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Effect of Chlorhexidine Incorporation on Acrylic Reline Resins – Release Studies

Neuza Isabel Fernandes Marcelino

Dissertação

Mestrado Integrado em Medicina Dentária

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Dissertação orientada pela Professora Doutora Maria Cristina Bettencourt Neves e coorientada pela Professora Doutora Ana Francisca Bettencourt

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Resumo

Nos últimos anos tem havido esforços no sentido de manter o máximo de peças dentárias possível. No entanto, a ausência de dentes mantém-se numa percentagem significativa de idosos, em todo o Mundo. No futuro, é de prever a necessidade de próteses parciais ou totais para um número considerável de adultos, uma vez que nos deparamos com um gradual envelhecimento populacional.

A estomatite protética associada a infeção por *Candida* é uma forma de candidíase oral comum em pacientes idosos reabilitados com próteses dentárias removíveis, tendo uma prevalência de 45-70%. Esta condição, apesar de frequentemente assintomática, manifesta-se como uma inflamação difusa das áreas cobertas pela prótese. Sendo uma condição inflamatória crónica de origem multifatorial, está associada à utilização de próteses mal adaptadas, a infeção microbiana, higiene protética insuficiente, utilização contínua da prótese sem períodos de descanso, secreção salivar reduzida devido a medicação ou radioterapia, carências nutricionais, fatores sistémicos, utilização de antibióticos de largo espetro, entre outros factores.

Apesar de vários organismos contribuírem para o desenvolvimento desta doença, o fungo *Candida albicans* é o principal agente causal, sendo que a sua aderência constitui o primeiro passo na patogénese da estomatite protética. Este organismo, embora seja um fungo comensal na cavidade oral, pode atuar como patogénio oportunista. Uma vez que uma das suas características é a capacidade de formar biofilmes, a sua proteção contra defesas do hospedeiro e a resistência a antimicrobianos encontra-se aumentada.

Existem vários antifúngicos para o tratamento da estomatite protética. O fluconazol é um antifúngico sistémico frequentemente utilizado por ser bem tolerado, ter baixa toxicidade e poucos efeitos secundários. No entanto, para além da sua reduzida eficácia em biofilmes, algumas estirpes de *C. albicans* e outras espécies de *Candida* também envolvidas nesta doença, são resistentes a fármacos baseados em azóis.

A eficácia clínica dos antifúngicos tópicos está dependente da sua distribuição e retenção numa localização específica, bem como da adesão à terapia por parte paciente. No entanto, a maioria dos antifúngicos tópicos possuem tempos de distribuição reduzidos e a sua retenção pode ser inferior a uma hora.

A clorexidina é amplamente prescrita em Medicina Dentária como bochecho antisséptico devido a sua ação tópica como agente antimicrobiano de largo espetro. Tem sido demonstrado em vários estudos que a clorexidina suprime a capacidade de *C. albicans* aderir às células do epitélio bucal e às superfícies das próteses dentárias acrílicas, ambos pré-requisitos cruciais para a infeção fúngica. Tem também sido verificada uma eficácia superior da clorexidina contra biofilmes, quando comparada com o fluconazol, sendo que o aparecimento de estirpes resistentes a esta não foi verificada em estudos clínicos. Por estes motivos, a clorexidina pode ser considerada um agente antimicrobiano eficaz contra infeções fúngicas. No entanto, tal como outros antifúngicos tópicos, a maioria do fármaco é removido da cavidade oral devido ao efeito de diluição da saliva e à auto-limpeza provocada pela musculatura oral, que se adicionam ao facto de estar dependente da adesão à terapia por parte do paciente. Estes aspectos podem então ser responsáveis pela redução da sua eficácia terapêutica.

Assim, embora estejam disponíveis várias modalidades terapêuticas, a taxa de recidiva da estomatite protética é relativamente alta. Numa tentativa de melhorar a qualidade de vida destes pacientes, vários estudos e materiais têm sido desenvolvidos.

A utilização de veículos de fármacos e de agentes de libertação controlada tem sido proposta para o tratamento da estomatite protética. Este conceito consiste na incorporação de agentes antifúngicos ou antimicrobianos em resinas acrílicas, de modo a inibir a adesão e o crescimento microbiano, induzindo efeito terapêutico. Estas formulações têm demonstrado manter níveis terapêuticos ideais do fármaco no local de infeção, excedendo geralmente a Concentração Mínima Inibitória das espécies suscetíveis. Isto permite que uma menor quantidade de fármaco seja necessária para alcançar efeito terapêutico e, consequentemente, que exista uma menor probabilidade da ocorrência de efeitos secundários ou interações medicamentosas. Acrescenta-se o facto de estes sistemas requerem uma monitorização mínima, o que também é favorável para o paciente uma vez que não são dependentes da sua adesão à terapia.

Vários antifúngicos têm sido estudados para uma libertação controlada na cavidade oral, tendo-se verificado resultados favoráveis para diferentes agentes. No entanto, a incorporação de clorexidina em resinas acrílicas das próteses removíveis tem demonstrado resultados superiores tanto na libertação como em testes microbiológicos, quando comparada com outros fármacos, como o fluconazol.

O principal objectivo deste estudo é então avaliar a libertação da clorexidina a partir de resinas acrílicas, mediante diferentes composições de materiais e diferentes

percentagens de incorporação na libertação do fármaco. Para tal, foi utilizada saliva artificial a um pH 7 como meio de libertação, de modo a simular a libertação na cavidade oral.

Três materiais foram selecionados para avaliação no presente estudo: Kooliner, Ufi Gel Hard (ambas resinas acrílicas de rebasamento directo) e Probase Cold (resina acrílica de rebasamento indirecto). Para cada material foram produzidos seis grupos de espécimes, sendo um de controlo e cinco experimentais com concentrações de incorporação de clorexidina de 1%, 2.5%, 5%, 7.5% e 10%. Foram avaliados um total de 54 espécimes em forma de cilindro (12mm de comprimento e 6mm de diâmetro). De modo a estudar a libertação da clorexidina pelos espécimes, os cilindros foram armazenados individualmente em frascos graduados de 5mL e cobertos por saliva, num rácio de 1g/5mL. Estes foram posteriormente incubados a 37 ^{o}C e, em intervalos de tempo específicos (1, 2, 4, 7, 24, 48, 72, 96, 168, 240, 360, 528, 672 horas), foram pipetados 900µL a partir de cada frasco para uma placa de micropoços (foram pipetados 300µL para cada poço). As amostras foram de seguida analisadas num espectrofotómetro a 255nm e as absorvâncias foram convertidas em concentrações. Nos mesmos intervalos de tempo, 900µL de saliva artificial foram renovados em cada frasco, de modo a simular a constante renovação salivar.

Foi ainda realizada a análise estatística dos dados dos espécimes incorporados com 1% de clorexidina, de forma a verificar a existência de diferenças significativas entre os materiais. Tendo em conta que os dados não apresentavam uma distribuição normal para as variáveis em estudo, os resultados foram submetidos a testes não-paramétricos pelo método de Kruskall-Wallis. Para tal, foi considerado um nível de significância igual a 5%.

Relativamente ao efeito das diferentes composições dos materiais na libertação da clorexidina, os resultados demonstraram que para todos estes, uma elevada libertação inicial foi seguida por uma libertação mais lenta e controlada, a qual permaneceu durante todo o tempo do estudo. Isto vai de encontro a outros estudos que associam esta alteração no padrão de libertação com o facto de a libertação de clorexidina ser controlada por um processo de difusão dependente da concentração. Os estudos referem também que enquanto a primeira fase poderá refletir uma libertação de superfície, a segunda estará associada a uma difusão a partir do interior do polímero. Verificou-se ainda que a maior libertação de clorexidina ocorreu nas primeiras 24-48h e que, para todas as percentagens de incorporação, o Ufi Gel Hard libertou a maior quantidade de

clorexidina quando comparado com os restantes materiais. Mais uma vez, os estudos corroboram estes resultados e associam-nos à diferente composição dos materiais.

No que diz respeito ao efeito da diferente percentagem de incorporação de clorexidina na libertação da mesma, os resultados demonstraram que esta é dependente da percentagem incorporada, sendo tanto maior quanto maior a quantidade de clorexidina presente no material. Outros estudos chegaram à mesma conclusão e correlacionam esta relação com a absorção de água por parte do material, que é maior à medida que a percentagem de clorexidina incorporada aumenta, criando porosidades e facilitando a dissolução do fármaco para a saliva.

No presente estudo, a libertação cumulativa máxima foi de apenas 1.77%, o que significa que apenas uma pequena porção da clorexidina inicialmente incorporada, foi libertada para a saliva artificial. Este resultado poderá dever-se ao facto dos espécimes serem de dimensões reduzidas e ao meio de libertação utilizado. No entanto, após comparação das concentrações cumulativas de clorexidina libertada com a respetiva concentração mínima inibitória, verificou-se que mesmo os materiais incorporados com 1% de clorexidina apresentavam concentrações superiores aos valores da MIC. Assim, os resultados sugerem que 1% de clorexidina é o suficiente para inibir *C. albicans*, o que reduz o risco do desenvolvimento de reações alérgicas pelo hospedeiro, dado se tratar de uma pequena concentração.

Relativamente a limitações do estudo, os espécimes não reproduzem a superfície protética, pelo que futuros estudos deverão ter este aspecto em conta. Para além disso, uma outra forma de misturar a clorexidina no pó da resina acrílica deverá ser utilizada, de modo a permitir uma melhor homogeneização. Serão também necessários estudos que avaliem a libertação em saliva com diferentes pH, estudos microbiológicos e de biocompatibilidade.

Desta forma, o presente estudo conclui que os sistemas de libertação de clorexidina baseados em resinas acrílicas de rebasamento poderão vir a ser uma potencial alternativa no tratamento da estomatite protética, uma vez que a libertação controlada deste fármaco mantém concentrações eficazes no local de infecção.

Palavras-chave: Estudos de libertação; Clorexidina; Resinas acrílicas; Estomatite protética; Incorporação de fármacos

Abstract

The use of drug carriers and controlled-release agents are a promising strategy to treat denture stomatitis, since it has been claimed that they maintain ideal therapeutic levels of the drug at the site of infection. Chlorhexidine incorporation into denture acrylic resins has shown good results, both on releasing and microbiological tests.

The main purpose of this study is to evaluate the release of chlorhexidine from acrylic reline resins, specifically the effect of different materials composition and drug loading on the drug release, with artificial saliva as media solution.

