Enkephalin Leucine Microspheres Suspension as a Sustained Antiplaque Antimicrobial Delivery System

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الخلاصة

الغرض من هذه الدراسة هو لتحضير صيغة علاجية تحتوي على الانكفالين ليوسين كمضاد للطبقلت الجرثومية/البكتريا . يتم تحضير الكرات المجهرية للانكفالين ليوسين باستخدام بوليمر متعدد احماض اللبن والكلايكوليك (PLGA) كنظام لايصال الدواء.

الكرات المجهرية حضرت بطريقة الانتشار/ استخلاص المذيب – تبخير ووصفت من حيث: كمية التحميل للدواء بواسطة جهاز تحليل الكروماتين السائل بالضغط العالي (HPLC)، وحجم الجسيمات بواسطة جهاز قياس الحيود الليزري (Laser diffractometry)، والشكل السطحي بواسطة المسح بالمجهر الالكتروني (SEM).

في خارج الجسم تم دراسة تحرير الانكفالين ليوسين باستخدام طريقة الديلزة. استقرارية الببتايد في اللعاب البشري كانت معتمدة على تركيزه في اللعاب. الكرات المجهرية للبوليمر متعدد احماض اللبن والكلايكوليك (PLGA) تحررت بعد التتقيع في العاب الصناعي. فعالية التغليف الدقيق كانت بحدود 83-97% مع شكل كروي. التحرر خارج الجسم تم بنجاح خلال 60 دقيقة بصورة مسيطر عليها مع نسبة تحرر تصل الى 70% للكريات الحرة (الغير متصل) و نسبة 25% للكريات المدمصة.

Abstract

The purpose of this study is to prepare a formulation containing enkephalin leucine as an antiplaque/antibacterial agent. Enkephalin leucine microspheres were prepared using a poly(D-L-lactide-co-glycolide) (PLGA) as a delivery system. The microspheres were prepared by a dispersion/solvent extraction-evaporation method and characterized for drug load by HPLC, particle size by laser diffractometry and surface morphology by scanning electron microscopy (SEM). In vitro enkephalin leucine release was studied using a dialysis method. The stability of peptide in human saliva was concentration dependent. PLGA microspheres had released after socking in the artificial saliva. The encapsulation efficiency was around 83% -97% with the spherical shape. The in vitro release was successfully controlled over 60 minute

or more with percentage of release reach to 70% for the free drug & 25% for the adsorbed drug.

Introduction

The most prevalent diseases affecting the oral cavity, dental caries and periodontal diseases, are associated with oral micro-organisms. The formation of dental plaque (biofilm) results from the adsorption of pellicle to bacterial accumulation and growth. Chemical agents for the control of the plaque formation are usually antimicrobials such as chlorhexidine, triclosan, and cetylpyridinium chloride [1-2]. The use of mouth wash as a vehicle for antiplaque agents is appealing from a practical and compliance standpoint. Promising results have been obtained by using this route for the delivery of chlorhexidine Smith et al. [3-5].

There is a great interest in the use of antimicrobial agents for the prevention and treatment of plaque-related oral diseases and many publications have reported the results of studies in which the minimum inhibitory concentration of agents fore cariogenic and periodontopathogenic bacteria have been determined [6].

Naturally occurring antimicrobial peptides have emerged as alternative classes of antimicrobilas. In general these antimicrobial peptides exhibit selectivity for prokaryotes and minimize the problems of engendering microbial resistance. As an alternative method to search for more effective broad-spectrum peptide antimicrobials, investigators have developed a pentapeptide by using synthetic combinatorial technology. A novel pentapeptide, Leucine enkaphline has been identified that shows a broad antibacterial activity in addition to its analgesic activity. The anti-plaque effect of the pentapeptide has been tested and documented [6,7].

Concannon et al. reported that Leucine enkephalin may be a useful antimicrobial agent for inhibiting the growth of oral bacteria that are associated with caries development and early plaque formation [8].

