model was chosen on the basis of goodness of fit test.

2.2.2. Biochemical Part

2.2.2.1 *In Vitro* Study

2.2.2.1.1 Specimens Isolation and Collection

The total samples were collected from the patients who were consulted in the Dept. of Dermatology, General Basrah Hospital. The collective samples were taken from skin (skin scraps and swabs from burn wounds) and mouth (swabs from oral mucous membranes) as follows: 1) Skin scraps: The patients with Tinea pedes, the surface was cleaned with 70% ethanol prior to the collection of the flakes of skin from the advancing border of the lesion by using two sterile glass slides. 2) Burn wounds swabs: The swab was taken from abscess of burn wound by sterile cotton swab stick. 3) Oral swabs: The patients with Candidiasis, the swab was taken from oral mucous membranes especially on the gingiva from the mouth by sterile cotton swab stick. It is worth mentioning that skin diseases which include Tinea pedes, candidiasis and in addition to burn wounds were clinically diagnosed by dermatologist.

The collective samples have been taken to Bacteria and Fungi Laboratories (Lab) in Dept. of Biology, College of Science, University of Basrah, Iraq in order to be examined, diagnosed and isolated. The collective samples from Tinea pedes and Candidiasis were planted on the sterile plate (peti dish's) surface of Sabouraud's dextrose agar (SDA) with 0.25g chloramphenicol and 0.5g cycloheximide. The former suppresses the growth of bacteria while the latter suppresses the contaminant fungi (that is, for isolating the fungi which can be a growth with this antibiotic existence from other fungi which cannot be a growth with the same antibiotic existence). The collective samples from burn wounds were planted on the sterile plate surface of Nutrient agar (NA). Triplicate sterile plates for each sample were prepared and were incubated at 37 ℃ for 1-3days and at 37 ℃ for 3-7days for bacteria and fungi, respectively.

Various types from bacteria and fungi were defined after diagnosis. Four clinical microbial isolates which include *Staphylococcus aureus* (Gram-positive), *Staphylococcus epidermidis* (Gram-positive), *Candida albicans* (yeast) and *Trichophyton* sp. (mold) were selected for the study. Bacterial isolates which were isolated from burn wounds grew on screw capped vials slant surface of NA , while fungal isolates which were isolated from Tinea pedes and Candidiasis grew on sterile screw capped vials slant surface of SDA. The vials were incubated at 37 ℃ for 1-3 days and at 37 ℃ for 3-7 days for bacteria and fungi, respectively. After the growth appearance, the vials were maintained at 4℃ (as a stock$_1$ cultures for each clinical microbial isolates) in the

refrigerator until the antimicrobial experiment assays.

2.2.2.1.2 Inoculum' Preparation[42]

Before the preparation of microbial inocula, pure isolate for each clinical microbial species was subcultured (as $stock_2$ culture). $Stock_2$ cultures were prepared by transferring a loopful of colonies from the $stock_1$ cultures which were maintained at 4 ℃ on NA slants (bacteria) and SDA slants (fungi) for all clinical microbial species to plates of NA for bacteria and SDA with chloramphenicol for fungi. The plates were incubated at 37 ℃ for 1-3 days and at 37 ℃ for 3-7 days for bacteria and fungi, respectively. For the preparation of suspension (as inoculum) for each clinical microbial isolate, active cultures ($stock_2$) for each clinical microbial species were prepared by transferring a loopful of colonies from the $stock_2$ cultures for all clinical microbial species to sterile test tubes of 5mL of sterile saline (isotonic solution) (0.85%NaCl). These test tubes were incubated without agitation for 24hrs at 37 ℃ for bacteria and fungi. A suspension for each clinical microbial isolate was prepared and was adjusted to 1×10^6 colony forming units (cfu) mL^{-1} standardized by (0.5 Mac-Farland)[43] and using haemocytometer[44].

2.2.2.1.3 *InVitro* Antimicrobial Activity

The prepared solid formulations of films which were loaded and not loaded with metronidazole in previous parts from the preparation, commercial gel (10mg of metronidazole loaded with 1g of gel) and in addition to the prepared liquid formulations of different concentrations of metronidazole, as shown in table(2), were used to test their antimicrobial activities.

Table (2) Liquid formulations of different concentrations of metronidazole

Metronidazole (mg)/1mL of solvent	0.0	2.5	5.0	10.0	15.0	20.0	25.0	33.0
Formulation code	S_0	S_1	S_2	S_3	S_4	S_5	S_6	S_7

2.2.2.1.4 Agar Diffusion Assay

The antimicrobial assay of all the formulations were evaluated by the zone of inhibition using the agar well diffusion assay[45], with slight modification according to the present experimental conditions. 0.2 mL of microbial inocula was placed on the surface of MHA media and then it was spread with L–shape glass rod. The plate was left for 10 min. Then individual plates were marked for the organism being inoculated. Each plate was punched to make one well of 9.0mm diameter with the help of a sterile cork borer at the central site of the plates. The formulations of antimicrobial were placed in a central pore which included 0.5g for solid formulations and 0.5mL for liquid formulations. A control experiment was set up using an equal amount of phosphate buffer pH 7.0 as control and sterile DMSO in central place of different antimicrobial formulations. These plates were incubated at 37 ℃ for 1-3 days and at 37 ℃ for 3-7 days for bacteria and fungi, respectively. The inhibition zones of each isolate were recorded in centimeter (cm) using ruler according to the growth of their controls. All formulations were tested in triplicates. It is worth mentioning that the process of subculture for each clinical microbial isolates and the preparations of inocula for antimicrobial assay were

happening in Biotechnology Lab, Dept. of Marine Biology. MSC, University of Basrah, Iraq.

2.2.2.1.5 Statistical Analysis

Data were presented as mean ± SD & standard error of the mean (SEM). The obtained data were subject to the analysis of variance (ANOVA) test to determine there LSD (Least Significant Difference) at probability level (p) < 0.05. Statistical analysis was performed with statistical software pakage of social sciences (SPSS) for windows (11.0 version)[46].

2.2.2.2 *In Vivo* Study

2.2.2.2.1 Animal Model

Twenty eight germany white male rabbits (young adult) weighing in range of (1.15-1.95 kg) were acclimatized for 50days before and during the study in Animal House of College of Science, University of Basrah, Iraq, under standard conditions at room temperature of (24±2℃), relative humidity (55±5%) and lighting (12hrs/day) with controlled care. The floor was spread with sawdust. Animals were fed with clover (trefoil), carrots, celery and lettuce, and they were supplied with water under strict hygienic conditions.

2.2.2.2.2 *In Vivo* Antimicrobials

The prepared formulations including F_0 and F_3 were topically applied to the animals for testing subacute toxicity and skin irritation. These formulations were placed in big papers as carriers before the application. The diameter of these papers were in range (4.6-4.8cm) and they were described with impermeable for the materials and resistant to the temperature. These papers and the cotton wool were firmly secured in place with gauze and adhesive plaster to be occlusion.

2.2.2.2.3 Biochemical Application

The study contained two types of tests including subacute toxicity and skin irritation tests. The determined area for the back of each animal was shaved with electrical clippers for removing hair till this area of the back became bare and smooth. The animals were left for one day before use.

2.2.2.2.3.1 Subacute Toxicity Test [47-50]

This test is sometimes also called LD_{50} (Median Lethal Dose or Lethal Dose 50%) dermal route. Twelve rabbits were used in this test. They were randomly divided into three groups containing four animals each as follows: The control group as group one was left untreated served. The prepared formulations including F_0 and F_3 were topically applied with doses (16g/kg) on the bare back of each animal to two groups as groups no. two and three, respectively. The animals were observed during three days. The data which including the happened death or any other effects were recorded.