Three different materials were evaluated in the present study, Kooliner, Ufi Gel Hard and Probase Cold. For each one, one control group and five experimental groups, incorporated with chlorhexidine 1%, 2.5%, 5%, 7.5% and 10% (*w/w*), were produced. A total of 54 cylinder-shaped specimens were evaluated. The cylinders were stored individually in graduated falcon tubes and covered with saliva at pH=7. The falcons were then placed into an incubator and, at specific time intervals, an aliquot were collected from each falcon and the same amount of artificial saliva was renovated, in order to simulate the constant salivary renovation. The samples were analyzed by UV-spectroscopy and the chlorhexidine content was determined.

The results showed that a high initial release was followed by a slower and steadier elution, during the entire study period. Besides it was demonstrated that the greatest amount of chlorhexidine release occurred within the first 24-48h of incubation, the results also showed that the release of chlorhexidine is affected by different materials composition, since that, for all chlorhexidine %, Ufi Gel Hard released the highest amount of chlorhexidine. It was also established that the release of chlorhexidine is drug loading-dependant. In addition, it was shown that the maximum cumulative release was 1.77%, so only a small amount of initial loaded chlorhexidine is liberated. However, all the materials, even with lower chlorhexidine % (1% *w/w*), presented a cumulative concentration of chlorhexidine superior than its MIC values.

Overall, the results indicate that chlorhexidine delivery systems based on acrylic reline resins are a potential approach in the treatment of denture stomatitis.

Keywords: Release studies; Chlorhexidine; Acrylic resins; Denture stomatitis; Drug incorporation

1. Introduction

Despite improvements in tooth retention over the last years, a substantial tooth loss remains common among the elderly worldwide. Complete or partial tooth loss is associated with reductions in physical, psychological and social functions and potential harm to general health (Jones *et al.* 2003; Pisani *et al.* 2011). As a consequence of the ageing of the population, it is expectable that an important proportion of older adults will need complete or partial dentures over the next years (Jones *et al.* 2003).

Candida-associated denture stomatitis is a common form of oral candidosis in denture wearers, with a prevalence of 45-70%. This condition, although usually asymptomatic, presents itself as a diffuse inflammation of the denture-bearing areas and it has a multifactorial etiology (Chandra *et al.* 2001; Amin *et al.* 2009; Cao *et al*. 2010; da Silva *et al.* 2011; Ryalat *et al.* 2011; Salim, Moore, *et al.* 2012a). As a chronic inflammatory condition, it is associated with trauma from ill-fitting dentures, microbial infection, poor denture hygiene, continuous denture wear, reduced saliva secretion due to medication or radiotherapy, nutritional deficiency, systemic factors, broad spectrum antibiotic drug use, among others (Redding *et al.* 2009; Rautemaa and Ramage 2011).

Even though several organisms may contribute to this disease, *Candida albicans* is the principal causative agent and its adherence is the first step in the pathogenesis of denture stomatitis (Chandra *et al.* 2001; Redding *et al.* 2009; Rautemaa and Ramage 2011; Ryalat *et al.* 2011; Salim, Moore, *et al.* 2012a). *C. albicans* is a commensal fungal organism, but it can act as an opportunistic pathogen (Patel *et al.* 2001; Bertolini *et al.* 2014). It is known for its ability to form biofilms, which are complex structured communities of microorganisms encased within an extrapolymeric matrix material, attached to biotic or abiotic surfaces, as teeth or dentures surfaces. These communities have unique characteristics that confer survival and pathogenicity, allowing protection from host defenses and an increased resistance to antimicrobials (Chandra *et al.* 2001; Redding *et al.* 2009; da Silva *et al.* 2011; Rautemaa and Ramage 2011; Salim, Silikas, *et al.* 2013b).

There are many antifungal agents available to treat denture stomatitis. Fluconazole is a systemic antifungal commonly used because is well tolerated, it has low toxicity and mild side effects. However, besides its poor efficacy on biofilms, in

elderly patients with reduced saliva production, there is a potential risk of low drug concentrations and the emergence of microbiological and clinical resistance. In addition, some *C. albicans* strains and other Candida species also present in this condition, are azole-resistant (Patel *et al*. 2001; Salim, Moore, *et al.* 2012a; Salim, Moore, *et al.* 2013a; Salim, Silikas, *et al.* 2013b).

The clinical effectiveness of topical antifungals is dependent upon its delivery and retention at a specific site, as well as patient compliance. Nonetheless, most of the topical antifungals have limited delivery times and the retention may be less than one hour. Nystatin, for example, is a highly effective topical antifungal and it has few drug interactions, but its four times daily dosage is a significant challenge for patient compliance (Addy and Thaw 1982; Salim, Moore, *et al.* 2012a; Salim, Moore, *et al.* 2013a).

Chlorhexidine is widely prescribed as an antiseptic mouthwash in dentistry because of its topical action as a broad spectrum antimicrobial agent. Its mechanism of action appears to be the binding to negatively charged groups in the candidal cell wall, inducing intracellular material leakage and cell death (Anusavice *et al.* 2006; Amin *et al.* 2009). It has been demonstrated in many studies that chlorhexidine suppresses the ability of *C. albicans* to adhere to buccal epithelial cells and to acrylic denture surfaces, crucial prerequisites to fungal infection (Patel *et al.* 2001; da Silva *et al.* 2011; Bertolini *et al.* 2014). It has also been found that chlorhexidine have a superior efficacy against candidal biofilms compared with fluconazole and the emergence of resistance strains has not been observed in clinical studies (S. J. Wilson and H. J. Wilson 1993; Salim, Silikas, *et al.* 2013b). For these reasons, chlorhexidine can be considered as an effective alternative antimicrobial agent against fungal infections (Salim, Moore, *et al.* 2012a). However, besides its unique substantivity, as other topical antifungals, most of the agent is removed from the oral cavity due to the diluent effect of saliva and the cleansing effect of the oral musculature, adding the fact that it is also compliance dependent. These aspects can reduce its therapeutic efficacy (Ryalat *et al.* 2011; Salim, Moore, *et al.* 2013a).

So, despite of many possible therapeutic modalities, the recurrence rate of denture stomatitis is considerably high. This is associated with poor access of antifungals onto the denture surfaces, their poor penetration into the biofilm or their

rapid clearance by saliva (Chandra *et al.* 2001; Amin *et al.* 2009; Cao *et al.* 2010; Salim, Moore, *et al.* 2012a). The search for a better quality of life for denture wearers led to the development of studies and materials (Pisani *et al.* 2011).

The use of drug carriers and controlled-release agents has been proposed as one strategy to treat dentures stomatitis (Riggs *et al*. 2000; Salim, Silikas, *et al.* 2013b). This concept consists in the impregnation of antifungal or antimicrobial agents into denture acrylic resins, in order to inhibit microbial adherence and growth, achieving a therapeutic effect (Amin *et al*. 2009). These formulations have been claimed to maintain ideal therapeutic levels of the drug at the site of infection, generally exceeding the Minimum Inhibitory Concentration (MIC) of the susceptible species (Gong *et al.* 2007; Cao *et al.* 2010). This allows that less amount of drug is needed to achieve the therapeutic effect, leading to less side effects or drug-drug interactions compared with the conventional forms (Bertolini *et al.* 2014). Moreover, the use of self-releasing systems requires minimal monitoring and it is also convenient for patients because it is not compliance-dependent (S. J. Wilson and H. J. Wilson 1993).

Many polymeric systems and antifungal agents have been study for the controlled release in the oral cavity. Favorable results were achieved with different antifungals. However, chlorhexidine incorporation into denture acrylic resins has shown better results than other drugs, like fluconazole, on releasing and microbiological tests (Gong *et al.* 2007; Amin *et al.* 2009; Redding *et al.* 2009; Salim, Moore, *et al.* 2012a; Salim, Silikas, *et al.* 2013b). Many studies that evaluated the chlorhexidine release from acrylic resins concluded that this is concentration dependent and that a high initial release is followed by a slower and steadier diffusion (Addy and Thaw 1982; Riggs *et al.* 2000; Patel *et al.* 2001; Hiraishi *et al.* 2008; Amin *et al.* 2009; Cao *et al.* 2010; Ryalat *et al.* 2011; Salim, Moore, *et al.* 2012a; Bertolini *et al.* 2014). Nonetheless, it appears that only a small proportion of the incorporated chlorhexidine is actually released (Addy and Thaw 1982) and that chlorhexidine incorporation compromise the physical properties of the resins, creating porosity and increasing water uptake (S. J. Wilson and H. J. Wilson 1993; Hiraishi *et al.* 2008).

There are various methods to measure the release of the chlorhexidine from the acrylic resins. Spectroscopy is widely used because not only is an easy method but it

also provides reliable results (Amin *et al.* 2009; Ryalat *et al.* 2011; Salim, Moore, *et al.* 2012a).

Despite there has been other authors studying the release of chlorhexidine from acrylic resins, most of them used distilled water as media solution (Hiraishi *et al.* 2008; Salim, Moore, *et al.* 2012a; Bertolini *et al.* 2014). In the present study it was used artificial saliva at pH 7, in order to simulate the release in the oral cavity. Also, the period of analysis was 28 days, that it is a relatively long time and allows comparisons with other studies (Hiraishi *et al.* 2008; Salim, Moore, *et al*. 2012a; Salim, Silikas, *et al.* 2013b). In addition, five different percentages of CHX incorporation were evaluated, as opposed to the other studies so far that only studied the release of CHX in one concentration.

2. Objectives

The main purpose of this study was to evaluate the release of chlorhexidine from acrylic reline resins, in particularly:

1. The effect of different materials composition (Kooliner, Ufi Gel Hard and Probase Cold) on the drug release;
2. The effect of different chlorhexidine loading percentages (1%, 2.5%, 5%, 7.5% and 10%) on the drug release.

3. Materials and Methods

3.1. Preparation of the specimens

In the present study, three auto-polimerizing acrylic resins (Table 3.1), presented in the powder-liquid form, were used: Kooliner (GC America Inc, Alsip, Illinois, USA) (Figure 3.1a), Ufi Gel Hard (Voco GmbH, Cuxhaven, Germany) (Figure 3.1b) and Probase Cold (Ivoclar Vivadent AG, Liechenstein) (Figure 3.1c). Kooliner is a non-crosslinking material composed of pre-polymerized poly(ethyl methacrylate) (PEMA) powder particles and the monomer isobutylmethacrylate (IBMA). Ufi Gel Hard is a crosslinking material composed of pre-polymerized poly(ethyl methacrylate) (PEMA) powder particles and the monomer 1,6-hexanodioldimethacrylate (1,6-HDMA). These are both direct reline resins. Probase Cold is an indirect reline resin and represents a poly(methyl methacrylate) (PMMA) based material which has methylmethacrylate (MMA) as the monomer (Arima *et al.* 1995).

Table 3.1 – Materials used in the study.