Gargle suspension of microspheres loaded with enkephalin leucine could constitute a valuable delivery system for establishment of a sufficient pentapeptide content to maintain an anti-plaque effect in the mouth. The advantage with chewing gum is that it is usually kept in the mouth longer than rinses and toothpastes. The active agent included in a suspension, if successfully released into the saliva, would thus have ample time to become retained a variety of reception sites [9].

This study aims at designing a novel Gargle suspension formulation and developing a technique capable of maintaining controlled release of medicine. leucine enkephalin has high water solubility [10,11]. This means that the drug release from gargle suspension is high if the drug is incorporated in the micro particles. To obtain better sustained release of the leucine enkephalin from

particles, a new preparation procedures involving incorporating or adsorbing the active principle into PLGA microspheres or adsorbed to the microsphere surface was adopted [12].

The gargal suspension formulations with variable contents of pentapeptide adsorbed and incorporated into PLGA microspheres were developed. The in vitro release profiles of enkephalin leucine from these formulations were measured to investigate the dissolution behavior and the release characteristics of the active principle after gargle suspension administration. Our effort was to evaluate if the PLGA microspheres incorporated gargle formulation delivers enkephalin leucine at a controlled release manner. The microspheres were dispensed as a unit single dose in aluminum package. Each package containing 100 mg of microspheres loaded with 20% peptide and mixed with 300 mg sweetening agent (sorbitol) and 100 mg of calcium phosphate as a abrasive agent. The microspheres suspended in ~ 4 table spoon of drinking water.

Materials and Methods
Materials

Enkephaline leucine (MW 555.63) were purchased from Sigma (St Louis, MO. Hydrophilic and hydrophobic 50:50 PLGA polymers (Resomer RG502H, RG502 and RG503H) were supplied by Boehringer Ingelheim (Ingelheim, Germany). Acetonitrile and tetrahydrofuran (THF) (HPLC grade) were supplied from Fisher Scientific (Fair Lawn, NJ). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL). All other chemicals were of analytical grade and used as obtained commercially.

Instrument

Magnatic stirrer, Silverson L4R homogenizer (Silerson Machines Inc., East Longmeadow, MA, USA), scanning electron microscope (SEM) (Hitachi model S800, Japan), Malvern 2600 Particle Sizer (Malvern Instruments, Malvern, England), HPLC using a Prosphere C-18 column (4.6 x 250 mm, Alltech, Deerfield, IL).

Preparation of Microspheres

Enkephaline leucine was incorporated into PLGA microspheres by a dispersion method followed by solvent extraction and evaporation [13,14]. In details peptide in methanol was combined with a solution of PLGA in methylene chloride and stirred using a magnetic stirrer for approximately 5 min. The solution was then slowly dispersed in 0.35% (w/v) solution of polyvinyl alcohol and stirred at 5000 rpm with a Silverson L4R homogenizer. The temperature of the reactor was maintained at 25° C for 30 min during extraction of the solvents from the microspheres and after that at 40°C for 60 min using a circulating water bath to facilitate evaporation. The hardened microspheres were recovered by vacuum filtration and washed with water. Finally, the microspheres were

freeze-dried. The solid in oil in water (s/o/w) method is represented in figure 1.

Characterization of Microspheres

Surface morphology

Surface morphology was assessed by scanning electron microscopy (SEM) after palladium/gold coating of the microsphere sample on aluminum stubs.

Particle size distribution

Particle size distribution was obtained by laser light scattering of microsphere suspensions in 0.1% aqueous Tween 80 (w/v) using a Malvern 2600 Particle Sizer.

Peptide content

Enkephalin leucin content in microspheres was determined as follow: The 20 mg of microspheres were dissolved in 5 mL of DMSO:acetonitrile (50/50, v/v) solution and the peptide was extracted with 10 mL of 0.1 M acetate buffer (pH 4.0) by agitation for 30 min. After centrifuge for 10 min, the supernatant was filtered with a PVDF 0.45 μm filter and the extract was analyzed by reversed-phase HPLC using a Prosphere C-18 colum. A gradient elution was performed with mobile phase A (0.1% TFA in water) and mobile phase B (0.1% TFA in acetonitrile). The enkephalin leucine was eluted with a linear gradient from 80:20 to 70:30 (mobile phase A:B) for 8 min at a flow rate of 1.0 mL/min. Total run time was 20 min and the injection volume was 50 μL. Chromatograms were recorded by UV detection at 220 nm.

