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Some Variables Affecting the Formulation of Pentoxifylline (PTX) as a Solid Sustained Release Dosage Form

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Abstract

An inert matrix that is used to control the release of (PTX) was prepared using Eudragit RL100 and RSPM types as matrix forming agent . The matrices were prepared by either dry granulation(slugging) , or wet granulation method using chloroform as a solvent evaporation vehichle. The cumulative release was adjusted by using polyvinylpyrollidone (PVP) or ethylcellulose (EC) polymers .The results indicated that both methods of preparation were valid for incorporation PTX as a sustained release granules .Moreover ,the results revealed that best polymer used was Eudragit RSPM in 3:20 polymer drug ratio .Besides to that , the results indicated that the release profiles were affected by pH- medium , PVP and EC addition as an enhancer or retardant polymer used respectively. As well as to the method of preparation .

Key words : Pentoxifylline Sustained Retease , Eudragit RS , RL .

الخلاصة

القالب الغير فعال المستخدم للسيطرة على تحرر عقار البنتوكسيفلين قد حضر بأستخدام اليودراجيت نوع ار.ال100 و كذلك ار.اسب م , لقد تم تحضير القالب بأستخدام طريقة الحبيبات الجافة (الاقراص) و الحبيبات الرطبة بأستخدام الكلوروفورم كمادة طيارة في طريقة التحضير . لقد اشارت النتائج الى أن كلا الطريقتين كانتا صالحة لأدخال العقار كحبيبات بطيئة التحرر علاوة على ذلك فأن النتائج أظهرت ان افضل بوليمر استعمل هو يودراجيت أر.اسب م بنسبة 20:3 بوليمر : عقار . كما اشارت النتائج الى ان الأوساط الحامضية مختلفة الأس الهايدروجينى لها تأثير على تحرر العقار . اضافة الى البولي فينيل بايروليدون و الاثيل سلليلوزكمواد مسرعة او مبطئة على التوالي . علاوة على طريقة التحضير .

Introduction

Tablet dosage form can be defined as a unit dose of medication containing one or more of medicinal agents, with or without diluents, made by molding the mixture in a suitable compressible shape(1).Sustained release dosage form having drug release features based on the time or location designed to accomplish convenience and therapeutic not offered by conventional release form (2).An inert matrix was used to control the drug release , and this can be adjusted by using the enhancers such as microcrystalline cellulose , polyvinylpyrrolidone or surfactants .Eudragit RL100 and RSPM ,are methacrylic copolymers introduced as a coating materials with different permeabilities (3) , depending on functional ionized or neutral groups .They are commonly used in sustained release dosage forms (4). The distinguishing letters RL and RS related to the initial letters of German words " Leihtduchlassig " freely permeable and Schwerduchlassig , slightly permeable . Pentoxifylline (PTX) also called oxpentifylline is one of the xanthine vasodilator derivative (5) ,that improve with its active metabolite peripheral aterial circulation, and enhance tissue oxygenation The apparent plasma half life of the drug and its metabolite is 2-3 hours . On the bases of using PTX as drug of choice in chronic occlusive aterial diseases , therefore ,it is of a wise candidate drug to be formulated in Sustained release oral dosage form. The usual dosage form of PTX in controlled release tablet from available maintained one is one tablet (400mg.) twice daily . The objective of this study is to prepare controlled release PTX matrix tablets , utilizing dry and wet granulation technique by using Eudragit RL and RS types with PVP and EC polymers as enhancer and retardant materials respectively , to control the release of PTX for extended period (6) .

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Materials and Methods

Materials

Pentoxifylline supplied by slovakopharma , Slovenia , Aceton,acetic acid,chloroform ,ethylcellulose , PVP,from BDH chemicals ,Ltd.Liverpool , England Disodium hydrogen phosphate , HCL ands Lactose from Reidal De Haen Ag Seelz Hanover , Germany (GER) , Eudrait RL100 and RSPM , from Rhom pharma GMBH Weiterstadt GER. ,All other reagents were of analytical grade .

Equipments

Dissolution apparatus ,Copley dissolution DIS 8000 , Copley Scientific , Ltd , UK , Hardness tester , Stokes Monsanto Corporation Limited ,England ,pH-meter ,Hanna Instrument pH 211 Microprocessor , Italy,Oven , Water bath , Memert UL 80 , Rostfrei, Schwach , Germany . Friabilator , Sieves , Roche type Moore and Wright ,Sheffield , England ,Sartorious Balance , Werk-GmbH ,Type 1265 MD , 2854 MD,Germany ,Spectrophotometer , Carry win UV , Varian , Australia ,Tablet Machine double punch , Korsch , type EKO , Erweka ,GmbH, Kr , Offenbach Main , Germany

Methods :

Preparation of Eudragit Solutions :

Eudragit solutions RL100 and RSPM were prepared separately at a concentrations 5 ,10 ,15% by dissolving the desired amount of Eudragit RL100 and RSPM with the desired volume of chloroform , the mixture was shacked for 15 minutes in 25°C until homogenous clear transparent solution was resulted [7]

Wet Granulation :

Different formulas (table 1) were prepared by weighing the drug and excipient equivalent to 30 tablets after drying , then blending the powder and mixed with binding solution of Eudragit polymers used gradually , until proper ball test consistency was result ,The wet mass was screened through 12 mesh sieve and dried in pre warmed oven maintained at 50°C for 2 hours , the dry granules were reduced in size by screening them through 16 mesh size sieve . Then an equivalent weight of granules that contain 400 mg. PTX was mixed with calculated amount of magnesium stearate 0.5% w/w for one minute and compressed into a tablets using 10mm. biconcave double punch compression tablets machine at 11ton compression force

Table (1) . Different Formula of Pentoxifylline with Different Physical Properties of Compressed Tablets

Frmula	PTX (gm)	Eudragit RL(mg)	Eudragit RS(mg.)	PVP (mg)	EC (mg)	Lactose (mg.)	Magnesium stearate (mg.)	Total weight (mg.)	Friability %	Hardness (mg.)	APPEARA
F1	400	20				127.25	2.75	550	3.0	17.1	Friable , Wdusty
F2	400	40				107.25	2.75	550	2.8	17.2	Friable , Wdusty
F3	400	60				87.25	2.75	550	1.5	19	Friable , Wdusty
F4	400	80				67.25	2.75	550	1.0	>20	Friable , Wdusty
F5	400		20			127.25	2.75	550	3.0	17.2	Friable , Wdusty
F6	400		40			107.25	2.75	550	2.7	17.4	Friable , Wdusty
F7	400		60			87.25	2.75	550	1.5	18.4	Friable , Wdusty
F8	400		80			67.25	2.75	550	0.9	>20	White Accepte
F9	400		60	13.8		73.45	2.75	550	0.67	>20	Gray Accepte
F10	400		60	27.6		59.65	2.75	550	0.54	>20	Faint yellow Accepte
F11	400		60	41.4		45.85	2.75	550	0.32	>20	Faint yellow Accepte
F12	400		60		13.8	73.45	2.75	550	0.66	20	White Accepte
F13	400		60		27.6	59.65	2.75	550	0.42	20	White Accepte
F14	400		60		41.4	45.85	2.75	550	0.34	20	White Accepte

Dry Granulation :

The method of dry granulation was introduced as an alternative method for a

selective formula (F14) , this was briefly done by weighing the drug and another excipients that equivalent to 30 tablets,then blending and compressed by means of wide single punch machine into 22mm. diameter slugs. These slugs were reduced in their sizes by milling and sieved by 16 mesh size sieves , the resulted particles were mixed with calculated amount of magnesium stearate 0.5% (w/w) and compressed into 10mm. double punch compression tablet machine at the same compression force .

Assay of Total PTX in Prepared Sustained Release (SR) Tablets:

An accurate weight of powder of triturated tablets equivalent to 400mg. of PTX was added to 500ml. of distilled water and shacked for 15 minutes and filter 1ml. of filtrate was diluted to the appropriate concentration (8µg/ml) with distilled water , the absorbance of the final solution was determined spectrophotometrically at 274nm wave length[5] This procedure was performed for those tablets prepared in all methods mentioned before .

Assay of Total PTX in Reference SR- Tablets (TRENTAL 400mg):

Ten tablets of the reference product were weighted individually to get the net weight of each tablet , and then triturated together , An accurate weight of triturated powder equivalent to 400mg. PTX was dissolved in 500ml. distilled water , the final solution was diluted to maintain PTX concentration at 8µg/ml by distilled water , the absorbance was measured using UV-spectrophotometer at 274nm. wave length [5] .

Friability and Breaking Strength Measurement

This measurement was carried out for all prepared formulas using Roche friabilator . the loss in weight of 20 tablets tested before and after rotation should not exceed 1% of total weight of tablet [8] .On the other hand the hardness of the prepared tablets were examined by means of Monsanto hardness tester .

Dissolution Behavior:

Dissolution of the PTX from prepared tablets was carried out using USP basket method maintained at 37°C temp. , and 75 r.p.m speed for 500ml.of buffer solutions (pH 1.2 ,4.6,and 6.8). Samples of 5ml. were withdrawn at 1 hour time intervals for 6-10 hours then the samples filtered and diluted, then cumulative drug release was measured at 274nm. for triplicate samples .

Results and Discussions

Formulation of PTX as A SR- Tablets :

All the powders blend and granules were intended for compression successfully into shiny look appearance . the breaking strength were varied from friable to hard (20kg.) hardness. This variation may be referred to the polymer type and concentrations [9, 10] . as shown in formula 4 and 8. On the other hand PVP presence gave harder granules (formula 12) compared with others [11] . The same results obtained when ethylcellulose was used as retardant material , since both PVP and EC act as binding and consolidating agents in tablets formulation [12,13] .

Dissolution Behavior

Effect of Eudragit Polymer :

Figures (1 and 2) illustrate the cumulative release of PTX from various polymer-drug ratios in phosphate buffer (pH6.8) , the results indicated that the drug release is increased as a function of increasing this ratio for both Eudragit types used , this behavior referred mainly to the thickness of the polymer around drug particles, that results in a delayed permeation of water inside drug particles and dissoluted drug outside stagnant layer of PTX particle [14] .On the other hand the cumulative release of PTX from two polymers used (fig.3) revealed that after 10h. of dissolution, the percent of drug release from formula 3 is 88% compared with 60% in formula 7 , while the reference tablet (Trental400) gave 51% drug release after same period of dissolution . This difference may be attributed to the presence of highly basic quaternary ammonium groups (1:20 and 1:40)ratios for both Eudrait RL and RS types ,respectively which they undergo more solubilization in acidic medium, then the over all solubility of the drug within deaggregated polymer is increased [15] .

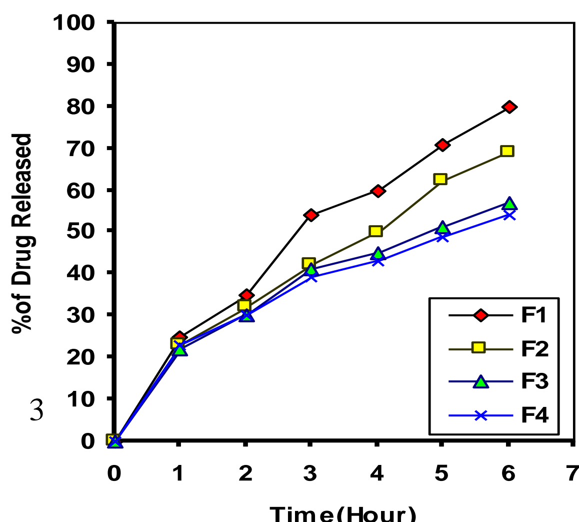

Figure 1. The percent of PTX released from different formulas at phosphate buffer pH 6.8 using Eudragit RL100 polymer.

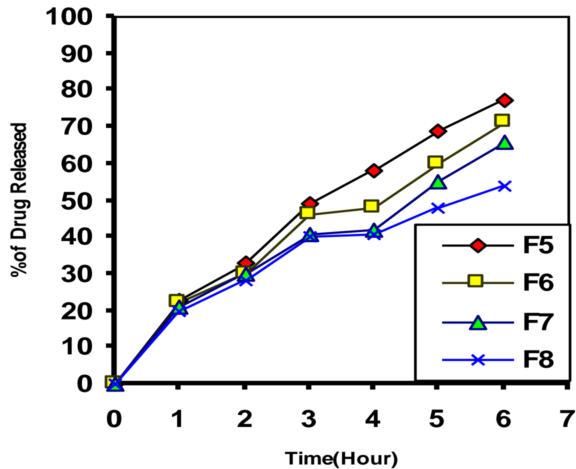

Figure 2. The percent of PTX released from different formulas at phosphate buffer pH 6.8 using Eudragit RSPM polymer.

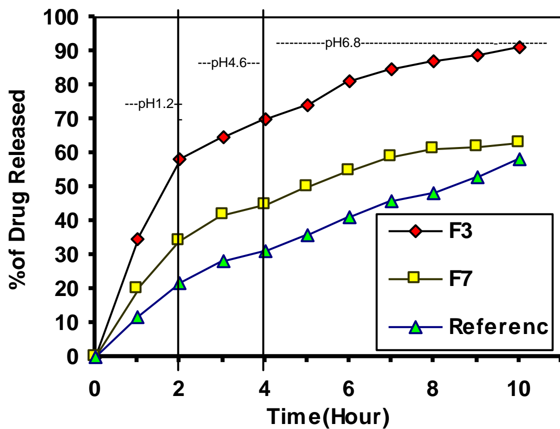

Figure 3. The cumulative percent of PTX released from formulas 3,7 and Reference (Trental 400 mg.) at different pH-medium.

Effect of Polymer Type :

Concerning the type of Eudragit used , fig.3 demonstrate that the time for 50% drug release extended for 2 hours compared with more than 5 hours for both Eudragit RS and RL , respectively . the illustration for this variation related to the higher permeability of Eudragit RL than that of RS polymer type[16] .

Effect of PVP Addition :

Based on the results obtained as shown in fig .4 , It was shown that addition of PVP in formula 7 made of Eudragit RS type , enhance the cumulative release to about 96% when 7.5% w/w PVP used , compared with 50% for reference one this effect may be referred to the higher solubility of PVP in different media mainly at pH 5-7 medium [17] .

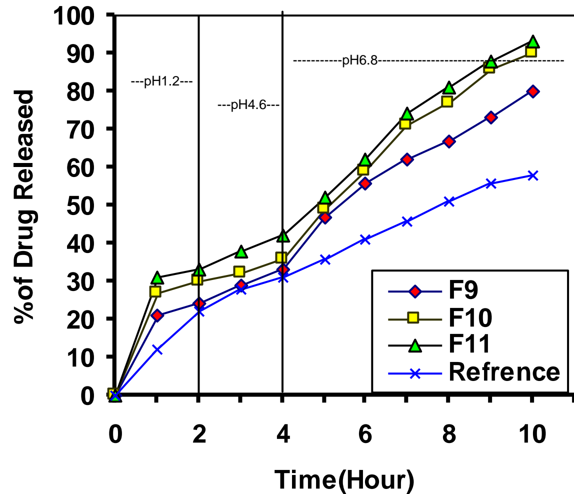

Figure 4. Effect of PVP addition on the cumulative released of PTX from tableted matrix at different pH-medium.

Effect of EC Addition :

The influence of addition 2.5 ,5 and 7.5% w/w of EC to the matrix formulation of formula 7 was illustrated in fig. 5 ,which represents the cumulative release of PTX ,It was seen that when EC incorporated as a retardant material, a slight less or more in drug release was resulted compared with that of reference one (Trental) , and in an appreciab decrease in drug in drug release compared with the other formulas free from EC , which is attributed to the insolubility of EC in water, besides rigid consolidation of Eudragit RS and EC polymer that retain drug in matrix , this phenomena is in a consistent with results obtained by Aldermann [18] and Sreubel [19] .

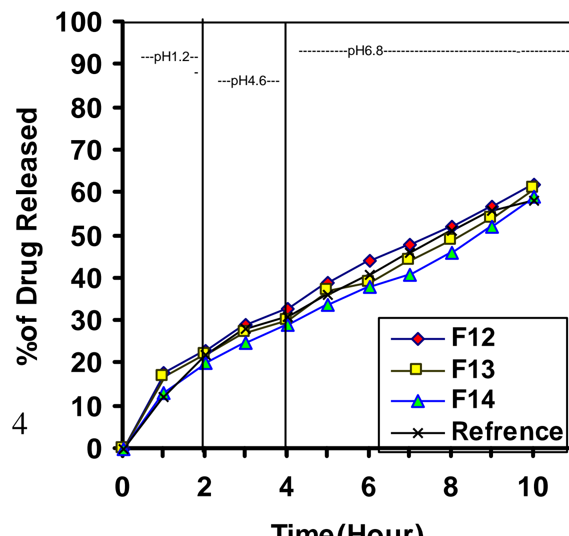

Figure 5. Effect of Ethylcellulose (EC) addition on the cumulative released of PTX from tableted matrix at different pH-medium.

Effect of Granulation Method :

The selected formula 14 was reformulated again using dry granulation method , the results indicated that cumulative drug release by this method gave 75% , compared with 59% for wet granulation after 10h. , as shown in fig. 6 , this behavior may be referred to the solvent effect (chloroform vehicle) that used in a later method help in a formation of more Van Der Waal"s forces among polymer used and PTX (20) . these bonds make a matrix more rigid and delayed drug release through them.

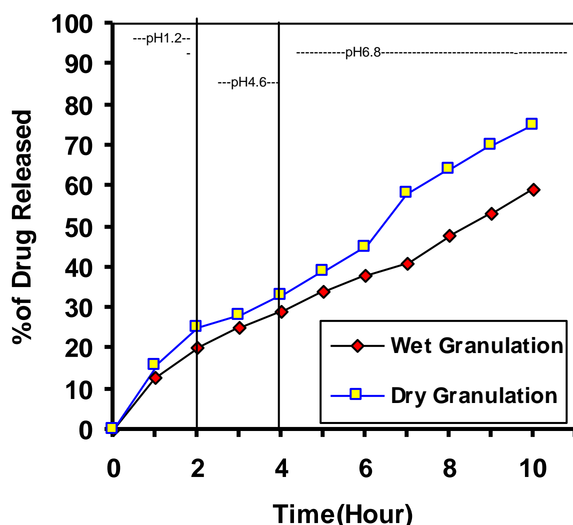

Figure 6. Effect of preparation method on the cumulative release of PTX from selected formula 14 at different pH medium.

Conclusion :

- Concerning the method used , the study demonstrate that both methods were valid for formulation of PTX as promised acceptable SR- granules. But wet method is preferred .
- Both Eudragits used RL100 and RSPM were succeeded in a formation of delayed release matrix .
- Eudragit RS is best to be used in 3:20 polymer :drug ratio for SR granules to utilize PTX as SR matrix similar to (Trental400) .
- The dissolution behavior of PTX from prepared granules varies with different pH-medium , and coating materials .
- Generally , the release of PTX is affected by addition of PVP and EC mainly for matrix made of Eudragit RS(PM) type in a concentration 7.5% w/w.
- Best formula when EC was added to formula 7 represented formula 14 .

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Synthesis and Preliminary Pharmacological Evaluation of Aminobenzensulfonamides Derivatives of Mefenamic Acid as a Potential Anti-inflammatory Agents

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Abstract

A group of amino derivatives [4-aminobenzenesulfonamide,4-amino-N¹ methylbenzenesulfonamide, or N¹-(4-aminophenylsulfonyl)acetamide] bound to carboxyl group of mefenamic acid a well known nonsteroidal anti-inflammatory drugs (NSAIDs) were designed and synthesized for evaluation as a potential anti-inflammatory agent. *In vivo* acute anti-inflammatory activity of the final compounds (9, 10 and 11) was evaluated in rat using egg-white induced edema model of inflammation in a dose equivalent to (7.5mg/Kg) of mefenamic acid. All tested compounds produced a significant reduction in paw edema with respect to the effect of propylene glycol 50% v/v (control group). Moreover, the 4-amino-N-methylbenzenesulfonamide derivative (compound 10) exhibited comparable anti-inflammatory activity to diclofenac (3mg/Kg) at times 180-300 minute with the same onset of action. The results of this study indicate that the incorporation of the 4-aminobenzenesulfonamide pharmacophore and its derivatives in to mefenamic acid maintain its anti-inflammatory activity.
Key ward: benzenesulfonamide, anti-inflammatory, paw edema, NSAIDs, mefenamic acid

الخلاصة

مجموعة من المشتقات الامينية[4- امينوبنزين سلفونامايد, 4-امينو-ان-مثيل بنزين سلفونامايد, ان-(4-امينوفنيل سلفونيل)اسيتامايد] متحدة بمجموعة الكاربوكسيل للميفانميك اسيد (mefenamic acid) الدواء غير الستيرويدي المعروف جيدا كمضاد للالتهاب, قد صممت وحضرت لتقييمها كمضادات قوية للالتهاب . في الجسم الحي, اجري تقييم الفعالية المضادة للالتهاب للمركبات النهائية(9, 10, 11) في الجرذ باستخدام زلال البيض مستحدثة وذمة التهابية تحت الجلد بجرعة مكافئة للميفانميك اسيد (7.5ملغم/كغم).كل المركبات المختبرة انتجت انخفاض مؤثرا للوذمة بالمقارنة مع البروبلين كلايكول(propylene glycol%50) كمجموعة ضابطة.علاوة على ذلك مشتق4-امينو-ان-مثيل بنزين سلفونامايد (مركب 10) اظهر فعالية مضادة للالتهاب مقارنة للدايكلوفيناك (3ملغم/كغم) في اوقات180 _300 دقيقة مع نفس الفعالية الابتدائية للدايكلوفيناك .نتيجة هذه الدراسة تشير الى ان اندماج الجزء العقاقيري 4- امينوبنزين سلفونامايد ومشتقاته مع الميفانميك اسيد حافظ على فعاليته المضادة.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat acute or chronic inflammation and offer symptomatic pain relief[1,2]. Conventional NSAIDs act by non selective inhibition of cyclooxygenase (COX) enzymes, which catalyze the formation of prostaglandins (PGs) from arachidonic acid[3, 4]. There are three isoenzymes of COX (COX-1, COX-2 and COX-3) have been identified[5,6]. COX-1 is expressed in most tissues of the body and largely governs the homeostatic production of arachidonic acid metabolites necessary to maintain physiologic integrity[7]. COX-2 is highly induced in settings of inflammation by cytokines and inflammatory mediators or physiological stress[8,9]. COX-3 activity in human has not been confirmed[10], but it may be implicated in fever[11]. All classic NSAIDs inhibit COX-2 as well as COX-1 to varying degrees; thus they can be considered nonspecific[12,13]. All classical NSAIDs are associated with an increased risk of gastrointestinal (GI) ulcers and serious upper GI complications, including GI hemorrhage, perforation, and obstruction[14,15].

In contrast many of the selective COX-2 inhibitors containing benzene-sulfonamide derivative, like valdecoxib(I) [16] , celecoxib(II) [17],or benzene-N-methyl sulfonamide like compound (III) [18] and benzene methylsulfonyl

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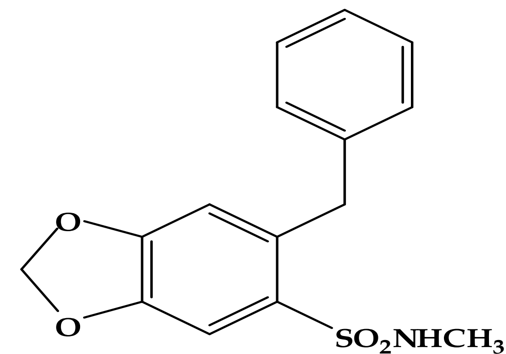
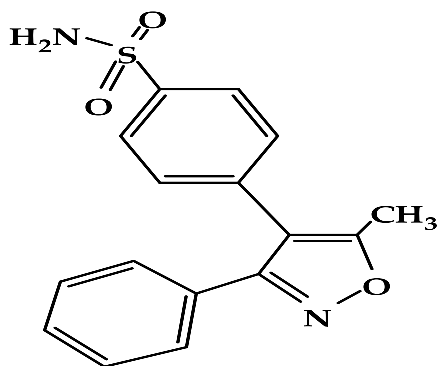
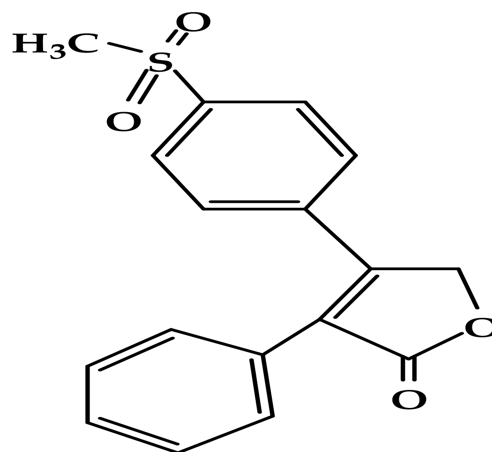
derivative, like Rofecoxib (IV) exert anti-inflammatory and analgesic activity in the clinic with markedly less GI toxicity than traditional NSAIDs[19]. In a recent study, it was shown that the incorporation of a para-N-acetylsulfonamido substitute on the C-3 phenyl ring of the Rofecoxib regioisomer provided a highly potent and selective COX-2 inhibitor (compound V) that has the potential to acetylate the COX-2 isozyme[20]. The improved GI tolerance of COX-2 selective inhibitors not withstanding, there is evidence to suggest that COX-2 selective inhibitors may inhibit COX-1 and induce GI irritation or ulceration with long term use or at higher doses[21,22]. Preclinical cardiovascular and renal liabilities of at least some COX-2 selective inhibitors have also been reported[23]. Thus there is still a need for new anti-inflammatory agents with an improved safety profile.