Product	Manufacturer	Batch number	Composition	P/L ratio (g/mL)	Curing cycle
Kooliner (K)	GC America Inc., Alsip, Illinois, USA	1406232 (P) 1404241 (L)	P: PEMA L: IBMA	1.4/1	10 minutes, at room temperature
Ufi Gel Hard (U)	Voco GmbH, Cuxhaven, Germany	1443063 (P) 1438417 (L)	P: PEMA L: HDMA	1.77/1	7 minutes, at room temperature
Probase Cold (PC)	Ivoclar Vivadent AG, Liechtenstein	S41038 (P) U03356 (L)	P: PMMA L: MMA	1.5/1	15 minutes, 40°C, 2-4 Bar

P – Powder; L – Liquid; PEMA – polyethylmethacrylate; IBMA – isobutylmethacrylate; HDMA – hexanedioldimethacrylate; PMMA – polymethylmethacrylate; MMA - methylmethacrylate.

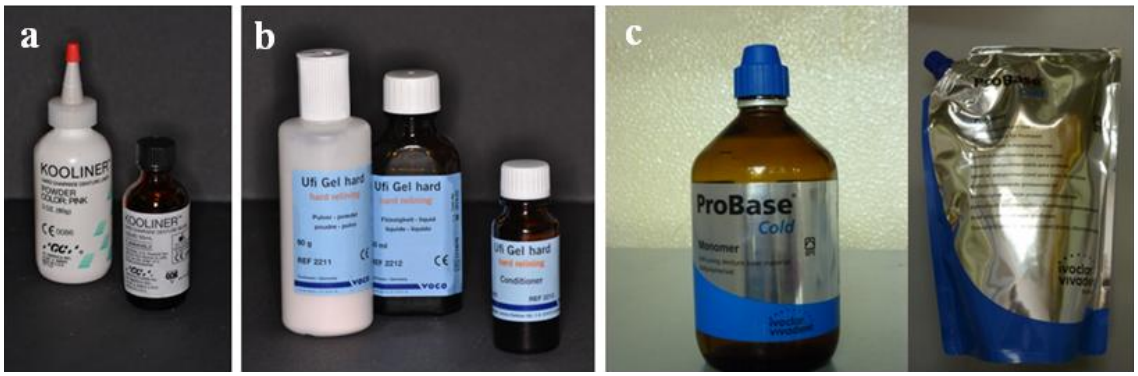

Figure 3.1 – Materials used in the study: a) Kooliner; b) Ufi Gel Hard; c) Probase Cold.

The acrylic resins were manipulated according to the manufacturer's instructions (Table 3.1). The powder was weighted using a precision balance (Mettler Toledo) and the liquid was measured using a graduated pipette. On the experimental specimens, 1%, 2.5%, 5%, 7.5% or 10% of the acrylic resin's powder weight (w/w) was replaced with chlorhexidine diacetate monohydrate (CHX) (Panreac Applichem, Darmstadt, Germany) (Figure 3.2a) and mixed using a mortar and pestle for homogenization (Figure 3.2b).

For each material six groups of specimens were produced (one control group without CHX and five experimental groups with the CHX percentages mentioned), resulting in eighteen specimens per material (n=18), three of each group (Table 3.2). The cylinder-shaped specimens (12 mm height and 6 mm diameter) (Figure 3.2c) were produced using stainless steel molds (Figure 3.2d). A total of 54 specimens were prepared for this study.

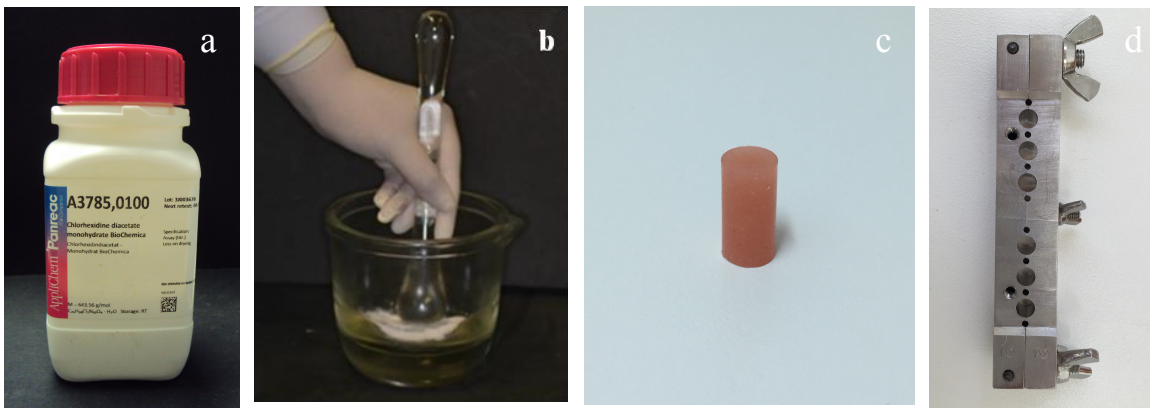

Figure 3.2 – Preparation of the specimens: a) Package of the Chlorhexidine diacetate monohydrate; b) Incorporation and homogenization of the CHX; c) Cylinder-shaped specimen; d) Cylinder-shaped mold.

In each preparation of Kooliner or Ufi Gel specimens, the materials dough was poured into the cylinder-shaped molds, maintained at room temperature during the recommended polymerization time (Table 3.1) and sealed with appropriate screws, providing compression in order to simulate the intraoral polymerization of direct reline resins. Polimerization of Probase Cold specimens was carried out in a pressure device (Ivomat, Ivoclar Vivadent, Liechenstein) (Figure 3.3) at recommended time, temperature and pressure (Table 3.1).

Figure 3.3 – Ivomat pressure device.

3.2. Analytical methodology

3.2.1. Standard stock and releasing solutions

A standard stock solution of 1000 µg/mL was prepared by dissolving approximately 10 mg of CHX into 10 mL of deionized water. This solution was kept out of light, at room temperature. On each new measurement of CHX, a series of dilutions of the standard stock solution were prepared (62.5, 31.25, 15.62, 7.81, 3.91, 1.95, 0.98 µg/mL).

The releasing solution used in the present study was artificial saliva at pH=7 (Figure 3.4), in order to understand how CHX would be released in the oral cavity. The artificial saliva was prepared according to a Faculty of Pharmacy University of Lisbon formula, courtesy of PhD student Joana Marto:

- Boiling 50 mL (F12-ED Refrigerated/Heating Circulator) of phosphate buffer pH=7.0 (Anhydride disodium phosphate, Monosodium phosphate anhydride and Deionized water) at 60^{o}C. Then sprinkled 0.05g of

Xanthan gum into boiling buffer and stirring until total of xanthan gum was dissolved.

- Dissolving 0.04g of Calcium chloride dihydrat (EW-N/EG-N balance), 0.08g of Sodium chloride and 0.08g of Potassium chloride in solution 1 and stirring until total of materials were dissolved.
- Dissolving 15 g of Propylene glycol in solution 2 and stirring until total of Propylene glycol was dissolved.
- Pouring the solution 3 into a graduated beaker and complete the solution with phosphate buffer pH=7.0 to 100 mL.

This solution was also kept out of light, at room temperature.

Figure 3.4 – Artificial saliva at pH=7.

3.2.2. Analytical technique

The absorbance of each solution was measured in a microplate reader (FLUOstar Omega – BMG LABTECH) (Figure 3.5) and the absorbance values were obtained using an Ultraviolet-Visible Absorbance Spectra detection mode, with a wavelength of 255nm, as recommended by other authors (Anusavice *et al.* 2006). The measurements were performed at room temperature of 25^{o}C.

The CHX release concentrations were determined based on the linear calibration methology, after subtracting the average of controls' absorbance, at the corresponding time interval.

Figure 3.5 – Microplate reader.

3.3. In vitro release studies

A preliminary study was conducted so further experimental protocols could be optimized.

To study the release of CHX from the specimens, the cylinders were stored individually in graduated falcon tubes of 5mL and covered with saliva pH=7, with a ratio of 1g/5mL (Figure 3.6a). The Falcons were then placed into an incubator at 37°C (Memmert), with constant gentle shaking (300 rpm) (Figure 3.6b). At specific time intervals (1, 2, 4, 7, 24, 48, 72, 96, 168, 240, 360, 528, 672 hours) (Table 3.2), and after the falcons were agitated in a mixer (VELP Scientifica, Vortex), 900µL were pipetted from each falcon to a polystyrene flat-bottom microplate wells (96-well microplates) (300µL were pipetted to each well). At the same time intervals, 900µL of artificial saliva at pH=7 were renovated in each falcon, in order to simulate the constant salivary renovation. The samples were analyzed as described above.

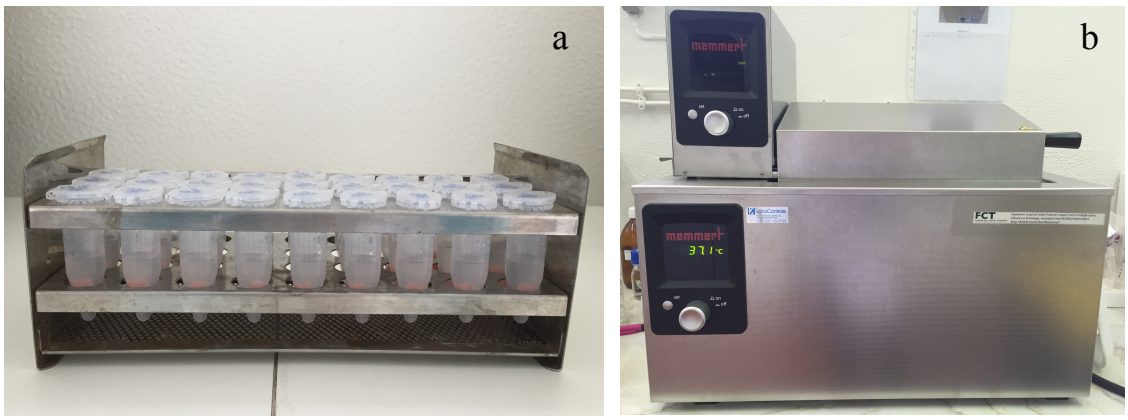

Figure 3.6 – Incubation of the specimens: a) in graduated falcon tubes, with saliva at pH=7; b) at 37°C, under constant gentle shaking by an incubator.

3.4. Statistic Analysis

Data from the specimens of the three materials with CHX 1% were statistically analyzed using SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA). Since data did not follow a normal distribution (verified by a Kolmogorov- Smirnov normality test), the results were submitted to nonparametric tests according to Kruskall-Wallis method, followed by multiple comparisons using Mann-Whitney tests with Bonferroni correction to determine whether there were specific significant differences among materials.

In all statistical tests, it was considered the 5% level of significance ($p<0.05$).