In vitro Release Studies

The in vitro release of enkephalin leucine from microspheres was determined by suspending 20 mg of microspheres in 5 mL of artificial saliva and rotating the suspensions at $37^{o}C$. The artificial saliva was composed of 0.844 g sodium chloride, 1.200 g potassium chloride, 0.193 g calcium chloride dihydrate, 0.111 g magnesium chloride hexahydrate, 0.342 g dibasic potassium phosphate per 1000 ml and the pH was adjusted with hydrochloride acid solution to 5.7 ± 0.1 [12]. At regular intervals (10 min), samples were centrifuged and the supernatant was analyzed by reversed-phase HPLC. Fresh replacement media was added to resuspend the microspheres.

Stability Study in Human Saliva

Stability of peptide was assessed in normal human saliva. The human saliva was collected from humans aged 25-50 years and filtered.peptide was added to human saliva at a concentration of 0.2, 0.5 and 1 mg/mL. The peptide in saliva was incubated at $37^{o}C$ and the intact peptide remained in saliva was analyzed by reversed-phase HPLC. The peptide in water at $37^{o}C$ was used as a control.

Peptide Binding

Blank microspheres were prepared by the same method described in Preparation of Microspheres without peptide. The blank microspheres were

suspended in vials contianing enkephalin leucine solution in 0.1 M sodium phosphate buffer pH 7.4 (PB) and and 0.1 M acetate buffer pH 4.0 (AB). The vials were mounted on a rotary wheel vertically spinning at a speed of 18 cycles/min. Kinetic analysis of the peptide and PLGA interaction was accomplished by allowing the interaction to proceed for 1, 2, 4, 6, 24, 48 h at room temperature. The adsorbed amount of leucine enkephalin was determined by analyzing the supernatant and the extract of microspheres with reversed-phase HPLC [15].

Results and Discussion
Preparation of Microspheres

Preparation of enkephalin leucine loaded microspheres was accomplished by the dispersion/solvent evaporation method as described in the experimental section. Various preparation conditions and materials were investigated in order to obtain the best results concerning loading and drug release where the preparation parameters for the peptide microspheres are shown in Table 1. A critical step at this point was the complete drug dispersion that is fundamental to have a uniform distribution of the drug inside the microsphere and higher encapsulation efficiency.

Characterization of Microspheres
Surface morphology and Particle size distribution

SEM analysis on enkephalin leucine microsphere showed that the microspheres were successfully fabricated with a spherical shape and smooth surfaces, a certain fragility and relatively low porosity. The SEM also showed homogeneous particle size distribution. Figures 4 (a - b are blank microspheres) and (c-d are the loaded microspheres).

Peptide content

Under the described HPLC conditions, the standard of enkephain leucine in D.W. was detected as a single peak at the retention time of 7.0 min (Figure 2a). The degradation compounds of peptide produced at each pH conditions ($55^{o}C$) could be resolved by the HPLC method (Figure 2b-d). The calibration curve of enkephalin leucine is shown in Figure 3. The result indicates that dispersion/solvent extraction-evaporation method has been used successfully in the incorporation of hydrophobic drug with good yield value loading percentage[13]. The remarkable encapsulation efficiency was around 83 - 97% for the formulations studied. Yield value is a function of the efficiency of preparation method and values up to 70% were accepted [16].

In vitro Release Studies

A pathway for enkephalin leucine 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 [17].

Figure 5 represents the in vitro release profiles of enkephalin leucine from microspheres formulation. In vitro release of microspheres formulations containing free enkephalin leucine showed 40% release at 10 min and then 67% at 40 min. Total 70% of the peptide was released for 60 min, Figure 6. The enkephalin leucine-adsorbed microspheres showed 19% release at 20 min and 25% release for 60 min, whereas the peptide incorporated microspheres showed initially 8% release at 5 min and then no release.

Stability Study in Human Saliva

Figure 7 shows the satiability of peptide in human saliva at 37°C. We see that the rate of degradation was concentration dependent.