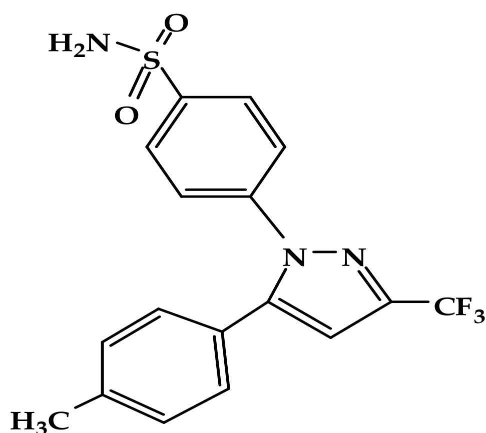
(III)



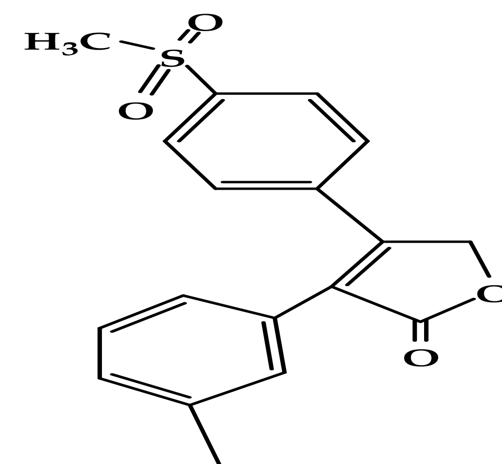
Valdecoxib (I)



Rofecoxib(IV)



Celecoxib (II)



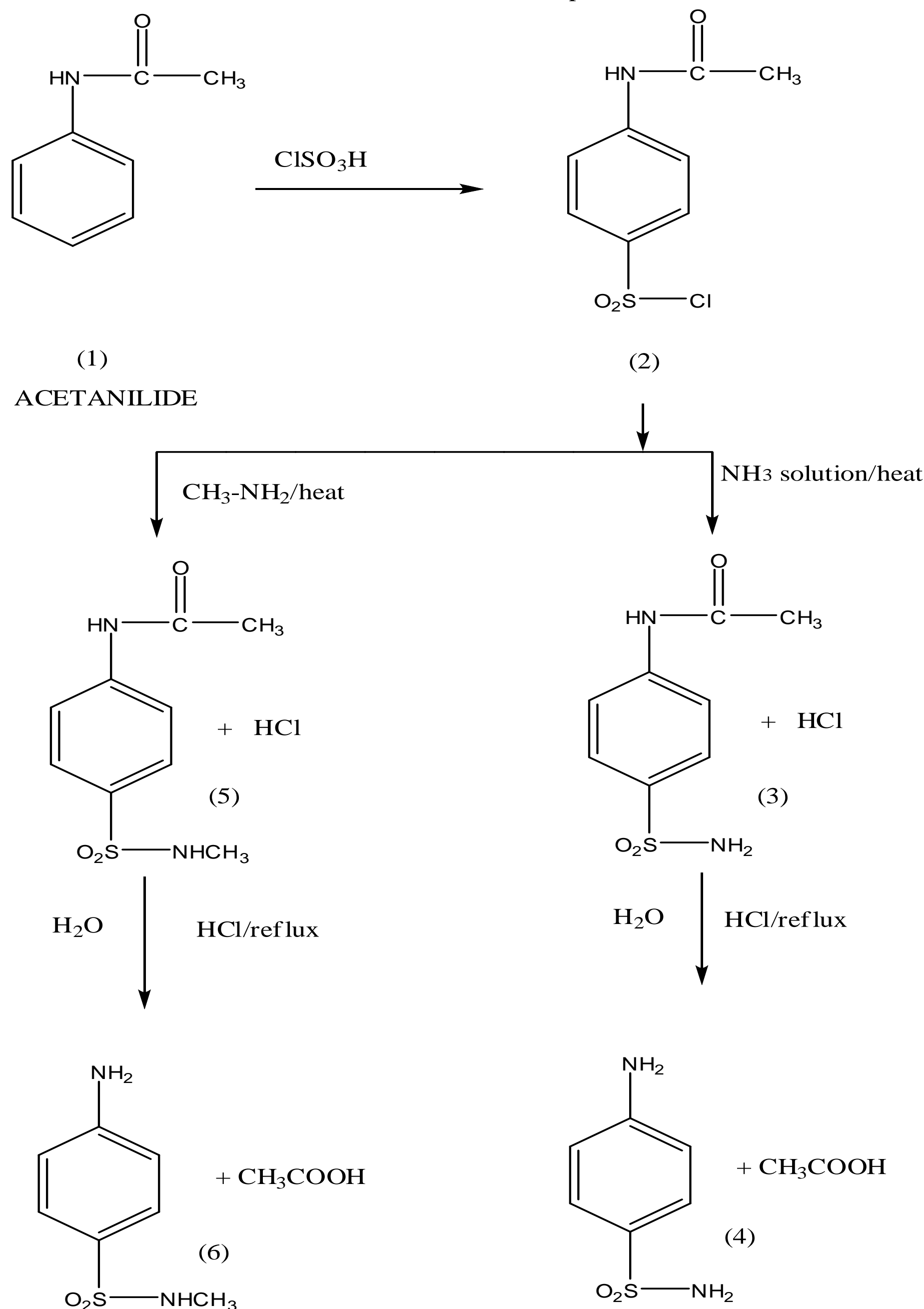
$SO_2NHCOCH_3$

(V)

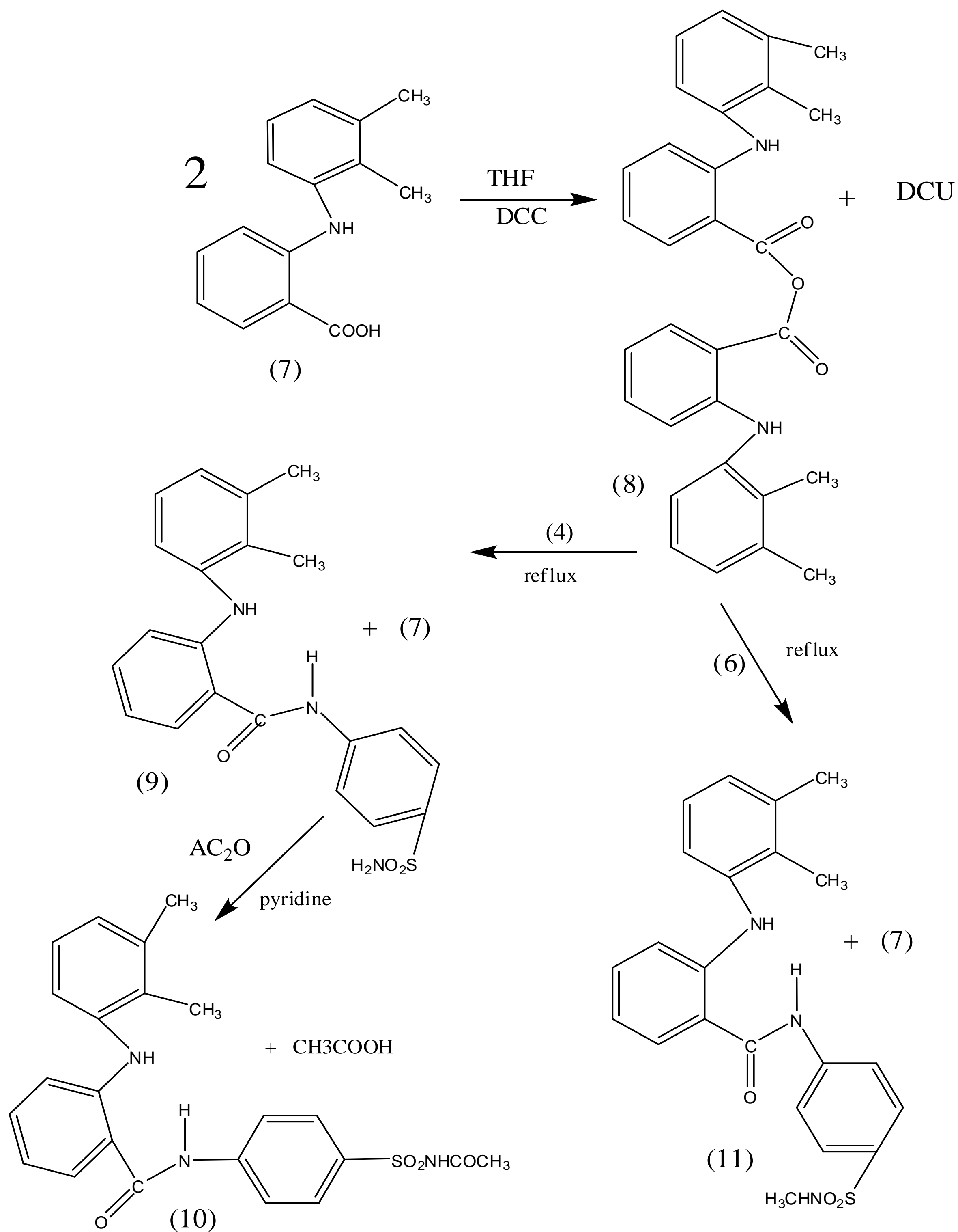
In the view of this background, the present study was conducted to design, synthesize and preliminarily evaluate new mefenamic acid derivatives as potential NSAIDs. [Future study: to measure their selectivity's on COX-2 enzyme.]

Chemistry

The general routes outlined in schemes 1 and 2 were used to synthesize all compounds described here. 4-aminobenzene-sulfonamide (4) and 4-amino-N-methylbenzene sulfonamide (6) was prepared as described by Vogel [24] starting from acetanilide as shown in scheme 1.Their characterization and physical data are presented in the table1.



Scheme 1: Synthesis of 4-aminobenzene sulfonamide (4) & 4-amino-N^1-methylbenzene sulfonamide (6)



DCU: dicyclohexyl urea

Scheme 2: Synthesis of compounds 9, 10, and 11

Table (1): The characterization and physical data of the compounds (3-6 and 8-11).

Compound	Empirical formula	Molecular weight	Description	% yield	Melting point Observed	Melting point reported	R_f value
3	$C_8H_{10}N_2O_3S_1$	214	Faint yellow crystals	53	213-214	216 [25]	0.45
4	$C_6H_8N_2O_2S_1$	172	White crystals	51	160-161	163-164 [24]	0.75
5	$C_9H_{12}N_2O_3S_1$	228	White crystals	62	179-181		0.52
6	$C_7H_{10}N_2O_2S_1$	186	White powder	44	107-108		0.68
8	$C_{30}H_{28}N_2O_3$	464	White powder	80	141-143		0.69
9	$C_{21}H_{21}N_3O_3S_1$	395	White crystals	40	198-199		0.82
10	$C_{23}H_{23}N_3O_4S_1$	437	White powder	48	169-171		0.76
11	$C_{22}H_{23}N_3O_3S_1$	409	White crystals	35	180-181		0.8

Solvent system: Methanol: Acetic acid: Ether: Benzene (2:18:60:20)

Experimental

All reagents and anhydrous solvents were of analar type and generally used as received from the commercial supplier(Merk-Germany,Reidel-Dehean-Germany ,Sigma-Aldrich-Germany and BDH-England).Mefenamic acid was supplied from Micro Company - Indian.Melting points were determined by capillary method on Thomas Hoover apparatus (England) and ascending thin layer chromatography (TLC) was run on DC-Kartan SI Alumina 0.2 mm to check the purity and progress of reaction. The identification of compounds was done using iodine vapor and the chromatograms were eluted by: **Methanol: Acetic acid: Ether: Benzene (2:18:60:20)**.

IR spectra were recorded on model 500 scientific IR spectrophotometer, Buck Company (USA) as a **KBr film**.**CHN** microanalysis has been done using exter TE micro-analyzer (Germany).The analysis was done in the micro analytical center faculty of science –University of Cairo.

Synthesis of 2-(2, 3-dimethylphenylamino) benzoic anhydride (8):

Mefenamic acid (comp.7) (5g, 20.7mmol) was dissolved in THF (30ml), and then DCC (2.12g, 10.35mmol) was added. The reaction mixture was continuously stirred at room temperature for 4 hours. A white precipitate of DCU was formed which then removed by filtration. The solvent was evaporated under vacuum to give comp.8[26] . The percent yield, physical data and R_f value were given in table (1). IR 3330(NH) of secondary amine 1814 and 1743 (C=O) of anhydride, 1618, 1515 and 1488 (C$\equiv$C st.v.), 1274, 1215 and 1172[C -(C=O) – O-(C=O) –C] cm^{-1} of anhydride.

Synthesis of 2-(2, 3-dimethylphenylamino)-N-(4-sulfamoylphenyl) benzamide (9):

Compound 8 (2.5g, 5.4mmol), compound 4 (0.93g, 5.4mmol), zinc dust (6mg), glacial acetic acid (0.5ml, 8.75mmol) and dioxane (20ml) were placed in a flask, equipped with refluxed condenser, boiling stones were added. The reaction mixture was refluxed gently for 90 minutes. The solvent was evaporated under vacuum, the residue was dissolved in ethyl acetate, washed with $NaHCO_3$ (10%, 3*10ml), HCl (1N, 3*10ml) and distilled water (3*10ml), filtered over anhydrous magnesium sulfate. The filtrate is evaporated under vacuum to give the product. The crystallization is carried out by dissolving the compound in ethyl acetate and petroleum ether (80-100 ^oC) is added to the filtrate until turbidity take place and it is kept in cold place over night. The mixture is filtered while it is cold and the precipitate is collected to give comp.9[27] . The percent yield, physical data and R_f value were given in table (1). IR 3376and3304 (N-H) of primary sulfonamide, 3227 (N-H) of secondary amine, 1660 (C=O) of secondary amide, 1598and1530(C$\equiv$C st.v.), 1327and1157 (SO2) cm^{-1} .

CHN Calculated (C21H21N3O3S1): C, 63.78; H, 5.35; N, 10.36; S, 8.11. Found: C, 62.55; H, 5.44; N, 10.51; S, 8.25.

Synthesis of N-(4-(N-acetylsulfamoyl)-2-(2, 3-dimethylphenylamino) benzamide (10):

Acetic anhydride (0.6ml, 6mmol), was added to a solution of compound 9 (0.79g, 2mmol) in pyridine (10ml) and the reaction was allowed to proceed then at 25 ^{o}C with stirring for 6 hours. Ethyl acetate (100ml) was added and this solution was washed successively with saturated aqueous ammonium chloride (2x20ml) followed by distilled water (2x20ml). The organic fraction was dried with anhydrous magnesium sulfate and the solvent was removed in vacuum to give comp.10 [28] .The percent yield, physical data and R_f value were given in table (1). IR 3350and3292 (N-H) of secondary amide andsulfonamide respectively, 1670 (C=O) of secondary amide, 1595, 1533, and1450(C⩵C st.v.) and1332 and1157(SO2) cm^{-1} . **CHN** Calculated (C23H23N3O4S1): C, 63.14; H, 5.30; N, 9.60; S, 7.33. Found: C, 62.25; H, 5.40; N, 9.83; S, 7.48.

Synthesis of 2-(2, 3dimethylphenylamino)-N-(4-(N-methylsulfamoyl) benzamide (11):

Compound 8 (2.5g, 5.4mmol), compound 6 (1g, 5.4mmol), zinc dust (6mg), glacial acetic acid (0.5ml, 8.75mmol) and dioxane (25ml) were placed in flask, equipped with reflux condenser, boiling stones were added. The reaction mixture was refluxed gently for 90 minutes, and then it was worked up as prescribed in section3.2 to liberate comp.11. The percent yield, physical data and R_f value were given in table (1). IR 3334and3201 (N-H) of secondary amide andsulfonamide, 1664 (C=O) of secondary amide, 1591, 1529 and 1496 (C⩵C st.v.) and1321 and1159(SO2) cm^{-1} . **CHN** Calculated (C22H23N3O3S1): C, 64.53; H, 5.66; N, 10.26; S, 7.83. Found: C, 65.20; H, 5.58; N, 10.45; S, 8.01.

Pharmacology:

Albino rats of either sex weighing (150 ± 10 g) were supplied by the National Center for Quality Control and Drug Research and were housed in the animal house of the College of Pharmacy, University of Baghdad under standardized conditions (12 light-12 dark cycle) for 7 days for acclimatization. Animals were fed commercial chaw and had free access to water ad *libitum*. Animals were brought 1 hour before the experiment to the laboratory, and were divided into five groups (each group consist of 6 rats) as follows: **group A**: served as control and treated with the vehicle (propylene glycol 50% v/v in water); **group B**: treated with sodium diclofenac (reference agent) in a dose of 3mg/kg suspended in propylene glycol [29]; **group C**, **D** and **E**: treated with tested compounds 9, 10 and 11 respectively in a dose equivalent to 7.5 mg/kg of mefenamic acid as finely homogenized suspension in 50% v/v propylene glycol[30] .

Anti-inflammatory activity:

The anti-inflammatory activity of the tested compounds was studied using egg-white induced edema model [31]. The drugs or the vehicle were administered i.p. at time zero and acute inflammation was induced by a subcutaneous injection of 0.05ml of undiluted egg-white into the planter side of the left hind paw of the rats at time 15 minutes. The paw thickness was measured by vernier at eight time intervals (0, 15, 30, 60, 120, 180, 240 and 300 minutes) after vehicle or drugs administration. The data are expressed as mean ± S.E.M. and results were analyzed for statistical significance using Student *t*-test (Two-Sample Assuming Equal Variances) for comparisons between mean values. While comparisons between different groups were made using ANOVA: Two-Factor Without Replication. Probability (P) value of less than 0.05 was considered significant.

Results and Discussion

The most widely used primary test to screen new anti-inflammatory agents is based on the ability of a compound to reduce local edema induced in the rat paw following injection of an irritant agent [32]. When egg-white is injected into the paw of rats, a substantial induction of COX-2 is observed at 2 hours coinciding with enhanced PGs and local edema [33]. Tables 2 and 3 show the effect of tested compounds on egg-white induced edema as an indicator for their anti-inflammatory activity. The intraplanter injection of egg-white into rat hind paw induces a progressive edema, which was reached maximum (measured by millimeter) after 2 hours of injection. Table 2 showed the effect of tested compounds (9, 10 and11) in respect to control group. All tested compounds were effectively limited the increase in paw edema, with the effect of compounds 9 and 10 started at time 30 minute (significantly difference compared to control), while compound 11 started at time 120 minute. However, the effect of all tested compounds continued till the end of the experiment with statistically significant ($p< 0.05$) reduction in paw edema. The differences among the

compounds started at time 30 minute in which the compounds 9 and 10 significantly difference at time 30 and 60 minute compared to compound 11. However, the differences among the compounds continued from the time 180 to 300 minute with statistically significant ($p< 0.05$) reduction in paw edema in the following orders 10, 11, and 9 respectively.

Table 2: Effect of Control and Compounds 9, 10 and 11 on egg-white induced paw edema in rats.

	Treatment groups				
	Time (min)	Control (n=6)	Compound9 (n=6)	Compound 10 (n=6)	Compound11 (n=6)
Paw thickness (mm)	0	4.46 ± 0.16	4.39±0.10	4.41±0.08	4.38±0.13
	15	5.41 ± 0.18	5.45±0.07	5.42±0.12	5.35±0.11
	30	6.05 ± 0.16	5.80±0.05*a	5.76±0.13*a	6.01±0.10 b
	60	6.35 ± 0.07	6.00±0.05*a	6.00±0.13*a	6.33±0.09 b
	120	6.50 ± 0.09	5.73±0.05*a	5.66±0.08*a	5.70±0.10*a
	180	5.93 ± 0.11	5.40±0.05*a	5.09±0.05*b	5.30±0.07*c
	240	5.38 ± 0.09	5.13±0.05*a	4.86±0.07*b	4.95±0.07*c
	300	5.20 ± 0.10	5.05±0.04*a	4.56±0.08*b	4.68±0.05*c

Non-identical superscripts (a, b, and c) among different groups are considered significantly different ($P<0.05$).

* significantly different compared to control ($P<0.05$).

Table 3 shows the effect of tested compounds (9, 10 and11) with respect to the reference group (diclofenac). As seen in this table; at time 0 and 15 minute there are no differences among different groups; at time 30, only compound 11 is significantly different than diclofenac; at time 60 and 120 all compounds are significantly different than diclofenac; while at time 180 to 300 compounds 9 and 11 are significantly different than diclofenac. The differences among the compounds started at time 30 minute in which the compounds 9 and 10 significantly difference at time 30 and 60 minute compared to compound 11 while at time 120 compound 10 is significantly different than compounds 9 and 11. However, the differences among the compounds continued from the time 180 to 300 minute with statistically significant ($p< 0.05$) reduction in paw edema in the following orders 10, 11, and 9 respectively.

Table 3: Effect of Diclofenac and Compounds 9, 10 and 11 on egg-white induced paw edema in rats.

Treatment groups

	Time (min)	Diclofenac (n=6)	Compound9 (n=6)	Compound 10 (n=6)	Compound11 (n=6)
Paw thickness (mm)	0	4.38±0.14	4.39±0.10	4.41±0.08	4.38±0.13
	15	5.37±0.41	5.45±0.07	5.42±0.12	5.35±0.11
	30	5.78±0.11	5.80±0.05 ^{a}	5.76±0.13 ^{a}	6.01±0.10^{*b}
	60	5.60± 0.10	6.00±0.05^{*a}	6.00±0.13^{*a}	6.33±0.09^{*b}
	120	5.35±0.10	5.73±0.05^{*a}	5.66±0.08^{*b}	5.70±0.10^{*a}
	180	5.07±0.10	5.40±0.05^{*a}	5.09±0.05 ^{b}	5.30±0.07^{*c}
	240	4.87±0.10	5.13±0.05^{*a}	4.86±0.07 ^{b}	4.95±0.07^{*c}
	300	4.61±0.10	5.05±0.04^{*a}	4.56±0.08 ^{b}	4.68±0.05^{*c}

Non-identical superscripts (a, b, and c) among different groups are considered significantly different (P<0.05).
* Significantly different compared to control (P<0.05).

Conclusion

The *in vivo* anti-inflammatory study showed that the incorporation of 4-aminobenzenesulfon-amide, 4-amino-N-methylbenzenesulfonamide, or N-(4-aminophenylsulfonyl) acetamide into well known anti-inflammatory drug (mefenamic acid) maintains its anti-inflammatory activity. Compound 10 showed more potent anti-inflammatory effect than compound 9 or 11 and have a comparable effect to that of diclofenac at time 180 to 300 minute with the same onset of action.

Acknowledgments

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Hepatoprotective Effect of *Echinops tenuisectus* (*Compositae*) on CCl_4 Induced Hepatic Damage in Rats

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Abstract

Flavonoids are known to play a vital role in the management of various liver disorders.They are a large family of compounds synthesized by plants; they belong to a group of natural substances with variable phenolic structures. In this study we aim to scan the types of flavonoids in a newly studied, wild Iraqi plant named *Echinops tenuisectus of Compositae* family. The medicinal importance of flavonoids on one hand, and the absence of any phytochemical investigation on tenuisectus species of Echinops genus on the other hand, acquired this study it's importance. Three flavonoids were identified in the seed's extract of this plant (Silymarin, Rutin, Quercetin) by two chromatographic methods, first Thin layer chromatography (TLC) using TLC ready made GF254 plates, UV detector at 254 nm, and two different solvent systems in which the R_f value of the standards (Silymarine, Rutin, Quercetin) matched with the R_f value of the Silymarin, Rutin and Quercetin found in the plant seed's extract. High pressure liquid chromatography (HPLC) was the other chromatographic method that used to identify the presence of these flavonoids in the plant seed. The plant seed 's aqueous extract was evaluated for its efficacy in rats by inducing hepatotoxicity with CCl_4.Single oral dose of 250mg/kg of Seeds Extract was given to rat's for 7 days. Serum activities of transaminases (ALT and AST) were used as the biochemical marker of hepatotoxicity. Histopathological changes in rats liver section were also examined. The results of the study indicated that, the pretreatment of rats with Echinops extract before the hepatotoxins agent (CCl_4) offered a hepato- protective action.

Key words: Echinops, Flavonoids

الخلاصة

الفلافينويدات تلعب دور مهم و حيوي في معالجة و تنظيم الكثير من امراض الكبد. الهدف من هذه الدراسة هو عمل مسح لمعرفة الأنواع المختلفة من الفلافينويدات في نبتة عراقية جديدة لم تدرس سابقا.نظرا للأهمية الطبية للفلافينويدات من جهة، وعدم وجود أي منشورات علمية تتناول المكونات الكيميائية لهذه النبتة، أخذت هذه الدراسة أهميتها. تم أكتشاف ثلاث أنواع من الفلافينويدات في مستخلص البذور للنبات (السليمارين، روتين، كوارستين) بواسطة طريقتين من طرق الكروماتوغرافيا، الأولى هي تقنية كروماتوغرافيا الطبقة الرقيقة(TLC) بأستخدام رقائق TLC ذات النوعية GF254 وكاشف الأشعة فوق البنفسجية U.V بالطول الموجي 254nm وثلاثة محاليل ناقلة مختلفة، حيث أن قيمة R_f للفلافينويدات القياسية طابقت قيمة R_f للفلافينويدات الموجودة في المستخلص النباتي. ثم طريقة كروماتوغرافيا تحت الضغط العالي (HPLC) التي أكدت وجود الفلافينويدات في المستخلص النباتي بتطابق retention times لكل من الفلافينويدات القياسية و الفلافينويدات في المستخلص النباتي. تم تقييم الفعالية العلاجية لهذه النبتة على كبد الفئران التي تم استحداثها بواسطة CCl_4 حيث تم اعطاء المستخلص المائي عن طريق الفم بجرعة قدرها 250 ملغم/كلغم ولمدة سبعة أيام وتم قياس مستوى الأنزيم ALT وAST الذي يبين الفعالية الوقائية والعلاجية لهذه النبتة ضد امراض الكبد.

Introduction

The *Echinops tenuisectus* belong to the *Family Compositae* (Fig1) is a wild, Iraqi plant first studied in Iraq. The Echinops genus consist of 100 spp.[1] which are widely distributed in Sharaban, Diyalah, Badrah ((Upper Tigris Plain))[2] . Preliminary investigation indicated that, this plant contain different types of flavonoids in accepted amount. Among these flavonoids: Silymarin (figure 2) which is a flavonolignan that has been introduced fairly recently as a hepatoprotective agent[3,4,5,6,7]. Silymarin has been found to provide hepatoprotection through its antioxidants properties (scavengers and regulators of the intracellular content of glutathione)[8,9,10] , as cell membrane stabilizers and permeability regulators that prevent hepatotoxic agents from entering hepatocytes[11,12]. Also as inhibitors of the transformation of satellite hepatocytes in to myofibroblasts, the process responsible for the deposition of collagen fibers leading to cirrhosis [13, 14, 15].

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Figure 1: Photography of *Echinops tenuisectus*

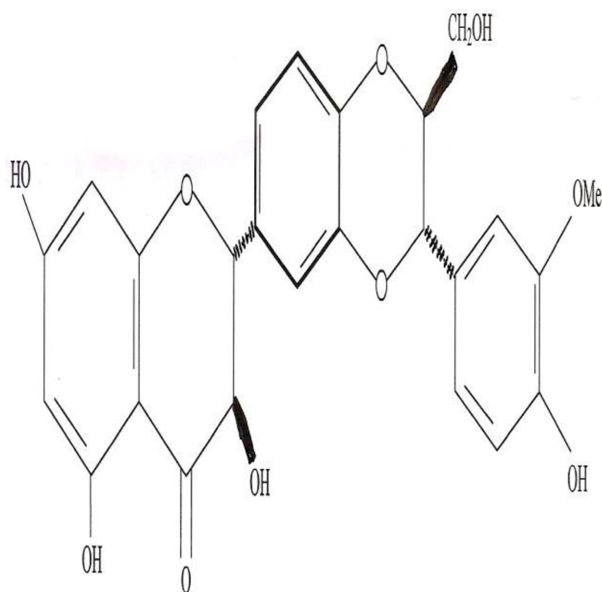


Figure 2: Structure of silybin (commercially called silymarin)

The other flavonoids found in this plant are the Quercetin and Rutin (Figure 3,4), both of them possess a powerful antioxidant activity which help to prevent free radical oxidative damage to cells, also help in the treatment and prevention of alcohol and chemical - induced hepatotoxicity by increase glutathione in the liver(16). Glutathione responsible for detoxifying a wide range of hormones, drugs, and chemicals. High level of glutathione in the liver increases its capacity for detoxification. Quercetin and Rutin increase the level of the important antioxidant enzyme superoxide dismutase in the cell cultures (17). In addition they stimulate protein synthesis in the liver, which results in an increase in the production of new liver cells to replace the damaged one (18). Shoskes 1999 demonstrate that Quercetin and Rutin also inhibit the synthesis of leukotrienes (mediators of inflammation, which can result in psoriasis) (19).Recently, flavonoids can help in prevention of cancer through several pathway: inhibiting proliferation and inducing apotosis(20,21)or through inhibiting tumor invasion and angiogenesis(22,23). This wide variety of beneficial health effects of these flavonoids acquired this study its importance in finding a new uninvestigated source of these important flavonoids , contained within *Echinops tenuisectus* of the Family *Compositae* and evaluate their possible protective effect against the experimentally- induced liver damage in rats by CCl_4 . Liver, the largest organ in vertebrate body, is the major site of intense metabolic activities. Liver injury caused by toxic chemicals and certain drugs has been recognized as a toxicological problem. Herbal drugs are playing an important role in health care programs world wide, and there is a resurgence of interest in herbal medicines for treatment of various ailments including hepatopathy(24). CCl_4 is reported to produce free radicals which affect the cellular permeability of hepatocytes and it causes massive histopathological changes like necrosis, congested vessel and fatty changes (steatosis). So, the reverse of these phenomenon can be considered as the index of hepatoprotective(25).