2.2.2.2.3.2 Skin Irritation Tests

2.2.2.2.3.2.1 Back Irritation Test [14, 51-59]

It is sometimes also referred to as the primary dermal irritation. In this test, twelve rabbits were used. They were randomly divided into three groups containing four animals each as follows: The control group as group no. one was left untreated served. The prepared formulations which including F_0 and F_3

were topically applied with doses 1g on $9cm^2$ subarea of the bare back of each animal to two groups. The animals were observed for 24hrs for any sign of edema and erythrema.

2.2.2.2.3.2.2 Rectal Irritation Test [60, 61]

In this test, twelve rabbits were used. They were randomly divided into three groups containing four animals each as follows: The control group as group no. one was left untreated served. The prepared formulations including F_0 and F_3 were topically applied with doses 0.5g into the rectum of each animal to two groups. The animals were observed for 72hrs for any sign of sensitization (allergic reactions), photosensitization, edema and excess redness.

3. Characterization

3.1 DSC Analysis of Metronidazole NDI®[62, 63]

DSC thermogram of metronidazole NDI® was shown in figure (1). A single sharp endothermic peak (melting endotherms) corresponds to T_m of pure drug metronidazole in the range of (159-163°C), especially at 162.56 °C and fusion heat (melting enthalpy) at 184.9 J/g . After the fusion, the DSC curve indicated the initiation of an exothermic process with a positive slope, resulting in a complete degradation of the drug at 283.14°C and degradation enthalpy at 181.67J/g.

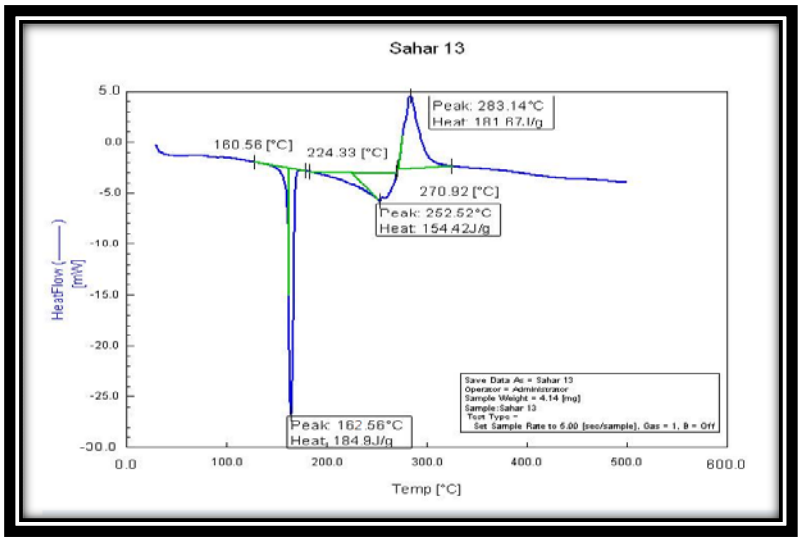


Fig (1) DSC thermogram of metronidazole NDI®

3.2 DSC Analysis of Pure PVA

DSC thermogram of of pure PVA was shown in figure (2). PVA reveals relatively a large and sharp melting (endothermic) peak at 222.57°C and a broad peak at 58.8°C corresponds to T_m and T_g, respectively. The temperature corresponding to the two transitions for pure PVA is good in accord with the reported values[64, 65]. Recent researches confirmed that pure PVA decomposes between 250-400°C[66].

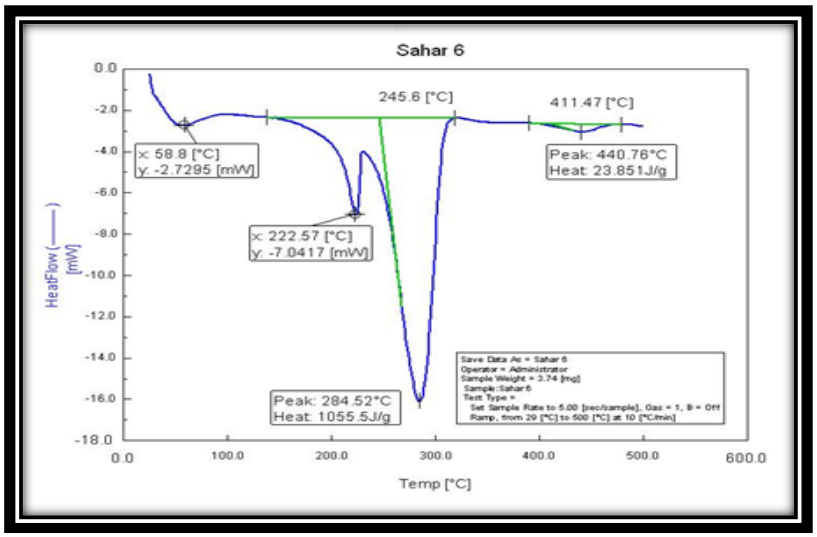


Fig (2) DSC thermogram of pure PVA

3.3 DSC Analysis of Pure C_{940}[67]

DSC thermogram of pure C_{940} was shown in figure (3). C_{940} that reveals endothermic peak at 62.83°C, exothermic peak at 124.65°C besides other peaks. These peaks have not an appropriate definition because of the amorphous nature of C_{940}.

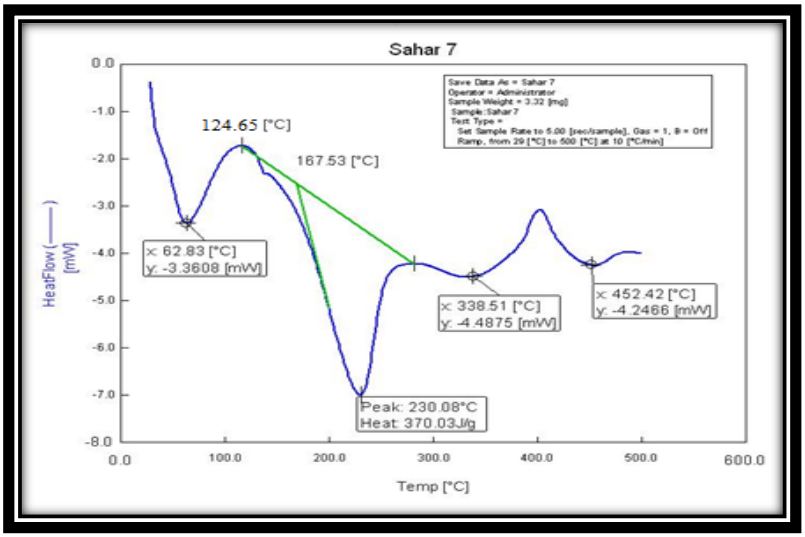


Fig (3) DSC thermogram of pure C_{940}

3.4 DSC Analysis of Pure HEC

DSC thermogram of pure HEC was shown in figure (4). It can be noticed that HEC showed two-thermal steps, as follows: the initial endothermic peak at 62.02°C corresponds to T_g according to the water content and the plasticizing effect in the composite[68]. At temperatures over 100°C the plot rises, possibly indicating the loss of moisture which is physically adsorbed. After the loss, the endothermic peak at 243.62°C showed and due to the absorbed moisture in determined rate. The steps of the loss and absorption of the moisture were correlated with the

decomposition process which continues to complete this process. This explanation of the thermal analysis for HEC agrees with the recent researches[68-70]. These researches confirmed that at temperatures over 550^{o}C the plot rises, possibly showing the decomposition of a charred polyaromatic residue originated from the cellulose cyclic structure. In addition, similar chars generally have a good thermal stability and they decompose at 600-800^{o}