Peptide Binding

Peptide binding to PLGA and PLA and non-specific adsorption to blank PLGA microspheres were dependent upon pH and were markedly higher in PB than in AB [15]

Figure 8 is a plot of the adsorption and desorption data for peptide on the PLGA microspheres. Although approximately 73% of the adsorbed peptide was released from the microspheres at 5 minutes, at equilibrium after 30 minutes, there was almost 1.6 mg still adsorbed. This represent 40 % of the total peptide adsorbed on the microspheres before desorption.

Conclusion

This is a first Iraqi study to report that antimicrobial peptide can be useful source for controlled delivery system. Enkephalin leucine showed high affinity to PLGA microspheres. In the release study of *in vitro* from microspheres (loaded and adsorbed) showed promising release profiles for appropriate time. This study suggests that the enkephalin leucine will be released from the microspheres in a controlled manner and effectively retain in oral cavity to act for inhibiting the formation of dental plaque. This study also suggests that the loaded PLGA microspheres could be incorporated in another oral delivery system such as suspension, chewing gums, and other gargle preparations. This study requires further clinical trials to show the effectiveness of the formula.

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17. Capan, Y.; Jiang, G.; Giovagnoli, S.; Na, K-H. and DeLuca, P.P. (2003). Preparation and Characterization of Poly(D,L-lactide-co-glycolide) Microspheres for Controlled Release of Human Growth Hormone. AAPS PharmSciTech; 4(2): article 28.