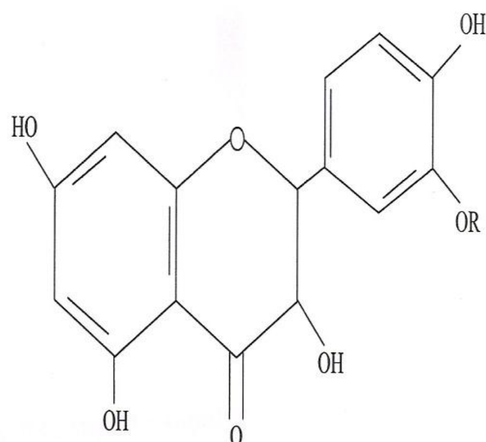


Figure 3: Quercetin; R = H
Figure 4: Rutin ; R= rhamno-glucosyl

Materials and Methods

A. Plant materials:

The plant material was collected during July 2005 From Sharaban,Dyala city. The plant was identified by the Department of Pharmacognosy, College of Pharmacy /University of Baghdad; and authenticated by the Herbarium of Baghdad University. Fifty grams of the powdered plant material (seed part) were first defatted by reflux with 100 ml of petroleum ether (60°-80°C) for one hour and filtered. The defatted dried plant material was then extracted by reflux using 100 ml of 70% ethanol for three hours.This step was repeated for four times, then the combined filtrates were evaporated under reduced pressure using Buchi rotatory evaporator attached to vacuum pump at 40°C, to a thick residue of ethanol extract (F1). This residue was then hydrolyzed with 2N HCl in aqueous methanol (1:1) under reflux for three hours; the resultant solution was then partitioned with 100 ml of ethyl acetate (F2). This fraction was evaporated under reduced pressure to dryness, as shown in the following diagram (Figure 5).

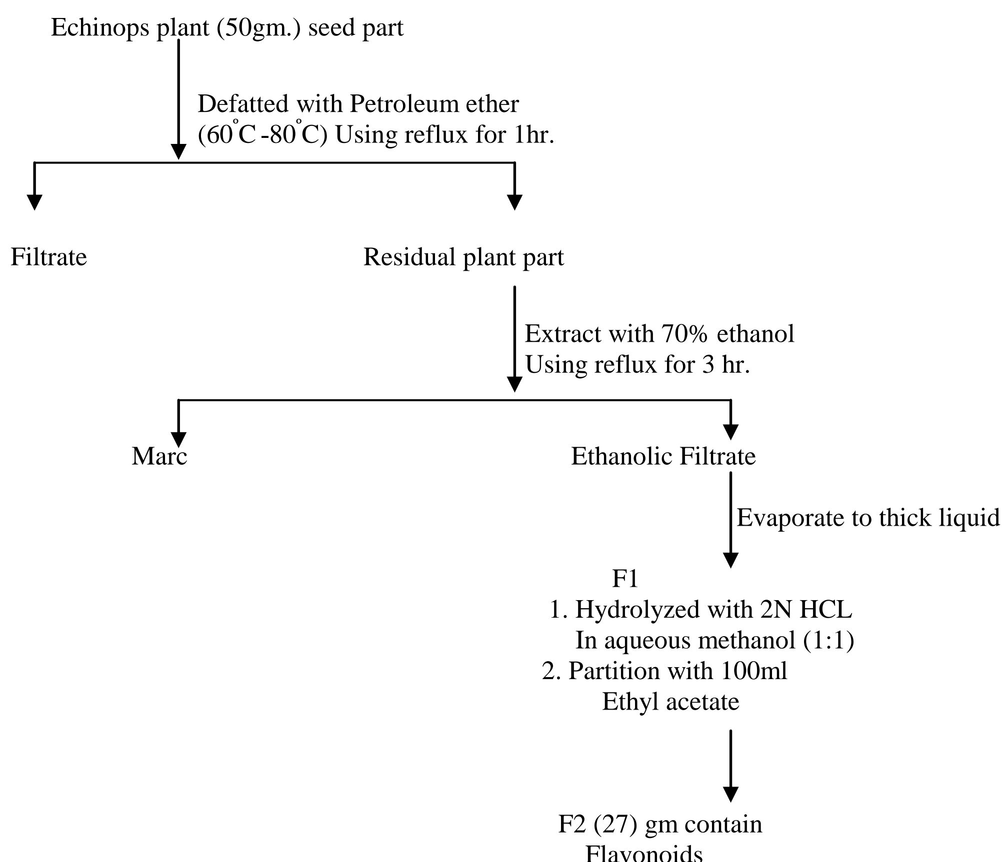


Figure-5 [Schematic representation of flavonoids extraction From *Echinops tenuisectus*]

F2 (ethyl acetate fraction) → evaporation to dryness under reduce pressure → black- greenish oily residue, TLC and HPLC indicated that this fraction contain three compounds which are silymarin, rutin and quercetin and by preparative thin layer chromatography and HPLC we can separate each one and calculate the percentage of each one by weighting.

F2 (oily residue fraction) → dissolved in water → suspention (ready for hepato- protective study)

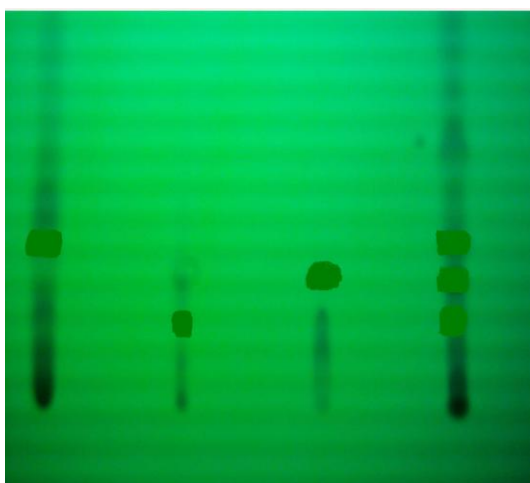
B. Identification of Silymarin,Quercetin and Rutin in the plant seed extract.

The Identification of these flavonoids in the seed extract was performed by:

1. Identification of Flavonoids by TLC:

Using TLC ready made Gf254 plates, UV detector at 254 nm, Standard flavonoids and two different solvent systems that were [26] :

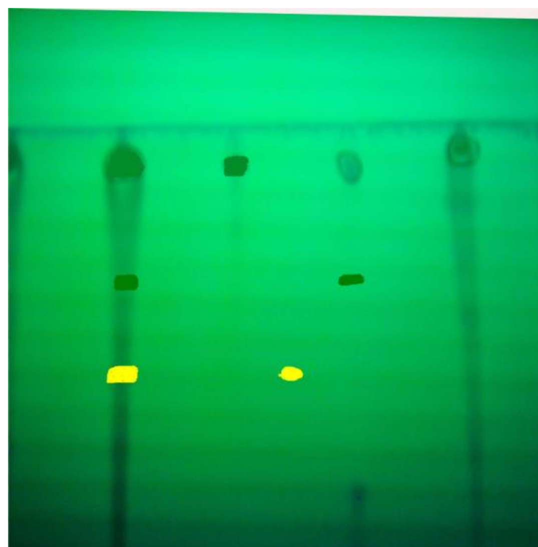
Solvent (1): chloroform: acetone: formic acid (75:16.5:8.5) (Figure 6)
Solvent (2): n.butanol: glacial acetic acid: water (40: 10:50) (Figure 7)
(Table-1) showed the R_f values of the standards Silymarin, Quercetin and Rutin, and the Rf value of plant seed part extract.



B D C A

Figure 6: TLC Gf254 plate of the seed extract and standards using S1 mobile phase.

A Plant seed extract C Quercetin standB Silymarin standard D Rutin standard



A C B D

Figure 7: TLC Gf254 plate of the seed extract and standards using S2 mobile Phase

A Plant seed extract C Quercetin standard
B Silymarin standard D Rutin standard

Table 1: R_f values of standard silymarin, rutin and quercetin and seed extract.

Solvent system	Standard silymarin	Standard Quercetin	Standard Rutin	Seed extract
S1	0.43	0.35	0.28	0.4,0.33,0.2
S2	0.2	0.81	0.56	0.21,0.8,0.5

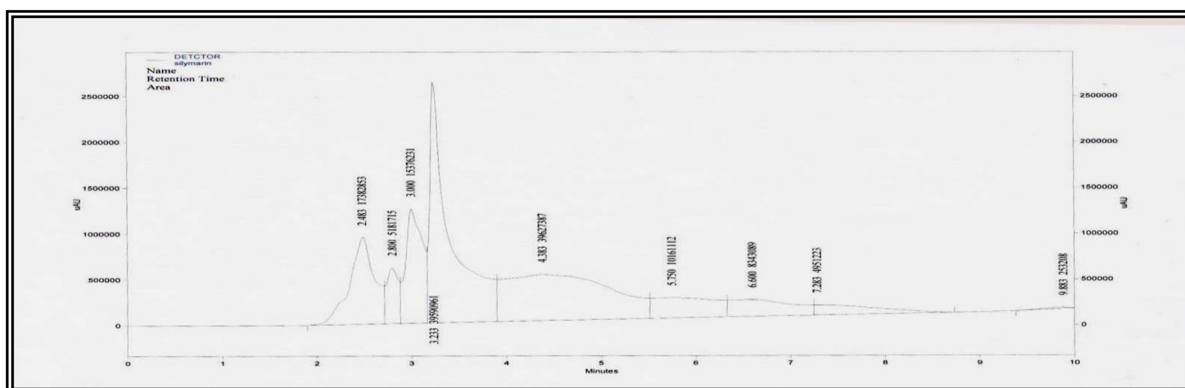


Figure 8: HPLC of plant seed extract of *Echinops tenuisectus*.

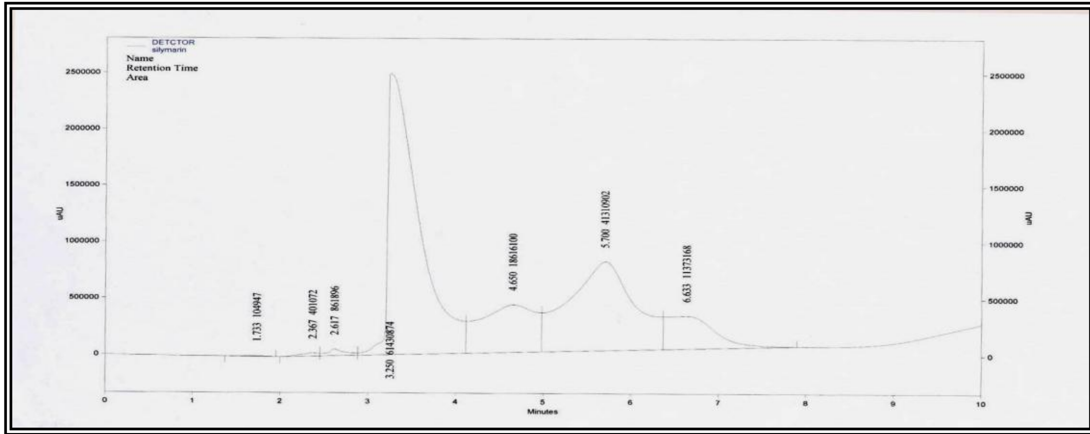


Figure 9: HPLC of standard Silymarin.

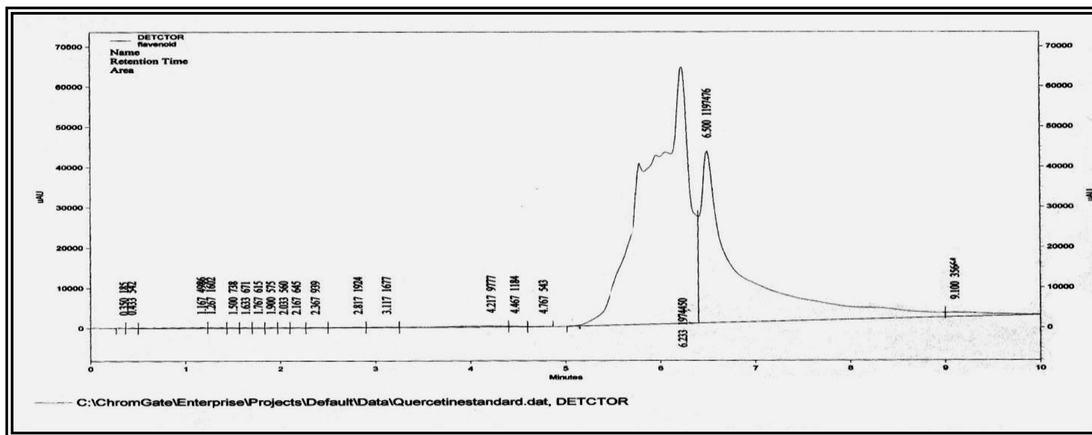


Figure 10: HPLC of standard Quercetin.

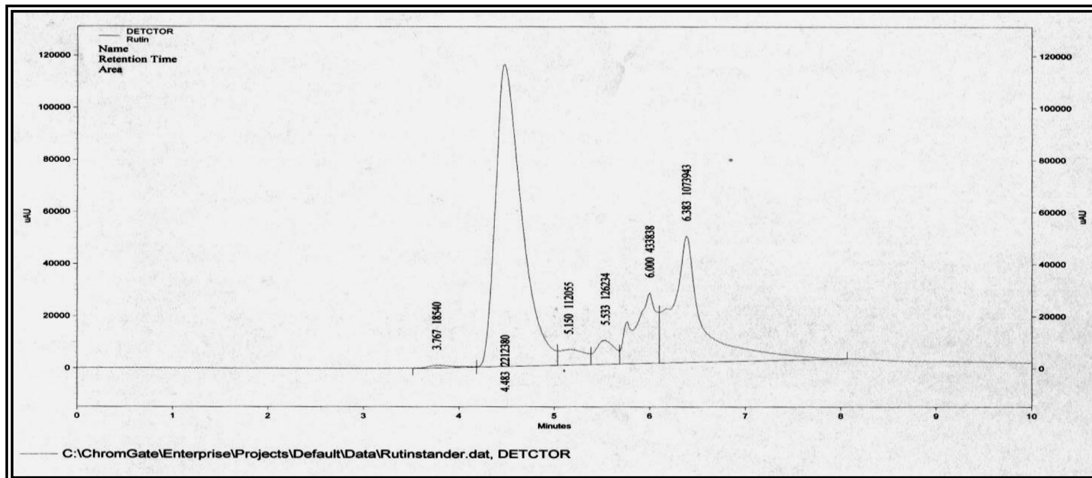


Figure 11: HPLC of standard Rutin.

2. Identification of Flavonoids by HPLC:
Silymarin, Quercetin and Rutin were authenticated by HPLC . (Figures 8-11) The HPLC conditions are listed in the following table. (Table-2)

Table 2: HPLC conditions.

HPLC Conditions	
Mobile phase	Methanol:water (50:50)
Column	C18 25cm
Flow rate	1ml/min
Detector	288 nm

C. Hepatoprotective studies:
1. Experimental animals:
Eighteen – Albino rats of both sexes weighting 150-200 gm (both sex) were used in this study. Animals were kept in the animal house of the College of Pharmacy/ University of Baghdad, under standardized condition (12 hr light dark cycle at room temperature). The animals were fed standard chow and given water ad libitum.
2. Experimental design:
The animals were divided in to three groups (each group have 6 animals) and treated as follows:
Group (1): Six rats received normal saline for 7 day orally and secreted at along 7, saved as control
Group (2): Six rats received single oral dose of CCl_4 (1mg/kg) diluted by corn oil in ratio of 1:1 v/v for the induction of liver damage and animals were sacrificed after 24 hr of CCl_4 administration.
Group (3): Six rats received oral dose of the seed extract of *Echinops tenuisectus*__Plant in amount equivalent to 250mg/kg by gavages tube for 7 days, befor CCl_4 (1mg/Kg diluted by corn oil in a ratio of 1:1 v/v), then the rats were sacrificed after 24 hr, after CCl_4 administration.

3. Biochemical estimation:
Serum was prepared from the collected blood and subjected to biochemical estimation of ALT and AST.

4. Histopathology:
Portion of liver tissue in each group was fixed in 10% formalin (Formalin diluted to 10% with normal saline) and proceeded for histopathology. After paraffin embedding and block making, serial section of 5μ thickness were made, stained with Haematoxylin and Eosin and examined under microscope.

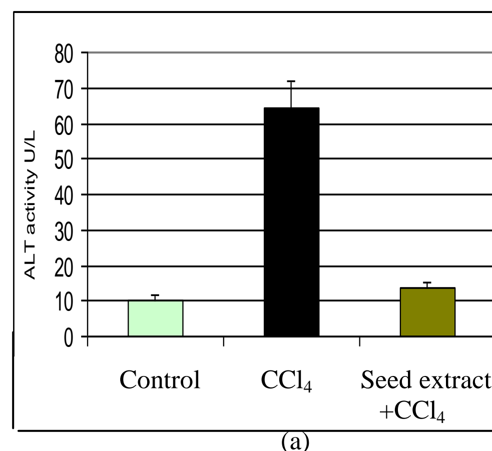
5. Statistical analysis:
The significance of difference between the mean values was calculated using unpaired student's t-test. P-value less than 0.05 were considered significant for all data showed in our results.

6. Results:
A) Biochemical parameters:
Table-3 showed a significant elevation in the activities of both ALT and AST in CCl_4-treated rats compared to control group. Pre-treatment rats with seed extract of *Echinops tenuisectus* (250mg/kg) showed a significant decline in the activities of ALT and AST compared with CCl_4 treated rats (Table 3 , Figure 12 and 13).

Table 3: Effects of seed extract of *Echinops tenuisectus* on the activities of serum ALT and AST in rats treated with CCl_4.

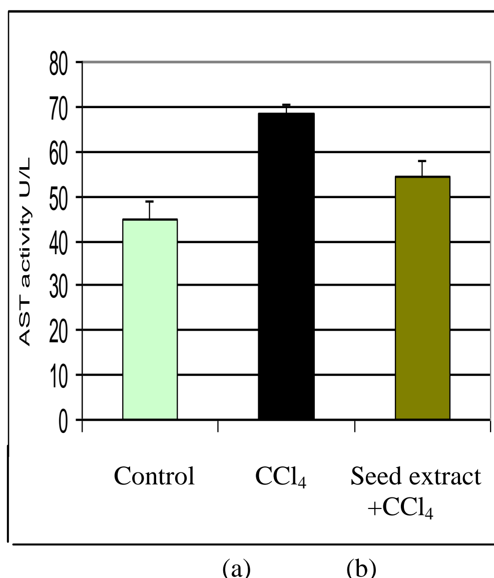
Treatment	Serum ALT U/L	Serum AST U/L
Control N=6	10.24±1.21	45±3.8
CCl_4-treated N=6	64.4±7.53[a]	68.6±1.67[a]
Seed extract + CCl_4 N=6	13.6±1.34	54.4±3.28[b]

- Each value represents Mean ± standard deviation.
- Values with non=identical superscripts (a,b) within each parameter are significantly different (P< 0.05)
- N= Number of animals.



(a)

Figure 12: Bar chart comparing the effects of seed extract of *Echinops tenuisectus* pre-treated with CCl_4 on serum ALT activity.



(a) (b)

Figure 13: Bar chart comparing the effects of seed extract of *Echinops tenuisectus* pre-treated with CCl_4 on serum AST activity.

B) Histological examination:

Histological examination of rats liver treated with CCl_4 showed that, there was centrilobular hemorrhage, with heavy inflammation and necrosis. In addition to steatosis and individual necrosis were observed compared with control (Figure 14 and 15). Pre-treatment of rats with seed extract of *Echinops tenuisectus* before CCl_4, exhibit variable degrees of recovery with slight centrilobular congestion, marked reduction in inflammatory reaction. Furthermore, neither necrosis nor steatosis was observed in rat's liver section (Figure 16).

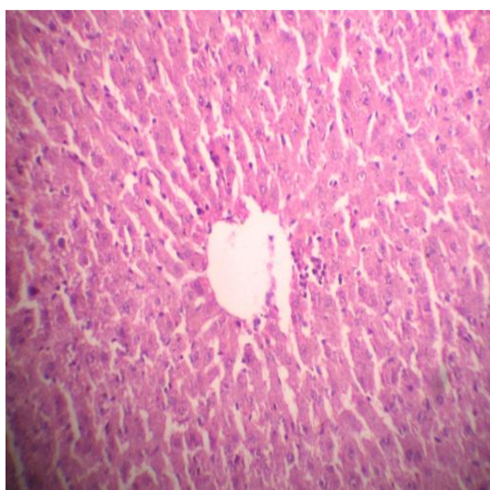


Figure 14: Section showing normal rat's liver. Magnification: 40X, staining: haematoxylline and eosin.

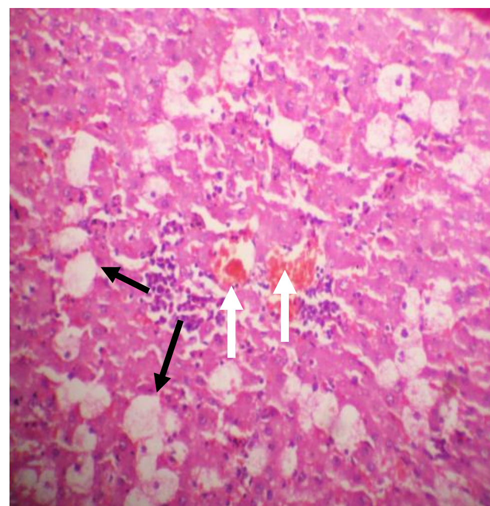


Figure 15: Section showing morphological alteration of liver from CCl_4-treated rats. Black arrow represents fatty changes (steatosis), white arrow represent haemorrhage. Magnification: 40X, staining: haematoxylin and eosin.

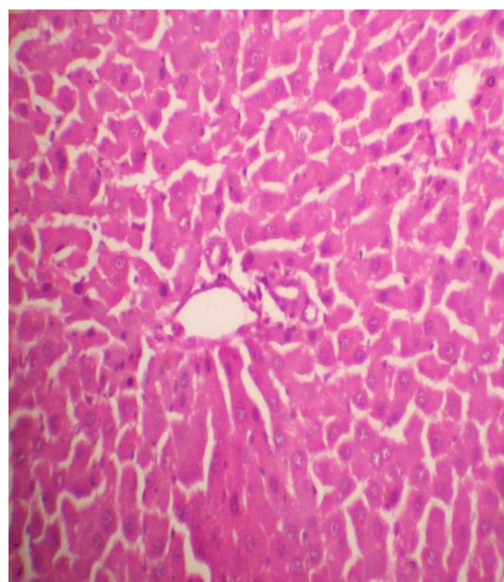


Figure 16: Section showing the administration of seed extract of *Echinops tenuisectus* improved CCl_4-induced hepatic damage. Magnification: 40X, staining haematoxylin and eosin.

Discussion:

Many compounds exhibit hepatoprotective activity, demonstrated either by decreasing the harmful effect of hepatotoxic compound or by maintaining the normal hepatic physiology. The present study showed that, the seed extract of *Echinops tenuisectus* have good hepatoprotective effect against CCl_4-induced hepatotoxicity in rat manifested by attenuating the increases in the serum activities of ALT

and AST (Table 3, Figure 12and13) and by reversing the histological damage induced by CCl_4, this was attributed to the presence of flavonoids, especially the silymarin, rutin and quercetine which possess antioxidant properties [8,16] which can improve the normal physiology of hepatocyte [17,18,19].

Conclusion

The present study showed that, seed extract of *Echinops tenuisectus* improve the hepatic damage and steatosis induced by CCl_4 toxicity.

Acknowledgment

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Antiplatelts Activity of Vitamin E in Relation to Dose and Duration of Therapy

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Abstract

Vitamin E, having the well known antioxidant activity through scavenging free radicals و it occurs in several isomeric forms , these isomers have relatively different functions . One of these actions is related to its ability to inhibit platelets aggregation and hence thrombosis. The present study included a total number of apparently healthy 62 males . 11of them served as standard group , treated with 100 mg aspirin /day for more than one month . Another 31 subjects were randomly grouped into 5 groups that received different daily doses of α – tocopherol : 400 IU , 800 IU and 1200 IU for 2-6 months.The remainder (20) subjects served as a control group (received no therapy) . Platelets function was assessed based on measuring bleeding time and Slide Platelets Aggregation Time (SPAT) Meanwhile , thiobarbituric acid reactive substances (TBARS) were measured as a marker for oxidative stress. The results showed that the commercially available vitamin E preparations (α-tocopherol) could exert anti-coagulant effect ,such effect is more dependant on duration of therapy , rather than dose related action .In addition to it's antioxidant effect, which seems to be significantly correlated to it's antiplatelets effect ($r=0.994$,$p<0.05$).Hence , long term administration of high doses of vitamin E could be effective in decreasing the incidence of thrombosis ,which in turn depends on platelets function. Such effect might not affect bleeding time obviously , but it could reduce chances for platelets recruitment , which might represent an additional advantageous action for vitamin E over other antioxidants.

Key Words : Vitamin E , α - Tocopherol , Antiplatelets .

الخلاصة

ان فيتامين أي معروف بخاصيته المضادة للاكسدة من خلال اقتناصه للجذور الحرة المتكونة .ان هذه الجزيئة توجد في عدة صور جزيئية وهذه الاشكال المختلفة للجزيئة تميز كل منها بخصائص ووضائف مختلفة .احدها يرتبط باحباط قابلية الصفيحات الدموية للتكتل وتكوين الخثرة الدموية .تضمنت هذه الدراسة 62 ذكرا سليما ، اعتبر احد عشر منهم مجموعة قياسية للمقارنة حيث تم علاجها بالاسبرين 100 ملغم يوميا ولمدة اكثر من شهر واحد . وتم تقسيم 31 شخصا اخر وبصورة عشوائية الى مجاميع تتناول الفا – توكوفيرول بجرع مختلفة (400 و800 و1200 وحدة دولية يوميا) ولمدد مختلفة تراوحت بين الشهرين والستة أشهر . اما باقي المشاركين بالدراسة فقد تم اعتبارهم مجموعة مقارنة لم يتناولوا أي علاج .تم اعتماد الفحوصات المختبرية الخاصة بفعالية الصفيحات الدموية مثل قياس مدة النزف و الوقت اللازم للصفيحات الدموية للتجمع على السلايداضافة الى قياس مستوى الموادالمتفاعلة مع حامض الثايوباربيجورك كدليل لدرجة الاجهاد التاكسدي.اظهرت النتائج ان النوع المتوفر في اسواق العراق من الفيتامين E وهو في الاغلب يتكون من نوع الفا توكوفيرول فقط يمكن ان يحدث تأثيرات على عملية تجلط الدم . وان هذا التأثير فيما يبدو يعتمد بصورة اكبر على مدة استمرار العلاج اكثر من ارتباطه بالجرعة المعطاة من هذا الفيتامين .لذلك ان تناول فيتامين أي -الفا توفيرول – وبجرع كبيرة نسبيا ولمدة طويلة –اكثر من خمسة اشهر – يمكن ان يعطي تأثيرا مفيدا من خلال تقليل احتمالية الاصابة بتخثرات الدم من خلال تأثيرها على فعالية الصفيحات الدموية وهذا التأثير الايجابي يمكن ان يكون تأثيرا اضافيا لتفضيل فيتامين أي على غيره من المواد المضادة للاكسدة .

Introduction

Vitamin E has been known as an essential nutrient to maintain normal reproduction since 1922 [1].The large scale studies have shown an inverse correlation between its high dietary intake and the incidence of coronary heart diseases [2,3] . However,these studies did not provide sufficient evidences , that vitamin E administration can prevent cardiovascular events , nor the subsequent cardiac deaths[4]. Such confliction could be attributed to the fact that , clinical studies usually utilizes the commercially available vitamin E preparations , which almost contain α- tocopherol alone , whereas vitamin E in food occurs in several other forms.Thus ,the absence of other tocopherols – than α-tocopherol –in the pharmaceutical preparations (utilized in the previously mentioned studies) may account for such unexplained results (5,6). Naturally vitamin E occurs as a family of eight members , four of them are referred to as tocopherols and the other four members are known as trienols . Both tocopherols and tocotrienols are subdivided into 4 types : alpha (**α**), beta (**β**) , gamma (**γ**) and delta(**δ**) , according to their substitutions on the molecule[7],as shown in figure (1) and table 1 .