Table 3.2 – Schematization of distribution of the specimens.

Material	Group	CHX incorporation	Releasing solution	Time intervals
Kooliner	1 control group	Without CHX (n=3)	Artificial saliva at pH=7	1, 2, 4, 7, 24, 48, 72, 96, 120, 168, 240, 360, 528, 672 hours
	5 experimental groups	With CHX 1% w/w (n=3) With CHX 2.5% w/w (n=3) With CHX 5% w/w (n=3) With CHX 7.5% w/w (n=3) With CHX 10% w/w (n=3)		
Ufi Gel Hard	1 control group	Without CHX (n=3)		
	5 experimental groups	With CHX 1% w/w (n=3) With CHX 2.5% w/w (n=3) With CHX 5% w/w (n=3) With CHX 7.5% w/w (n=3) With CHX 10% w/w (n=3)		
Probase Cold	1 control group	Without CHX (n=3)		
	5 experimental groups	With CHX 1% w/w (n=3) With CHX 2.5% w/w (n=3) With CHX 5% w/w (n=3) With CHX 7.5% w/w (n=3) With CHX 10% w/w (n=3)		

4. Results

4.1. CHX quantification

It was established, for artificial saliva at pH 7, a linear relationship between CHX concentrations and the absorbance peak areas obtained with a microplate reader UV-Visible Spectrophotometer at 255 nm (Figure 4.1). The absorbance peak areas of the release solutions at 255 nm were converted to the CHX release concentrations based on the linear calibration methology, after subtracting the average of controls' absorbance, at the corresponding time interval. The analytical method showed good linearity (Figure 4.1).

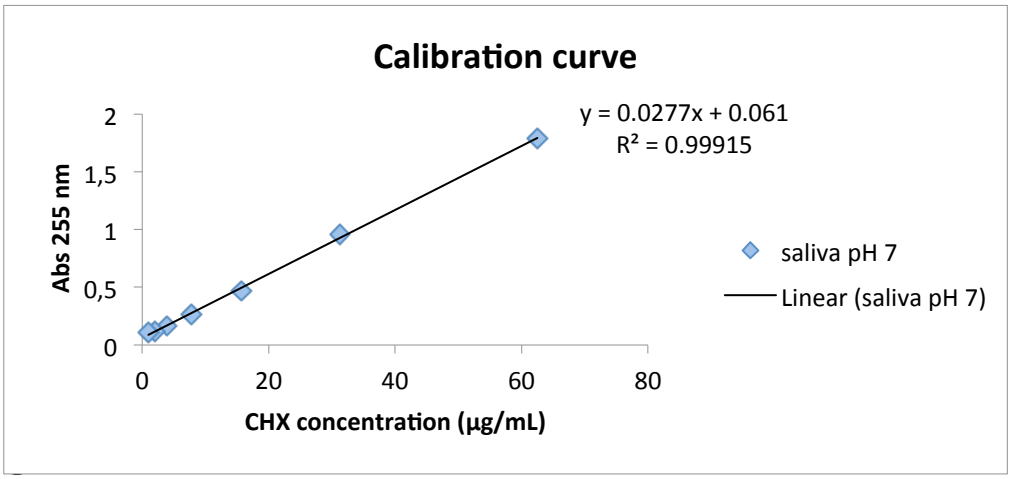

Figure 4.1 – Linear relationship between CHX concentrations and the absorbance peak areas for artificial saliva at pH 7.

4.2. The effect of the different materials composition on the drug release

Specimens of three different materials (Kooliner - K, Ufi Gel Hard - U and Probase Cold - PC) were evaluated in the present study, with CHX 1%, 2.5%, 5%, 7.5% and 10% (*w/w*).

For all the evaluated acrylic reline resins, a high rate of initial release was followed by a slower and steadier release, during the entire study period of 28 days. The greatest amount of CHX release occurred within the first 24-48h of incubation. In

addition, for all CHX % and for the majority of time intervals, Ufi Gel Hard released the highest amount of CHX, followed by Kooliner and Probase Cold.

The results of CHX release from the different materials are showed below.

For CHX 1%, 29.14 µg/mL from Ufi Gel Hard, 11.74 µg/mL from Kooliner and 8.62 µg/mL from Probase Cold were released until 48 hours of incubation and, at the end of the study (672 hours), a total of 35.36 µg/mL from U, 13.71 µg/mL from K and 12.40 µg/mL from PC were released (Figure 4.2).

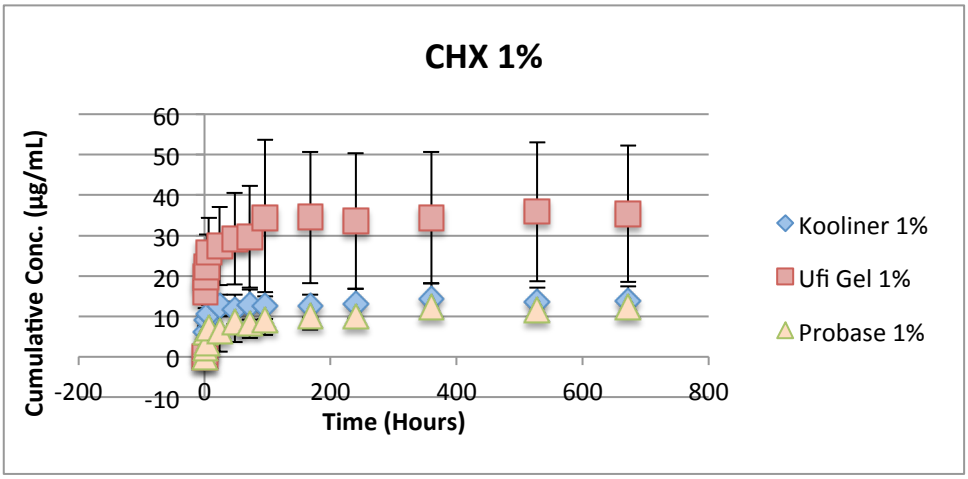

Figure 4.2 – Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 1%.

For CHX 2.5%, 79.01 µg/mL from U, 20.47 µg/mL from K and 11.34 µg/mL from PC were released until 48 hours of incubation and, at the end of the study, 90.39 µg/mL from U, 25.19 µg/mL from K and 19.40 µg/mL from PC were released (Figure 4.3).

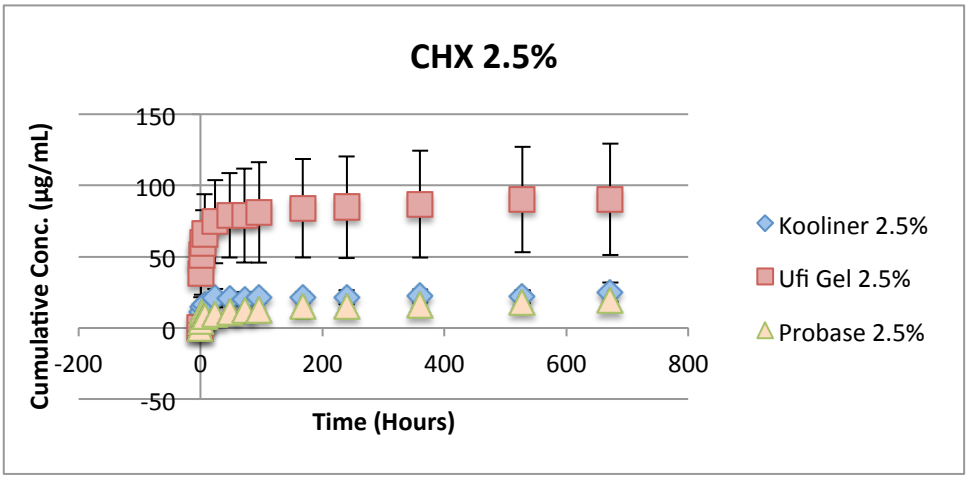

Figure 4.3 – Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 2.5%.

For CHX 5%, 103.92 µg/mL from U, 30.06 µg/mL from K and 19.62 µg/mL from PC were released until 48 hours of incubation and, at the end of the study, 129.02 µg/mL from U, 43.41 µg/mL from K and 35.47 µg/mL from PC were released (Figure 4.4).

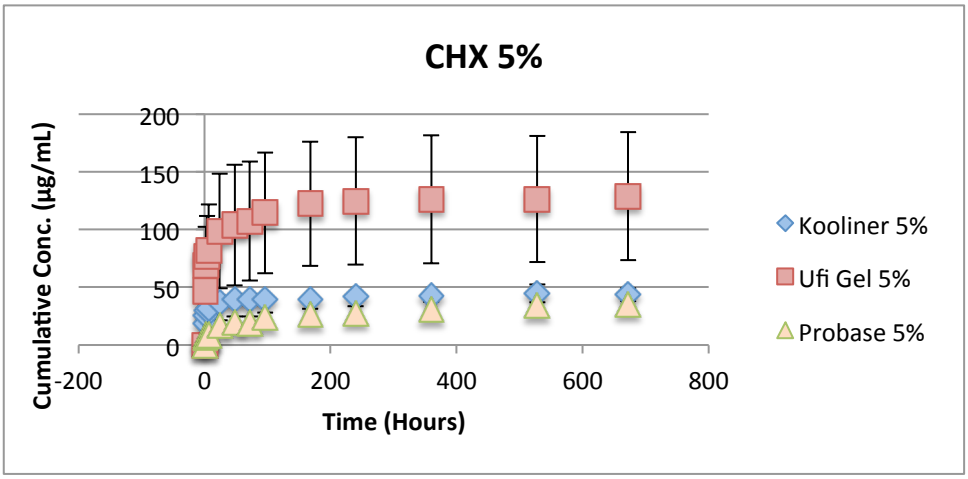

Figure 4.4 – Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 5%.

For CHX 7.5%, 68.30 µg/mL from U, 60.90 µg/mL from K and 20.86 µg/mL from PC were released until 48 hours of incubation and, at the end of the study, 97.24 µg/mL from U, 65.88 µg/mL from K and 44.08 µg/mL from PC were released (Figure 4.5).