Figures and tables legend:
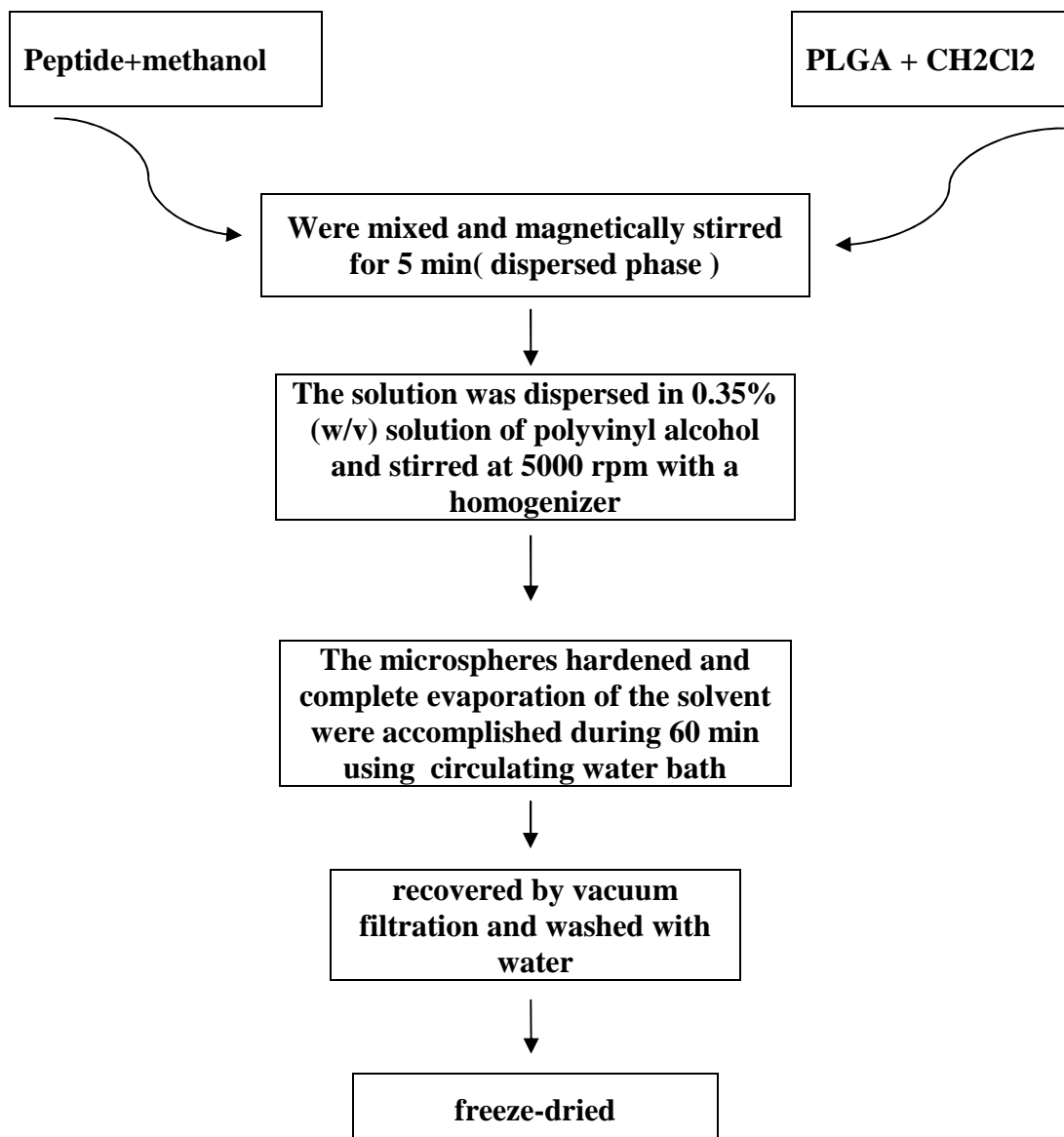**Table 1**:   Characterization of Enkephalin Leucine loaded microspheres.
Figure 1:   preparation of PLGA enkephalin leucine microspheres using dispersion/ solven extraction-evaporation method
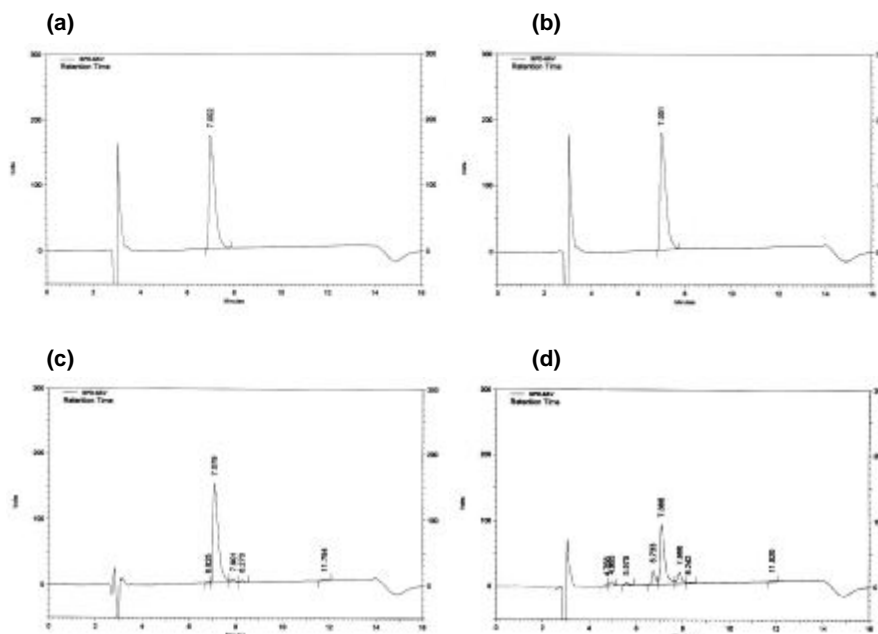**Figure 2**:   HPLC chromatograms of enkephalin leucine in various aqueous solutions (a: enkephalin leucine 20 µg/mL in D.W., b: enkephalin leucine incubated at pH 4 (55°C) for 3 days, c: enkephalin leucine incubated at pH 7.4 (55°C) for 3 days, d: enkephalin leucine incubated at pH 9 (55°C) for 3 days).
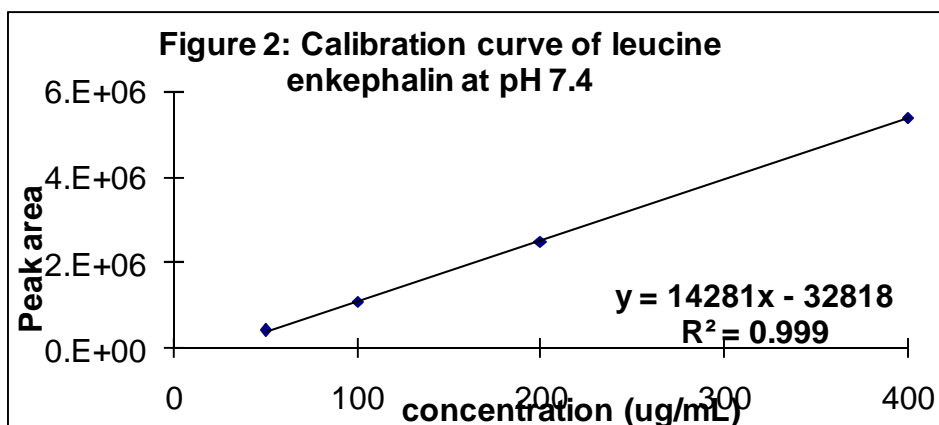**Figure 3:**   Calibration Curve of Enkephalin Leucine using the previous HPLC method
Figure 4:   SEMs of Enkephalin Leucine Blank and loaded microspheres
Figure 5 and 6: *In vitro* release of the Enkephalin Leucine loaded PLGA microspheres
Figure 7:   Stability of Peptide in Human Saliva
Figure 8:   Adsorption and Desorption of Enkephalin Leucine from Microspheres