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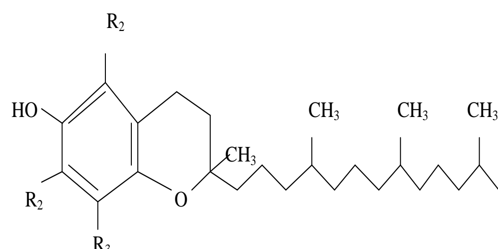
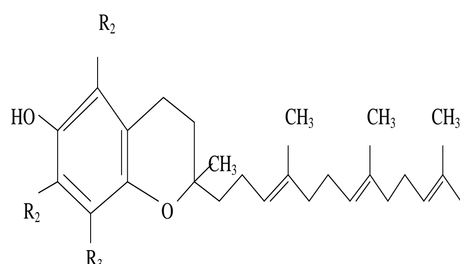
Table - 1-
Different types of vitamin E

	R 1	***R 2***	***R 3***
Alpha	C H3	CH3	CH3
Beta	CH3	H	CH3
Gamma	H	CH	CH3
Delta	H	H	CH3

R = Substituted groups in the general structure of Tocotrienols



General structure of Tocopherole



General structure of Tocotrienols

Figure 1

The most abundant forms of vitamin E in nature are alpha and gamma – tocopherols [8] .Hence , some members of vitamin E was shown to exert specific functions , that may not be found in others [9] .Considering vitamin E action , it can act as scavenger of free radicals , thereby it can provide protection from free radicals – produced damage [10] .Also , it had been reported that vitamin E could exert an antiinflammatory effects through inhibiting lipooxygenase action , thus inhibiting leucotrienes release (a powerful mediator of inflammation) [11] .Meanwhile , vitamin E can decrease the cyclooxygenase cascade in leukocytes which interferes with inflammatory process [12] .Some observations by ***Chan and Leith*** (1981) and ***Gilber et al*** (1983), demonstrated that vitamin E enhances the release of prostacyclin – a potent vasodilator and inhibitor of platelets aggregation – in a dose-dependent manner. This study was designed to evaluate the antiplatelets action of **α –tocopherol** in the commercially available vitamin E preparations in Iraqi market , in relation to dose and duration of therapy .

Subjects and Methods :

The study included 62 male subjects with age ranged between 32 and 55 years old (45 ±4.2) . The contributing subjects were selected to have no past history of cardiovascular disease or thrombotic disorder, from those attended a private clinic for inferlility at Al-Sadoon Street /Baghdad , under supervision of a senior physician for the period July-November 2005 . Twenty of them served as a control group
(received no therapy) .Another group of 11 subjects were treated with a daily dose of 100 mg aspirin for more than one month (1-3 months) . The remainder (31 subjects) were subdivided into 5 groups to be treated with vitamin E (**α-tocopherol**) as follows :

Group A : included 6 subjects treated with 400 IU /day for less than 5 months (2-4 months).

Group B : included 7 subjects treated with 400 IU / day for more than 5 month (5-6 months) .

Group C : included 6 subjects treated with 800 IU/ day for less than 5 months (2-4 months).

Group D : included 6 subjects treated with 800 IU / day for more than 5 months (5-6 months).

Group E : included 6 subjects treated with 1200 IU / day for less than 5 months (2-4 months) .

The treatment in all groups did not exceed six months . Blood specimens were obtained by venipuncture , to perform the platelets assessments anticoagulant(EDTA-K2) was added ,whereas those aliquots used to assess serum TBARS were obtained by centrifugating blood specimens after clotting .

Platelet function was evaluated by measuring **Slide platelet aggregation time** SPAT TM , based on measuring time required by platelets to aggregate on a slide in the presence of a potent soluble aggregating agent (30 micromol propylgallate) , purchased from Analytical Control System ACS Inc [15] .

Bleeding time was measured for each subject at the end of treatment period according to **TVY method** [16].

Oxidative stress was assessed by measuring **thiobarbituric acid reactive substances TBARS** in serum according to Beuge and Auest method(1978).

Results was expressed as mean ± SD , student t-test (unpaired) were used considering P values less than 0.05 to be significant [18].

Results

Effects of α –tocopherol therapy on bleeding time :

Bleeding time values showed no significant change in subjects treated with daily dose of 400 IU of α –tocopherol (group A), even in those continued therapy for more than five months (group B) figure (2) . Whereas , the effect of a dose of 800 IU /day was time–dependent , as shown by results of the group treated for more than 5 months (group D).Higher doses (1200 IU/day) of α –tocopherol for the same period (less than 5 month) also failed to produce significant change in bleeding time values (group E) . While , the standard therapy with antiplatelet agent(aspirin) produced a significant elevation .

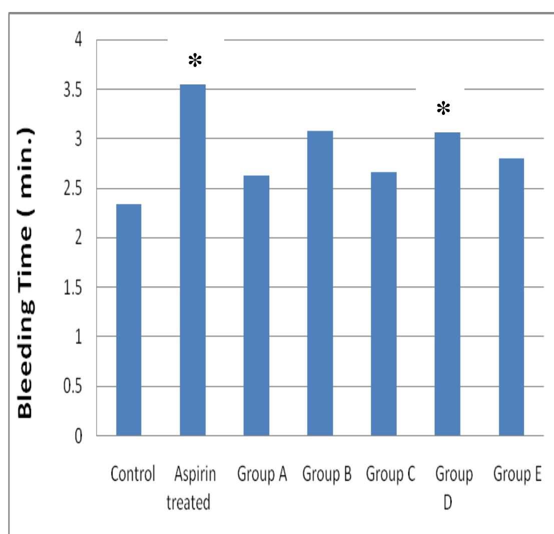


Figure 2 . Effects of Alpha – Tocopherol on Bleeding Time

Group A : included subjects treated with 400 IU /day less than 5 months (2-4 months) .
Group B : included subjects treated with 400 IU / day more than 5 month (5-6 months) .
Group C : included subjects treated with 800 IU/ day less than 5 months (2-4 months) .
Group D : included subjects treated with 800 IU / day more than 5 months (5-6 months) .
Group E : included subjects treated with 1200 IU / day less than 5 months (2-4 months).
Aspirin group : included subjects treated with 100 mg / day (1-3months) .
* = significantly different from control (< 0.05)

Effects of α –tocopherol therapy on SPAT values :

The results of SPAT test for the studied groups are illustrated in figure (3) . Significant alterations in SPAT values were observed in those subjected to therapy that continued more than 5 months , by either doses : 400 or 800 IU α –tocopherol /day – i.e. groups Band D , respectively .

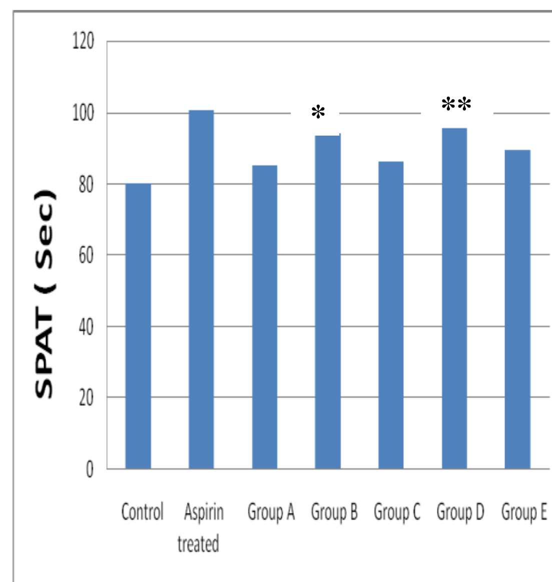


Figure 3 . Effects of alpha –tocopherol on Slide Platelet Aggregation Test (SPAT) values

Group A : included subjects treated with 400 IU /day less than 5 months (2-4 months) .
Group B : included subjects treated with 400 IU / day more than 5 month (5-6 months) .
Group C : included subjects treated with 800 IU/ day less than 5 months (2-4 months) .
Group D : included subjects treated with 800 IU / day more than 5 months (5-6 months) .
Group E : included subjects treated with 1200 IU / day less than 5 months (2-4 months).
Aspirin group : included subjects treated with 100 mg / day (1-3months) .
*=significantly different from control(p< 0.05)
** =significantly different from control (p< 0.01) .

Effects of α –tocopherol on serum TBARS levels :

Figure (4) summarizes the changes in serum TBARS levels in response to tested therapy regimens .A significant reduction was detected in groups treated with aspirin and those given α –tocopherol in doses of 400 or 800 IU/day for more than five months.

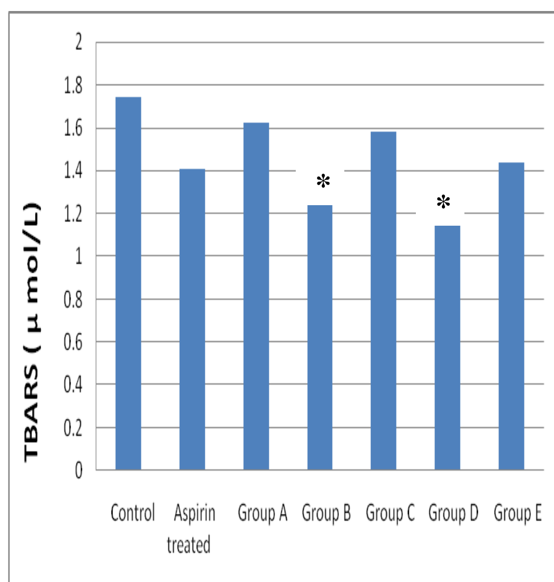


Figure 4 . Serum thiobarbituric acid reactive substances (TBARS) concentration in various groups

Group A : included subjects treated with 400 IU /day less than 5 months (2-4 months) .
Group B : included subjects treated with 400 IU / day more than 5 month (5-6 months) .
Group C : included subjects treated with 800 IU/ day less than 5 months (2-4 months) .
Group D : included subjects treated with 800 IU / day more than 5 months (5-6 months) .
Group E : included subjects treated with 1200 IU / day less than 5 months (2-4 months)
Aspirin group : included subjects treated with 100 mg / day (1-3months) .
* = significantly different from control ($p < 0.05$) .

Discussion

One of the reported actions for vitamin E is the anti-coagulant and antiplatelets activity. Such effect is mainly presented by the gamma (γ) form of tocopherols , as prolongation of bleeding time and abnormal platelet aggregability results after ingesting high doses of vitamin E for long period of time [19,20]. In vitro , alpha (α) , gamma (γ) and delta (δ) – tocopherols have similar effects on human platelet aggregation and a combination of these tocopherols has a synergestic platelets inhibitory effect over the α –tocopherol alone [21,22]. One of the proposed mechanisms for vitamin E antiplatelet activity could be through its interference with vitamin K activity with subsequent disturbance of the cascade of reactions for clot formation ,that predispose to thrombosis . However, such interruption depends on the isomer of vitamin E used , dose and period of administration[23] . Another proposed mechanism to explain the platelet antiaggregability effect of vitamin E is related to NO bioactivity [24,25] . Decreased bioavailability of NO is a characteristic feature in patients with coronary artery disease and impaired platelet NO production which predicts acute coronary syndrome [26] . Platelets -derived NO has been found to inhibit platelets aggregation and to reduce recuiment to grow to thrombus [27] . Incorporation of α – tocopherol might increase NO producion in platelets by its free radicals scavenging activity and by preventing NO quenching by peroxyl radicals [28,29]. The results of the present study shows that α –tocopherol when administered alone could exert significant modifications in platelets function as presented by changes in SPAT values (figure -3 -).Such effect seems to be related to duration of therapy rather than to dose administered . Although , such effect was less obvious in bleeding time values (figure -2-) .However , the concomitant changes in serum TBARS in the studied groups (figure 4)could strongly suggest a relationship to exist between antioxidant activity of vitamin E with it's antiplatelets activity [30] , indicated by a significant correlation between TBARS and SPAT values ($r=0.994, p<0.05$) .Although some reported that antiplatelets activity of vitamin E is independent on it's antioxidant effect [31].The lowering effect of TBARS by vitamin E may represent an index for delivering vitamin E to membrane structures of different cells including the platelets, which is reflected by a decrease in platelets aggregability upon longer time of exposure to these doses of α –tocopherol , through increasing the amount of α –tocopherol inside body with possible participation of it's antioxidant activity to affect SPAT values. Aspirin administration for more than one month could lower TBARS levels (figure -3-) through increasing the apoferritin level , whose duty is to quench free iron in plasma , since free iron catalyses free radicals generation through Fenton's reaction [32,33]. Long-term ingestion of α –tocopherol (more than 5 month) is needed to exert it's antiplatelts activity which may be explained on the bases of its pharmacokinetic behavior , because it is stored initially in adipose tissues before its action appears in circulation[34,35]. Thus to get greater benefit from vitamin E administration , it may be preferable to take other forms of tocopherols (i.e. γ–tocopherol) with a pharmacokinetic behavior that does not require to build up a concentration after accumulation in adipose tissues [36,37]. However , similar studies including larger number of subjects and longer duration of therapy could provide more clear picture about such effects of different

isomeric forms of tocopherols. In conclusion , vitamin E administration can produce significant effects in those patients with high risk of thrombus formation to be preferred over other antioxidanats like vitamin C .

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Formulation and *in vitro* Evaluation of In-situ Gelling Liquid Suppositories for Naproxen

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Abstract

In-situ gelation is a process of gel formation at the site of application, in which a drug product formulation that exists as a liquid has been transformed into a gel upon contact with body fluids. As a drug delivery agent, the in-situ gel has an advantage of providing sustained release of the drug agent. In-situ gelling liquid suppositories using poloxamer 188 (26-30% W/W) as a suppository base with 10% W/W naproxen were prepared, the gelation temperature of these preparations were measured and they were all above the physiological temperature. Additives such as polyvinylpyrrolidin "PVP" ,hydroxylpropylmethylcellulose "HPMC", sodium alginate and sodium chloride were used in concentration ranging from (0.25-1%W/W) to modulate the gelation temperature and gel strength .The best preparation was obtained through using a combination of poloxamer 188, sodium alginate, naproxen and distilled water (29,0.5,10and 60.5 % W/W respectively)with gelation temperature of 33.6ºC±0.2 and gel strength of 28±2 seconds. The release of drug from this preparation was sustained for about 12 hours and it was faster than conventional solid suppository (Proxen® 500) and oral tablets (Naproxen®500) using dialysis tubing method.

Key words: - naproxen, in-situ gelation, liquid suppository**,** poloxamer188

الخلاصة

إن عملية التحول الذاتي للمستحضر الدوائي الى هلام في موضع الإستعمال تتضمن إعطاء المستحضر بشكل سائل والذي يتحول الى هلام عند تماسه مع سوائل الجسم ومن فوائد هذه العملية إنها تساعد على تحرر الدواء بشكل طويل الأمد. لذلك تم تحضير التحاميل السائلة الموضعية التحول الى هلام للنابروكسين باستعمال البولوكسامر ١٨٨(٢٦-٣٠% وزن/ وزن) كمادة أساسية لتحضير التحاميل مع ١٠% وزن/ وزن من النابروكسين وعند قياس درجة حرارة التحول الى هلام لهذه المستحضرات وجد انها فوق درجة حرارة الجسم الفزيولوجية. ولتغيير درجة حرارة وقوة التحول الى هلام لهذه التحاميل تم إستخدام مواد إضافية مثل بولي فنيل بايرولدون ، هيدروكسي بروبيل مثيل سليلوز، الجنيت الصوديوم وكلوريد الصوديوم بتراكيزتتراوح بين (٠,٢٥- ١,٠% وزن/وزن) .وإن أفضل مستحضر تم الحصول عليه من مزج البولوكسامر ١٨٨، الجنيت الصوديوم ، كلوريد الصوديوم و ماء مقطر (٢٩ ، ٠,٥ ، ١٠ ، ٦٠,٥ % وزن/ وزن بالتعاقب) بدرجة حرارة تحول الى هلام ٣٣,٦ ± ٠,٢ مº وبقوة ٢٨± ٢ ثانية. ولقد أظهرت الدراسة الخارجية للتحرر والتي اجريت بإستخدام قناة الميز الغشائي بأن تحرر الدواء من هذا المستحضر كان طويل الأمد بمدة ١٢ساعة تقريبا كما وإنه أسرع من التحاميل الصلبة التقليدية والحبوب الفموية.

Introduction

Naproxen is a non steroidal anti-inflammatory drug used for painful and inflammatory rheumatic arthritis, osteoarthritis, non articular rheumatism, in acute injury, migraine and tension headache, postoperative pain and pain associated with gynecological procedures.[1] Its main adverse effects are gastrointestinal, of which peptic ulcer, with or without bleeding is the most sever effect. In addition esophageal ulceration may rise due to incorrect consumption. Therefore rectal delivery has been explored as a potential method of avoiding gastric irritation that may occur when this drug is administered orally. [2] The ideal suppository would be easy to administer without any pain during insertion and would remain at the administered site avoiding the first-pass effect in the liver and the gastrointestinal tract. Several problems are associated with the solid suppositories such as giving the felling of alien, discomfort and refusal to the patient with the possibility of lowering patient compliance. Furthermore, solid suppositories might reach the end of the colon allowing the carried drug to undergo the first-pass effect. [3] To solve these problems, there have been several attempts to develop suppositories which exist as liquid at room temperature but gels at physiological one so they are easy to administer to the anus. [4, 5] Suitable gel strength with suitable bioadhesive force is required in liquid suppositories so as not to leak out from the anus after administration and not to reach the end of the colon [6, 7]

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Poloxamer series is a group of non ionic surface active compounds of polyoxyethylene-polyoxypropylene-polyoxyethylene block copolymer .[8] It is the most common base of liquid suppositories in which their solutions were known to exhibit the phenomenon of reverse thermal gelation; remaining as a solution at low temperature and gelling when the temperature increases. By modulating the gelation temperature of different poloxamer solutions, liquid base can be formulated which form a gel in the rectum at body temperature with suitable gel strength [4, 9, 10] Furthermore, poloxamers were reported not to cause any damage on mucosal membrane. [11] This study was carried out to prepare an acceptable in-situ gelling liquid suppository for naproxen through studying different variables affecting the physicochemical properties and the in vitro release of the drug from this preparation.

Materials and Instruments

Materials

Naproxen, Hydroxypropylmethylcellulose (HPMC) supplied by Samara Drug Industry (SDI), Poloxamer 188(BASF, United Pharmaceuticals, Jordan), Polyvinylp-yrrolidone (PVP), Disodium Hydrogen Phosphate , Potassium Dihydrogen Phosphate (BDH Chemicals, LTD, Liverpool, England) , Sodium Alginate (Hopkins and Williams, LTD, England), Sodium Chloride (Evans Medical , LTD, Liverpool, England) Dialysis tubing 36/32 with clips (Medicell International LTD, Liverpool, England), Proxen®500 Suppositories (Grünenthal Pharma AG, Suisse), Naprox®500 Tablets (Medical Bahri, Syria)

Instruments

Hot Plate with Magnetic Stirrer (IKA®-WEKE, Copley,MBH and Co.KG, D-7921, Germany), Manually Modified Gardener Can-type Mobilometer , Sartorius balance (Werke-GMBH, type 2842, Germany),pH-meter (Hanna Instruments pH 211 Microprocessor, Italy), USP Dissolution apparatus, Type II (Copley Scientific LTD, England), UV.Visible Spectrophotometer (Carrywin UV.Varian,100i spectrum, Australia), Water bath (Mammert, Germany)

Method

Preparation of Liquid Suppository

Naproxen and various amounts of excipient, except the liquid suppository base "poloxamer 188", were completely dispersed in the specified amount of distilled water with continuous agitation at room temperature and cooled down to 4°C. Poloxamer 188 was then slowly added to the solution with continuous agitation. The liquid suppository was left at 4°C until a clear solution was obtained. [4] Several preparations were prepared according to the factors that affect the physicochemical properties of the resultant liquid suppositories in order to get an acceptable formula.

Evaluation of In-situ Gelling Liquid Suppositories:-

1. Gelation Temperature:-

A 20-ml transparent vial containing a magnetic bar and 10 g of the liquid suppository was placed in a low-temperature thermostat water bath.A digital themosensor connected to a thermositor was immersed in the liquid suppository. The liquid suppository was heated at a constant stirring "100 (rpm)".When the magnetic bar stopped moving due to gelation, the temperature displayed on the thermistor was determined as the gelation temperature. [4] Only those liquid suppository preparations that pass the gelation temperature test were subjected to the next tests.

2. Gel Strength:-

The gel strength of the liquid suppositories was measured using manually modified Gardener Can type mobilometer (figure 1).[12] A 50 g of the liquid suppository was transferred to a 100 ml graduated cylinder, and the cylinder was kept at 36.5°C for 30 minute in a water bath.A "35 g" weight of an appropriate size was then placed on the surface of the liquid suppository in the cylinder, and the time in second(s) required for this disc to move 5 cm down through the gelled suppository was measured and taken as an arbitrary index of gel strength. [13] Only those liquid suppository preparations that pass the gel strength test were subjected to the next test.

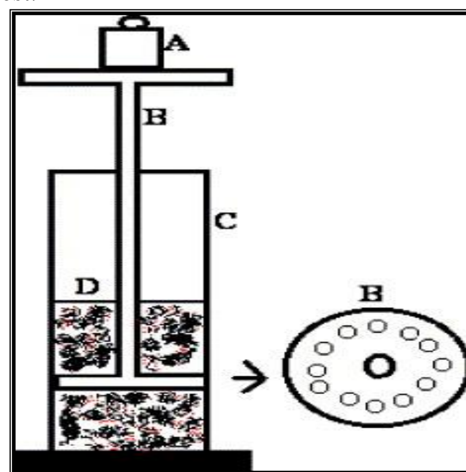

Figure 1.[11]: Gel-Strength Measuring Device
(A): weight, (B): device, (C): mess cylinder , (D): Liquid

3. Dissolution Test:-

To perform the in vitro release test for liquid suppositories a semipermeable membrane "Dialysis tubing 36/32"[14] of 7 cm in length with clips [15] was used. A 5 g weight of the liquid suppository containing 500 mg naproxen was inserted into the semipermeable membrane tubing and both sides of the tube were closed with the clips to prevent leakage. The semipermeable membrane tube was then placed in a dissolution tester. Drug release test was performed at 36.5°C using the USP dissolution apparatus type II at 100 rpm with 500 ml Sorensen's phosphate buffer pH 6.8 as a dissolution medium.At one hour intervals, 5 ml of the dissolution medium was sampled and filtered. The volume inside the jar was kept constant by addition of an equal volume"5 ml" of the same buffer solution. [6, 9] The filtrate was analyzed spectrophotometrically for naproxen content at 331 nm.

Factors Affecting the Physicochemical Properties of the In-situ Gelling Liquid Suppositories

1. Effect of Poloxamer 188 Concentration

Different concentration of poloxamer 188 ranging from 26-30%w/w [4] were used to prepare a plain liquid suppository, and the effect of poloxamer 188 concentration on the gelation temperature and gel strength was studied.

2. Effect of Addition of Naproxen

Naproxen in a dose of 500 mg (10% w/w), the usual rectal dose, was added to the different concentrations of poloxamer 188 solutions to study its effect on the physicochemical properties of the resultant medicated liquid suppository.

3. Effect of Addition of Additives

Different additives including "PVP", "HPMC", sodium alginate and sodium chloride were added in different concentrations ranging from (0.25-1.0% w/w)[4,9,10] to the different concentrations of poloxamer solutions and their effect on the gelation temperature, gel strength and dissolution behavior of the resultant liquid suppositories were then studied.

Factors Affecting the in vitro Release of Naproxen

1. Effect of Formulation Components

To study the effect of the liquid suppository components; "the base and the additives", on the naproxen release profile, an in vitro release of the drug powder was performed and compared with that from the liquid suppository. A 500 mg naproxen powder was inserted into the semipermeable membrane tubing and then the release test was performed as mentioned previously.

2. Effect of Dosage Forms

To study the effect of the dosage forms on the in vitro release of naproxen a comparison was done between the liquid suppository and other dosage forms. A commercial solid suppository (Proxen®500) or commercial oral tablet (Naprox®500) containing 500mg naproxen were inserted into the semipermeable membrane and then the dissolution test was performed as mentioned previously. The release profile of these dosage forms were then compared with that of the liquid suppository.

Statistical Analysis

Results are given as a mean ± S.D for triplicate samples. The results were statistically analyzed by using analysis of variance (ANOVA) table and t-test, P-values less than 0.05 was considered significant

Results and Discussion

Physicochemical Properties of the In-situ Gelling Liquid Suppositories

The temperature dependent gelation of poloxamer solutions could be explained to be due to desolvation of hydrophilic chains of the polymer as a result of the breakage of the hydrogen bonds that have been established between the solvent and these chains. This phenomenon favors hydrophobic interaction among the hydrophobic chains of the polymer[16] and the polymer self-assemble spontaneously, forming micelles. [17, 18] Raising the temperature of poloxamer solutions will be accompanied by an increase in the micellar aggregation number and a decrease of a critical micelle concentration allowing the formation of a more closely packed and more viscous gel. [19] An accepted liquid suppository must have a gelation temperature in the range of (30-36°C) and gel strength of (10-50 seconds), so as to be in a liquid form at room temperature and to form a gel phase instantly in the rectum without leakage. [4, 9, 10]

Factors Affecting the Physicochemical Properties of the In-situ Gelling Liquid Suppositories

1. Effect of Poloxamer 188 Concentration

Table (1) shows the effect of poloxamer 188 concentration on the gelation temperature of the prepared liquid suppositories. The results indicated that increasing the poloxamer 188 concentration from 26 % to 30 % w/w was accompanied with a decrease in the gelation temperature. These results were in agreement with those reported by Choi.H.G. et al. [4] The increment in poloxamer 188 concentration led to an apparent dramatic

increase of micellar size and polydispersibility which could be the reason for such reduction in the gelation temperature . It was suggested that such changes were a consequence of interactions between polyoxyethylene chains of adjacent micelles which, as a result of their dehydration, experience increased friction with a resulting tendency to form multimolecular units leading eventually to gel formation [20]

Table (1): Gelation temperature of different naproxen suppositories

Concentration of Poloxamer 188 % w/w	Mean Gelation Temperature °C± S.D of poloxamer solutions	
	Plain	Medicated
26%	>50	45. 6 ± 0.1
27%	>50	43.1 ± 0.2
28%	>50	40.9 ± 0.2
29%	>50	37.7 ± 0.2
30%	48.0±0.1	35.2 ± 0

2. Effect of Addition of Naproxen

The incorporation of 10 % w/w (500 mg) of naproxen into the poloxamer 188 solutions of different concentrations decreased the gelation temperature of the resultant liquid suppositories as compared with the plain one, as shown in table (1). As a possible mechanism by which naproxen affected the gelation temperature of these preparations, it may be speculated that placing naproxen in the gel matrix would make its carboxyl group bonded strongly with the cross-linked reticular poloxamer gel through hydrogen bonding. This suggestion was based on the results obtained by El-Kamel AH. who found that if hydrogen bonding is supplemented to the poloxamer solutions by adding compounds containing a hydrogen-bonding forming group, the gelation temperature will decrease.[21]

3. Effect of Addition of Additives

Tables (2a-2e) show that the addition of any of the used additives PVP, HPMC, sodium alginate and sodium chloride, would lower the gelation temperature and reinforce the gel strength of the resultant liquid suppositories. The impact of additives on the gelation temperature and gel strength was found to be depending on their nature and concentration. Increasing the concentration of any of the used additives from 0.25 to 1.0 % w/w produced a gradual decrease in the gelation temperature and increase in the gel strength of the corresponding liquid suppositories. The gelation temperature lowering and gel strength increasing effect of PVP, HPMC and sodium alginate could be explained by their ability to bind to the polyoxyethylene chains present in the poloxamer molecules through hydrogen bonds. This will promote dehydration causing an increase in entanglement of adjacent molecules which will lead to gelation at lower temperature and reinforce the gel strength. [5, 9, 22] On the other hand, the reduction in the gelation temperature with the increase in gel strength by addition of sodium chloride could be attributed to its salting-out effect which results in dehydration of the polyoxyethylene chains, causing an increase in the entanglement of adjacent molecules.[17]

Table (2): Effect of addition of additives on the physicochemical propertie of liquid suppositories containing 10% w/w naproxen and different concentrations of poloxamer 188.