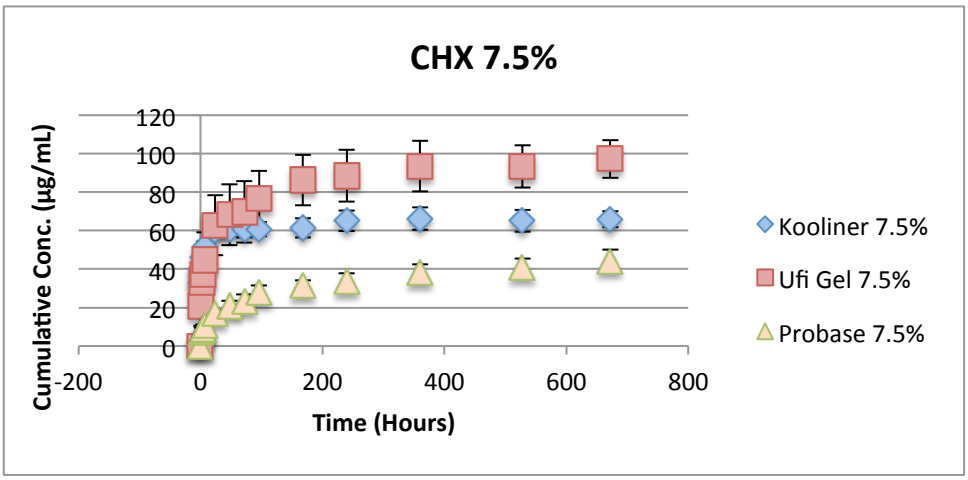

Figure 4.5 – Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 7.5%.

For CHX 10%, 96.71 µg/mL from U, 101.16 µg/mL from K and 45.04 µg/mL from PC were released until 48 hours of incubation and, at the end of the study, 126.72 µg/mL from U, 115.66 µg/mL from K and 72.53 µg/mL from PC were released (Figure 4.6).

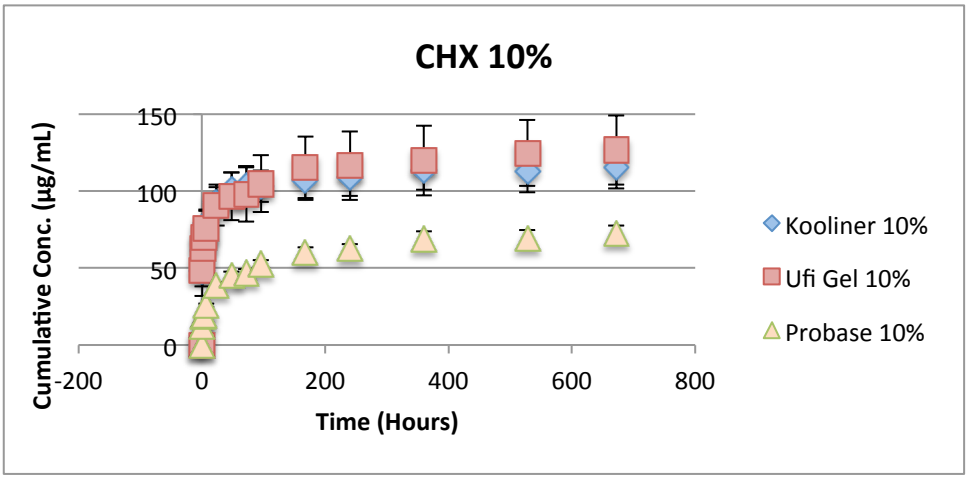

Figure 4.6 – Line diagram plotting the cumulative CHX concentration (µg/mL) versus time (hours) for the different materials with CHX 10%.

In addition, it can be observed that the pattern of release from U and K is similar at all times, however, PC release appears to continue in higher rates until the end of the study. This can be seen especially in CHX 7.5 and 10% (Figures 4.5 and 4.6).

Since it was not possible to perform microbiological studies, it was assessed a comparison between the cumulative concentrations of CHX and its mean minimum inhibitory concentration at 24h and 48h of incubation, achieved in Salim *et al*. 2013a study for 32 *C. albicans* isolates. As it can be observed in Figure 4.7, all the materials, even with lower CHX % (1% *w/w*), present a cumulative concentration of CHX superior than *C.albicans* MIC levels, at 24h and 48h.

In order to compare the three materials with CHX 1%, data analysis was applied and showed that the release of CHX from U was significantly higher compared to both K and PC ($p<0.001$). However, it wasn't found significant differences between CHX release from K and PC ($p>0.05$).

The maximum cumulative release (% *w/w*) from the three materials incorporated with CHX 1% (*w/w*), at the end of the study, is shown in Table 4.1. The results show that U had the highest CHX release, followed by K and PC. However, the maximum

cumulative release is only 1.768%, meaning that only a small amount of initial loaded CHX is liberated from the specimens to the artificial saliva.

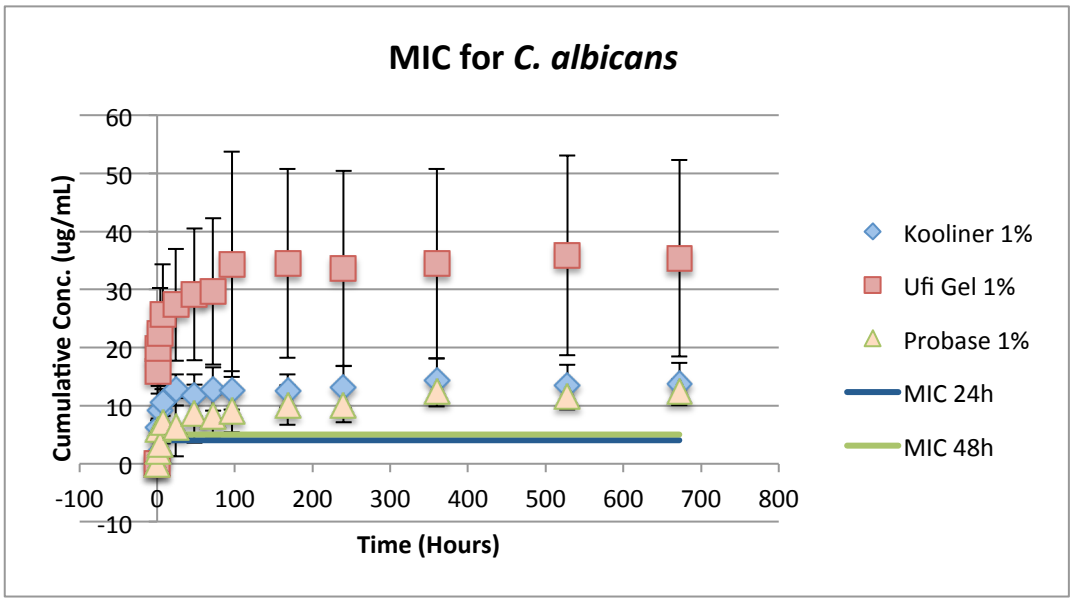

Figure 4.7 – Cumulative concentration of CHX 1% and MIC results for *C. albicans* achieved in Salim *et al.*, 2013a study.

Table 4.1 – Maximum cumulative release of CHX for each material with CHX 1% (*w/w*), at the end of the study. Results are presented as M±SD.

Material	CHX released (% *w/w*) at 672h
K 1%	0.685±0.183
U 1%	1.768±0.844
PC 1%	0.620±0.105

4.3. The effect of drug loading on the drug release

Once again, for all materials and CHX %, it is observed a initial high release followed by a controlled and sustained release, that continued throughout the 28-day test period.

The results show that, at higher drug loading concentrations, higher CHX release is detected, which means that the release of CHX is drug loading-dependant.

The release from Kooliner (Figure 4.8) and Probase Cold (Figure 4.9) was similar, however, Ufi Gel Hard release (Figure 4.10) showed some variations. In this material, it can be noticed that CHX 5% have a superior release than all the other CHX %, at all time intervals.

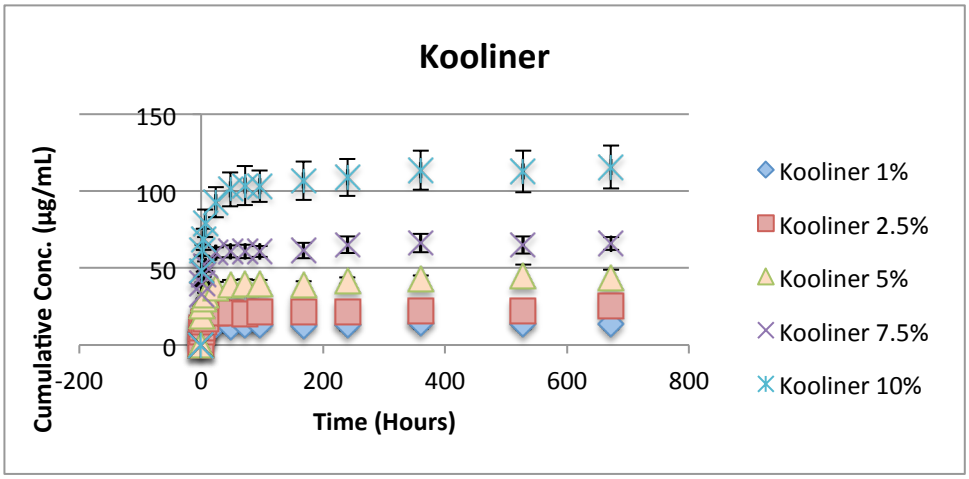

Figure 4.8 – Cumulative concentration of CHX from Kooliner specimens as a function of drug loading (1%, 2.5%, 5%, 7.5% and 10%).

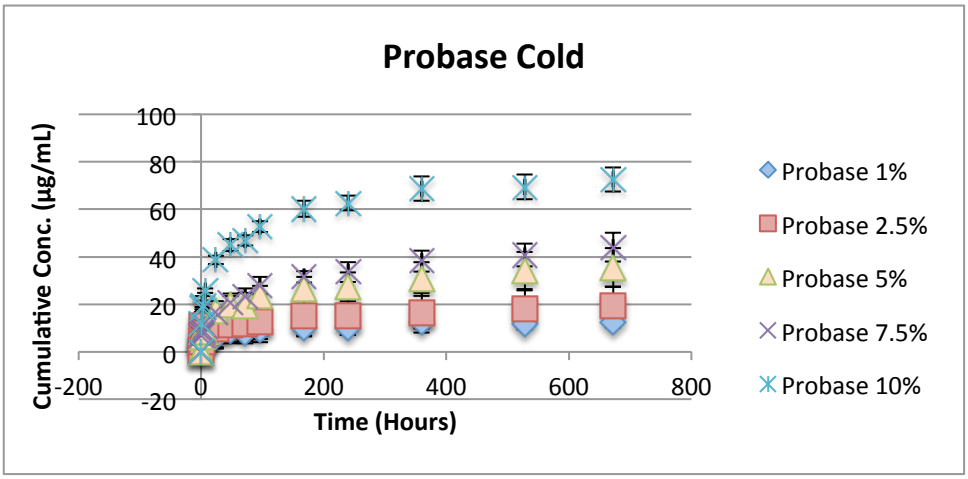

Figure 4.9 – Cumulative concentration of CHX from Probase Cold specimens as a function of drug loading (1%, 2.5%, 5%, 7.5% and 10%).