parameters	
Mw of polymer	45KD
Drug Load	20%
Encapsulation Efficiency	83%
Mean Particle size	24.6 μm
Dose of peptide	20 mg

Table 1: Characterization of microspheres

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┌──────────────────────┐                    ┌──────────────────────┐
│  Peptide+methanol    │                    │   PLGA + CH2Cl2      │
└──────────────────────┘                    └──────────────────────┘
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Were mixed and magnetically stirred for 5 min(dispersed phase)

The solution was dispersed in 0.35% (w/v) solution of polyvinyl alcohol and stirred at 5000 rpm with a homogenizer

The microspheres hardened and complete evaporation of the solvent were accomplished during 60 min using circulating water bath

recovered by vacuum filtration and washed with water

freeze-dried

Figure-1: preparation of PLGA enkephalin leucine microspheres using dispersion/ solvent extraction-evaporation method

Figure-2: HPLC chromatograms of enkephalin leucine in various aqueous solutions (a: enkephalin leucine 20 ㎎/mL in D.W., b: enkephalin leucine incubated at pH 4 (55°C) for 3 days, c: enkephalin leucine incubated at pH 7.4 (55°C) for 3 days, d: enkephalin leucine incubated at pH 9 (55°C) for 3 days).

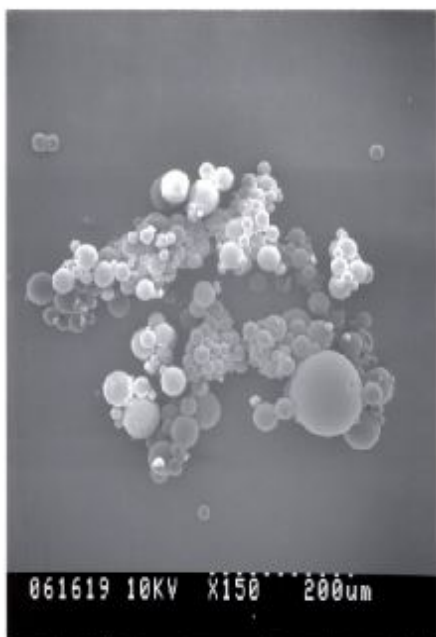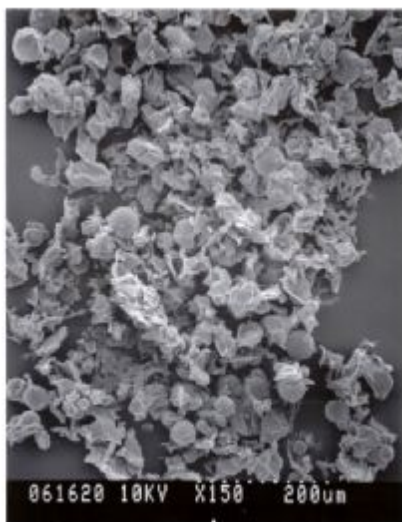**Figure-3: Shows the calibration curve of the peptide using the previous HPLC method.**