(a): with 30% w/w poloxamer 188

Types of additives	Conc.of additives	Mean Gelation Temperature (°C) ± S.D	Mean Gel Strength (seconds) ±S.D
No	——	35.2 ± 0	102 ± 2.3
PVP	0.25	29.8 ± 0.1	/**
	0.5	/	/
	0.75	/	/
	1.0	/	/
HPMC	0.25	34.7 ± 0.1	200 ± 1.8
	0.5	33.1 ± 0.4	/
	0.75	31.8 ± 0.4	/
	1.0	30 ± 0.1	/
Sodium alginate	0.25	33.7 ± 0	146 ± 2.04
	0.5	31.4 ± 0.1	/
	0.75	29 ± 0.4	/
	1.0	/	/
Sodium chloride	0.25	34.2 ± 0.5	Not fall*
	0.5	32.4 ± 0.1	/
	0.75	31.1 ± 0.1	/
	1.0	30.2 ±0.3	/

***not full up to 300seconds**
****not done**

(b) : with 29% w/w poloxamer 188

Types of additives	Conc.of additives	Mean Gelation Temperature (°C) ± S.D	Mean Gel Strength (seconds) ±S.D
No	——	37.7 ± 0.2	/**
PVP	0.25	33.8 ± 0	Not fall*
	0.5	30.2 ± 0.1	/
	0.75	27.1 ± 0.5	/
	1.0	/	/
HPMC	0.25	37 ± 0.1	/
	0.5	36.6 ± 0	/
	0.75	36.3 ± 0	/
	1.0	34.2 ± 0.1	253 ± 2.3
Sodium alginate	0.25	36.8 ± 0	/
	0.5	33.6 ±0.2	28 ± 2
	0.75	30.5 ± 0	83 ± 3.72
	1.0	27.7 ± 0.1	/
Sodium chloride	0.25	35.4 ± 0	150 ± 1.5
	0.5	34 ± 0	/
	0.75	32.7 ± 0.1	/
	1.0	31 ± 0.1	/

***not full up to 300seconds**
****not done**

(d): 27% w/w poloxamer 188

Type of additives	Conc. of additives	Mean Gelation Temperature (°C) ± S.D	Mean Gel Strength (seconds) ± S.D
No	——	43.1 ± 0.2	/**
PVP	0.25	36.1 ±0.1	/
	0.5	32.4 ± 0	348 ± 3.77
	0.75	28.3 ± 0	/
	1.0	/	/
HPMC	0.25	/	/
	0.5	/	/
	0.75	/	/
	1.0	/	/
Sodium alginate	0.25	/	/
	0.5	37.2 ± 0	/
	0.75	34.3 ±0	2 ± 4.25
	1.0	34.6 ± 0	3 ± 2.86
Sodium chloride	0.25	39.5 ±0	/
	0.5	36.1 ± 0	/
	0.75	35.2 ± 0.1	98 ± 1.79
	1.0	33.5 ± 0.2	/

****not done**

(c): 28% w/w poloxamer 188

Type of additives	Conc. of additives	Mean Gelation Temperature (°C) ± S.D	Mean Gel Strength (seconds) ± S.D
No	——	40.9 ± 0	/**
PVP	0.25	34.1 ± 0.1	Not fall*
	0.5	30.6 ± 0.58	/
	0.75	27.6 ± 0.3	/
	1.0	/	/
HPMC	0.25	37.4 ± 0	/
	0.5	/	/
	0.75	/	/
	1.0	/	253 ± 2.3
Sodium alginate	0.25	37.7 ± 0.4	/
	0.5	34.8 ±0.2	3 ± 1.96
	0.75	33.9 ± 0	14 ± 2.03
	1.0	33.6 ± 0.1	99 ± 1.43
Sodium chloride	0.25	35.9 ± 0	95 ± 0.9
	0.5	34.9 ± 0.3	/
	0.75	33.7 ± 0.58	/
	1.0	32 ± 0.2	/

***not full up to 300seconds**
****not done**

(e): 26% w/w poloxamer 188

Type of additives	Conc. of additives	Mean Gelation Temperature (°C) ± S.D	Mean Gel Strength (seconds) ± S.D
No	——	45.6 ± .1	/**
PVP	0.25	/	/
	0.5	33.4 ± 0.1	212 ± 1.53
	0.75	29.8 ± 0	/
	1.0	/	/
HPMC	0.25	/	/
	0.5	/	/
	0.75	/	/
	1.0	/	/
Sodium alginate	0.25	/	/
	0.5	/	/
	0.75	35.9 ± 0.2	1 ± 1.63
	1.0	35 ±0	2 ± 1.46
Sodium chloride	0.25	/	/
	0.5	/	/
	0.75	36 ± 0	46 ± 1.32
	1.0	34.2 ± 0.1	63 ± 2.61

****not done**

Dissolution Test

Three naproxen liquid suppositories passed the gelation temperature and gel strength tests (nomenclated as A, B and C) were subjected to the dissolution test. Table (3) summarizes the constituents of each liquid suppository. In addition, these liquid suppositories (A, B and C) may have a mucoadhesive force that prevent the gelled suppositories from reaching the end of the colon, the pathway for the first-pass effect, which may be related to their ability to bind strongly to the oligosaccharide chains of the rectal mucous membrane through the hydrophilic groups of poloxamer and the additives.[4,6,9]The percentage of naproxen released from these preparations to the dissolution medium for 12 hours [23 , 24], was selected for the comparison study. In addition, the time required for 100 % release for the drug from these three preparations were also measured, table (4). It was found that there is no significant difference in the percentage of drug released among these three preparations, (P > 0.05), and all of them gave sustained drug release, since they released about 70 % of the drug within 12 hours as shown in figure (2) and table (4). The in-situ gelling liquid suppository (A), which had suitable gelation temperature and an intermediate gel strength was selected for further study, since it may has a lower chance to be leaked from the rectum and easier to be administered than the others.

Table (3): The constituents of the in-situ gelling liquid suppositories with their physicochemical properties

Name of the liquid suppository	Constituents of the liquid suppository		Physicochemical properties	
	Component	Conc. % w/w	Mean Gelation temperature (°C)± S.D	Mean Gel Strength Sec. ± S.D
A	Poloxamer 188	29	33.6 ± 0.2	28 ± 2
	Sodium alginate	0.5		
	Naproxen	10		
	Distilled water	60.5		
B	Poloxamer 188	28	33.9 ± 0	14 ± 2.03
	Sodium alginate	0.75		
	Naproxen	10		
	Distilled water	61.25		
C	Poloxamer 188	26	36 ± 0	46 ± 1.32
	Sodium chloride	0.75		
	Naproxen	10		
	Distilled water	63.25		

Table (4): Percentage released of naproxen from in-situ gelling liquid suppositories and the time required for 100% release

Liquid Suppository	%Released for 12 hours	Time for 100 % release (hours)
A	66.64	20
B	72.86	17
C	69.73	21

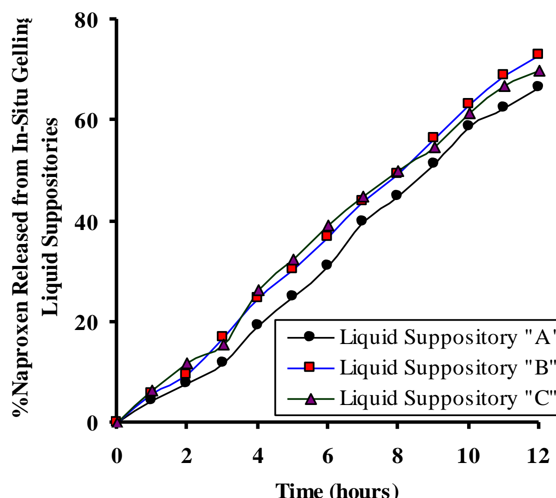

Figure 2. Percentage of naproxen released from three different in- situ gelling liquid suppositories in Sorenson's phosphate buffer pH 6.8 at 36.5°C using dialysis tubing method

Factors Affecting the in vitro Release of Naproxen

1. Effect of the Formulation Components

To study the effect of the formulation components on the in vitro release of naproxen from the liquid suppositories, the percentage of drug release from the powder (500 mg) was compared with that from in-situ gelling liquid suppository (A), using the dialysis tubing method. The release profile for in-situ gelling liquid suppository (A) was similar to that for naproxen powder at the first five hours and it was significantly faster (P<0.05) after hour five as shown in figure (3). This could be attributed to the solubilizing effect of poloxamer 188, especially for the poorly water soluble drug. [8, 25]

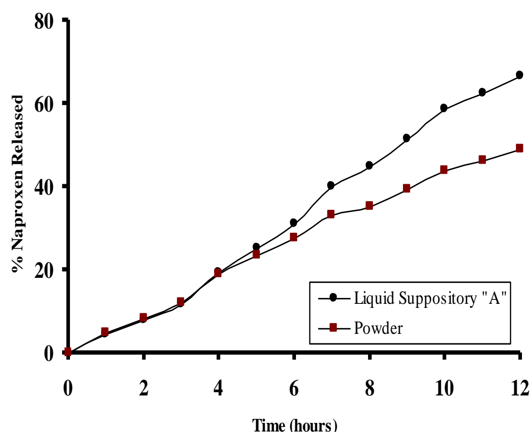

Figure 3. Effect of formulation components on the in vitro release of naproxen in Sorenson's phosphate buffer pH 6.8 at 36.5ºC using dialysis tubing method

2. Effect of the Dosage Form

To study the effect of the dosage form on the in vitro release of naproxen, the percentage released of the drug from the liquid suppository (A), commercial solid suppository and commercial oral tablet were compared by using dialysis tubing method. As shown in figure (4), the in-situ gelling liquid suppository had the faster release, followed by the commercial oral tablet and finally the commercial solid suppository. These results indicated that the dosage form design would affect the in vitro release of naproxen. A statistical analysis were done and showed a significant differences ($P<0.05$) for the percentage released of naproxen among these three dosage forms.The faster release from the in-situ gelling liquid suppository (A) could be due to the fluidity of the liquid suppository [7,9], and the solubilizing effect of poloxamer 188 for the poorly water soluble drug.[8, 25]

Error! Not a valid link.Figure 4. Effect of dosage Form on the in vitro release of naproxen in Sorenson's phosphate buffer pH 6.8 at 36.5ºC using dialysis tubing method

Future work

Further study on the stability, mucoadhesive property, clinical and pharmacokinetic study on human subjects for naproxen –loaded poloxamer in-situ gelling liquid suppositories is to be done.

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Synthesis of New Opioid Analgesic Peptide Analogues to Enkephalin (Leucine- and Methionine-Enkephalin)

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Abstract

A small number of researches were done in the design and synthesis of enkephalin analogues that are able to resist degradation effect of proteolytic enzymes with good bioavailability and half-lives.Through studying structure activity relationships we tried to incorporate phthalyl group, tryptophan and lysine amino acids in different positions in the basic backbone structure of the naturally occurring opioid Leu^5- and Met^5- enkephalin, in the hope that such insertion of these amino acids could induce interesting addition in the biological activity of these analogues with enhancement of their bioavailability, in addition to decrease side effects as addiction liability.

These synthesized peptides are:

1- Analogue I: phthalyl-tyrosyl-glycel-tryptophan methyl ester.

2- Analogue II: Boc-tyrosyl-glycyl-phenylalanyl-lysine ethyl ester. HBr.

According to the designed structures, the analogues were synthesized following the conventional solution method and they were identified using the following techniques: melting point, optical rotation, thin layer chromatography (TLC), infrared spectroscopy (IR), elemental analysis (CHN) and amino acid analysis.

Key words: peptide, phthalyl, opoioid analgesics

الخلاصة

إن عدداً قليلاً من البحوث تم إنجازها في مجال تصميم وتحضير مركبات جديدة مشابهة للببتيدات المسكنة الطبيعية (الانكفالين)، والتي لها القدرة على مقاومة تأثير الأنزيمات المحللة لها في داخل الجسم مع توافر حيوي وعمر نصفي جيد. وهذا البحث تم إنجازه على هذا الأساس. من خلال الدراسة المستفيضة للعلاقة بين الفعالية الحيوية والتركيب الكيمياوي لهذه المركبات تم اجراء تحويرات على البنية الجزيئية للببتيدات المورفينية بادخال مجموعة الفثاليل والاحماض الامينية التربتوفان واللايسين في مواقع مختلفة للبنية الجزيئية للببتيد المسكن الخماسي ليوسين^5- وميثيونين^5-انكفالين أملاً بالحصول على مركبات شبيهة بالانكفالينات لم يسبق تحضيرها من قبل ولها فعالية حياتية وفسيولوجية مهمة مع زيادة توافرها الحيوي، على أمل ايضاً من تقليل الأعراض الجانبية مثل الإدمان.

والمركبات المحضرة هي:

1- المشتق الاول: فثاليل-تايروسيل-كلايسيل-تربتوفان مثيل استر.

2- المشتق الثاني: بوك-تايروسيل-كلايسيل-فنيل الانيل-لايسين اثيل استر هيدروبرومايد.

والتي خلقت بطريقة الطور السائل التقليدية، وتم استخدام التقنيات التالية بهدف التوصل الى الخواص المميزة لتلك الببتيدات ولإثبات بنيتها التركيبية: قياس درجة الانصهار، كروماتوغرافيا الطبقة الرقيقة، الاستدارة البصرية، التحليل الدقيق للعناصر، تحليل الأحماض الامينية، مطياف الأشعة تحت الحمراء.

Introduction

The opium group of narcotic drugs is among the most powerfully acting and clinically useful drugs producing depression in the central nervous system [1]. Opiates are drugs derived from opium (*Papver somniferum F. Papaveraceae*), and include morphine, codeine and a wide variety of semisynthetic congeners derived from them and thebain (another compound of opium) [2]. The term opioid is more inclusive, applied to all agonists and antagonists with morphine-like activity as well as to naturally occurring (endogenous) and synthetic opioid peptides [3]. Opioid peptides defined as peptides with opiate-like pharmacological effect [4,5]. The discovery of endogenous opioids has been followed shortly after the identification of opioid receptors [6]. The main clinical uses of opioid peptides are as analgesics (enkephalins) [7,8], as antioxidants (enkephalins) [9], as anticancers (dalargin) [10], as antibacterials and antifungals (phthalyl serine, phthalyl arginine) [11].

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Materials and Methods:

All amino acids and their derivatives were optically active and of L-configuration and supplied from Fluka AG/Switzerland. All of the solvents and materials used were of Analar type and used without further purification. The method used for synthesis of these analogues was conventional solution method in which we used *N,N′*-dicyclohexyl carbodiimide (DCCI) and 1-hydroxy-benzotriazole (HBT) as a coupling agent and to prevent racemization, respectively. We used *tert*-butyoxy carbonyl (Boc) group and benzyloxy carbonyl (Z) group as terminal amino protecting group. Boc-tyrosine and lysine ethyl ester-N^{ε}-Z were obtained fully protected from Fluka AG/Switzerland; while we use methyl and ethyl ester to protect the carboxyl moiety in peptide synthesis. The final analogues were purified using gel filtration on sephadex LH-20 column eluted with 0.1N acetic acid. The synthesis of analogues (I and II) include the following general procedures:

a. N-terminal protection: the phtalylamide derivative of amino acid was used for the intermediate cpd (1.3) in analogue I by fusion method [12], and the common amino protecting group which is tert-butoxycarbonyl (Boc), (Boc-Tyrosine) and N-benzyloxycarbonyl (Lys-OEt-N-Z)were obtained fully protected for analogue II.

b. C-terminal protection: methyl and ethyl ester were used to protect the carboxyl group of amino acid in peptide synthesis.

c. Coupling method: conventional solution method was used as a coupling method between the protected amino acid for peptide synthesis. DCCI was used in the peptide bond formation as a coupling agent, while HBT was used to decrease racemization and to increase the yield.

d. De-protection of C-terminals: the removal of methyl or ethyl ester (de-esterification) was carried out with 1.5 equivalent of 1N sodium hydroxide solution (saponification).

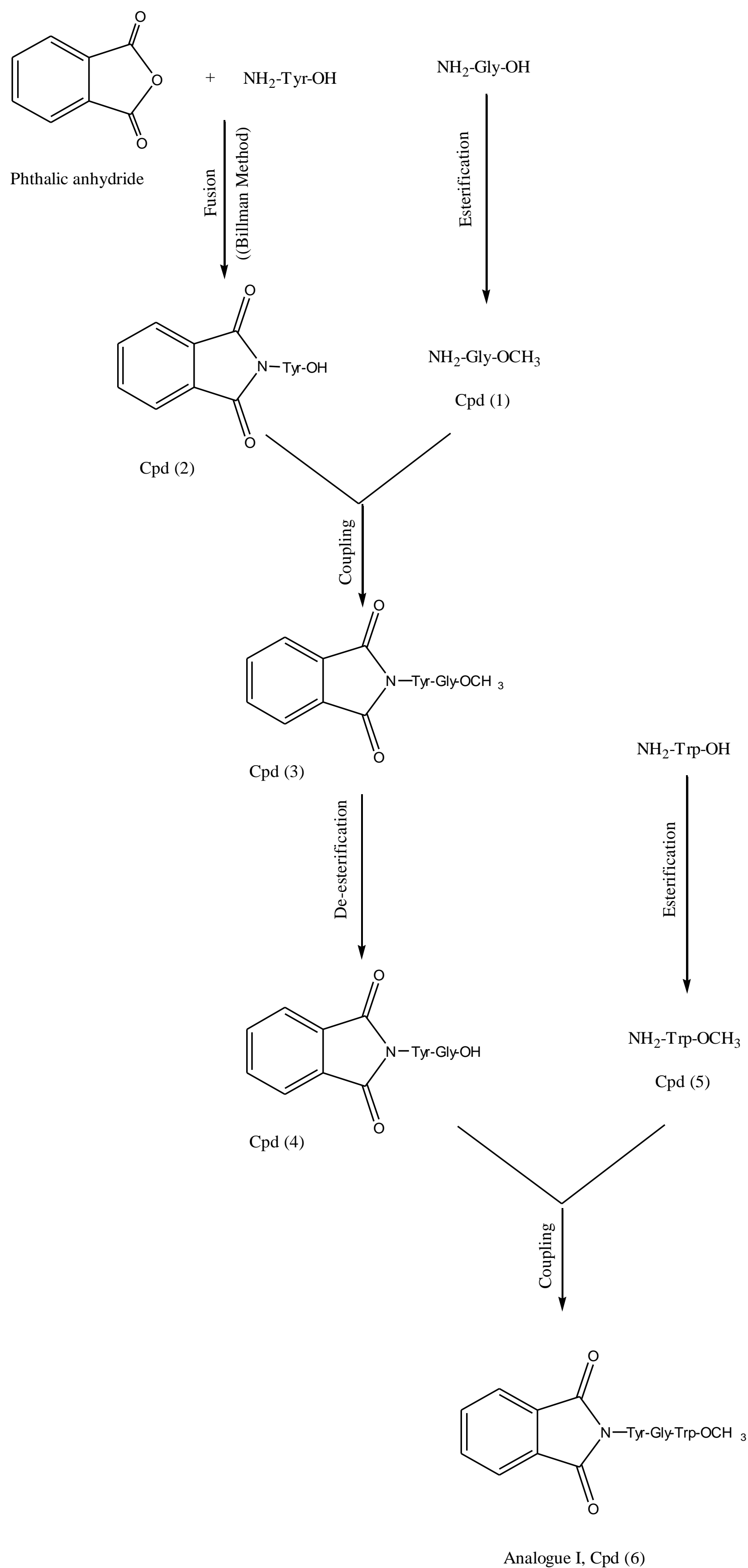
e. De-protection of N-terminal protecting groups: this step was performed with strong acidic conditions in which the benzyloxycarbonyl (Z) groups were removed with equimolar quantities of HBr in glacial acetic acid.

f. Peptide purification: the intermediates and the peptides have been purified by repeating re-crystallization several times (2-4 times) using different solvents as diethyl ether, petroleum ether, ethyl acetate, absolute ethanol, distilled water and chloroform. The final analogues were purified using gel filtration on sephadex LH-20 column eluted with 0.1N acetic acid.

Synthesis of analogue I:

Scheme (1) shows the steps of synthesis of analogue I which include:

a- Synthesis of glycine-methyl ester (cpd. 1): a suspension containing (1.5gm, 0.02 mol) of glycine in methanol (15ml) was cooled down to (-15 ^{o}C), then thionyl chloride (1.46ml, 0.02 mol) equimolar was added drop wise to the suspension, keeping the temp below (-10 ^{o}C). Then the reaction mixture was kept at (40 ^{o}C) for (3 hrs), followed by refluxing for (3 hrs), and left at room temperature overnight. After solvent evaporation to dryness in vacuum, the product was purified by re-crystallization from methanol-diethylether (1:10) mixture. Physical appearance, melting point, and R_f value are listed in table (1) [13].



Scheme (1): Synthesis of analogue I.

Table (1): Physical appearance, M.Ps, and R_f values of intermediates and final analogues.

Compound No.	Physical appearance	Melting points (°C) Found	Melting points (°C) Reported	R_f values *
5	White crystals	209-210	213-214	0.88 (D) 0.71 (A)
1	White crystals	171-173	175	0.78 (D) 0.86 (E)
2	Off-white powder	188-190	-	0.84 (B) 0.85 (C) 0.76 (D)
3	Off-white Powder	140-142	-	0.39 (B) 0.68 (D)
4	White powder	180-182	-	0.8 (C) 0.92 (D)
6 (Analogue I)	White powder	170-173	-	0.75 (D) 0.9 (E)
9	White crystals	154-155	157-158	0.76 (A) 0.94 (D)
7	White powder	115-118	-	0.69 (D) 0.88 (E)
8	White powder	155-158	-	0.68 (C) 0.95 (D) 0.83 (E)
10	White powder	148-150	-	0.8 (A) 0.36 (B) 0.6 (D)
11	Faint yellow powder	218-220	-	0.7 (C) 0.8 (D)
12	White powder	158-160	-	0.55 (B) 0.67 (C)
13 (Analogue II)	Needle shaped crystals	196-198	-	0.45 (B) 0.37 (C)

* Solvent system used in TLC were: A: Butanol: Acetic acid : D.W. (4:1:5).
B: Chloroform: Methanol: Acetic acid (4:5:1). C: Chloroform: Methanol: Ether (5:3:3).
D: Chloroform: Methanol (7:3). E: C: Chloroform: Methanol: Benzene (4:3:2).

Table (2): Infrared values for analogue I and II.

Analogue number	IR value
Analogue I	3600-3460, 3340, 3074, 2983, 2858, 2806, 1738, 1677, 1569, 1504, 1440, 1373, 1244, 846 and 786
Analogue II	3690-3384, 3328, 3031, 2927, 2850, 1736, 1670, 1628, 1610, 1569, 1508, 1448, 1340, 1311, 1277 and 640

b- Synthesis of phthalyl-tyrosine (cpd. 2): Compound 2 is prepared by Billman *et al.* method [14], in which L-tyrosine (18.119gm, 0.1mol) and phthalic anhydride (114.8gm, 0.1mol) fused together to give cpd. 2. Physical appearance, melting point, and R_f value are listed in table (1). Elemental analysis, amino acid analysis and optical rotation are listed in table (3), (4) and (5), respectively.

c- Synthesis of phthalyl-tyrosyl-glycin methyl ester (cpd. 3): a stirred solution of cpd. 2 (1 mmol) in DMF (5ml) and NMM (1 mmol) were added with stirring for (10 min). Then eqimolar amount of cpd. 1 previously dissolved in DMF (5 ml) was also added, the mixture was cooled down to (-10 °C). HBT (2 mmol) and DCCI (1 mmol) were added with stirring. Stirring was continued for (3 days) at (0 °C) and then at room temperature for (7 days). Then DCU was filtered, the filtrate was concentrated under vacuum, and then the residue was re-dissolved in ethyl acetate washed several times. The product was collected after solvent evaporization. Physical properties, elemental analysis, amino acid analysis and optical rotation

are listed in table (1), (3), (4) and (5), respectively.

d- Synthesis of Phthalyl-tyrosyl-glycine (cpd 4): to a stirred solution of cpd 3 (0.5mmol) dissolved in dioxan (5 ml): water mixture (5:1) at (18 ^{o}C), sodium hydroxide solution (1N, 0.75ml) was added drop wise over (30 min). The reaction was allowed to proceed for additional (3 hrs). Then the reaction mixture was acidified with equimolar quantity of hydrochloric acid. After the addition of ice-water, a precipitate was obtained. The physical properties are listed in table (1).

e- Synthesis of Trptophane-methyl ester (cpd 5): a suspension of tryptophan (9.8 mmol) in methanol (20ml) was cooled down to (-15°C), and continue the procedure as in the synthesis of cpd 1.

f- Synthesis of analogue I (Phthalyl-glycyl-tryptophane methyl ester (cpd 6)): the same procedure was performed as in the synthesis of cpd 3. Physical properties, elemental analysis, amino acid analysis and optical rotation are listed in table (1), (3), (4) and (5), respectively. IR values for analogue I are shown in table (2).

Table (3): Elemental analysis of some intermediates and final analogues.