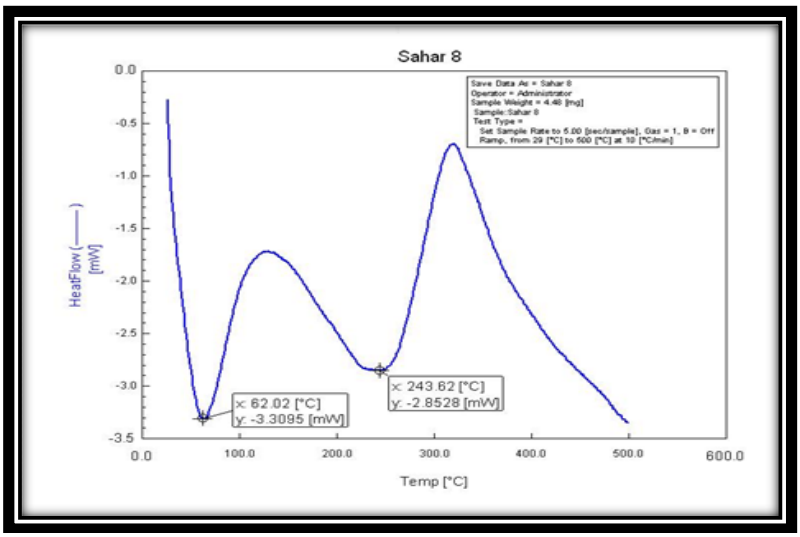


Fig (4) DSC thermogram of pure HEC

3.5 DSC Analysis of F_0 Formulation

DSC thermogram of F_0 was shown in figure (5). It revealed the following data: endothermic peaks at 84.38^{o}C and 218.82^{o}C which corresponds to T_g and T_m, respectively and in addition to endothermic peak at 355.22^{o}C. When there is a comparison between the DSC thermograms of pure PVA, pure C_{940}, pure HEC and F_0, the peaks of F_0 were resulting from the shifting process of the peaks from pure PVA, pure C_{940} and pure HEC. T_g of F_0 is higher as being compared to T_g of pure PVA (in the capacity as the biggest quantity) and pure HEC. This behaviour was attributed as a result of ester and intermolecular hydrogen bonding formation between the units of these polymers. Because the mobility of the chains decreases due to the presence of hydrogen bonds that act as crosslinks[71]. Finally, DSC thermogram of F_0 confirms that F_0 is considred semi or pseudo interpenetrating polymer networks (Semi-IPNs) hydrogel.

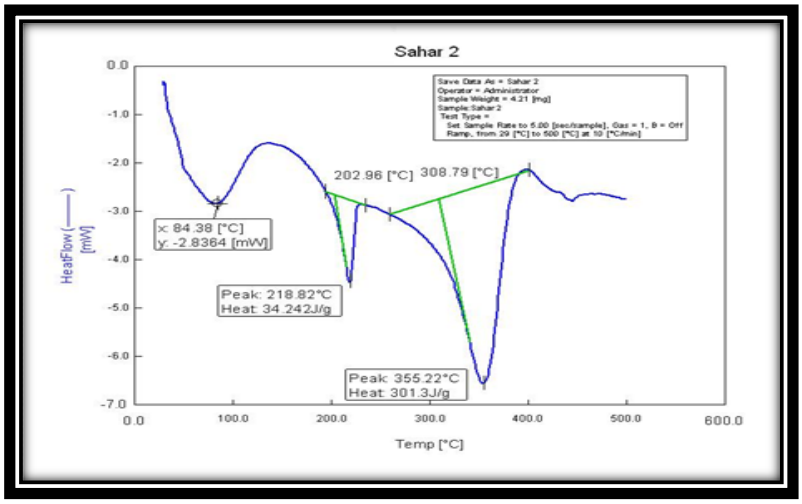


Fig (5) DSC thermogram of F_0 formulation

3.6 DSC Analysis of F_3 Formulation

DSC thermogram of F_3 was shown in figure (6). It revealed the following data: endothermic peaks at 77.85°C and 211.37°C corresponds to T_g and T_m, respectively, endothermic peak at 162.34°C corresponds to T_m of metronidazole and in addition to two endothermic peaks at 351.29°C and 443.61°C. When there is a comparison between the DSC thermograms of metronidazole NDI®, F_0 and F_3, DSC thermogram of F_3 confirmed that the mixture between metronidazole NDI® and the polymers was physical because the appearance endothermic peak at 162.34°C and which corresponds to T_m of pure metronidazole in the range of (159-163°C)[62, 63]. This result indicates that metronidazole is compatible, molecular dispersion and does not show any interaction with the chosen polymers.

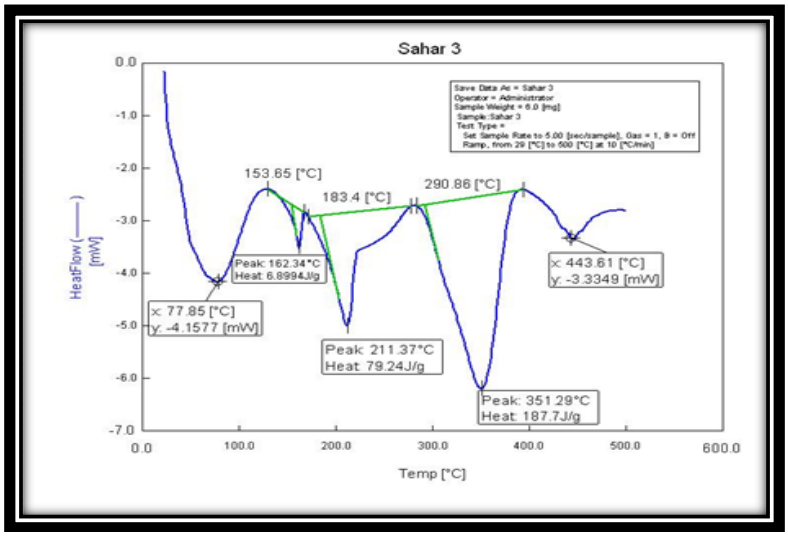


Fig (6) DSC thermogram of F_3 formulation (physical mixing)

4. Results and Discussion

4.1 Chemical Part

4.1.1 Evaluation of the Films

4.1.1.1 Weight Uniformity of the Films

The formulations of F_0 and F_3 show the uniformity in weights. The formulation of F_0 was having a minimum average of the weight while the formulation of F_3 was having the maximum average of the weights. Results are shown as mean ± SD.; n=3 in table (3).

4.1.1.2 Thickness Uniformity of the Films

The formulations of F_0 and F_3 show the uniformity in thickness. The formulation of F_0 was having a minimum thickness while the formulation of F_3 was having a maximum thickness. Results are shown as mean ± SD.; n=3 in table (3).

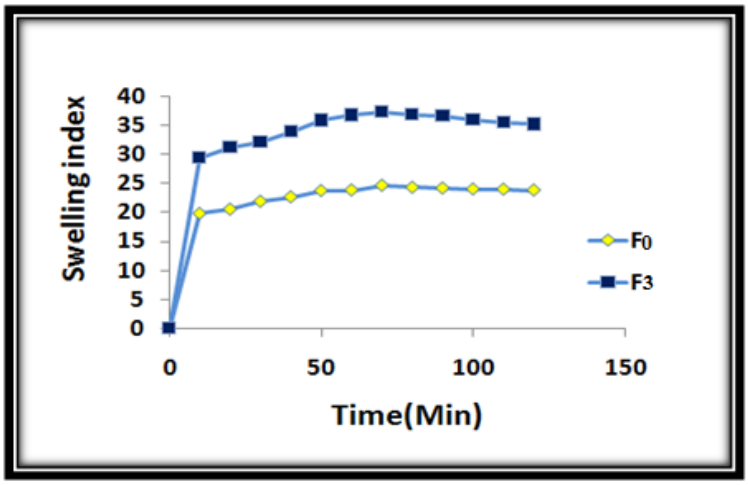
Table (3) Bioadhesive film weight and thickness (1cm^2) of F_0 and F_3 formulations

formulationCode	Bioadhesive film weight* (mg)	Bioadhesive film thickness* (mm)
F_0	183.58 ± 8.74	0.1 ± 0.0187
F_3	196.37 ± 5.92	0.1008 ±0.01207