a- Blank Microspheres

b- Blank Microspheres

c- Loaded Microspheres
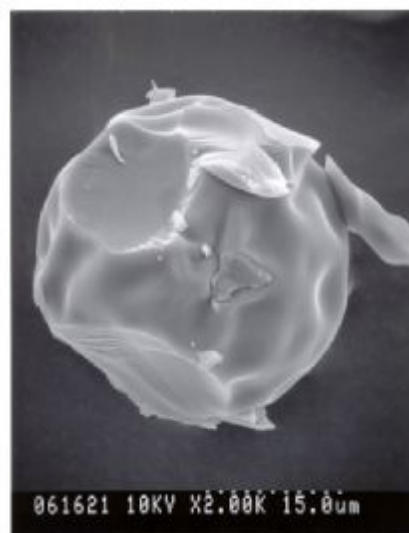
d- Loaded Microspheres

Figure 4: SEM of Blank and Loaded Microspheres
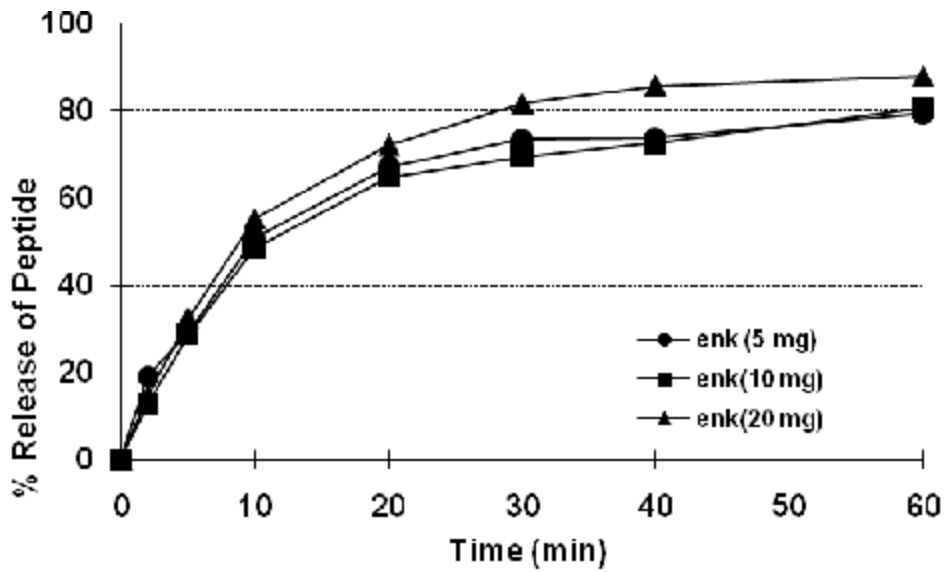
Figure 5: Effect ofEnkephalin Leucine Amount on
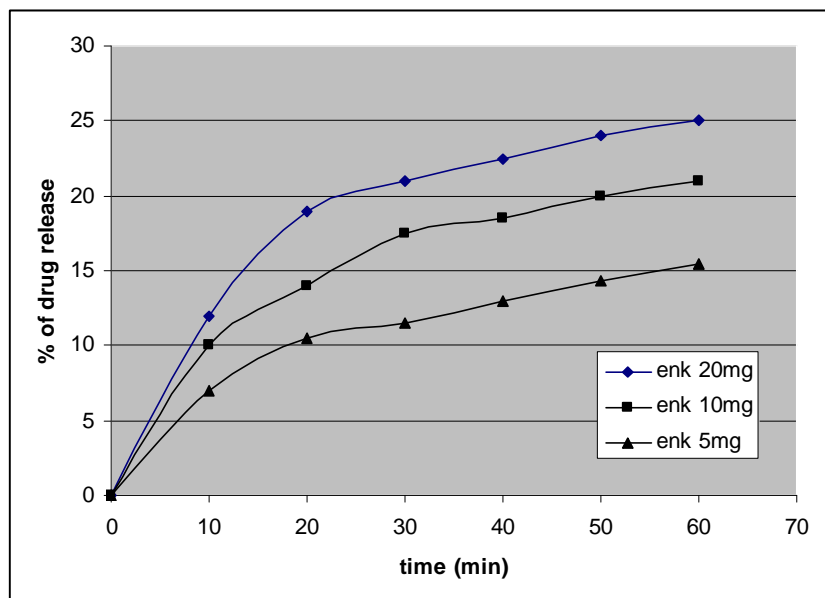In Vitro Release from Microspheres