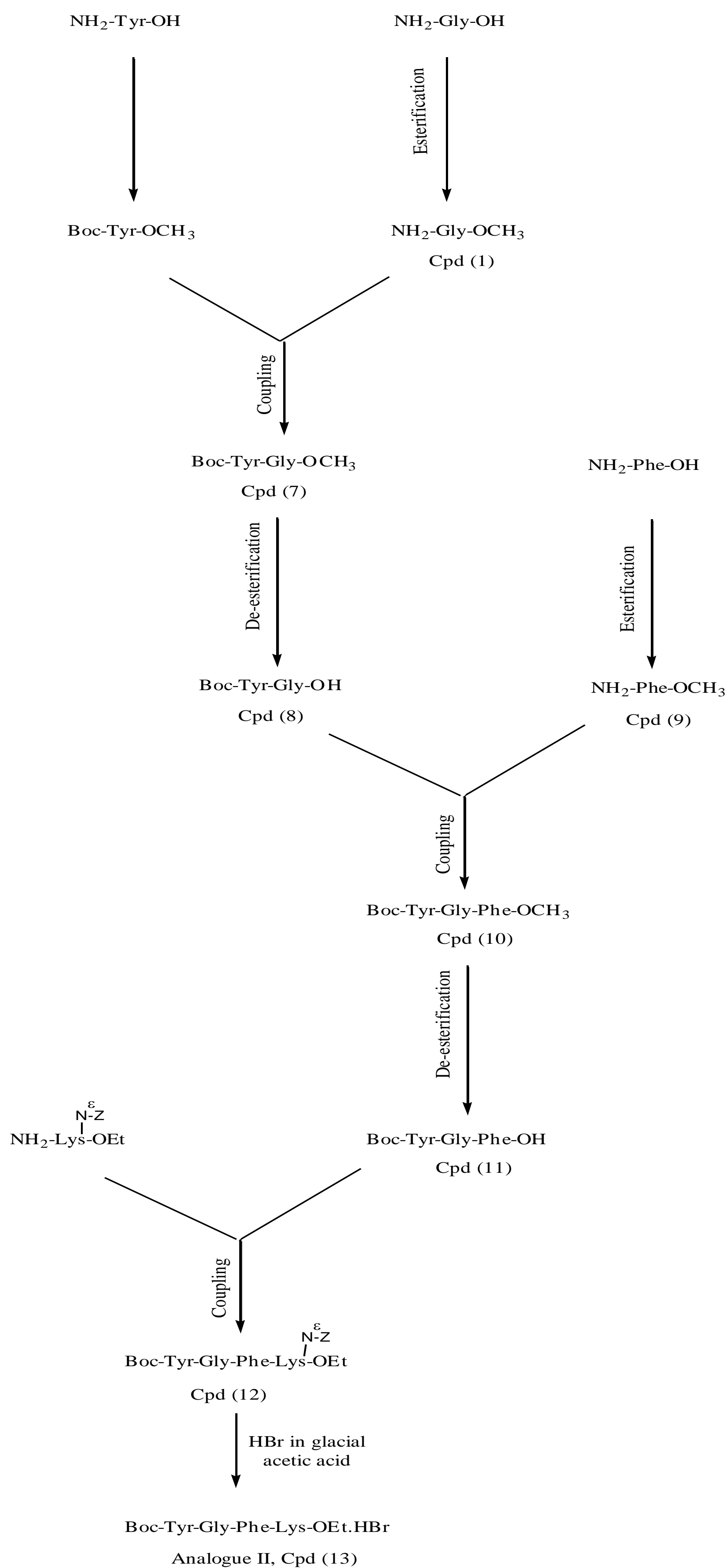
Cpd. no.	Compound	Chemical formula	Calculated/Found		
			C%	H%	N%
2	Phthalyl-N-Tyr	$C_{17}H_{13}N_1O_5$	65.59 65.89	4.21 4.45	4.49 4.31
3	Phthalyl-N-Tyr-Gly.OMe	$C_{20}H_{18}N_2O_6$	62.82 63.2	4.74 4.55	7.32 7.59
6	Phthalyl-N-Tyr-Gly-Trp.OMe (Analogue I)	$C_{31}H_{22}N_2O_6$	65.48 66.01	4.96 5.50	9.85 9.66
8	Boc-Tyr-Gly	$C_{16}H_{22}N_2O_6$	56.79 57.45	6.55 6.11	8.27 8.75
10	Boc-Tyr-Gly-Phe.OMe	$C_{26}H_{33}N_3O_7$	62.51 62.81	6.65 6.60	8.41 8.91
12	Boc-Tyr-Gly-Phe-Lys.OEt (-N$^{\varepsilon}$-Z)	$C_{41}H_{53}N_5O_{10}$	63.46 66.1	6.88 6.79	9.02 9.35
13	Boc-Tyr-Gly-Phe-Lys.OEt.HBr (Analogue II)	$C_{33}H_{48}N_5O_8Br$	54.84 54.81	6.55 5.99	9.69 9.21

Table (4): Amino acid analysis of some intermediates and final analogues.

Cpd. no.	Compound	Amino acids				
		Tyr	Gly	Phe	Lys	Trp
2	Phthalyl-N-Tyr	1.1				
3	Phthalyl-N-Tyr-Gly.OMe	0.99	1.02			
6	Phthalyl-N-Tyr-Gly-Trp.OMe (Analogue I)	0.96	0.99			1.01
8	Boc-Tyr-Gly	1.09	1.11			
10	Boc-Tyr-Gly-Phe.OMe	1.06	0.98	1.08		
12	Boc-Tyr-Gly-Phe-Lys.OEt (-N$^{\varepsilon}$-Z)	1.01	0.95	1.1	0.89	
13	Boc-Tyr-Gly-Phe-Lys.OEt.HBr (Analogue II)	0.89	1.13	1.1	1.18	

Table (5): Optical rotation of some intermediates and final analogues.

Cpd. no.	Compound	Optical rotation $[\alpha]_D^{25}$, c=1 in DMF
2	Phthalyl-N-Tyr	-32 ^{o}
3	Phthalyl-N-Tyr-Gly.OMe	-16 ^{o}
6	Phthalyl-N-Tyr-Gly-Trp.OMe (Analogue I)	-36 ^{o}
8	Boc-Tyr-Gly	+48 ^{o}
10	Boc-Tyr-Gly-Phe.OMe	+80 ^{o}
12	Boc-Tyr-Gly-Phe-Lys.OEt (-N$^{\varepsilon}$-Z)	-29 ^{o}
13	Boc-Tyr-Gly-Phe-Lys.OEt.HBr (Analogue II)	-27 ^{o}



Scheme (2): Synthesis of analogue II.

Synthesis of Analogue II:

Scheme (2) shows the steps of synthesis of analogue II which include:

a- Synthesis of phthalyl alanine methyl ester (cpd. 9): the same procedure was carried out as in synthesis of cpd. 1 and cpd. 5.

b- Synthesis of Boc-tyrosyl-glycine methyl ester (cpd. 7), Boc-tyrosyl-glycyl-phenylalanyl-lysine etheyl ester-N^{ε}-Z (cpd. 12): again the same procedure was applied as in the synthesis of cpd. 3 and cpd. 6.

c- Synthesis of Boc-tyrosyl-glycine (cpd. 8) and Boc-tyrosyl-glycyl-phenylalanine (cpd. 11): The de-esterification was performed as in the synthesis of cpd. 4 and the physical results are listed in table(1).

d- Synthesis of analogue II (Boc-tyrosyl-glycyl-phenylananyl-lysin ethyl ester. HBr (cpd. 13)): cpd. 12 (0.337 mmol) was dissolved in HBr (2.5N, 3ml) in glacial acetic acid, the mixture was left at room temperature for (30 min), then it was poured into (200ml) of diethyl ether with stirring. The separated oily material was evaporated under reduced pressure. The precipitated HBr salt was re-crystallized from ethyl acetate-petroleum ether mixture (1:7). The physical properties, elemental analysis, amino acid analysis and optical rotation are shown in tables (1), (3), (4) and (5), respectively. IR values are shown in table (2) .

Results and Discussion:

The results of our work were been shown in schemes (1 and 2), figures (1 and 2) and tables (1, 2, 3, 4 and 5). The methodology that has been adapted in this work seems to be successful according to the results indicated previously, in addition a biological activity study has been done on cpd. 4 (phthalyl-tyrosyl-glycine) and give significant positive results for analgesic activity relative to morphine [15].The structural modification that has been made on the backbone of Leu-enkephalin in this work to synthesize these new analogues and the positive results mentioned indicate that an enhanced analgesic activity has been achieved from this modification and this would open the door for further modifications or studies of these two analogues to discover a new biological activities as antimicrobials and anticancers.

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Evaluation of the Role of Interleukein-2 and Interleukein-4 in the Immunopathogenesis of Steroid Therapy Resistance in Iraqi Asthmatic Patients

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Abstract

Interleukins (IL-2 and IL-4) are increased in asthmatics and were reported to induce resistance to steroid therapy in some patients who fail to get benefit from glucocorticoids when used in full dose and for long period of time. In this context, the present study was conducted on Iraqi patients to provide additional laboratory mean, beside the clinical diagnosis, for the decision whether the asthma is steroid sensitive or resistant by monitoring the level of immunoglobulins, complement proteins and interleukins among asthmatic patients (steroid sensitive or resistant) and the possible contribution of other factors like age, sex and environments in the development of steroid resistance. A total number of 55 asthmatics and 28 normal subjects were enrolled in the study. Patients were diagnosed clinically as steroid sensitive (SSA) and steroid resistant (SRA) and blood samples were taken from all subjects included in the study for the measurement of immunoglobulins (IgA, IgG, IgM and IgE), complement proteins (C3 and C4), interleukins (Il-2 and Il-4), and total and differential WBC counts.The results showed no age, sex and residence dependency of acquired steroid resistance, while smoking habit (and may be the atopic allergy) constitute marked predisposing factors. The level of IgA and IgE were high in both SRA and SSA, while IgG level was low in SRA. Complement proteins (C3 and C4) were not differ in asthmatic patients in comparison with control group. The interesting results were those concerning interleukins. The levels of IL-2 and IL-4 were very high in SRA than in SSA. These are parallel with high lymphocyte and neutrophil counts in blood samples of those patients.In conclusion, beside clinical diagnostic features concerning the dose and duration of therapy with glucocorticoids, monitoring the levels of IL-2 and IL-4 could provide additional laboratory diagnostic measures for the convincing decision that asthma is steroid resistant.

Key words: steroid resistant asthma, steroid sensitive asthma, IL-2, IL-4.

الخلاصة

يزداد مستوى بعض الوسائط الالتهابية والمناعية عند مرضى الربو وقد يكون ذلك سببا لظهور بعض العلامات المرافقة أو المهيجة للمرض. وقد لوحظ أن مستوى الانترليوكينات ومن بينها interleukin-2 and interleukin-4 يزداد عند مرضى الربو وقد يُنتج مقاومة ضد العلاج بالستيرويدات عند بعض المرضى الذين اظهروا فشل الاستجابة للستيرويدات عند استخدامها بجرعها القصوى ولفترة طويلة. وفي هذا السياق فقد أجريت الدراسة الحالية لإضافة وسيلة مختبرية بالإضافة إلى التشخيص السريري للتمكن من التقرير فيما إذا كانت حالة الربو مستجيبة أو مقاومة للعلاج بالستيرويدات وذلك من خلال مراقبة مستوى الغلوبينات المناعية (imunoglobulins)، البروتين التكميلة (complement proteins) والانترليوكينات (interleukins) بين مرضى الربو (مستجيبون أو مقاومون للستيرويدات) بالإضافة لدراسة المساهمة المحتملة للعوامل الأخرى مثل العمر والجنس والعوامل البيئية في تطوير المقاومة للستيرويدات. أجريت الدراسة على 55 مريضاً بالربو و28 من الأصحاء في مستشفى الناصرية العام وللفترة من شباط 2005 إلى تشرين أول 2005. تم اخذ بيانات كاملة عن المرضى والذين كانوا قد شخصوا سريرياً كمقاومين أو مستجيبين للعلاج بالستيرويدات. وتم اخذ عينات الدم من كل الأشخاص المشاركين بالدراسة وذلك لقياس مستوى الغلوبينات المناعية (IgA, IgG, IgM, IgE)، والبروتينات التكميلية (C3 وC4)، والانترليوكينات (IL-2 و IL-4) بالإضافة لقياس عدد كريات الدم البيضاء وإحصائها التفاضلي (Differential count). أظهرت نتائج هذه الدراسةِ أن مقاومة العلاج بالستيرويدات غير معتمدة على العمر أو الجنس أو منطقة السكن (إن كانت حضرية أم قروية) ، بينما كان للتدخين (وربما للحساسية المفرطة) تأثيرا واضحا كعواملَ مهيئة لحدوث المقاومة. إن مستوى IgA وIgE كان عالياً عند كلا الصنفين من مرضى الربو (المقاومين والمستجيبين للستيرويدات)، بينما كان مستوى IgG منخفضاً عند المجموعة المقاومة للستيرويدات. وقد وجد أيضاً بان مستوى البروتينات التكميلية (C3 وC4) غير مختلف عند كلا الصنفين من المرضى بالمقارنة مع مجموعة الأشخاص الأصحاء. من النتائج المثيرة في هذه الدراسة تلك المتعلقة بالانترليوكينات (IL-2 وIL-4) والتي كانت مستوياتها عالية جداً عند المجموعة المقاومة للستيرويدات. وهذه النتائج جاءت متوازية مع العدد المرتفع لكريات الدم البيضاء اللمفاوية والمعتدلة عند أولئك المرضى. يمكن الاستنتاج من هذه الدراسة انه بالإضافة إلى التشخيص السريري فيما يتعلق بالجرعة ومدة العلاج بالستيرويدات لمرضى الربو فان مراقبة مستوى تركيز الانترليوكينات (IL-2 و IL-4) يمكن أن يضيف وسيلة مختبرية للمساعدة في تقرير فيما إذا كانت حالة الربو من النوع الذي يستجيب أو يقاوم العلاج بالستيرويدات.

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Introduction

Increased responsiveness to a variety of stimuli is a common feature involved in the one of the interesting lung diseases, asthma [1]. Whether hypersensitivity to inhaled allergens is present or not, asthma is categorized broadly into extrinsic and intrinsic types, respectively[2,3]. The incidence of extrinsic asthma occurs most frequently between the age of 3 and 45 years, although its onset may be at any age [4]. Serum immunoglobulin E (IgE) antibody level is raised in most of those patients [5]. Intrinsic asthma, on the other hand, has a primary onset before the age of 3 years, or after 35 to 45 with, however, some hypersensitivity to drugs, particularly aspirin and some other manifestations including nasal polyposis and urticaria [6]. Other category of asthma are causally related to different inducing factors including occupational, exercise-induced and aspirin-induced asthma[7,8]. The role of immunity and related inflammatory cytokines in the pathogenesis of asthma has been extensively studied. Interleukin-2 (IL-2) is a known T-cell growth factor which induces clonal expansion of T-lymphocytes [9]. Activated T-cells, B-cells and mast cells have the ability to synthesise IL-2 [10,11]. The second important T-cell growth factor is interleukin-4 (IL-4), which is synthesized by some types of T-lymphocytes, basophils, eosinophils and mast cells to function as mitogenic and lymphocyte differentiation factor[11]. These cytokines have, beside these functions, anti-tumor effects on some types of tumors[12,13]. In addition to activation of immune system during the pathogenesis of asthma, neuropeptides (substance P and neurokinine A) and nitric oxide have shown to play major roles in the initiation of cascade of responses including vasodilation, mucous secretion, plasma protein extravasation, leukocyte adhesion and activation, and bronchoconstriction that comprise the classical signs and symptoms of asthma [14]. These pathogenic mechanisms are controlled to some extent by the use of glucocorticoids (GC) [15]. However, some resistance to this mode of therapy is continuously increased and is manifested by failure to improve baseline morning (AM) pre-bronchodilator forced expiratory volume during the first second (FEV1) by greater than 15% following 7-14 days of 20mg twice daily oral prednisolone [16]. Some patients do not have an absolute resistance, but rather GC insensitivity and some might respond to higher doses of prednisolone or require longer period of therapy [17]. Patients with steroid resistant asthma (SRA) have higher level of immune activation (raised levels of T-cells and eosinophils with high level of IL-2 and IL4)[18]. This accompany by persistent respiratory symptoms, nocturnal exacerbations and chronic airway obstruction together with poor clinical and physiologic responses to oral glucocorticoids (GCs) therapy [17]. In clinical practice, setting any patient not responding to 40-60mg/day prednisolone after 3 weeks of therapy should be suspected to be SRA [16]. The clinical efficacy of GCs therapy is the result of the combinations of inhibitory effects on the process of inflammation including decreased trafficking of inflammatory cells and inhibition of inflammatory cytokines production [19]. This efficacy has shown to be altered in SRA [20]. The correlation between increased inflammatory cytokines (IL-2 and IL-4) and the development of steroidal resistance have been studied. Cytokines were reported to induce activation of transcriptional factors that interfere with GC binding to their nuclear recognition sites [21,22]. IL-2 and IL-4 were shown to promote the synthesis of altered GC binding protein (GCRβ), which reported to be a dominant negative inhibitor of the classic ligand binding protein for GC (GCRα) [23,24,25]. Factors contributing to GC insensitivity via immune activation may include allergen exposure which is reported to decrease GC receptor binding affinity and steroid responsiveness in atopic asthmatics [26] possibly by IL-2 and IL-4 dependent mechanisms[17]. On the other hand, superantigen secretion by bacterial or viral agents my contributed to poorly controlled asthma and reduce GC sensitivity. In this context, staphylococcal enterotoxin B is a potent inducer of GCRβ isoform in T-cells [27]. According to previously mentioned immunopathogenic features of steroid resistant asthma, this study was conducted to monitor the role of IL-2 and IL-4 in the immunopathogenesis of steroidal resistance asthma among Iraqi patients; and to monitor any role for immunoglobulins, complement proteins, age, sex, residence and smoking habit on the incidence of SRA.

Patients, Materials and Methods

This study was conducted on 55 asthmatic patients (25 females and 30 males) and another 28 healthy persons (18 females and 20 males) in a single-blind technique. The study was carried out in AL-Nasseriya General Hospital from February 2005 till October 2005. The age range of healthy subjects and patients was 16-73 years with average age ± SD (39.4 ± 14.67). Steroid sensitive asthmatics (SSA) were 28 (10 females and 18 males) and

SRA were 27 (12 female and 15 males). Patients' selection was based on special criteria including (a) presence of no acute infection at the time of study, (b) presence of no any other chronic infection, (c) steroidal therapy must be discontinued at least for 2 weeks and (d) presence of no systemic disease that my be associated with steroid resistance. Patients were diagnosed for steroidal resistance depending on the history of steroidal therapy and the clinical decision. Other patient's information were collected in a specially prepared sheet including: age, sex, chief complain, type and dose of steroid used, predisposing factors, associated symptoms, medical history, family history of steroid resistance, smoking habit, residence (civilian or rural), and presence of atopic allergy.

Materials:

IL-2 and IL-4 Elisa Kit (Immunotech, Marseille, France), Single Radial Immuno-diffusion Test Kit for immunoglobulins (BINDARIDTM The Binding Site Ltd., Birmingham, UK), Single Radial Immunodiffusion Test Kit for Complements (BINDARIDTM The Binding Site Ltd., Birmingham, UK), IgE Elisa Kit (Immunotech, Marseille, France).

Methods:

Blood samples were drawn, left for clotting and then centrifuged for 5-10 minutes at 2000 rpm (using Centrifuge, K24, Coold With Rotor Number 905, WIR 12x10 ML, Janetzki, Germany) for separation of serum, which was kept frozen unless analyzed immediately. Serum levels of IL-2 and IL-4 were determined using ELISA kits (Immunotech, Marseille, France), based on the interaction between monoclonal antibody bound to the wells of a microtiter plate to the IL-2 and IL-4 found in the serum. The antigen-antibody complex was detected by the addition of a chromogenic substrate, and the intensity of color was recorded colometrically (using Spectrophotometer SP6-500, Pye-Unicam, England); accordingly serum levels of IL-2 and IL-4 were calculated utilizing a standard curve prepared for this purpose [28]. Serum level of the Immunoglobulins (IgG, IgA, and IgM) and the complement proteins (C3 and C4) were determined using SRID kits (BINDARIDTM The Binding Site Ltd., Birmingham, UK). Equal volumes of reference sera and test samples were added to wells in an agarose gel containing a monospecific antiserum. The samples diffused radially through this gel and the tested compound (antigen) being assayed by forming a precipitin ring with the monospecific antiserum; rings diameters were measured (using Microwell System Reader 2305, Organon Teknika, Austria), and concentrations were determined using standard curve prepared for this purpose[29]. IgE was determined using IgE ELISA kit (Immunotech, Marseille, France)

Statistical Analysis

All results were presented as a mean ± SEM. Comparisons were made using Chi-square, Student's *t*-test and ANOVA. P values less than 0.05 were considered significant.

Results

In this study, age distribution of the volunteers enrolled in the study revealed that the incidence of SRA was varied but generally is great in the middle and older ages (Table 1).

Table (1): Distribution of patients according to their age among steroid resistant and sensitive asthmatic patients.

Age (years)	SRA (%) (n=27) (32.5%) of total (83) patients	SSA (%) (n=28) (33.73%) of total (83) patients
10-20	---	3.57
21-30	11.11	17.85
31-40	33.33	28.57
41-50	11.11	21.42
51-60	11.11	14.28
> 61	33.33	14.28

SRA = Steroid resistant asthma.
SSA = Steroid sensitive asthma

However, no significant difference (P>0.1) was noticed in the incidence of SRA among female (55.56%) and male (44.44%) with male/female ratio of 0.33 (Table 2). On the other hand, the incidence of SRA was shown to be high in those with negative family history (77.77%) in comparison to those with positive family history (22.23%) (P<0.01) as shown in table (2). In addition, steroid resistance was more pronounced in civilian (66.67%) than in rural areas (33.33%); but however the difference was not significant (P>0.1) (Table 2). It is clearly shown that smoking may comprise significant predisposing factor for steroid resistance (55.56%) when smoking SRA patients were compared to non-smoking

SRA patients (44.44%) (Table 3). Atopic allergy, on the other was more pronounced in patients with SRA (66.66%) in comparison to SRA patients with no symptoms of atopic allergy (34.34%); however, the difference was not significant, P>0.5 (Table 3).

Table (2): Sex distribution, family history and residence among SRA and SSA patients included in the study.

Asthm-atic groups	Sex		Family history		Residence	
	Male (%)	Female (%)	Positive (%)	Negative (%)	Civilian (%)	Rural (%)
SSA (n=28)	64.28	35.72	71.42	28.57	82.14	17.85
SRA (n=27)	44.45	55.55	22.22	77.78	66.67	33.33
P-values	>0.1		<0.01		>0.1	

Table (3): Smoking habit and atopic allergy among steroid resistant and steroid sensitive asthmatic patients.

Asthmatic groups	Smoking		Atopic allergy	
	Non-smokers (%)	Smokers (%)	Present (%)	Absent (%)
SSA (n=28)	82.15	17.85	71.43	28.57
SRA (n=27)	44.45	55.55	66.66	33.34
P-values	<0.05		>0.5	

SRA group of patients exhibited varying patterns of immunoglobulin levels as shown in figure (1). There is significant elevation in IgA level (3105.55 ± 225.12) and IgE level (295.17 ± 61.5) in comparison to control group (2346.11 ± 142.7 and 39.05 ± 4.5, respectively) (P<0.05). However, the levels of these immunoglobulins also were significantly high in SSA group (3815.71 ± 302.61 and 387.85 ± 52.5, respectively) (P<0.05). Moreover, only IgA levels were shown to be significantly different between SRA and SSA. The level of IgG level did not differ significantly in SRA (13615.56 ± 993.23) over that in control group (12798.33 ± 746.27) (P>0.05); but it was significantly high in SSA (15890.36 ± 892.08) (P<0.05). Lastly, the level of IgM did not differ significantly among control (1626.89 ± 139.6), SRA (1407.11 ± 187.9) and SSA (1593.82 ± 136.38) groups (P>0.05) as shown in figure (1).

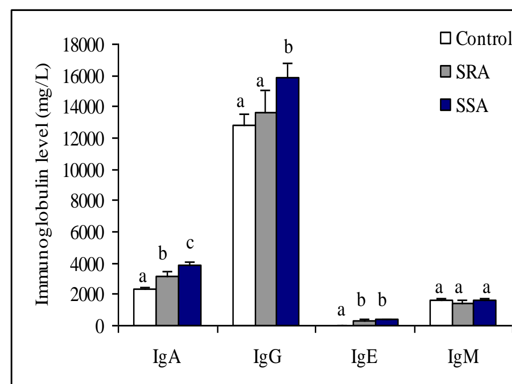

Fig. (1): Immunoglobulins level among SSA (n=28) and SRA (n=27) patients. Values are (mean ± SEM). Non-identical superscripts (a, b, c) considered significant, P<0.05 analyzed by ANOVA.

In this study also, the levels of complement protein (C3) did not differ significantly in SRA (1834.44 ± 46.48) and SSA (1666.78 ± 58.21) in comparison with control group (1699.44 ± 46.48), P>0.05 (Fig. 2). The same profile was seen in the second complement protein (C4) who its level show comparable values in SRA (398.44 ± 20.3) and SSA (389.82 ± 25.5) to that in control group (375.72 ± 23.5), P>0.05 as shown in figure (2).

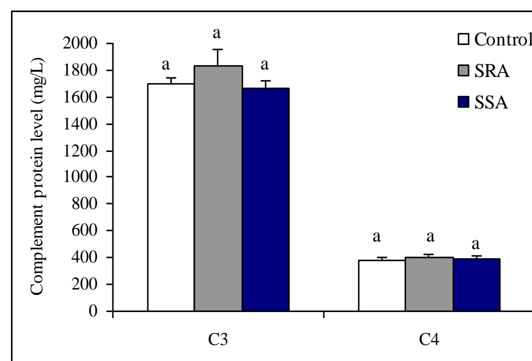

Fig. (2): Complement proteins level among SSA (n=28) and SRA (n=27) patients. Values are (mean ± SEM). No significant difference among groups, P>0.05 analyzed by ANOVA.

The interesting results in this study were those related to cytokines (IL-2 and IL-4) levels. Both cytokines were significantly high in SRA (46.11 ± 2.31) and SSA (18.28 ± 0.398) in comparison to control group (8.38 ± 0.63), P<0.05; however, its level among SRA was significantly higher than that in SSA group (P<0.05) as shown in figure (3).

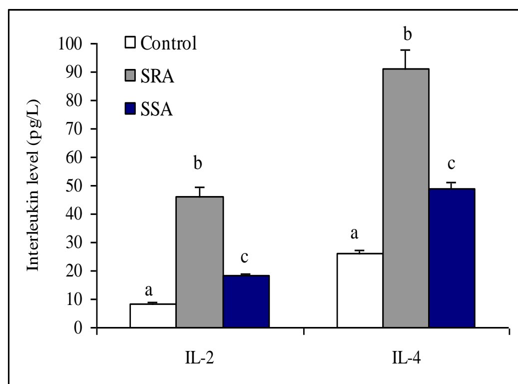

Fig. (3): Interleukins level among SSA (n=28) and SRA (n=27) patients. Values are (mean ± SEM). Non-identical superscripts (a, b, c) considered significant, P<0.05 analyzed by ANOVA.

On the other hand, the same profile was seen for IL-4 who its level was significantly high in SRA (91.33 ± 4.42) and SSA (49.03 ± 2.13) in comparison to control group (26.05 ± 11), P>0.5; with significant difference among the two groups of asthmatics, P<0.05 as shown in figure (3). Total WBC counts were significantly high in SRA and SSA in comparison to control group, P<0.05 (P<0.05) as shown in figure (4).

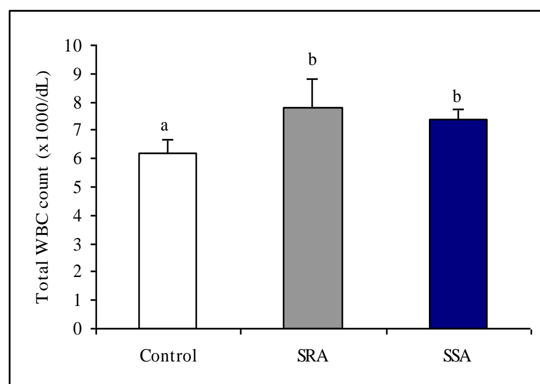

Fig. (4): Total WBC count among SSA (n=28) and SRA (n=27) patients. Values are (mean ± SEM). Non-identical superscripts (a, b) considered significant, P<0.05 analyzed by ANOVA.

Further, the count did not differ significantly among SRA and SSA groups (P>0.05). Neutrophils were significantly high in SRA and SSA groups in comparison to control group (P<0.05). However, their level did not differ significantly among SRA and SSA as shown in figure (5), P>0.05. Lymphocyte levels were low in both groups of asthmatics using steroidal therapy in companion to control group; however it was slightly lower in SSA than in SRA, but the difference was not significant (P>0.05). Differential count of other WBCs did not show any significant difference in SRA and SSA in comparison to control group (P>0.05) as shown in figure (5).