*Each data represent mean ± SD.; n=3

4.1.1.3 Swelling Study

Hydration is required for the mucoadhesive polymer to expand and create a proper bond between the polymer and the mucin. The capability of polymer to swell governs the release rate of incorporated drug and also bioadhesiveness of the formulation[72, 73]. Polymer swelling permits the mechanical entanglement by exposing the bioadhesive site for hydrogen bonding and/or electrostatic interaction between the polymer and mucin network of mucus[74]. These various physicochemical interactions occur to consolidate and strengthen the adhesive joints, leading to prolonged adhesion[75]. Several important characteristics of S.I are also shown in figure (7). It is worth mentioning that F_3 has higher thickness compared to F_0. The initial swelling values were rapidly increased, and thereafter they increase gradually reach a plateau at minute 70. This observed trend of increasing in the swelling values at a later stage suggests that the degradation of these hydrogels is facilitated through hydrolytic cleavage of the ester bonds in the crosslinked structure, the network crosslinking density decreases with degradation, resulting in an increase in the S.I [76].



Fig(7) Swelling index of metronidazole films

The high initial uptake of water was also due to the faster hydration rate of HEC[77]; PVA and the property of C_{940} is attributed to retain water and to increase the swelling degree to form thick swollen mass[77] because these polymers have more hydroxyl groups. After 70 minutes, the swelling values started to decrease. This observed trend of decrease in the swelling values at a later stage suggests that PVA has the ability to be soluble in water without using any chemical crosslinking agent[78-81], the crosslinked hydrogels might undergo an erosion process with a physical disintegration and mass loss[82].

4.1.2 Standard Calibration Curve of Metronidazole[83-86]

After the scanning of $1x10^{-4}$ M of metronidazole so lution (n=3) in UV region (200-400nm) by spectrophotometer, the maximum absorbance gave the λ_{max} for the drug at 325nm. So the standard calibration curve of metronidazole was developed at this wave length. The calibration curve was linear between (0.3-60µg/mL) concentration ranges. The manual standard calibration curve graph of metronidazole was determined in phosphate buffer of pH7.0 by plotting absorbance (y_1) against concentration (x) at 325nm by Microsoft Excel 2007, and it follows the Beer's law. Results were tabulated in table (5). The correlation coefficient (R^2) was found to be at 0.996. The slope (b) and intercept (a) were found to be at 0.045 and at 0.013, respectively according to the regressed line which was represented in the equation [1] and as shown in figure (8).

$y_1=bx+a$……[1]

where, y_1=practically calculated

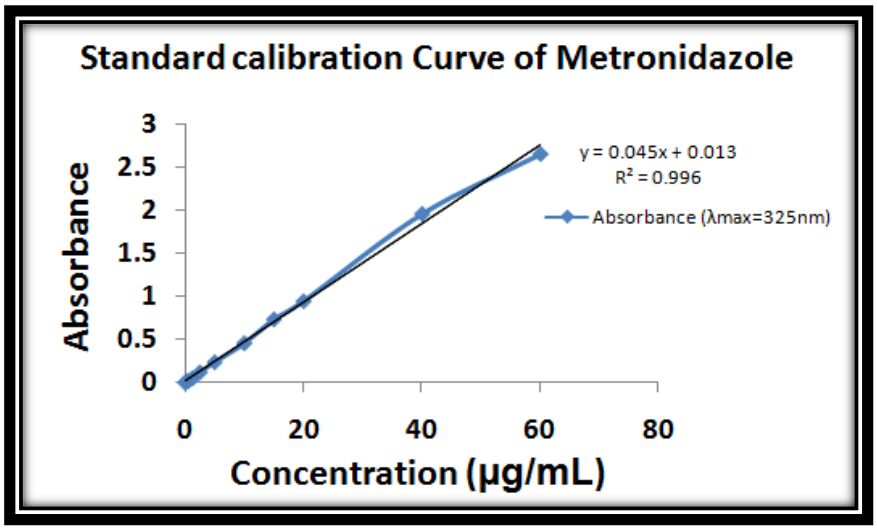


Fig (8) Standard calibration curve graph of metronidazole

In mathematics, the slope or gradient of a line described its steeper, incline. The slope is (in the simplest terms) the measurement of a line, and is defined as the ratio of the "rise" divided by the "run" between two points on a line, or in other words, the ratio of the altitude changes to the horizontal distance between any two points on the line. But in case of multiple points here is the method to calculate the slope and value of the intercept.

For the representation of standard regression curve graph, a least square method was used to calculate the slope (b) and intercept (a), as follows:

Step (1): Calculation of slope (b)

By the fomula:

$$b = \frac{n\sum xy - \sum x.\sum y}{n\sum x^2 - (\sum x)^2} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots [2]$$

where,

n = no. of observation (10)

Table (4) shows observation charts for the calculation of the slope (b) and intercept (a).

Table (4) observation charts for calculating the slope and intercep

S.NO	Concentrations (µg/mL)	Absorbance λ_{max}=325nm		
	x	y_1	x^2	xy_1
1.	0.3	0.016	0.09	0.0048
2.	0.6	0.023	0.36	0.0138
3.	1.25	0.052	1.5625	0.065
4.	2.5	0.121	6.25	0.3025
5.	5.0	0.238	25	1.19
6.	10.0	0.459	100	4.59
7.	15.0	0.737	225	11.055
8.	20.0	0.948	400	18.96
9.	40.0	1.964	1600	78.56
10.	60.0	2.660	3600	159.6
∑	154.65	7.218	5958.2625	274.34
n	10	10	10	10

The values in table (4) were put in equation [2] to calculate (b) value and which was equal to 0.0456.

Step (2): Calculation of intercept (a)

By the fomula:

$$a = \bar{y} - b\bar{x} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots[3]$$

where,

$\bar{y}$ = mean of y_1 (sum of observations / no. of observations)

$\bar{x}$ = mean of x (sum of observations / no. of observations)

b = slope

$\bar{x} = \Sigma X / 10 \longrightarrow \bar{x} = 15.465$

$\bar{y} = \Sigma y_1 / 10 \longrightarrow \bar{y} = 0.7218$

The values of $\bar{y}$ and $\bar{x}$ were put in equation [3] to calculate (a) value and which was equal to 0.0165.

Step (3): Calculation of regressed values

In the table (5), the calculation of regressed values of y_2 was followed according to the regressed line:

$y_2 = bx + a \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots[4]$

where, y_2 = theoretically calculated absorbance (regressed y). Then the graph was plotted between the regressed values obtained from table (5).