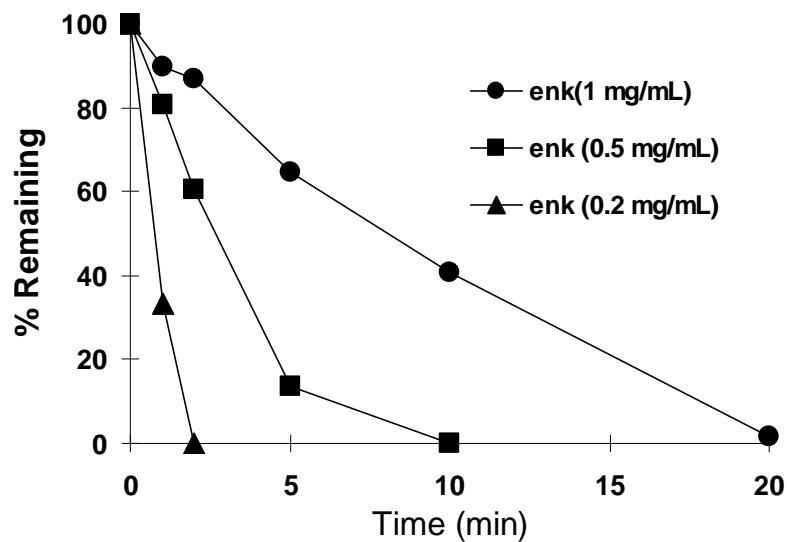**Figure- 6: the in vitro release of enkephalin leucine–adsorbed microspheres**

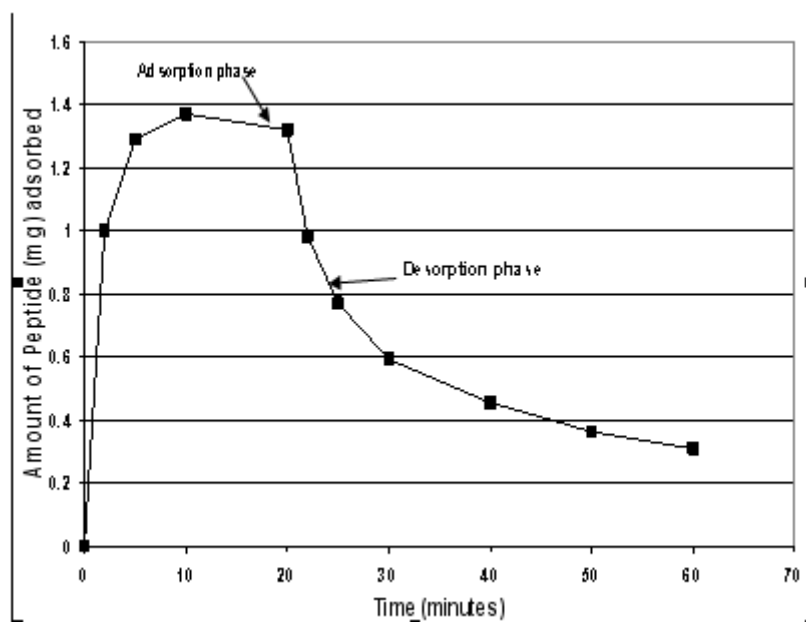**Figure-7: Stability of Peptide in Human Saliva**

Figure-8: Amount of Enkephalin Leucine Adsorbed anb desorbed From the Microspheres.