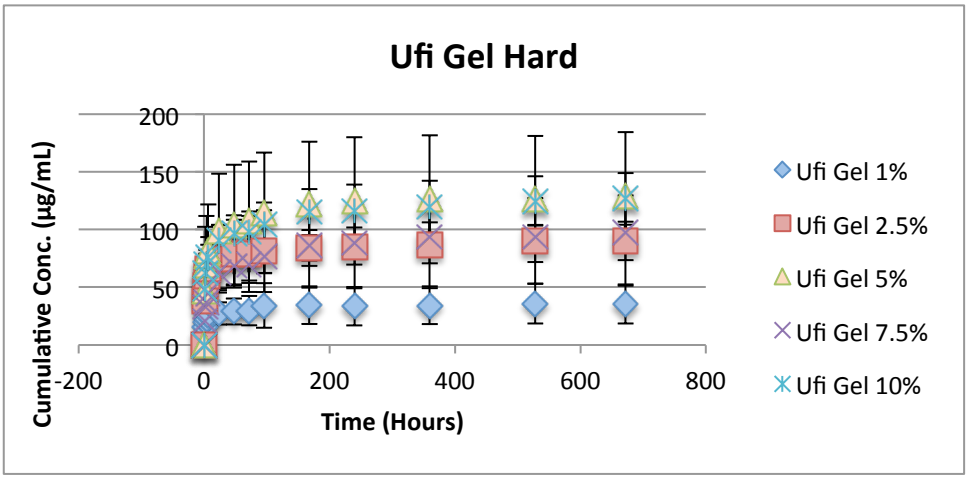

Figure 4.10 – Cumulative concentration of CHX from Ufi Gel Hard specimens as a function of drug loading (1%, 2.5%, 5%, 7.5% and 10%).

After analysis of the CHX release at 24h, it is conclusive that only for Kooliner it is observed a linear relation between drug loading and release (Figures 4.11, 4.12 and 4.13).

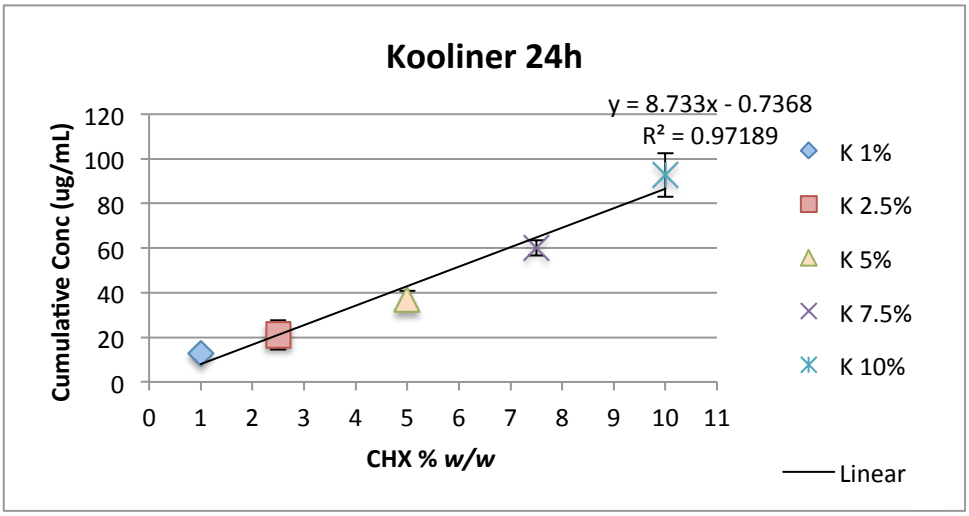

Figure 4.11 – Linear relationship between drug loading and CHX release, with Kooliner specimens at 24h.

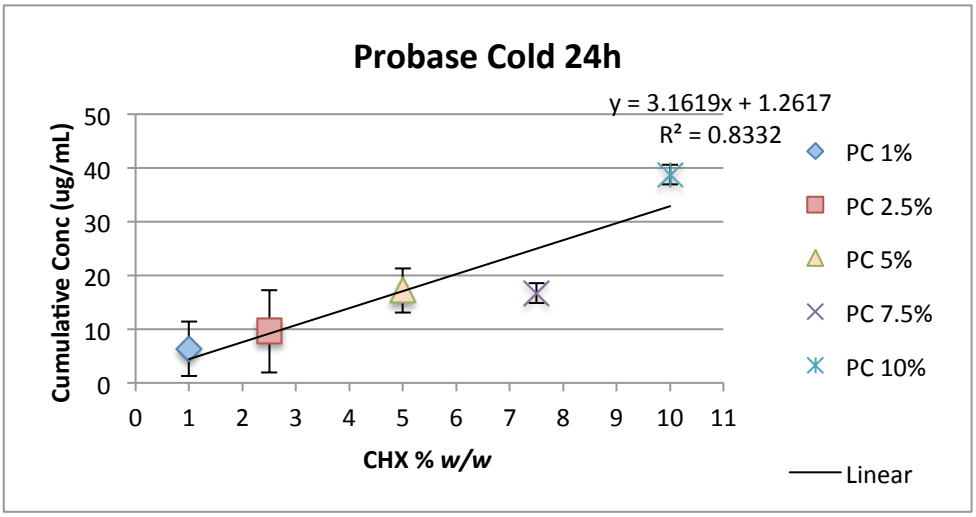

Figure 4.12 – No linear relationship between drug loading and CHX release, with Probase Cold specimens at 24h.

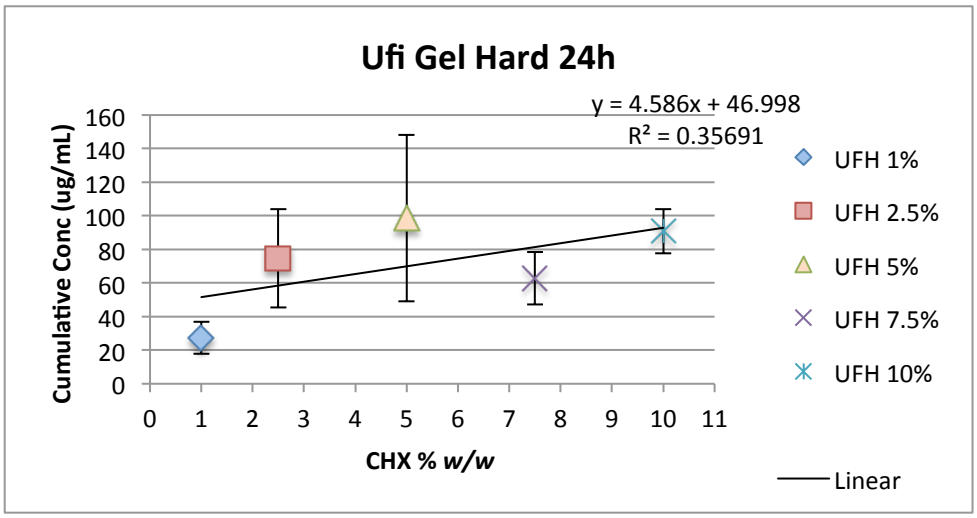

Figure 4.13 – No linear relationship between drug loading and CHX release, with Ufi Gel Hard specimens at 24h

5. Discussion

The use of polymers as drug delivery sistems for slow release of antifungal drugs, in order to treat oral infections, is an ongoing area of research (Li *et al.* 2009; Salim, Satterthwaite, *et al.* 2012b). Several studies investigated the release of antifungal agentes, such as Fluconazole, Nystatin or Chlorhexidine, from denture acrylic resins and its effect on the inhibition of *C.albicans* (Riggs *et al.* 2000; Patel *et al.* 2001; Hiraishi *et al.* 2008; Ryalat *et al.* 2011; Salim, Moore, *et al.* 2012a; Salim, Moore, *et al*. 2013a; Salim, Silikas, *et al.* 2013b; Bertolini *et al.* 2014). Chlorhexidine has been refered to be superior to others antifungal drugs, both on releasing and microbiological tests (Amin *et al.* 2009; Redding *et al.* 2009; Salim, Moore, *et al.* 2012a; Salim, Moore, *et al.* 2013a; Salim, Silikas, *et al.* 2013b).

However, most of the studies use distilled water as media solution and there weren't found studies that evaluate the release of CHX to saliva (Hiraishi *et al.* 2008; Salim, Moore, *et al.* 2012a; Bertolini *et al.* 2014). In the present study, artificial saliva at pH 7 was used in order to simulate oral cavity conditions, since it has properties that can influence the results, such as viscosity. In addition, it is a 28-day study, which allow comparations with other investigations. Five different CHX % are evaluated, as opposed to other studies that only assessed the release of CHX in one concentration. This point is very important in order to find the minimum concentration that is effective against *C. albicans* and, at the same time, prevents an alergic reaction by the host.

The purpose of this study was to evaluate the effect of different materials composition and drug loading on the release of chlorhexidine from acrylic reline resins.

In the present study, for all the evaluated acrylic reline resins, a high rate of initial release was followed by a slower and steadier release that continued until the end of the study period. This is in agreement with previous studies that associate this change in the rate of drug release with the fact that CHX release is controlled by a concentration dependent diffusion process (Anusavice *et al.* 2006; Gong *et al.* 2007; Hiraishi *et al.* 2008; Amin *et al.* 2009; Li *et al.* 2009; Ryalat *et al.* 2011; Salim, Moore, *et al.* 2012a; Bertolini *et al.* 2014). Some studies suggest that the mechanism of release seemed to have two phases: a rapid behavior obeying Fick's law, followed by the development of discrete clusters of the immersion liquid at unidentified osmotically active sites (Riggs

et al. 1999; Patel *et al.* 2001; Anusavice *et al.* 2006; Amin *et al.* 2009; Ryalat *et al.* 2011). Studies have also reported that the rapid elution phase from the drug containing specimens probably reflects surface release, since CHX there is most readily released. The subsequent slow phase of controlled release may be the result of the drug diffusion from the core of the polymer by complex processes involving fluid cluster formation around the CHX molecules and the interaction of these clusters with the fluid uptake process (Addy and Thaw 1982; S. J. Wilson and H. J. Wilson 1993; Riggs *et al.* 1999; Riggs *et al.* 2000; Patel *et al.* 2001; Amin *et al.* 2009; Salim, Moore, *et al.* 2012a;).

In the present study, the results showed that in all the materials and CHX %, the greatest amount of CHX release occurred within the first 24-48h of incubation. For example, analyzing the three materials with CHX 1%, of the total amount of CHX leached, 82.41% from U, 85.63% from K and 69.52% from PC was released during the first 48h of incubation. Patel *et al.*, 2001 also had similar results, refering that, in the first 24h, it was released between 50-80% of the total amount of CHX leached.