Table (5) The calculation of regressed values of y_2

S. NO.	Slope * conc. (bx)	Intercept (a)	Regressed values (y_2=bx+a)
1.	0.3*0.0456(x_1)=0.01368	0.0165	0.01368+0.0165=0.318(y_1)
2.	0.6*0.0456(x_2)=0.02736	0.0165	0.02736+0.0165=0.0438(y_2
3.	1.25*0.0456(x_3)=0.057	0.0165	0.057+0.0165=0.0735(y_3)
4.	2.5 *0.0456(x_4)=0.114	0.0165	0.114+0.0165=0.1305(y_4)
5.	5.0*0.0456(x_5)=0.228	0.0165	0.228+0.0165=0.2445(y_5)
6.	10.0*0.0456(x_6)=0.456	0.0165	0.456+0.0165=0.4725(y_6)
7.	15.0*0.0456(x_7)=0.684	0.0165	0.684+0.0165=0.7005(y_7)
8.	20.0*0.0456(x_8)=0.912	0.0165	0.912+0.0165=0.9285(y_8)
9.	40.0*0.0456(x_9)=1.824	0.0165	1.824+0.0165=1.8405(y_9)
10.	60.0*0.0456(x_{10})=2.736	0.0165	2.736+0.0165=2.7525(y_{10})

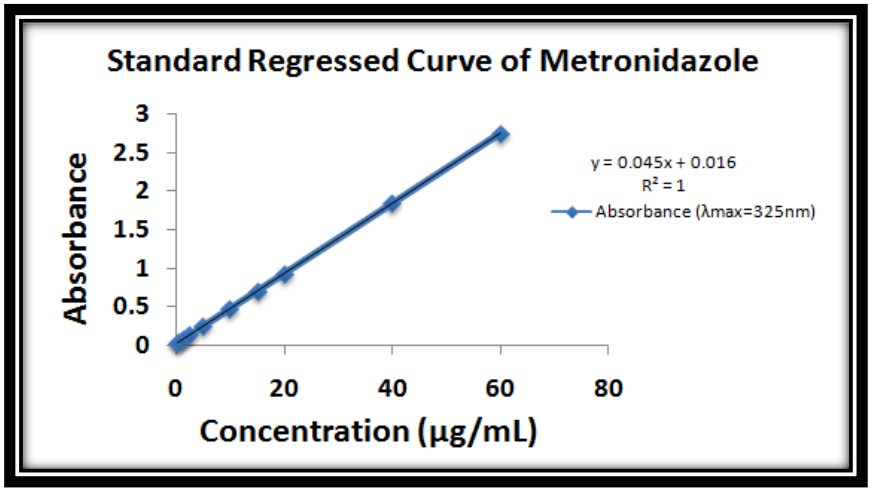


Fig (9) Standard regression curve graph

The absorbance (y_2) was found to be the line of regression and the curve was found to be the standard regressed curve. In figure (9), the value of R^2 (regression coefficient) was found to be equal to 1, and this means that the line is perfectly straight.

The above work is done because the values of slope and intercept was directly calculated from the software and the manual calculation found a tedious job. Also the improper way to handle the glassware and some minor negligence in plotting the graph can make the results differ. Finally, the goal of the representation of the standard regression curve graph {figure (9)} was to obtain the regressed line {y_2=bx+a, equation[4]} which was useful to calculate the metronidazole released (%) in *in vitro* release studies and *in vitro* metronidazole release kinetic studies.

4.1.3 *In Vitro* Release and Kinetics of Metronidazole

After the application of dialysis tube method[87] which was followed in *in vitro* metronidazole release study, the data were obtained and they were plotted between % cumulative drug release (y-axis) against the time (hrs) (x-axis) by Microsoft Excel 2007 as shown in figure (10) to explain the behaviour of metronidazole release (µg/mL) from the prepared formulations with an increasing time. Figure (10) included a number of the prepared formulations with the same method which were different in metronidazole concentrations (mg/mL).

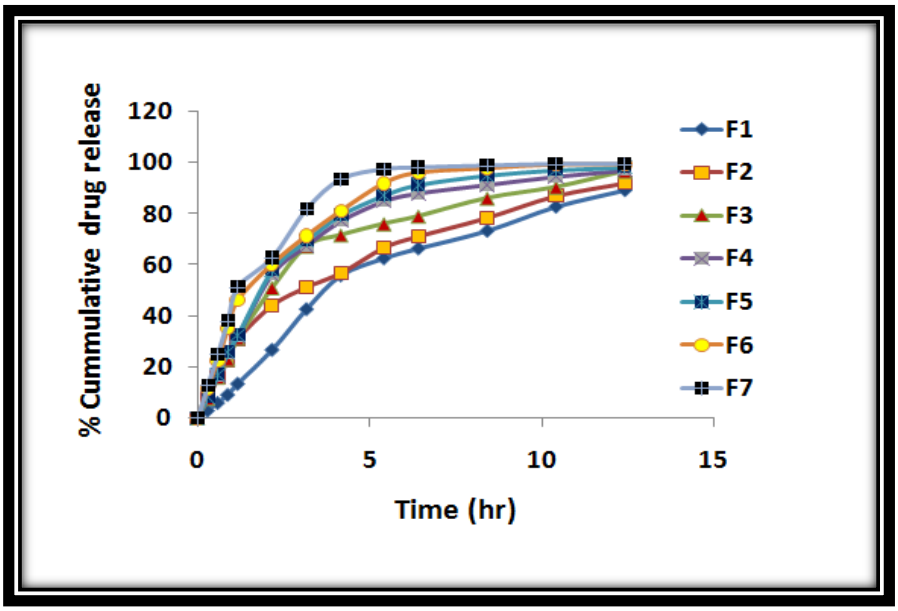


Fig (10) *In vitro* release profile metronidazole from film F_1 to F_7

Generally, figure (10) showed that the metronidazole release (µg/mL) from each prepared formulation increased with increasing the time. Also figure (10), the released metronidazole concentration (µg/mL) from the prepared formulation with a minimum concentration (mg/mL) of metronidazole at the determined time was lower as compared to the released metronidazole concentration (µg/mL) from the prepared formulation with a maximum concentration (mg/mL) of metronidazole at same the time. These results corresponded to the results obtained from the inhibition

zones of *in vitro* biochemical studies of same the prepared formulations in the biochemical part. Figure (11) showed an initial burst release of F_3 within first 15min. This observation was expected to inhibit the growth of the microorganisms assaied in *in vitro* biochemical study. It is worth mentioning that F_3 was described with good adhesiveness without any difficulty of administration and other handling problem.

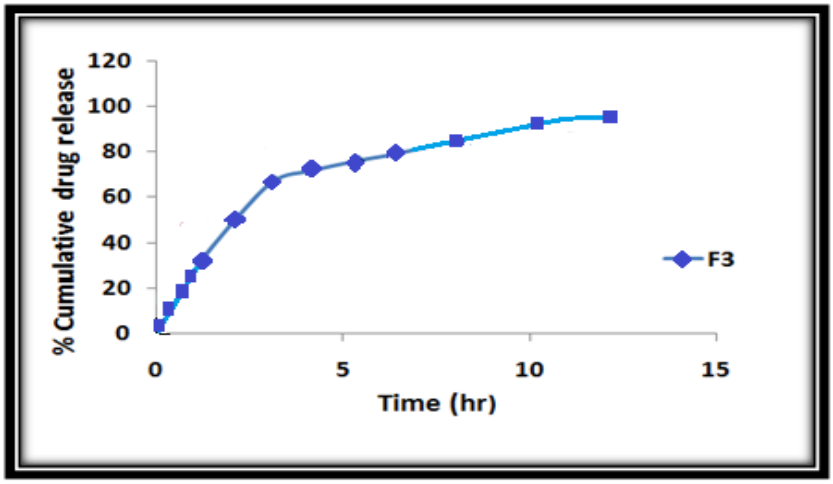


Fig (11) *In vitro* release profile metronidazole from the formulation F_3

The release (dissolution) of metronidazole from F_3 was evaluated by different kinetic models. The release patterns of these formulations were evaluated and calculated manually by Microsoft Excel 2007 using different equations including zero order, first order, Higuchi plot and korsmeyer-Peppas plot. These patterns were shown in figures (12-15).