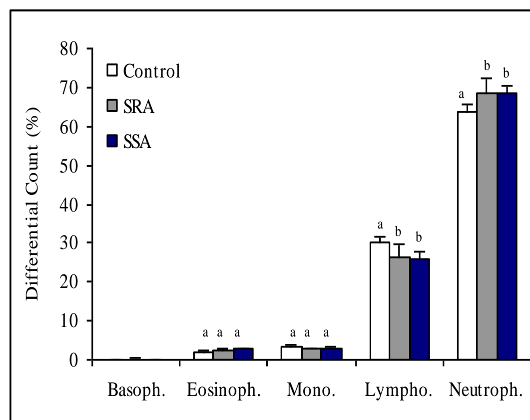

Fig. (5): Differential WBC count among SSA (n=28) and SRA (n=27) patients. Values are (mean ± SEM). Non-identical superscripts (a, b) considered significant, P<0.05 analyzed by ANOVA.

Discussion

The present study showed that the incidence of steroid resistance is increased among all patients' age rang. This is come in agreement with that reported by Ishioka and his co-workers[30]. However, resistance to steroid did not significantly affected by sex variation, but may be linked to other factors such as smoking habit and presence of atopic allergy, a finding supported by what reported by others [31,32]. Atopic allergy and immune activation is clearly suggested in this study to underlay steroid resistance and this is supported further by finding in this study that the levels of IgA and IgE were high in those patients [33]. Furthermore, the low or un increase level of IgG in SRA in comparison to SSA group suggest that the decrease in this immunoglobulin to be one factor contributing to steroid insensitivity. This has been solidified by the question; why Nathan and Erwin introduced IgG as intravenous immunoglobulin in the treatment of steroid resistance; this in turn based on the ability of IgG to decrease the levels of IL-2 and IL-4 *in vitro*, an effect thought to be involved in the potentiation of inhibitory effect of glucocorticoids on cell proliferation and cytokine secretion[34].The link between the high concentrations of IL-2 and IL-4 and the development of SRA relay on the increased resistant of lymphocytes to the action of GCs in a theory suggest altered splicing of the GCR

pre-mRNA genes induced by these cytokines [35]. The results is the generation of a second GCR, termed GCRβ, which does not bind GC but antagonizes the transactivating activity of the classic GCR [25]. Thus, increased expression of GCRβ could account for glucocorticoid insensitivity among asthmatic patients [36,37]. For this reason, the use of high dose of glucocorticoids might make down regulation to the classical glucocoricoid receptors (GCRα), leaving the inhibitory isoform (GCRβ) to be predominate, and that is why resistance occurs to GCs [23]. The levels of C3 and C4 did not differ significantly in this study among SRA and SSA in comparison with baseline. For this reason we suppose monitoring the level of these complements is without benefit to decide wither the patient has steroid sensitivity of resistance. This speculation was come in agreement with that reported by Liao and his associates [38].

The elevated levels of IL-2 and IL-4 seen in this study are correlated well with the acquired resistance to steroid therapy. Positive correlation was existed between the increased levels of IL-2 and IL-4 among SRA patients although the correlation failed to reach the level of statistical significance (data not shown). These results came in agreements with those reported by Kam and his co-workers (1993) in that the combination of IL-2 and IL-4 induced T cell resistance to GCs and increase GCRβ expression in the T cells of normal subjects [39]. In mice IL-2 alone can induce T cell resistance to GC [40]. The mechanism of such resistance involves a defect in nuclear translocation of the GC receptors. This in turn depends upon the phosphorylation of GC receptors [41]. Thus, the results obtained in this donate a possible usefulness of IL-2 and IL-4 as predictive immune markers for the development of steroid resistance and to be a possible underling cause for such resistance [23, 42]. The levels of total WBC were increased in both groups of asthmatic patients. Further, the percentage of lymphocytes and neutrophils were high in SRA group in comparison to SSA group, although the change was not significant. The high level of these leukocytes in SRA based on the theory that glucocorticoids intake could inhibit cell proliferation because of the high dose of steroid used in asthmatic patients as what happen in SSA patients [43].In conclusion, beside clinical diagnostic features concerning the dose and duration of therapy with glucocorticoids, monitoring the levels of IL-2 and IL-4 could provide additional laboratory diagnostic measures for the convincing decision that asthma is a steroid resistant.

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Factors Affecting the Formulation of Carbamazepine Extended Release Tablet

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Abstract

Carbamazepine is an anticonvulsant agent which acts on the central nervous system and used for the treatment of epilepsy. Carbamazepine was formulated as an oral extended release tablets using ethyl cellulose as retardant substance. Different types of tablets additives such as cellulose materials (sodium carboxymethyl cellulose and microcrystalline cellulose), lactose, calcium phosphate and solubilizing agents (sodium lauryl sulphate and polyethylene glycol 6000) were utilized to study their effect on the release profile of drug from ethyl cellulose matrices. It was found that sodium carboxymethyl cellulose increased the carbamazepine release and the same effect was obtained when the same amount of microcrystalline cellulose used. The result also showed that sodium lauryl sulphate greatly enhanced the release of the drug compared to polyethylene glycol 6000. Also incorporating lactose led to an increase in the release of the drug while utilization of calcium phosphate slowed down the release of the drug. The results of this study revealed that formula which composed of 4% ethyl cellulose, 5% sodium carboxymethyl cellulose, as well as 25.6% of lactose and 1% magnesium stearate is comply with United State Pharmacopea XXVIII and showed best release profile comparable to that of the brand product Tegretol CR®. The shelf life was 3.6 years for the selected formula.

Key word: Carbamazepine, Ethyl cellulose, Extended release.

الخلاصة

كاربامازبين هو عامل مضاد للاختلاج يعمل على الجهاز العصبي المركزي ويستعمل لمعالجة الصرع. لقد تم تصييغ كاربامازبيين كحبوب فموية ممتدة التحرر باستخدام اثل سليلوز كمادة مثبطة للتحرر. تم استخدام انواع مختلفة من المواد المضافة للحبوب مثل المواد السليلوزية(كاربوكسي مثل سيليلوز الصوديوم والسليلوز مجهري التبلور) ، لاكتوز ، فوسفات الكالسيوم ، والمواد المحفزة للذوبان(لوريل سلفات الصوديوم وبولي اثيلين كلايكول 6000) لتقييم تأثير هذه المواد على شكل تحرر الدواء من قوالب اثل سليلوز. لقد وجد ان الصوديوم كاربوكسي مثل سليلوز يزيد من تحرر الكاربامازبيين ونفس التأثير على التحرر قد وجد عند استخدام نفس الكمية من السليلوز مجحهري التبلور . اضهرت النتائج ايضا ان اللوريل سلفات الصوديوم حفز بشكل كبير تحرر الدواء مقارنة مع بولي اثلين كلايكول 6000 . لقد وجد ان اضافة اللاكتوز يؤدي الى زيادة اكبر من تحرر الدواء بينما استخدام فوسفات الكالسيوم قلل من تحرر الدواء. كشفت نتائج هذه الدراسة ان الصيغة المكونة من 4 % اثل سليلوز و 5% كاربوكسي مثل سليلوز الصوديوم، بالاضافة الى 2.5% لاكتوز و 1% ستيارات الصوديوم تتطابق مع دستور الأدوية الامريكي واعطت افضل شكل تحرر يمكن مقارنته مع المنتج القياسي تكريتول. ثابت التحلل للصيغة المنتجة هو 4.95×10^{-4} اسبوع^{-1} وعمر الرف 3.6 سنة.

Introduction

Extended release tablets are those which formulated in such a manner to make the contained medicament available over an extended period of time after ingestion. Expressions as ″prolonged-action″, ″repeated-action″, and ″sustained release″ have also been used to describe such dosage forms. Extended release delivery systems mostly allow at least a twofold reduction in the dosing frequency compared to the conventional immediate release formulations and increase patient compliance as well as therapeutic performance(1). Matrix systems appear to be very attractive approach from economic as well as process development and scale up points in the controlled release systems(2). Matrix tablets are classified according to the type of materials used for retarding the release of drugs (3). In case of fat **lipophilic** matrices, the drug which is incorporated into a melt of fat and waxes will be released by leaching out and/or dissolution of the carrier during passage throughout GIT. Among these lipophilic materials are carnauba wax, cetyl alcohol, hydrogenated vegetable oils, triglycerides, stearic acid, and polyethylene glycols(4). Meanwhile **hydrophilic** matrices, a dispersed drug is released as the retarding polymer swell in the gastric fluid, forming a gel barrier through which drug will be released by diffusion or dissolution of the matrix. These hydrophilic materials include cellulose Derivatives as hydroxypropyl cellulose,sodium carboxymethyl cellulose,

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hydroxylpropylmethyl cellulose, methyl cellulose as well as carbopols, sodium alginate, xanthan and guar gums[5]. In case of **plastic** matrices which they are composed of materials characterized by their capability to form insoluble, sponge-like skeletons from which the drug is released by diffusion. Examples of such materials are the acrylic/methacrylic coplymers, ethyl cellulose, polyvinyl acetate, and polyvinyl alcohol [6]. Ethylcellulose is usually combined with water-soluble additives to impart some hydrophilic nature for films or matrices, altering its structure by virtue of pores and channels through which drug can diffuse more easily[7]. In the present study, ethyl cellulose was used as retardant substance to formulate carbamazepine matrix tablet dosage form. Extended release formulation of CBZ should be considered in patients receiving high doses of CBZ and who are suffer from intermittent adverse effects such as diplopia, nausea, dizziness, and drowsiness, offering the opportunity for converting the three or four times daily regimen to twice, or even once daily administration[8].

Materials and Methods

Materials:

Carbamazepine and microcrystalline cellulose (Avicel PH 101) kindly supplied by Samara Dug Industry (SDI). Ethylcellulose, magnesium stearate, , sodium carboxymethylcellulose and sodium lauryl sulphate from(BDH, England). Lactose from (Riedel-DeHaen, Germany). All other chemicals and solvents were of analytical grade.

Methods:

Formulation of Carbamazepine as Extended Release Tablet:

Formulas 1-9 shown in table (1) were prepared by mixing CBZ with lactose for 10 minutes, then the granulating solution (5% ethyl cellulose in absolute ethanol) was added gradually until a wet ball mass was obtained. The resultant mass was screened through 12-mesh sieve and the resultant granules were dried at 50°C for 2 hours. A second screening through 18-mesh sieve was done, followed by mixing the granules with magnesium stearate as lubricant for 2 minutes, then the resultant granules were compressed into tablets using double punch tablet machine (Korsch EKO, Germany).

Table 1: Formulation of CBZ tablet using ethyl cellulose with different additives.

Substance (mg)	Formula No								
	F1	F 2	F 3	F 4	F 5	F 6	F 7	F 8	F 9
Carbamazepine	200	200	200	200	200	200	200	200	200
Ethyl cellulose	8.61	11.6	14.7	12.08	12.08	12.08	12.08	12.08	12.08
Sodium carboxyme-thyl cellulose				15.25				15.25	15.25
Microcrystalline cellulose					15.25				
Sodium lauryl sulphate						4.43			
PEG6000							4.43		
Calcium phosphate									75
Lactose	75	75	75	75	75	75	75	95	
Magnesium stearate	3	3	3	3	3	3	3	3	3
Total Weight of Tablet	286.6	289.6	292.7	286.6	289.6	292.7	286.6	289.6	292.7

Evaluation of Carbamazepine Extended Release Tablet :-

Hardness Test

The hardness of 3 tablets from each of the prepared formulas was measured individually by using Pharma Test equipment. An anvil driven by electric motor presses the tablet at a horizontal position and constant load until the tablet breaks [9].

Friability Test

This test was done for 20 tablets, starting by weighing them and then operating the friabilator at 25 r.p.m for 4 minutes, re-weighing the tablets to determine the loss in their weight[9].

Uniformity of Dosage Units

Five tablets were individually subjected to the following procedure. One tablet was finely powdered and quantitatively transfered, with the aid of methanol, to 100-ml volumetric flask. About 70 ml of methanol was added, shaked by mechanical means for 60 minutes. The mixture was sonicated for 15 minutes, diluted with methanol to volume, allowed to stand for 10-15 minutes, and then the clear supernatant was analyzed spectrophotometry to determine the amount of CBZ , using methanol as a blank[1].

Drug Release

This test was done for all formulas according to the USP XXVIII specifications stated in the monograph “Carbamazepine Extended-Release Tablets”. The test carried out as triplicate for each formula using apparatus I (basket) at 100 r.p.m and 900 ml water as dissolution medium at 37±0.5°C under sink conditions. At each time interval 5 ml sample was withdrawn, filtered and suitably diluted to be the absorbance within the calibration curve level. The amount of CBZ dissolved at one hour intervals was measured spectrophotometrically at maximum absorbance wavelength, 285 nm (Cecil, England). The withdrawn samples were replaced with water. The percentages of CBZ released at the specified times must conform to the following:

Time (hr.)	Amount released (%)
3	10-35
6	35-65
12	65-90
24	Not less than 75

Determination of the Release Kinetics

To study the mechanism of drug release from the matrix tablets, the release data were fitted to zero-order, first order, and Higuchi equations. Furthermore, to characterize the release behavior, i.e. to understand the mechanism, Korsmeyer-Peppas model (equation 7) was applied:

$$Q_t / Q_\infty = kt^n$$

Where, Qt is the amount of drug release at time t ; Q∞ is the amount of drug release after infinite time; k is a release rate constant incorporating structural and geometric characteristics of the tablet; and n is the diffusional exponent indicative of the mechanism of drug release [10].

Effect of Temperature:

The effect of temperature on the degradation of CBZ in the selected formula was studied according to accelerated stability study. The study was done by storing the tablets in ovens at different temperatures of 40, 50, and 60ºC for four months. Samples were withdrawn at weekly intervals to determine the total content of CBZ by measuring UV absorbance at λ max at 285nm.

Statistical Analysis

The results of the experiments are given as a mean of triplicate samples ± standard deviation and were analyzed according to the one way analysis of variance (ANOVA) at the level of ($P < 0.05$).

Results and Discussion

Evaluation of Carbamazepine Extended Release Tablet :-

Hardness of Tablets

The hardness of prepared tablets which is shown in table (2) revealed variation which may be attributed to the differences in amount of retarding polymer, in addition to the other exciepient added. For formulas 1-3, the hardness increased as the amount of retarding polymer increased, this result may be attributed to the increase in the compressibility of the matrix resulting from the higher polymer proportion [11]. Formula 6 and 7 showed lower hardness values in comparison with other formulas and this is originated from the extremely poor compatibility of the surfactant or PEG containing granules, which may assume a wax-like physical property [12]. For other formulas in which several types of additives were included, they have higher hardness values depending on the nature of these additives. The inclusion of sodium carboxymethyl cellulose and microcrystalline cellulose may result in the consolidation of granules due to the plasticity of these materials and increase intraparticulate bonding during

compaction in addition to the homogenous distribution of bonds in the compact [13].

Table 2: Hardness of Tablets (expressed as mean ± SD).

Formula No.	Hardness (Kp)
F 1	11.2±0.65
F 2	12.3±0.36
F 3	12.8±0.14
F 4	13.0±0.24
F 5	11.9±0.07
F 6	11.5±0.20
F 7	11.4±0.33
F 8	12.2±0.12
F 9	13.5±0.06

Friability of Tablets

All formulas have lost not more than 1% of their weights. The incorporation of sodium carboxy methylcellulose and microcrystalline cellulose to the formulations resulted in improved skeleton integrity and acceptable friability as seen in table(3)[13].

Table 3: Friability of Tablets.

Formula No.	Friability (%)
F 1	1.215
F 2	0.856
F 3	0.835
F 4	1.334
F 5	0.848
F 6	0.822
F 7	0.779
F 8	0.521
F 9	0469

Uniformity of Dosage Units

Table (4) shows that all the prepared formulas which were subjected to this test complied with USP specification which is 85-115% of CBZ content in each individual tablet[1].

Table 4: Uniformity of dosage units for CBZ tablet (expressed as mean± standard) deviation.

Formula No.	Content of CBZ (%)
F 1	94.2±2.5
F 2	98.5±3.6
F 3	98.1±3.3
F 4	96.3±2.3
F 5	95.7±3.1
F 6	96.2±4.0
F 7	93.7±2.5
F 8	94.1±3.4
F 9	95.0±4.7

Variables Affecting CBZ Release From Extended Release Tablets :-

The Effect of Retarding Polymer Concentration

The release of CBZ from formulas 1-3 which they are formulated using concentrations 3%, 4% and 5% of ethyl cellulose is shown in figures (1). It appears that there is a significant difference (P < 0.05) in the release of CBZ from matrices of ethyl cellulose (F 1-3) when the polymer concentration was changed. These results indicated that increasing the concentration of polymer tends to decrease the drug release since the amount of CBZ released was decreased from 79% to 13% for when the concentration of polymer was increased from 3% to 5% in the matrices.

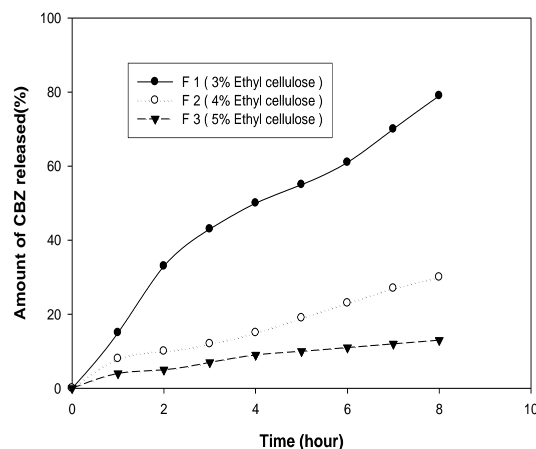

Figure 1: The effect of ethyl cellulose concentration on the release of CBZ.

This difference can be attributed to the decrease in the porosity with a concomitant increase in the tortuosity of matrix [14]. At the concentration of 4% and 5%, ethyl cellulose exhibits an extremely prolonged release as only 30% and 13% of CBZ was released after 8 hours from these matrices respectively. These findings may be due to the limited

solubility of CBZ and ethyl cellulose in water, therefore it is difficult for dissolution medium to penetrate the matrix [15]. The same effect was produced by ethyl cellulose matrices on the release of caffeine and pseudoephedrine hydrochloride [16].

The Effect of Cellulose Polymers Addition

Since ethyl cellulose is a hydrophobic insoluble polymer, two different hydrophilic, cellulose materials [the water-soluble sodium carboxy methyl cellulose and the water-insoluble microcrystalline cellulose (Avicel PH 101)] were incorporated (F 4 and F 5). Figure (2) shows that the time courses of release from formulas 4 and 5 which contain these additives are not significantly different ($P < 0.05$) although both materials enhance the release of CBZ. At first, CBZ release from formula 4 was enhanced due to the high solubility of sodium carboxymethyl cellulose; however, a relative slower release is followed when the glassy nature of this swellable polymer was changed to the rubbery state upon the contact of matrix tablet with water [17]. Recent developments in the analytical techniques as Raman/IR spectroscopy and scanning electron microscopy had revealed that such hydrophilic polymers are adsorbed on CBZ compacts through hydrogen bonding [18]. Such results are consistent with those obtained for the release of propranolol hydrochloride from matrix tablets [19]. Avicel PH 101 increased the release of CBZ from formula 5 which has a MDT comparable to that of formula 4 as shown in figure (2). Although it is water insoluble, Avicel PH 101 can absorb water to some extent; thus it acts as a pore-forming agent, enhancing the permeation of dissolution medium through the stress relaxation of the polymeric matrix, resulting in a rapid release of the drug [20].

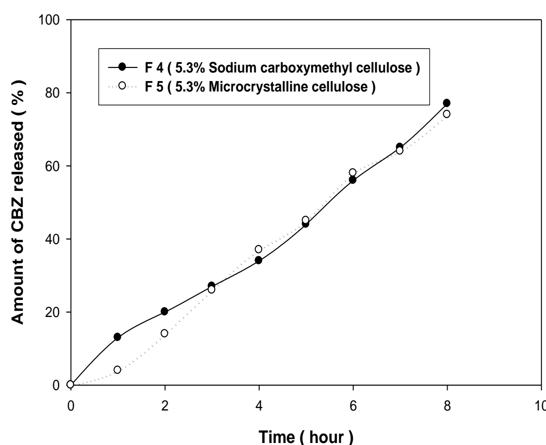

Figure 2: The effect of cellulose derivatives addition on the release of CBZ from ethyl cellulose matrix

The Effect of Adding Solublizing Agents

Since the absorption of insoluble drugs from the GIT is controlled via their dissolution, then utilization of solublizing agents in their formulation could improve the oral bioavailability of such drugs [21]. The release of CBZ from formulas 6 and 7 in which sodium lauryl sulphate and polyethylene glycol 6000 were included respectively as solublizing agents is shown in figure (3). Both agents increase the release rate of CBZ, however; a significant difference ($P < 0.05$) in the release rate between the two surfactants was observed due to the higher solublizing activity of sodium lauryl sulphate. This is may be attributed to the higher hydrophilic-lipophilic balance (HLB) value for sodium lauryl sulphate compared with polyethylene glycol 6000[22]. Moreover, cationic drugs dissolve better in anionic surfactants depending on the degree of dissociation, due to ionic interaction [23].

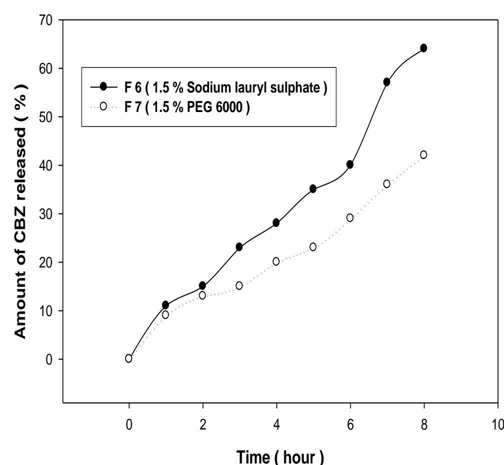

Figure 3: Effect of solubilizing agents on the release of CBZ from ethyl cellulose matrix.

The Effect of Diluent Quantity and its Type

Diluents or bulking agents are frequently added to the formulations in order to give the tablets their appropriate size. Although diluents are usually thought to be inert ingredients, they can significantly change the physical or biopharmaceutical properties of dosage forms [24]. Increasing the quantity of lactose (which is one of the mostly used diluents in tablets formulation) to 95 mg in formula 20 compared to 75 mg in formula 4 resulted in a significant increase ($P < 0.05$) in the release of CBZ as shown in figure (4) and this may be due to the high solubility of lactose so that it will act as channeling agent, permitting a rapid ingress of dissolution medium into the matrix tablets, thus facilitating drug release [25]. On the other hand,

when calcium phosphate was incorporated in formula 9, a significant reduction ($P < 0.05$) in the release rate of CBZ was observed compared to the release rate from formula 4 in which lactose was included. The slower release rate of CBZ which is the direct result for the presence of an insoluble additive in the matrix, therefore slowing down the drug diffusion and/or the medium infiltration[26]. Sodium sulphadiazine, Ketoprofen, and theophylline showed the same observations when formulated as matrix tablets with HPMC [25].

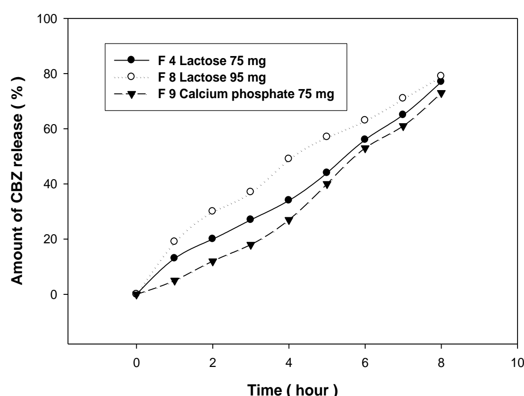

Figure 4: The effect of diluents addition on the release of CBZ from ethyl cellulose.

Determination of the Release Kinetics

Table (5) shows that n values of formulas 1-3, equals to 0.725-0.931, pointing to an anomalous (non-Fickian) diffusion mechanism with a trend toward higher values as the polymer content increased. On the other hand, higher values of kkp was determined for formula 1 with the least content of retarding polymers, suggesting the possibility of occurrence of burst effect. Formula 2 and 3 show a better fitness to zero-order and first-order respectively. It can be considered that decrease of CBZ release through increased polymer content produces a change in the release mechanism moving away from diffusion as n values becomes closer to 1.0, which also confirms that the kinetics go in the direction of zero-order release[27]. Incorporation of sodium carboxymethyl cellulose into ethyl cellulose matrices (formula 4) produced n value of 0.656 with a remarkable fitness to first-order kinetics. Meanwhile, microcrystalline cellulose in formula 5 gave an approximately unity value for n, indicating zero-order release. This variation may reflect the different behavior of each polymer since the sodium carboxymethyl cellulose will form a viscous gel through which diffusion occurs [28], and the microcrystalline cellulose has a disintegrating properties so that resulting matrix erosion [29]. Although n values for formulas 6 and 7 had revealed an anomalous diffusion, sodium laurylsulphate gave a higher kkp value indicating fast initial release with first-order kinetics due to its powerful solubilizing effect compared to polyethylene glycol 6000 which have low solubilizing ability and moderate release restriction properties [30]. In addition, increasing the quantity of lactose in formula 8 produced a lower n value with an elevated kkp value due to the high solubility of this material, thus stimulating water penetration into the matrix [24]. In contrast, calcium phosphate in formula 9 gave matrices in which CBZ release is controlled by erosion because this diluent is insoluble in water [26].

Table 5: Fitting results of formulas 1-9 for CBZ release data to different kinetic model.

Formula No.	Model								
	Zero-order		First-order		Higuchi		Korsmeyer-Peppas		
	K_0 (%h^{-1})	R^2	K_1 (h^{-1})	R^2	K_H (h$^{-1/2}$)	R^2	K_{KP} (h^{-n})	n	R^2
F1	9.229	0.9557	0.2575	0.8348	32.638	0.9869	16.748	0.788	0.9677
F2	3.646	0.9893	0.1792	0.9865	14.734	0.9378	6.437	0.725	0.9467
F3	3	0.9707	0.2303	0.9889	12.368	0.9034	2.989	0.931	0.9463
F4	9.25	0.9773	0.273	0.9902	33.247	0.9247	4.109	0.656	0.981
F5	9.21	0.9764	0.319	0.9793	33.505	0.9239	8.167	1.008	0.982
F6	11.6	0.9885	0.281	0.9931	37.04	0.9584	20.811	0.693	0.9755
F7	6.055	0.9915	0.212	0.9691	25.293	0.9474	7.466	0.876	0.9711
F8	8.943	0.9915	0.234	0.9468	31.096	0.9897	18.703	0.678	0.9948
F9	8.988	0.9804	0.346	0.9429	41.84	0.9278	4.855	1.238	0.996

Selection of the Best Formula

Although several prepared formulas met the release specifications of USP, formula 4 showed a release profile comparable to that of the brand product Tegretol CR® . The similarity factor (f 2) introduced by Moore and Flanner is used as criterion for assessment of the similarity between two dissolution profiles [31]:

$$f_2 = 50 \times \log\left\{\left[1+\frac{1}{n}\sum_{t=1}^{n}\left|R_t - T_t\right|^2\right]^{-0.5} \times 100\right\} \quad \text{..........(1)}$$

Where n is the number of dissolution time points, Rt and Tt are the reference and test dissolution values at time t. For the conventional tablets, a difference not exceeding 10% at any sampling time point between reference and test products may be acceptable and f 2 value of 50-100 indicates similarity in the dissolution profiles, while for sustained release tablets, the lower limit of 50 is very liberal especially for drugs with narrow therapeutic index. Therefore, the generalized equation to estimate the lower acceptable value of f 2 (f 2LX) is:

$$f_{2LX} = 50 \times \log\left\{\left[1+\frac{1}{n}\sum_{t=1}^{n}\left|R_t - \left(R_t \pm \frac{X}{100}R_t\right)\right|^2\right]^{-0.5} \times 100\right\} \quad \text{........(2)}$$

Where X is the percent deviation for the reference product [32]. If 10% deviation is allowed for the dissolution profiles to be similar, then the calculated f 2LX value using equation (2) will be 61.18 for Tegretol CR®. For the prepared formulas, the highest calculated f 2 value according to equation (1) is 85.22 for formula 4. Figure (5) shows the non significant difference ($P < 0.05$) in the dissolution profiles of CBZ from Tegretol CR-® and formula 4.