Between the materials evaluated in the present study, U released the highest amount of CHX, followed by K and PC. This was verified for all CHX % and can be associated with the different composition of the acrylic resins. Both U and K are PEMA based materials, which are known for them anomalous water uptake behaviour (Riggs *et al.* 2000; Patel *et al.* 2001; Salim, Moore, *et al.* 2012a; Salim, Satterthwaite, *et al.* 2012b) that makes them have superior drug release characteristics compared with methylmethacrylate based materials, as PC (Patel *et al.* 2001). Studies suggest that the addition of CHX to the PEMA based materials greatly increases the water uptake and that this is probably due to the formation of droplets around CHX particles. Then, the droplets expand because of water diffusing through the polymer, along with an osmotic gradient between the internal droplet and external solutions. Another factors that may influence the water uptake are the hydrophilicity of the materials (Hiraishi *et al.* 2008) and the solubility of the impregnated drug. CHX is characterized by higher water solubility when compared with other drugs, like fluconazole (Salim, Satterthwaite, *et al.* 2012b). In respect of PMMA based materials, it has been shown that when droplet expansion occurs in excess, cracks are formed, which enable the release of the drug from the material (Addy and Thaw 1982; Riggs *et al.* 2000; Patel *et al.* 2001; Amin *et al.* 2009; Salim, Satterthwaite, *et al.* 2012b). The same mechanism can be presumed to happen with artificial saliva as media solution, however, water absorption from saliva is much lower than from distilled water due to the increased osmolarity of this external

solution (Patel *et al.* 2001; Amin *et al.* 2009; Salim, Satterthwaite, *et al.* 2012b). Bertolini *et al.*, 2014 also achieved higher CHX release values from a PEMA liner when compared with a PMMA based material.

Our results also showed that, despite the pattern of release from U and K was similar at all times, PC release appears to continue in higher rates until the end of the study. This is probably associated with the different materials composition as well. So, although PC has a lower release of CHX, it seems to be longer than the other materials.

In addition, the results showed that, with an increase on drug loading concentrations, higher CHX release was detected. This indicates that the release of CHX is drug loading-dependent. Other studies also came to the same conclusion (Patel *et al.* 2001; Bertolini *et al.* 2014). This can be related to the fact that the water uptake from saliva increases with an increase in the amount of CHX loading (Patel *et al.* 2001) and, as refer above, a higher water uptake is associated to a higher CHX release (Hiraishi *et al.* 2008). Some authors also report that the drug incorporation increases porosity of the material, which encourages water uptake and, consequently, CHX diffusion (Addy and Thaw 1982; Anusavice *et al.* 2006; Gong *et al.* 2007; Hiraishi *et al.* 2008; Amin *et al.* 2009).

Although the release from K and PC, with respect to CHX % was similar, U specimens with CHX 5% had a superior release than all the other CHX % of the same material, at all time intervals. This is probably related to the fact that the mixture of CHX into de resin's powder was not the ideal, leading to a higher CHX incorporation of some cylinders rather than others.

The results also showed that only for K was observed a linear relation between drug loading and release. Other author (Bertolini *et al*. 2014) reported a linear trend line between these two variables as well.

Besides CHX release, residual unpolymerized monomer has been reported to be released from the acrylic resins as well (Riggs *et al.* 2000; Anusavice *et al.* 2006; Amin *et al.* 2009) and, likewise, it appears to increase with CHX loading. Studies correlate this to a slightly inhibition of polymerization because of the addition of CHX (S. J. Wilson and H. J. Wilson 1993; Riggs *et al.* 2000; Anusavice *et al.* 2006; Hiraishi *et al.* 2008).

In the present study, the maximum cumulative release was only 1.77% from U, which means that only a small amount of initial loaded CHX was released to the artificial saliva. This percentage is lower than other studies and it may be due to the smaller dimensions of the specimens and to the releasing solution used (Patel *et al.* 2001; Salim, Moore, *et al.* 2012a). Since saliva has an increased viscosity when compared to distilled water, it is possible that, at some point, the saliva fills the porosity present in the acrylic resins and hinders the CHX diffusion to the media.

However, the amounts of CHX released appear to be enough. Since it was not possible to perform microbiological studies, it was assessed a comparison between the cumulative concentrations of CHX and its mean minimum inhibitory concentration at 24h and 48h of incubation, achieved in Salim *et al.* 2013a study for 32 *C. albicans* isolates. This comparison showed that, for all the materials, even with lower CHX % (1% *w/w*), the cumulative concentration of CHX was superior than its MIC values. Salim *et al.*, 2012a also reports that the amount of CHX released exceeded the MIC values of all isolates. This is an interesting finding, since it indicates that CHX 1% could be enough to inhibit *C. albicans*. This result encourages the use of CHX in low concentrations, reducing the risk of developing an allergic reaction by the host, yet possessing an effective antifungal potencial (Amin *et al.* 2009; Bertolini *et al.* 2014). It can also reduce the release of residual monomer (Riggs *et al.* 2000). When compared the three materials with CHX 1%, data analysis showed that the release of CHX from U was significantly higher than both K and PC and that, between K and PC, there were no differences. This means that, in one hand U with CHX 1% have higher releases but, on the other hand, PC with CHX 1% appears to continue the release with higher rates. So, U with CHX 1% is an efficient choice for an acute denture candidosis because it will provide higher amounts of CHX released. Then, it can be further replaced by PC with CHX 1%, in order to maintain the release and prevent relapses.

In respect of study limitations, despite the specimens used in this *in vitro* study are smaller than the actual denture surface, the CHX is released from all the cylinders 'superficies, while in the clinical situation only one surface is releasing the drug. So, although it is known that a smaller surface area reduces the drug release by exposing less drug particles to the saliva (Salim, Moore, *et al.* 2012a), this can be compensated by the higher number of surfaces releasing CHX. In addition, as referred above, the mixture of CHX into the resin's powder was not perfect and brought some modifications to the results. Despite some studies refer that the polymerization of the

acrylic resins don't adversely affect the efficacy of the antifungal agents (Patel *et al.* 2001; Amin *et al.* 2009; Salim, Moore, *et al.* 2012a), this could only be possible to conclude with the addition of a microbiological study. So, future studies should be carried out with specimens more similar to the denture surfaces, should test the release at different pH values (since in case of infection, the oral cavity pH is lower) and should associate microbiological and biocompatibility tests.

To resume, CHX delivery systems based on acrylic reline resins could potentially be used for the treatment of denture stomatitis, since a sustained and controlled elution of CHX maintains an effective and gradually increasing concentration of the drug, at the exact site of pathology. Clinical studies are essential, in order to guide the implementation of this systems in clinical practice.

6. Conclusions

Within the limitations of this study, the main conclusions are:

- For all the evaluated acrylic reline resins, a high rate of initial release was followed by a slower and steadier release that continued until the end of the study period.
- The greatest amount of chlorhexidine release occurred within the first 24-48h of incubation, in all the materials and chlorhexidine percentages.
- Different acrylic reline resins composition affects the drug release. Ufi Gel Hard revealed the highest amounts of chlorhexidine released.
- The different chlorhexidine loading percentages affect the drug release, since CHX release was shown to be drug loading-dependent.
- The maximum cumulative release was only 1.77%, which means that only a small amount of initial loaded chlorhexidine was liberated to the media.
- The three materials incorporated with chlorhexidine 1%, had cumulative concentrations superior than MIC values against *C. albicans* isolates.

Overall, the results indicate that CHX delivery systems based on acrylic reline resins are a potential approach in the treatment of denture stomatitis.

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Appendices

Appendix 1 – List of Tables

Appendix 2 – List of Figures

Appendix 3 – List of Abbreviations

1,6-HDMA	1,6-hexanedioldimethacrylate
C. albicans	*Candida albicans*
CHX	Chlorhexidine
h	Hours
IBMA	Isobutylmethacrylate
K	Kooliner
L	Liquid
M	Mean
MIC	Minimum inhibitory concentration
MMA	Methylmethacrylate
P	Powder
PC	Probase Cold
PEMA	Polyethylmethacrylate
PMMA	Polymethylmethacrylate
SD	Standard deviation
U	Ufi Gel Hard

Appendix 4 - Experimental Data

1. Kooliner results

Time intervals (hours)	Kooliner 1%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	1,747894103	0,754028829	0,087394705	
2	6,235018903	1,535665796	0,311750945	0,07678329
4	9,235599342	5,133754597	0,461779967	0,25668773
7	10,56826405	2,317854164	0,528413202	0,115892708
24	12,69407734	2,661258737	0,634703867	0,133062937
48	11,73519134	3,623285195	0,586759567	0,18116426
72	12,85841213	3,716730891	0,642920606	0,185836545
96	12,62957452	3,32681913	0,631478726	0,166340956
168	12,51318268	2,833189212	0,625659134	0,141659461
240	13,14121715	3,644735427	0,657060858	0,182236771
360	14,37873712	3,758331359	0,718936856	0,187916568
528	13,55369744	3,504592471	0,677684872	0,175229624
672	13,70879839	3,66010204	0,685439919	0,183005102

Time intervals (hours)	Kooliner 2.5%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	8,130766145	3,72231364	0,162615323	
2	11,25767284	0,711261814	0,225153457	0,014225236
4	14,73055142	0,698208591	0,294611028	0,013964172
7	16,79400839	0,964196024	0,335880168	0,01928392
24	21,05995509	6,511459536	0,421199102	0,130229191
48	20,4688187	3,865993205	0,409376374	0,077319864
72	20,43514487	4,554676407	0,408702897	0,091093528
96	21,39144308	3,70842711	0,427828862	0,074168542
168	21,45384278	3,736824767	0,429076856	0,074736495
240	21,62212546	4,850920522	0,432442509	0,09701841
360	22,50651566	4,617873478	0,450130313	0,09235747
528	21,90734526	4,455094196	0,438146905	0,089101884
672	25,18853258	6,793366024	0,503770652	0,13586732

Time intervals (hours)	Kooliner 5%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	18,59606899	2,597466722	0,18596069	
2	25,77941687	1,898389453	0,257794169	0,018983895
4	29,97893538	0,309133926	0,299789354	0,003091339
7	32,14402338	1,890172669	0,321440234	0,018901727
24	36,85872492	4,044406703	0,368587249	0,040444067
48	39,06062191	3,190442544	0,390606219	0,031904425
72	39,77930599	3,096868114	0,39779306	0,030968681
96	39,97363047	2,437297273	0,399736305	0,024372973
168	39,01761138	2,5405866	0,390176114	0,025405866
240	41,69405626	2,061022009	0,416940563	0,02061022
360	42,61055655	2,758300382	0,426105566	0,027583004
528	44,6783584	7,649507496	0,446783584	0,076495075
672	43,40620932	5,553973363	0,434062093	0,055539734