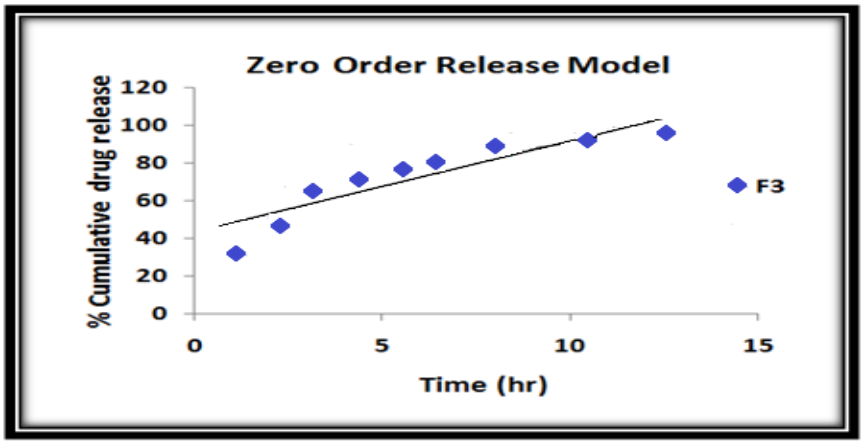


Fig (12) Zero order release plot of F_3 formulations

The release data were analyzed according to these equations depicted in table (6). The interpretation of data was based on the value of the resulting regression coefficients. F_3 followed zero order.

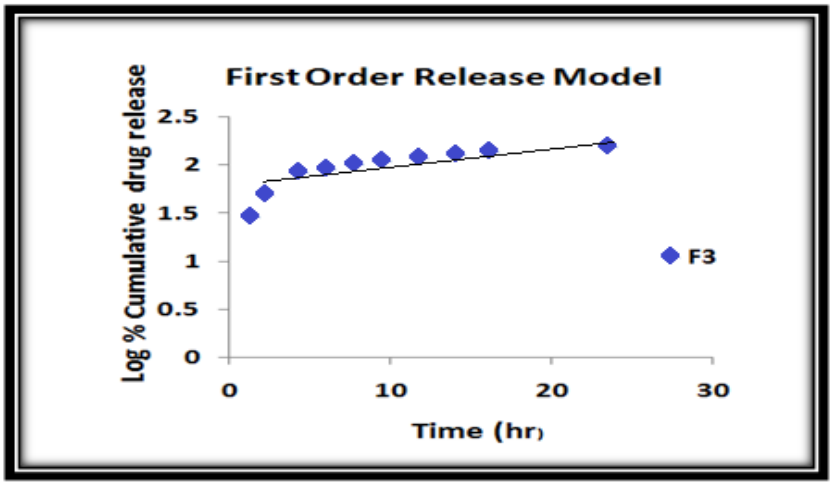


Fig (13) First order release plot of F_3

To understand the mechanism of release of metronidazole from the formulations F_3, the drug release data were fitted into Higuchi model and korsmeyer-Peppas model. The drug release data showed that the highest regression coefficient values were for korsmeyer-Peppas model.

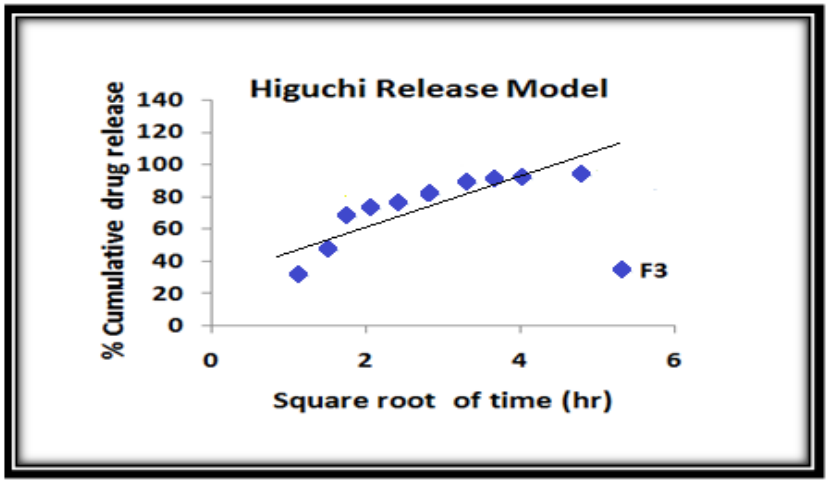


Fig (14) Higuchi plot of F_3 formulations

Also table (6) showed that all the formulations followed Fickian (n<0.5) transport mechanism (release mechanism), viz., diffusion controlled release .

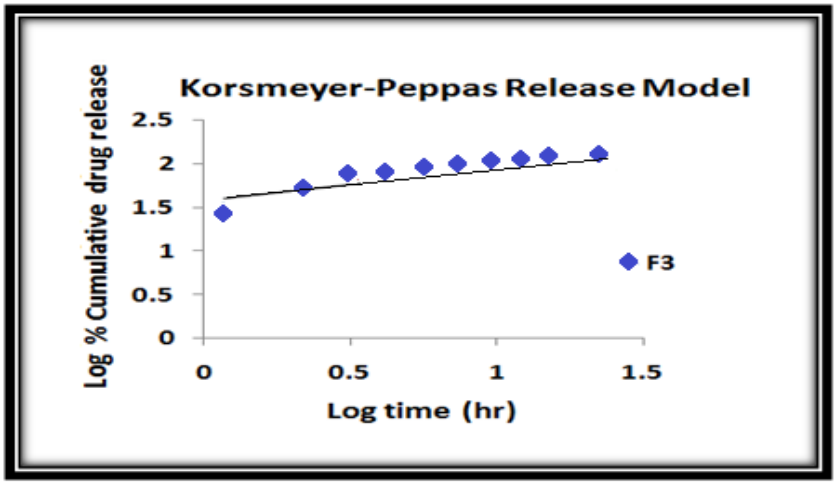


Fig (15) Korsmeyer-Peppas plot of F_3 formulations

Table (6) Correlation coefficient and constants of different kinetic models

Formula code	Zero order		First order		Higuchi model		Korsmeyer-peppas model	
	R^2	K_0	R^2	K_1	R^2	K_H	R^2	n
F_3	0.823	4.852	0.493	0.01	0.809	17.04	0.868	0.368

Whenever R^2 values were high, the rates of drug release were slow. This explanation conformed with the explaination of figure (15).

4.2 Biochemical Part

4.2.1 *In Vitro* Study

4.2.1.1 The Influences of S_1 to S_7 on the Microbial Isolation Growth

The values of inhibition zones (cm) were different from one isolate to another toward the different concentrations of metronidazole solutions (S_1 to S_7) (liquid formulations). Statistical analysis results indicated the existence of a significant difference between these concentrations as shown in figure (16). The highest inhibition value was 4.52cm for *Staphylococcus aureus* isolate by S_7 and the lowest inhibition value was 1.94cm for *Staphylococcus epidermidis* isolate by S_3. *In vitro* biochemical activity of S_0 and S_3 against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Candida albicans* and *Trichophyton* sp. isolates . *In vitro* biochemical activities indicated the existence of a significant difference at $p<0.05$, viz., S_7 which was used against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Trichophyton* sp. and S_6 which was used against *Candida albicans* showed high significant difference as compared to remaining concentrations as shown in table (7).