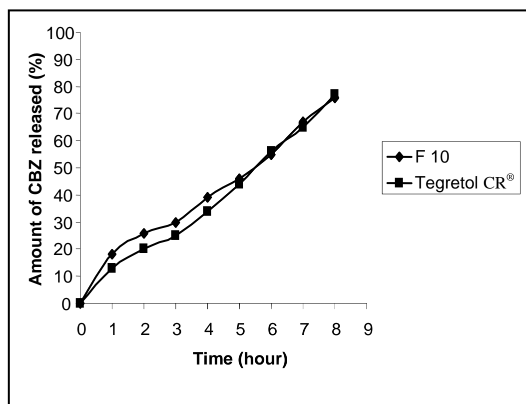

Figure 5: The release of CBZ from formula 10 compared to Tegretol CR® in water.

Stability Study: Accelerated Temperature Effect

The stability of the selected formula 4 was studied at three different temperatures ; 40ºC , 50ºC , and 60ºC for 16 weeks.The degradation of CBZ followed first-order kinetics because straight lines were obtained when logarithm of percent remaining of the drug was plotted versus time[33]. Figure (6) shows the degradation curves of CBZ at 40ºC, 50ºC and 60ºC, from which the degradation rate constant (K) at each temperature was determined from the slope of each line. The values of rate constants are summarized in table (6). The date of expiration for CBZ was determined through constructing Arrhenius plot as shown in figure (7) in order to estimate the degradation rate constant (K25) at 25ºC which was equal to 5.623 × 10-4 week-1 The following equation is used for calculating the expiration date [34]:

$$t_{10\%} = \frac{0.105}{K_{25}}$$

where t10% is the time required for a drug to lose 10% of its potency and it was found to be 185 weeks (about 3.6 years for CBZ).

Table 6: Degradation Rate Constants for CBZ in Formula 10 at 40ºC, 50ºC and 60ºC.

Temperature (ºC)	K (week^{-1})
40	1.8×10^{-3}
50	3.7×10^{-3}
60	5.9×10^{-3}

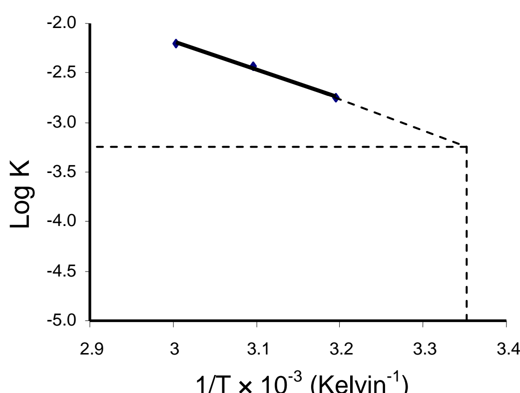

Figure 6: Accelerated degradation of CBZ in formula 10 at 40ºC, 50ºC and 60ºC.

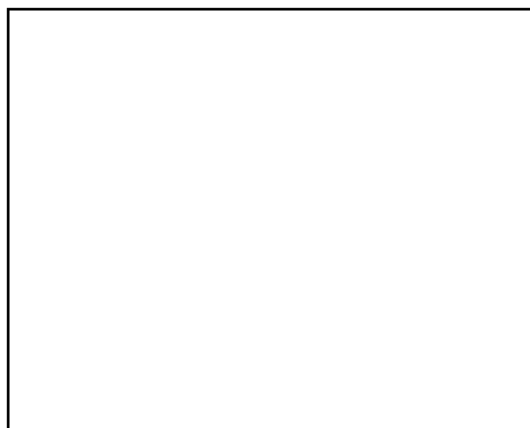

Figure 7: Arrhenius plot of CBZ in formula 10 for the estimation of the expiration date.

Conclusion

Carbamazepine extended release tablet has been successfully fabricated by using ethyl cellulose as retardant substance. In vitro release of carbamazepine was correlated with types and concentrations of the additives used in the formulation.

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Preparation and Characterization of Biodegradable Microspheres Containing Sertraline Hydrochloride

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Abstract

Four batches of sertraline HCl microspheres were prepared using a poly (D-L-lactide-co-glycolide) (PLGA) polymer (Mw. 9, 27, 30 and 83 KDa) as a delivery system. The microspheres were prepared by a dispersion/solvent extraction-evaporation method and characterized for drug loading by UV, particle size by laser diffractometry and surface morphology by scanning electron microscopy (SEM). The in vitro sertraline HCl release was studied. Spherical microspheres with a mean diameter of 21 to 26 µm loaded with 24.6 – 38.2% were produced. The in vitro drug release was shown to be depend on polymer molecular weight and also on the drug loading. Differential scanning calorimetry (DSC) was employed to investigate the physical state of sertraline HCl inside the microspheres and stability and polymer interaction study were performed in solution.
Key words: Sertraline HCl , Microspheres , PLGA polymer

الخلاصة

اربعة صيغ من الكرات المجهرية للسيرترالين هايدروكلورايد (Sertraline HCl) حضرت بأستخدام بوليمر متعدد احامض اللبن والكلايكولك(PLGA) (الوزن الجزيئي 9 , 27 , 30 , 83 كيلو دالتون) كنظام لايصال الدواء. الكرات المجهرية حضرت بطريقة الانتشار/ استخلاص المذيب- تبخير , ووصفت من حيث : كمية التحميل للدواء بواسطة الاشعة فوق البنفسجية , حجم الكرات بواسطة جهاز مشتت الليزر(Laser diffractometry) , والشكل السطحي بواسطة المسح بالمجهر الالكتروني (SEM) . تم دراسة التحرر للسيرترالين هايدروكلورايد خارج الجسم. كرات مجهرية ذو معدل قطر 21-26 µm محملة بنسبة 24.6-38.2% قد انتجت. التحرر خارج الجسم للدواء قد اظهر انه يعتمد على الوزن الجزيئي للبوليمر وكذلك على كمية تحميل الدواء. تحليل المسح التفريقي للسعرات(DSC) قد استخدم للتقصي عن الحالة القيزياوية للسيرترالين هايدروكلورايد داخل الكرات المجهرية , كذلك تم دراسة الاستقرارية وتفاعل البوليمر داخل المحلول.

Introduction

Sertraline HCl is the second most potent inhibitor of serotonin reuptake and the second most selective blocker of serotonin over noradrenaline uptake. It has been approved in 1997 in France, and is currently widely prescribed in Europe and the United states[1]. It has been also used for the treatment of depression, obsessive-compulsive disorder (OCD), depression relapse and social phobia[2,3]. It is the only selective serotonin reuptake inhibitor (SSRI) that binds to dopamine transporters[4]. Sertraline HCl exhibits linear pharmacokinetics[5]. After single doses between 50 and 200 mg, t1/2 is similar for single dose and steady-state conditions[6]. The elimination rate constant is higher in young males than in females or subjects 65 years old or older[7]. The hepatic metabolism is the most important pathway, with only 0.2% of an oral dose being excreted unchanged in the urine[8]. The N-demethylation is the main metabolic step in the biotransformation of sertraline[9]. Drug absorption from the GIT is slow, but complete with maximum plasma concentrations (Cmax) attained within 6-8 hr and compared to other SSRIs, a relevant portion of oral sertraline is excreted in the feces (~50%) [10]. Increasing evidence from randomized controlled trials of SSRIS show their efficacy in treating pediatric depression. The number of prescriptions for sertraline HCl use in pediatric populations has exploded recently with figures ranging from 600,000 children and adolescents[11 - 13].

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The oral administration of this drug to children is hard to control compared to adults. In addition, to frequent administration, chances of drug misuse following oral administration are high. This necessitates administration of the drug via a different route. Therefore, there is a strong need for a non-oral controlled delivery dosage form for this drug. This paper investigates the feasibility of formulating sertraline HCl into biodegradable microspheres using PLGA polymer to be used as injectable dosage form. In addition, drug stability and drug-polymer interactions were studied. Finally, in vitro release efficacy from the formulations was also assessed.

Experimental

Materials

Poly(D,L-Lactic-co-glycolic acid) (PLGA); Resomer® 502H (9 kD), 503 H (27 kD), 503 (30 kD), PLGA 50:50 and 6535DL (83 kD) PLGA 65:35 ;were supplied by Boehringer Ingelheim (Ingelheim, Germany). Polyvinyl alcohol (M.wt. 30000-70000; PVA) and sertraline HCl were supplied by Sigma (St. Louis, MO, USA). All other chemicals were obtained commercially as analytical grade reagents.

Preparation of Microspheres

Four batches of sertraline HCl microspheres (MS) were formulated with polymers at increasing molecular weight. The microspheres were prepared by dispersing the homogenous suspension of polymer and drug into a 0.35% PVA solution continuous phase followed by solvent extraction / evaporation /dilution as already described(14). In detail sertraline HCl was sieved through a 150 mesh sieve and amount corresponding to 25, 40 and 45% loading of the sieved powder was dispersed in methylene chloride and properly sonicated. The polymer in the proportion of 12% was then added to the resulting suspension and after its complete dissolution the suspension was slowly injected into phosphate buffer saline (pH 8.9) containing 0.35% poly vinyl alcohol (PVA) and mixed at 900 rpm. at 4°C. The microspheres hardening and complete evaporation of the solvent were accomplished increasing slowly the temperature up to 20°C in on hour. At the end of the process the microspheres have been recovered by filtration through a 0.65µm harvesting filter and freeze-dried overnight .Table 1 outlines the preparation parameters for sertraline HCl microspheres. The s/o/w method is represented in figure 1.

Table 1 - Preparation parameters and particle size of Sertraline HCl MS.

Polymer	M.W. (kDa)	Method	pH of CP	Target Load % w/w	Drug Content % w/w	Encapsulation Efficiency (%)	Particle size (µ)
503H	27	s/o/w	8.8	45	38.2	84.9	19.6
502H	9	s/o/w	8.8	25	24.6	98.4	21.0
503	30	s/o/w	8.8	40	34.3	85.8	26.0
6535DL	83	s/o/w	8.8	40	34.5	86.3	24.0

Particle Characterization:-

Particle Size Distribution

The prepared particles were sized by laser diffractometry using a Malvern 2600 laser sizer (Malvern 2600 particle sizer, Malvern, UK). The average particle size was expressed as the volume mean diameter Vmd in microns (µ).

Surface Morphology

Surface morphology was examined by scanning electron microscopy (SEM) (Hitachi Model S800 Japan) after palladium/gold coating of the microspheres sample on an aluminum stub.

Drug Content

10 mg of microspheres were dissolved in dimethyl sulfoxide (DMSO). The Sertraline HCl was extracted, since polymer and drug were soluble in DMSO. In detail, triplicate samples of 10 mg of the microspheres were quantitatively transferred to 12 ml glass test tube. The microspheres was solubilized in 2 ml of DMSO, then 10 ml of 0.1M acetate buffer pH 5 was added and the tubes were agitated by

a wrist action shaker for 1 hr. The sample were centrifuged at 3000 rpm and the aqueous layer was analyzed spectrophotomery at 273 nm. Absorbance measurements were made at a selected wavelength (λ max = 273 nm).Absorbance measured values were fitted against a calibration curve based on a Lambert–Beer law[15].

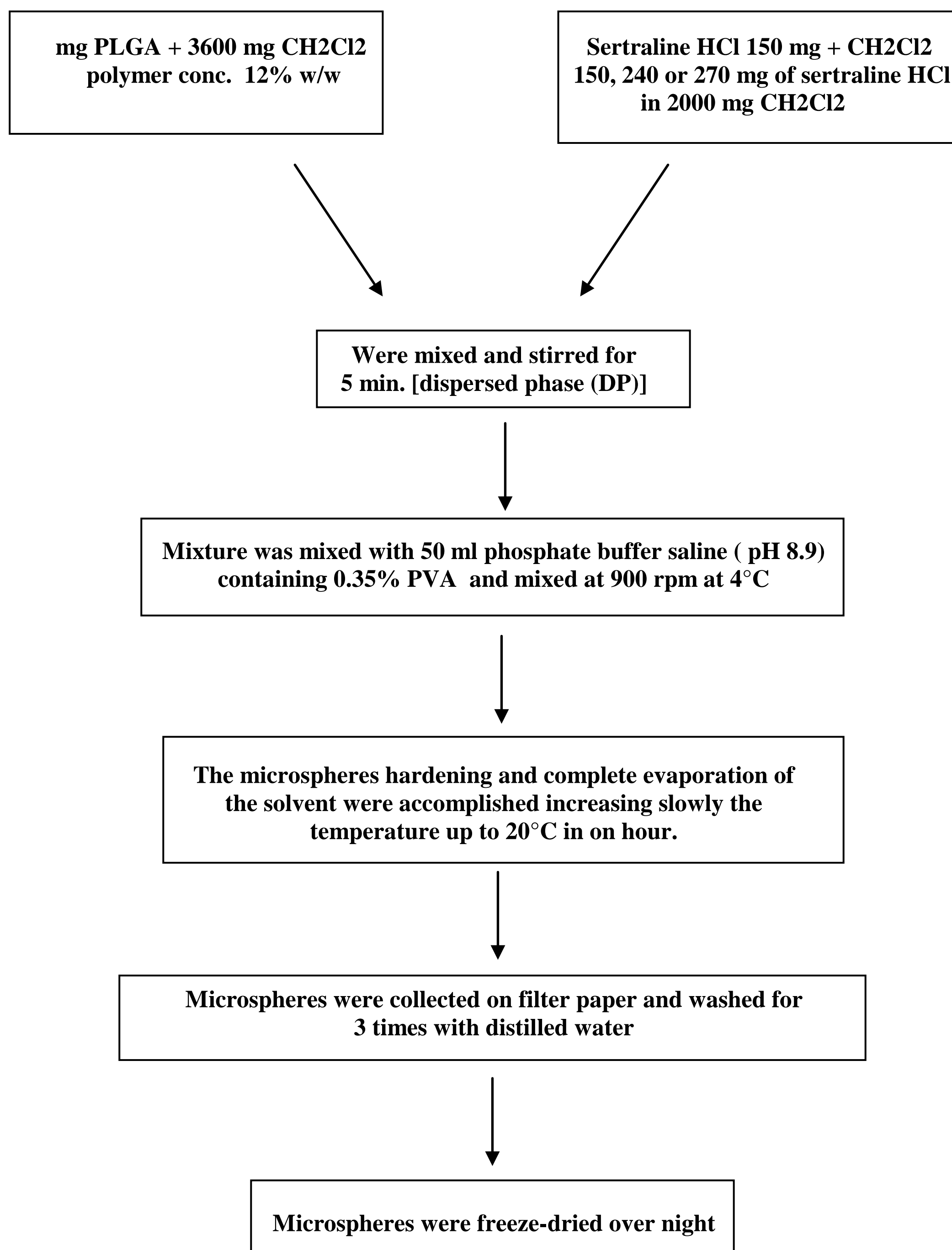

Figure 1: Preparation of PLGA Sertraline hydrochloride microspheres using dispersion/solvent extraction-evaporation method.

DSC Analysis

Sertraline HCl thermotropic behavior inside the microspheres was investigated by a DSC 2920, DE differential scanning calorimeter. Samples of sertraline loaded microspheres and blank microspheres were scanned at 5 °C/min heating rate in the range –10°C to 300°C. In addition, DSC scans were run on the drug, polymers and physical mixtures of the drug with polymers used in the preparation of microspheres. Further measurements were carried out on drug powder after suspension and sonication for 20 seconds in dichloromethane, then evaporation of dichloromethane and on the drug, post-sieving, to detect any structural modification due to the preparation process. All the samples were freeze-dried dried over night before the analysis(16).

Drug-polymer Interaction

Drug-polymer interaction studies were carried out in solutions containing lactic acid, glycolic acid and with mixtures of lactic acid and glycolic acid (50:50 LA : GA) at 37°C. Sampling was performed periodically (5, 10, 20, 30, 40 and 50 days) followed by UV analysis at 273 nm. All analysis were performed in duplicate(17).

In Vitro Drug Release Study

Long-term (48 days) in vitro drug release was carried out in 0.1M acetate buffer, pH 5 at 37 °C. The pH of this buffer is close to that of an acidic microenvironment that form within the PLGA matrix(18). Briefly, 10 mg of microspheres were suspended in 10 mL of the buffer. At each time point(1, 3 , 8, 10, 15, 20, 27, , 34, 41, and 48 days) 1 mL of supernatant was withdrawn from each tube after centrifugation (2min, 3000 rpm) and an equivalent volume of fresh buffer was then added to replace the amount collected. Analyses were carried out using UV spectrophotometry at 273 nm on triplicate or duplicate samples.

Results and Discussion

Preparation of Microspheres

Preparation of sertraline HCl loaded microspheres was accomplished by the s/o/w method already described in the experimental section. The reason for choosing such procedure is the low solubility shown by the drug into most of the solvents commonly used in microsphere formulation. Various preparation conditions and materials were investigated in order to obtain the best results concerning loading and drug release. Microspheres morphology and size distribution and in vitro release behavior to test the feasibility of sertraline formulation. The results shown in Table 1 reveal the remarkable encapsulation efficiencies (84.9% - 98.4%). A critical step at this point was the complete drug dispersion that is fundamental to have a uniform distribution of the drug inside the microspheres and higher encapsulation efficiency. PLGA polymers were employed with increasing molecular weight (9-83 kDa.) and different glycolic/lactic ratio (50:50 and 35:65) in order to investigate the effect of these parameters on the release behavior of such formulations. The best batches resulted PLGA based preparations and especially microspheres with PLGA Resomer 503H and 502H polymers showed the best results in term of encapsulation efficiency and drug content.

Microspheres Characterization

SEM analysis on sertraline HCl microspheres showed that the microspheres were successfully fabricated with a spherical shape, a certain fragility and relatively low porosity (figure 2). The average particle size was approximately 22µm which is suitable for intramuscular or subcutaneous injections(19).

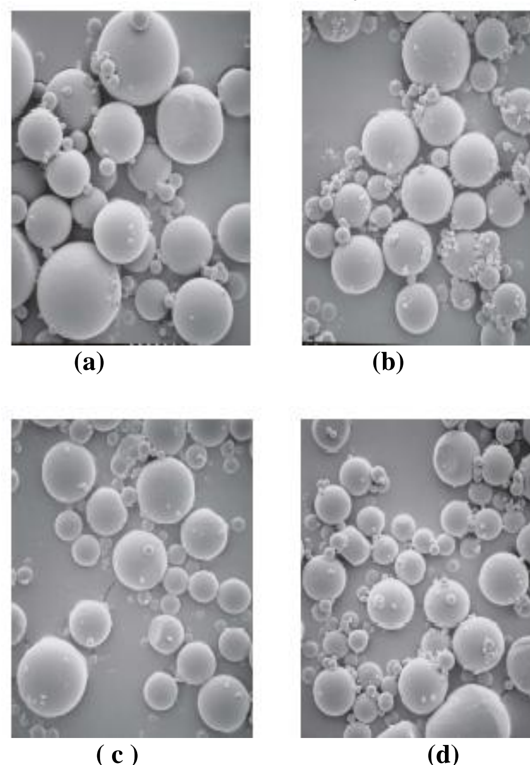

Figure 2: SEMs of Sertraline hydrochloride loaded microspheres.
503 (a), 502H (b), 6535DL (c) , 503H (d)

Drug Content and Encapsulation Efficiency

Dispersion /solvent extraction-evaporation method has been used succefully in the incorporation of hydrophobic drugs with good yield value loading percentage(20). Loading efficiencies ranged from 84.9-98.4 as illustrated in table 1. Yield value are function

of the efficiency of preparation method and values up to 70% were accepted[21]. Sertraline HCl , being a water insoluble molecule is better dispersed in organic solvent then emulsified in aqueous solution of the surfactant where minimum amount of the drug would be in the aqueous continous phase[22]. The loading efficiency of 502H microspheres was the highest among other batches(table 1). This result may be due to its lowest target load (25%), since a higher target load of bioactive material is likely to decrease the entrapment efficiency of drug in PLGA[23-25]. The drug content ranged from 24.6-38.2 % .

Drug-polymer Interaction

There was no detectable decrease in sertraline HCl concentration in 0.1M acetate buffer, pH 5.0 for the entire duration of study (50 days) at temperatures 37 °C. There is no significant change in drug levels when incubated with solutions of lactic acid, glycolic acid and a 50:50 mixture corresponding to the molar amount that would be obtained on complete hydrolysis of the PLGA polymers , 502H and 503H.

The DSC Analysis

The DSC analysis confirmed a high drug-polymer affinity. The comparison of thermal profiles of drug, polymer, physical mixture and drug loaded microspheres revealed that the drug was present as a dispersion in the polymeric matrix for all the microsphere batches as demonstrated by the lack of sertraline HCl melting peaks **(Figure 3 a-d)**. Differences in glass transition temperature (Tg) between drug loaded microspheres and raw polymer suggest that the drug has a plasticizing effect on the internal structure of the polymer[26]. The drop in the Tg was greater for microspheres prepared from high molecular weight polymers. Tg values of all the systems studied are shown in Table 2.

Table 2 : The Tg of the Sertraline bpowder, microsphere and the physical mixture of sertralin with polymer.

RG503H	**Tg (°C)**	**RG502H**	**Tg (°C)**
Sertraline-503H Phys. mix.	43.09	Sertraline-502H phys. mix.	34.90
Pure polymer	45.60	Pure polymer	33.62
503H MS	37.79	502H MS	32.27
RG503	**Tg (°C)**	**6535DL**	**Tg (°C)**
Sertraline-503 phys. mix.	46.25	Sertraline-6535 DL phys. Mix.	43.01
Pure polymer	47.05	Pure polymer	46.25
503 MS	30.15	6535 DL MS	33.80

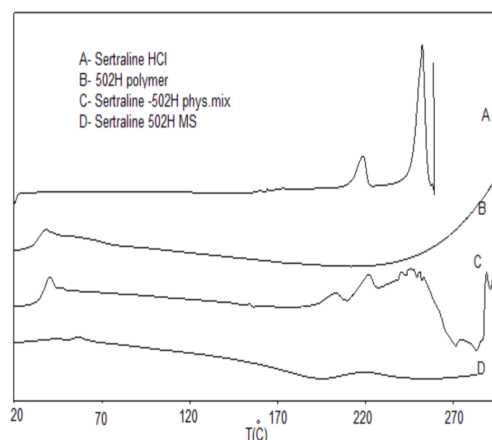

Figure 3a : DSC scan of Sertaline HCl, Sertaline HCl-502H polymer physical mixture, 502H polymer and Sertaline HCl 502H microspheres.

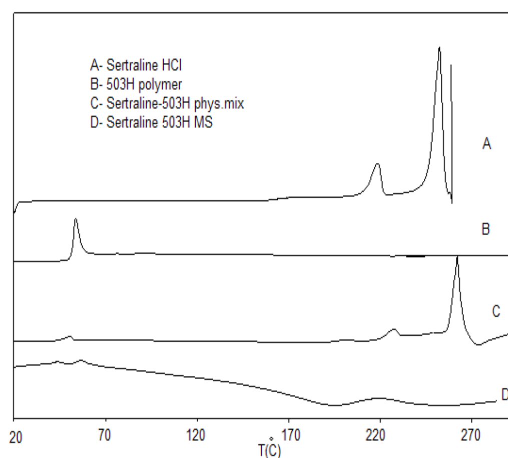

Figure 3b : DSC scan of Sertaline HCl, Sertaline HCl-503H polymer physical mixture, 503H polymer and Sertaline HCl 50 microspheres.

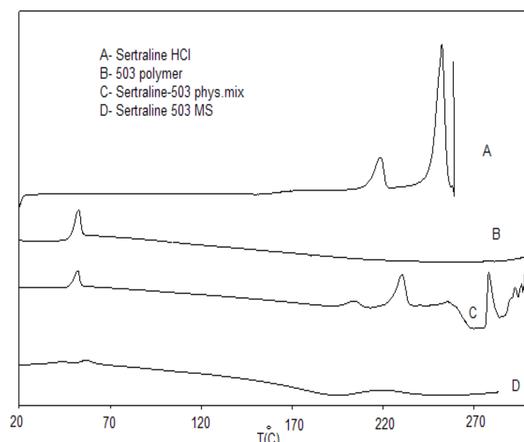

Figure 3c : DSC scan of Sertaline HCl, Sertaline HCl-503 polymer physical mixture, 503 polymer and Sertaline HCl microspheres.

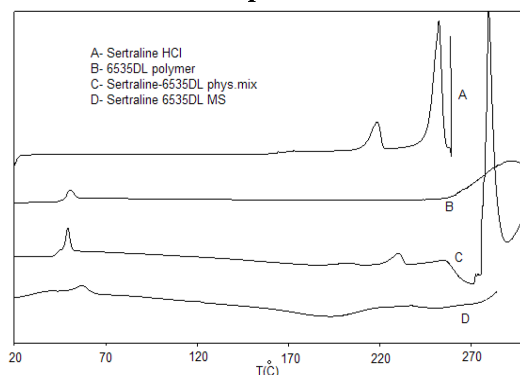

Figure 3d : DSC scan of Sertaline HCl, Sertaline HCl-6535DL polymer physical mixture, 6535DL polymer and Sertaline HCl 6535DL microspheres.

In Vitro Drug Release

A pathway for sertraline HCl release was provided by microsphere degradation where water-soluble degradation products (i.e. monomers and oligomers) leave the microspheres matrix for the surrounding aqueous medium. Since oligomers are close to the surface they can leach out faster than that located deeper within the matrix, carboxylic acid oligomers trapped within the matrix autocatalyze further ester bond hydrolysis, resulting in the increasing rate of mass loss[27]. Four batches of microspheres were subjected to long-term in vitro release (48 days) at 37°C in 0.1M acetate buffer, pH 5.0. The data in figure (4) showed complete sertraline HCl release from 503H and 502H microspheres throughout 35 days with no significant variation between them($P< 0.01$). On the other hand, 503 and 6535DL microspheres gave total drug release about 82% and 59% respectively within 35 days. The high drug release from 503H and 502H microspheres can be attributed to the highest loading percent of the drug for 503H microspheres and to the low molecular weight for 502H polymer, and these two effects may fasten the hydrolysis of microspheres[28,29]. In paired comparison (503H vs 503), where the overwhelming majority of structure are chemically identical, and the difference between them is whether the polymer end groups are a carboxylic function (503H) or a long-chain fatty ester(503), the more hydrophilic polymer , the greater amount of drug bound . In a similar study, release of bone morphogenetic protein-2 from hydrophilic PLGA microspheres was higher than that from hydrophobic one[30]. The slow release of sertraline HCl from DL6535 microspheres might be due to the slow hydration and degradation of the high molecular weight polymer[31]. This result was expected and similar results reported by researchers[32-34].

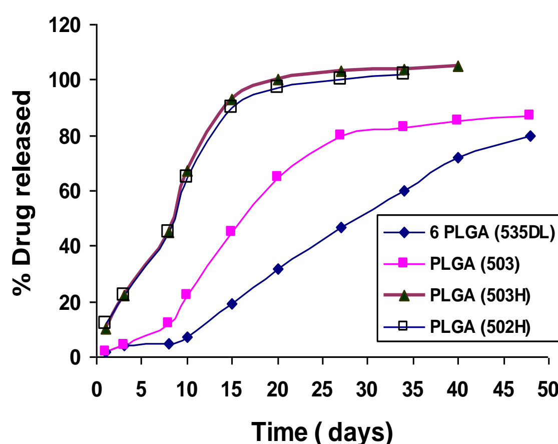

Figure 4: In vitro release of sertraline HCl from different polymer microspheres in acetate buffer pH 5 at 37°C.

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