Time intervals (hours)	Kooliner 7.5%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	32,83594063	6,29750371	0,218906271	
2	40,18586274	0,377985663	0,267905752	0,002519904
4	46,00155562	1,781532537	0,306677037	0,011876884
7	51,30056574	3,230680535	0,342003772	0,02153787
24	60,0943134	3,418746799	0,400628756	0,022791645
48	60,90341173	3,961593444	0,406022745	0,026410623
72	60,86570816	4,387241714	0,405771388	0,029248278
96	60,80089032	3,722340112	0,405339269	0,024815601
168	61,41562563	5,124053449	0,409437504	0,034160356
240	65,08048282	5,29118497	0,433869885	0,035274566
360	66,21886957	5,917030773	0,44145913	0,039446872
528	65,0612475	5,630502789	0,43374165	0,037536685
672	65,88146985	4,091834139	0,439209799	0,027278894

Time intervals (hours)	Kooliner 10%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	48,15483353	10,11113578	0,240774168	
2	61,2662179	3,758546688	0,306331089	0,018792733
4	68,65168438	6,894786211	0,343258422	0,034473931
7	78,94598896	9,001148447	0,394729945	0,045005742
24	92,66676824	9,791149215	0,463333841	0,048955746
48	101,1567532	11,02354512	0,505783766	0,055117726
72	103,5413112	12,76307377	0,517706556	0,063815369
96	103,1835348	10,2928425	0,515917674	0,051464212
168	106,8439489	12,54156102	0,534219745	0,062707805
240	108,8425615	11,93995234	0,544212808	0,059699762
360	113,5629112	12,7683469	0,567814556	0,063841735
528	112,8185133	13,6165222	0,564092566	0,068082611
672	115,6572897	13,9340737	0,578286449	0,069670368

2. Ufi Gel Hard results

Time intervals (hours)	Ufi Gel Hard 1%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	15,96470116	8,500976792	0,798235058	
2	19,8369067	6,456586735	0,991845335	0,322829337
4	22,38172317	7,920489481	1,119086159	0,396024474
7	25,74299263	8,564352269	1,287149631	0,428217613
24	27,37400322	9,636875384	1,368700161	0,481843769
48	29,14161858	11,31962057	1,457080929	0,565981028
72	29,65209038	12,57648589	1,482604519	0,628824294
96	34,32026856	19,36890249	1,716013428	0,968445125
168	34,47085785	16,25557499	1,723542893	0,812778749
240	33,63417762	16,77563916	1,681708881	0,838781958
360	34,43587582	16,30051672	1,721793791	0,815025836
528	35,87457926	17,23863833	1,793728963	0,861931916
672	35,36185013	16,88888376	1,768092506	0,844444188

Time intervals (hours)	Ufi Gel Hard 2.5%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	37,97833935	16,37284614	0,759566787	
2	51,33999022	17,40067616	1,026799804	0,348013523
4	59,63747778	23,01659121	1,192749556	0,460331824
7	65,94574111	27,71914001	1,318914822	0,5543828
24	74,62304273	29,18426949	1,492460855	0,58368539
48	79,0056433	29,53829757	1,580112866	0,590765951
72	79,00715807	33,04872259	1,580143161	0,660974452
96	81,21397787	35,3277049	1,624279557	0,706554098
168	84,02549337	34,53826369	1,680509867	0,690765274
240	84,82916599	35,75807334	1,69658332	0,715161467
360	86,82815729	37,57749046	1,736563146	0,751549809
528	90,19443497	37,01629656	1,803888699	0,740325931
672	90,38689731	39,14084594	1,807737946	0,782816919

Time intervals (hours)	Ufi Gel Hard 5%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	46,6706779	27,5613551	0,466706779	
2	69,09931926	33,46841957	0,690993193	0,334684196
4	76,89138182	35,14997447	0,768913818	0,351499745
7	82,09596349	39,69825009	0,820959635	0,396982501
24	98,58516155	49,6689541	0,985851615	0,496689541
48	103,9190286	52,27018598	1,039190286	0,52270186
72	107,2842489	51,41195041	1,072842489	0,514119504
96	114,3339837	52,15194131	1,143339837	0,521519413
168	122,3982903	53,89011358	1,223982903	0,538901136
240	124,6914492	55,21417216	1,246914492	0,552141722
360	126,053746	55,43299059	1,26053746	0,554329906
528	126,3416278	54,6638763	1,263416278	0,546638763
672	129,0178072	55,52292652	1,290178072	0,555229265

Time intervals (hours)	Ufi Gel Hard 7.5%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	20,93862816	17,19368799	0,139590854	
2	33,29892987	14,64382157	0,221992866	0,097625477
4	37,21490506	15,815095	0,248099367	0,105433967
7	44,71655771	14,26993226	0,298110385	0,095132882
24	62,75470921	15,52904956	0,418364728	0,103526997
48	68,30509286	15,89467874	0,455367286	0,105964525
72	69,80947478	16,03869364	0,465396499	0,106924624
96	76,61888608	14,34925648	0,510792574	0,09566171
168	86,29851042	13,13548613	0,575323403	0,087569908
240	88,49563558	13,44540077	0,589970904	0,089636005
360	93,440942	13,10561483	0,622939613	0,087370766
528	93,27789345	11,01179489	0,621852623	0,073411966
672	97,23820282	9,791908139	0,648254685	0,065279388

Time intervals (hours)	Ufi Gel Hard 10%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	48,61612515	16,77020626	0,243080626	
2	62,67008177	9,258544969	0,313350409	0,046292725
4	69,60578342	9,377113225	0,348028917	0,046885566
7	76,03186661	11,00192089	0,380159333	0,055009604
24	90,88865359	13,27419222	0,454443268	0,066370961
48	96,71095819	15,56218151	0,483554791	0,077810908
72	97,89386764	17,68430657	0,489469338	0,088421533
96	104,8608841	18,4223329	0,524304421	0,092111665
168	115,3412614	20,03365533	0,576706307	0,100168277
240	116,5243107	22,22738216	0,582621553	0,111136911
360	119,8518414	22,6055675	0,599259207	0,113027837
528	124,6734316	21,38273817	0,623367158	0,106913691
672	126,7208959	22,50506171	0,633604479	0,112525309

3. Probase Cold results

Time intervals (hours)	Probase Cold 1%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	2,174087445	2,620376664	0,108704372	
2	5,95490725	6,105868631	0,297745362	0,305293432
4	3,401211315	2,922820897	0,170060566	0,146141045
7	7,167757645	3,66420778	0,358387882	0,183210389
24	6,315936015	5,043916434	0,315796801	0,252195822
48	8,61611667	4,990425217	0,430805833	0,249521261
72	8,245497758	3,582760802	0,412274888	0,17913804
96	9,092608056	3,682365704	0,454630403	0,184118285
168	10,13960109	3,424735959	0,506980054	0,171236798
240	10,02666872	2,87663637	0,501333436	0,143831818
360	12,39195064	2,542592789	0,619597532	0,127129639
528	11,62009971	2,266925536	0,581004985	0,113346277
672	12,40143513	2,09408088	0,620071756	0,104704044

Time intervals (hours)	Probase Cold 2.5%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	4,356197353	2,920626208	0,087123947	
2	10,80148418	12,57093759	0,216029684	0,251418752
4	7,992886612	7,14734503	0,159857732	0,142946901
7	7,928282992	7,475509398	0,15856566	0,149510188
24	9,575843359	7,642018056	0,191516867	0,152840361
48	11,33985629	7,343314695	0,226797126	0,146866294
72	11,96386879	8,371945669	0,239277376	0,167438913
96	12,7697662	8,702115068	0,255395324	0,174042301
168	15,16897475	8,68732838	0,303379495	0,173746568
240	15,25912727	8,162560397	0,305182545	0,163251208
360	16,37713848	8,322632879	0,32754277	0,166452658
528	18,17990359	7,747439411	0,363598072	0,154948788
672	19,40386337	8,087466182	0,388077267	0,161749324

Time intervals (hours)	Probase Cold 5%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	6,317689531	2,403487575	0,063176895	
2	8,412374625	1,658677003	0,084123746	0,01658677
4	8,310924774	2,12248319	0,083109248	0,021224832
7	8,470254704	3,480465415	0,084702547	0,034804654
24	17,1917635	4,1077119	0,171917635	0,041077119
48	19,62165978	4,954768634	0,196216598	0,049547686
72	19,46118346	5,435107146	0,194611835	0,054351071
96	23,37603634	4,447190756	0,233760363	0,044471908
168	26,63883049	4,978043718	0,266388305	0,049780437
240	27,33769273	6,0319067	0,273376927	0,060319067
360	30,64321473	7,062490252	0,306432147	0,070624903
528	34,20745271	7,806724404	0,342074527	0,078067244
672	35,47355978	8,128105997	0,354735598	0,08128106

Time intervals (hours)	Probase Cold 7.5%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	6,84316085	3,550454796	0,045621072	
2	8,124226688	1,454085712	0,054161511	0,009693905
4	8,89546845	2,210369839	0,059303123	0,014735799
7	10,56785728	3,8038362	0,070452382	0,025358908
24	16,69464956	1,847906597	0,111297664	0,012319377
48	20,8629724	2,724128359	0,139086483	0,018160856
72	23,18142182	3,516319361	0,154542812	0,023442129
96	27,87205468	3,661584337	0,185813698	0,024410562
168	31,62863273	2,436432055	0,210857552	0,01624288
240	33,59481186	4,204358739	0,223965412	0,028029058
360	38,17370832	4,37729826	0,254491389	0,029181988
528	40,72353994	4,72791961	0,271490266	0,031519464
672	44,08114215	6,050734118	0,293874281	0,040338227

Time intervals (hours)	Probase Cold 10%			
	M (cumulative concentration)	SD (cumulative concentration)	M (CHX % released)	SD (CHX % released)
0	0	0	0	0
1	12,02567188	4,714352386	0,060128359	
2	18,45494524	0,50958368	0,092274726	0,002547918
4	19,38636488	2,182144464	0,096931824	0,010910722
7	25,73102608	0,9551934	0,12865513	0,004775967
24	38,7399678	1,799388113	0,193699839	0,008996941
48	45,03769576	2,560432966	0,225188479	0,012802165
72	46,96367763	2,230534393	0,234818388	0,011152672
96	52,75960865	2,303955641	0,263798043	0,011519778
168	60,25793997	3,372278728	0,3012897	0,016861394
240	62,60773205	3,060895572	0,31303866	0,015304478
360	68,71331279	5,179176362	0,343566564	0,025895882
528	69,40154549	5,141574304	0,347007727	0,025707872
672	72,52870929	5,018314845	0,362643546	0,025091574