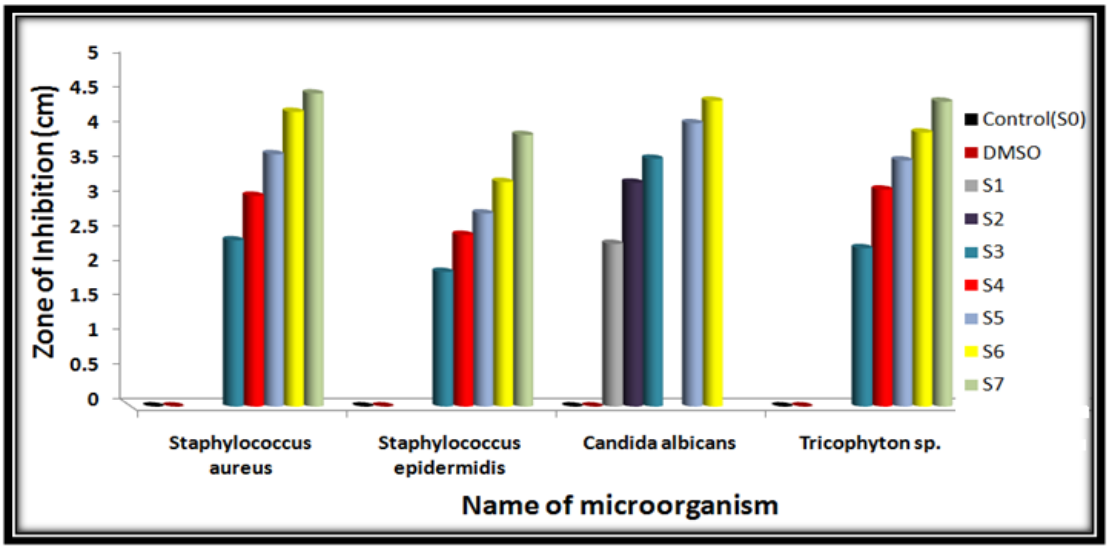


Fig (16) Histogram showing *in vitro* biochemical activities of S_0 as control, DMSO and

different concentrations of metronidazole (S_1 to S_7) (liquid formulations)

Table (7) In vitro biochemical activities of different concentrations of metronidazole (S_1 to S_7) (liquid formulations)

Mean diameter of inhibition zone in cm (mean ± S.D)*				
Formula code	**Microorganisms**			
	Staphylococcus aureus	***Staphylococcus epidermidis***	***Candida albicans***	***Trichophyton* sp.**
S_0	NZ	NZ	NZ	NZ
S_1	-	-	2.35±0.131e	-
S_2	-	-	3.233±0.180d	-
S_3	2.4±0.396e	1.944±0.357e	3.579±0.097c	2.287±0.100e
S_4	3.044±0.406d	2.478±0.290d	-	3.133±0.192d
S_5	3.644±0.710c	2.789±0.378c	4.092±0.092b	3.554±0.184c
S_6	4.256±0.731b	3.244±0.421b	4.417±0.109a	3.958±0.176b
S_7	4.522±0.882a	3.922±0.633a	-	4.404±0.151a
DMSO	NZ	NZ	NZ	NZ

*: (n=3); a, b, c, d and e: The means which had the same letter indicated non existence significant differences between them and vice versa ($p<0.05$) for the same isolate; NZ: No Zone of inhibition

4.2.1.2 The Influences of F_0 to F_7 as Compared to Commercial Gel on the Microbial Isolation Growth

The values of inhibition zones (cm) were different from one isolate to another toward F_0, F_1, F_2, F_3, F_4, F_5, F_6 and F_7 (solid formulations) and they were compared to the values of inhibition zones of commercial gel (semisolid formulation). Statistical analysis results indicated the existence of a significant difference between these formulations and as compared to the commercial gel as shown in figure (17). The highest inhibition value was 5.06cm for *Trichophyton* sp. isolate by F_7 and the lowest inhibition value was 2.07cm for *Candida albicans* isolate by F_1. When the observation of the figure (17), the inhibition values of the prepared formulations (F_1 to F_7) were approximated to the inhibition values of the commercial gel, viz., the prepared formulations (F_1 to F_7) are useful in the biochemical applications. F_0 was not active against all the microbial isolates. Also these results

indicated the existence of a significant difference at $p<0.05$, viz., F_7 which was used against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Trichophyton* sp. and F_6 which was used against *Candida albicans* showed high significant difference as compared to other formulations as shown in table (8).

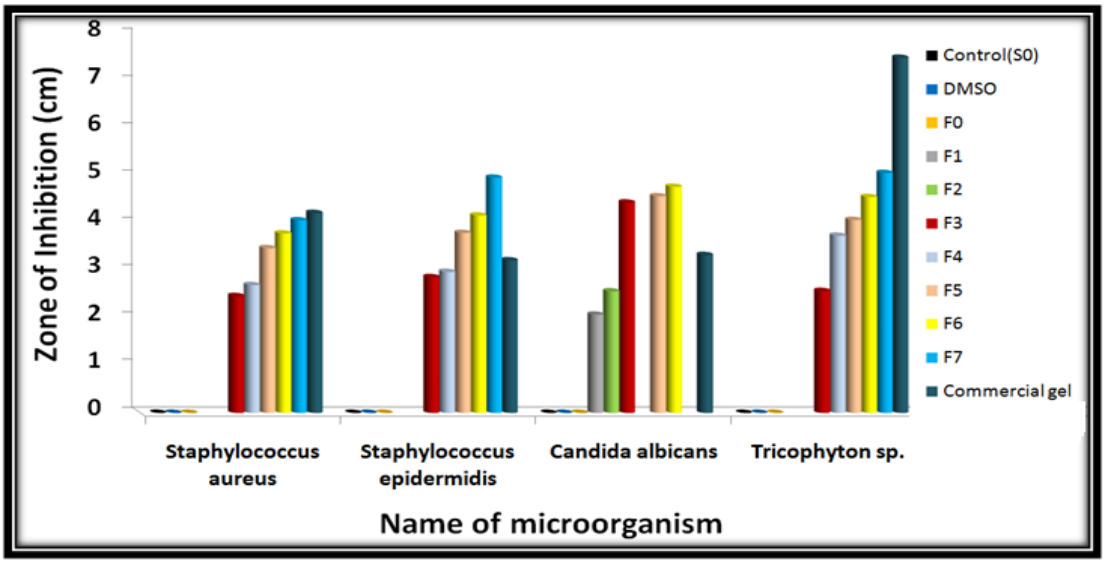


Fig (17) Histogram showing *in vitro* biochemical activities of S_0 as control, DMSO, the prepared formulations F_0 to F_7 (solid formulations) and commercial gel (semisolid formulation)

Table (8) In vitro biochemical activities of different concentrations of metronidazole (F_0 to F_7)(solid formulations)

Mean diameter of inhibition zone in cm (mean ± S.D)*				
Formula code	**Microorganisms**			
	Staphylococcus aureus	***Staphylococcus epidermidis***	***Candida albicans***	***Trichophyton* sp.**
F_0	NZ	NZ	NZ	NZ
F_1	-	-	2.075±0.0442e	-
F_2	-	-	2.567±0.048d	-
F_3	2.467±0.05e	2.867±0.05e	4.442±0.0653c	2.575±0.044e
F_4	2.7±0.00d	2.978±0.044d	-	3.742±0.065d
F_5	3.478±0.044c	3.8±0.086c	4.575±0.044b	4.075±0.044c
F_6	3.789±0.033b	4.167±0.05b	4.775±0.044a	4.558±0.065b
F_7	4.067±0.05a	4.967±0.05a	-	5.067±0.048a
Commercial gel	4.222±0.204	3.222±0.571	3.333±0.127	7.5±0.00
DMSO	NZ	NZ	NZ	NZ

*: (n=3); a, b, c, d and e: The means which had the same letter indicated non existence significant differences between them and vice versa ($p<0.05$) for the same isolate; NZ: No Zone of inhibition

Metronidazole was selected as the anti-inflammatory and the antimicrobial agent for this study because it has been shown to be highly effective toward dermatitides and microbes[88]. After drug application, metronidazole readily permeates mammalian cells as well as anaerobic and aerobic microbial cell membranes by diffusion, to achieve a steady state intracellular concentration. When the nitro group of the compound is reduced by nitroreductase (ferrodoxin-like electron

transport protein), a concentration gradient is created and more drug enters the cells. The reduction of metronidazole leads to the release of decomposition products with toxic properties (nitro, nitroso, nitroso-free radicals and hydroxylamine derivatives)[89]. It is believed that these intermediates interfere with deoxyribonucleic acid (DNA) synthesis. Once in the cell, metronidazole binds to the DNA strands and disrupts the helical structure of the molecule. DNA strands breakage and inhibition of nucleic acid synthesis occurs, which ultimately leads to cell death. This process results in a rapid killing of the microorganisms especially anaerobes. The hydroxymetabolite of metronidazole also exhibits antimicrobial activity. This suggests that there may be synergism between the parent drug and its metabolite, which may account for the greater than expected clinical efficacy of the drug in treating microbial infections[90]. It is worth mentioning that metronidazole is primarily metabolized in the liver and its metabolites are excreted in the feces[91].

HEC and C_{940} were considered as bio (muco) adhesive pharmaceutical polymers[92, 93], viz.,their bioadhesive property was high (+ + +). Whilst, glycerol was used in very little amounts and it was considered as inactive materials toward microbes. It was used as a plasticizer to increase the folding endurance for the films through lowering T_m and T_g, as well as increasing the softness and the flexibility.

Formulations

4.2.2 *In Vivo* Study

4.2.2.1 Subacute Toxicity Study

The results of subacute toxicity test (LD_{50} dermal route) did not shown any state of death or any other effects in all groups. That is, the behaviour of these animals was normal when they were compared with control group to period three days. These results proved that the prepared formulations which were topically applied on the bare back of animals were not toxic according to the scheme of toxicity classification. This scheme represented that the material is considered non-toxic when this material did not cause a state of death in animals with dose (>15g/kg)[94]. In addition, these results were in accord with the apparent results of the medical administrations of topical metronidazole as gel, cream and lotion. these medical administrations proved that the minimal absorption of metronidazole and consequently its insignificant plasma concentration which happened after topical administration[13, 51, 52, 95-97]. The hydrogels (C_{940}, PVA and HEC) which were used in the preparation of various formulations as films, they were considered biodegradable materials and their analyitical results were non-toxic according to the novel researches in this connection[98-101].

4.2.2.2 Skin Irritation Studies

4.2.2.2.1 Back Irritation Study

All the formulations were homogenous in the texture and showed no skin irritation (safety) for all groups of animals as compared to a control group upon the application for 24hrs.

4.2.2.2.2 Rectal Irritation Study

All the formulations were found to be non-irritant to rectums of animals.

Conclusions

After the observation of the results which were obtained from this study, a number of conclusions were determined, as follows: DSC analysis confirmed that ester bond was formed between the used polymers in preparing the films, the prepared films are considred semi-IPNs hydrogels and the mixture between the used polymers and metronidazole in preparing the films is considred physical mixing, viz., chemical, physical and therapeutical properties of drug was firm (stable). Weight uniformity of the prepared films (F_0 and F_3) showed that the average of the weight of F_3 was higher as compared to F_0. Thickness uniformity of the prepared films (F_0 and F_3) showed that the thickness of F_3 was higher as compared to F_0. Swelling study of the prepared films (F_0 and F_3) confirmed that the used polymers in the preparation of the films are considred biodegradable materials according to the cleavage of the ester bonds and erosion process with physical disintegration and mass loss. *In vitro* drug release study of all the prepared formulations confirmed that the drug release was increased linearly with increasing the time and in the same type of the prepared formulations, the released drug from the prepared formulation with minimum concentration of drug at determined time was lower as compared to the released drug from the prepared formulation with maximum concentration of drug at same the time. *In vitro* drug release kinetics study of the prepared formulation (F_3) followed zero order, Korsmeyer-Peppas model and Fickian ($n<0.5$) transport mechanism (diffusion controlled release). With increasing drug concentration (mg/mL) which was loaded with the used polymers in the preparation of the formulations, R^2 values will decrease and drug release will increase. This will cause to make these prepared formulations unuseful and are not obedient to controlled and slow drug release systems. The prepared formulation F_0 has not a good inhibition toward the selected microorganisms according to describe their polymers except PVA (with a big amount) with biochemical activities, while the prepared formulations F_1 to F_7 have high inhibition toward the same selected microorganisms as compared to pure drug and commercial gel at $p<0.05$. *In vivo* study confirmed that the prepared formulations including F_0 and F_3 were not poisonous and non irritant.

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تحضير بعض صيغ المترونيديزول كأفلام وتقييمها ودراسة فعالياتها الدوائية ضد الاحياء المجهرية المرضية المسببة للألتهابات الجلدية

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الملخص

في الدراسة الحالية, جرى العمل لتطوير أنواع من الهلاميات لنظام علاجي عبر الجلد مؤلفا من تراكيزمختلفة من المترونيديزول مع أتحادات بوليمرية آلفه (محبه) للماء بأستخدام تقنية تبخر المذيب. لقد أثبت تحليل المسح الحراري التفاضلي (DSC) تكون أواصر أسترية بين البوليمرات المستخدمة في تحضير الافلام, وأعتبرت الافلام المحضرة هلاميات من نوع أشباه بوليمرات شبكية التداخل والتي تعرف أيضا بأسم السبائك البوليمرية (semi–IPNs), كما وأعتبر مزيج البوليمرات والدواء المستخدمين في تحضير الافلام مزيجا فيزيائيا, أي إن الخصائص الفيزيائية والكيميائية والعلاجية للدواء بقيت نفسها دون أن تتغير. لقد تضمنت هذه الدراسة جانبين رئيسيين تمثلا بالجانب الكيميائي والجانب الكيموحياتي. ففي الجانب الكيميائي تم تقييم الافلام المحضرة F_0 و F_3 من خلال دراسة اتساق (انتظام) الوزن, واتساق السمك, وسلوك الانتفاخ. كما وتمت دراسة سرع تحرر الدواء وحركيات سرع تحرر الدواء (خارج الجسم الحي) وذلك لكل الصيغ الدوائية المحضرة. ولقد تبين بأن الصيغة المحضرة F_3 كانت أعلى من حيث معدل الوزن ومن حيث السمك مقارنة مع الصيغة المحضرة F_0 , وبالاضافة إلى إمتلاك تلك الصيغتين لنفس سلوك الانتفاخ والذي فسر على أساس الزيادة في نسبة الانتفاخ لغاية الفترة الزمنية عند 70 دقيقة والتي أتبعت بنقصان في تلك النسبة وفقا لكسر الاواصر الاسترية المتكونة في التركيب المتشابك, عملية تأكل مع تحطم فيزيائي, وفقدان كتلة. كما وتبين بأن الصيغة المحضرة F_3 أتبعت المرتبة الصفر, نموذج (Korsmeyer–Peppas), وميكانيكية الانتشار المسيطر (Fickian (n<0.5) transport) . أما في الجانب الكيموحياتي فقد تم دراسة الفعاليات الكيموحياتية (خارج الجسم الحي) لكل الصيغ المحضرة (كمضادات الجرثومية) وذلك لأربع عزلات جرثومية أشتملت *Staphylococcus aureus* (الموجبة لصبغة كرام), *Staphylococcus epidermidis* (الموجبة لصبغة كرام) , *Candida albicans* (خميرة), و *Trichophyton* sp. (عفان) بطريقة الأنتشارالمتبعة بالحفر. وقد تبين بأن إزدياد الفعاليات الكيموحياتية لهذه الصيغ تجاه تلك العزلات قد ترافق مع الزيادة في تراكيز الدواء المحمل مع البوليمرات المختارة في تلك الدراسة, وبالاضافة إلى إمتلاك تلك الصيغ لفعاليات كيموحياتية وضد جرثومية عالية لتثبيط نمو العزلات الجرثومية وذلك بالمقارنة مع الدواء لوحده ومع الهلام التجاري عند مستوى إحتمالية أقل من 0,05($p<0.05$). كما تم تطبيق الصيغتين المحضرتين F_0 و F_3 على جلد أرانب ذكور لدراسة السمية (subacute toxicity (LD_{50})), إختباري إثارة (تهيج) الجلد, وإختبار معالجة إثارة (تهيج) الجلد المستحدث. وقد تبين بأن تلك الصيغ لم تكن سامة أو مثيرة (مهيجة) للجلد.