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ORGINAL RESEARCH

Radioactive Permeability Studies of Doxycycline Hyclate from Microemulsion and Solution

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ABSTRACT

Doxycycline Hyclate (DOX) is an antibacterial drug which is member of the second tetracycline group and the absorption of DOX is reduced about 20% in the presence of skimmed milk. Therefore new drug delivery system can be advisable for DOX to reduce the food and drug interaction and improve the bioavailability. The aim of the present study is to prepare a microemulsion system of DOX which could result in reducing in food interaction and an improvement of oral bioavailability by increasing the drug's permeability. For this purposes, DOX was radiolabeled with ^{99m}Tc. Radiochemical purity was determined with radioactive thin layer chromatography (RTLC) studies. Permeability of DOX from ^{99m}Tc-DOX solution (^{99m}Tc-DOX-S) and ^{99m}Tc-DOX loaded microemulsion (^{99m}Tc-DOX-M) was investigated with *in vitro* cell culture studies by using human colonic adenocarcinoma cell line (Caco-2). The radioactivity of ^{99m}Tc-DOX-M for the apical to basolateral direction (Papp (A→B)) and basolateral to apical direction (Papp (B→A)) were found higher than ^{99m}Tc-DOX-S. Based on the *in vitro* cell culture studies, this dosage form is a promising formulation as an alternative for oral drug delivery of DOX.

Keywords: Doxycycline Hyclate, Permeability, Caco-2, Microemulsion, ^{99m}Tc.

Introduction

The tetracyclines including the glycylcyclines represent a large group of antibacterial which can be divided into three groups (1). Based on their pharmacokinetic and antibacterial properties, a broad-spectrum oxytetracycline synthetic derivative antibiotic Doxycycline Hyclate (DOX) is a member of the second tetracycline group (2). While first group consists of the older agents (like tetracycline, oxytetracycline) second group drugs' are 3–5 times more lipophilic than group 1. It was approved by the American Food and Drug Administration (FDA) in 1967 and is still used to treat a wide variety of bacterial infections and for the prophylaxis of malaria in short-term travelers (3).

Although the tetracycline antibiotics may be bactericidal to some microorganisms at high concentrations, their activity is primarily bacteriostatic (4). It is therefore important that circulating levels of these compounds be maintained above the minimum inhibitory concentration for pathogenic

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organisms during a course of treatment. Milk and other dairy products inhibit the absorption of tetracyclines to varying degrees. As an illustration Rosenblatt *et al*. reported that the absorption of DOX was reduced by about 20% in the presence of skimmed milk (4). Therefore new drug delivery system can be advisable for DOX to reduce the food and drug interaction and improve the bioavailability.

In recent years, there is a challenge for novel drug delivery systems to achieve improved bioavailability and safety. Microemulsions are currently of interest to the pharmaceutical scientist because of their considerable potential to act as drug delivery vehicles by incorporating a wide range of drug molecules. These systems are clear, stable, isotropic mixtures of oil, water and surfactant, frequently in combination with a cosurfactant (5-8). Microemulsions offer an interesting and potentially quite powerful alternative carrier system for drug delivery because of their high solubilization capacity, transparency, thermodynamic stability, ease of preparation, and high diffusion and absorption rates when compared to solvent without the surfactant system (9). Herein, we report on the preparation of a microemulsion system of DOX which could result in reducing in food interaction and an improvement of oral bioavailability by increasing the drug's permeability.

In the development of drugs radionuclides are applied as signal sources since they can be incorporated into the formulation without any change of their characteristics. The main benefit of using radiolabeled compounds in drug development is to be sensitive and detectable for minimal amounts. The development of powerful radiotracers requires careful consideration in the selection of the radionuclide (10). Choice of a suitable radionuclide for radiolabeling studies can be ascertained by considering factors such as the radiation energy, half-life, and extent of particulate radiation, cost and availability. Technetium-^{99m} (^{99m}Tc) is the most popular radionuclide with its versatile chemistry, near-ideal energy (140 keV), low radiation dose and short half-life (6 h) (11,12).

The aim of this study was to assess the permeability of DOX microemulsion and solution with radioactive studies. For this purposes, DOX was radiolabeled with ^{99m}Tc. Radiochemical purity was determined with radioactive thin layer chromatography (RTLC) studies. Permeability of DOX from ^{99m}Tc-DOX solution (^{99m}Tc-DOX-S) and ^{99m}Tc-DOX loaded microemulsion (^{99m}Tc-DOX-M) was investigated with *in vitro* cell culture studies by using human colonic adenocarcinoma cell line (Caco-2).

Materials and Methods

Materials

All chemicals and solvents were used without further purification. DOX was obtained from AppliChem (Germany). Stannous tartrate was purchased from Sigma-Aldrich (USA) and ascorbic acid was purchased from Sigma-Aldrich (United Kingdom). ^{99m}Tc was eluted from the Molybdenum-99 (^{99}Mo)/^{99m}Tc-generator (Nuclear Medicine Department of Ege University). Soybean oil, tween 80 and colliphor EL were obtained from Sigma Aldrich. Transcutol was obtained as a gift from GatteFosse. Cell culture reagents were obtained from Gibco Invitrogen (Grand Island, NY). Caco-2 was obtained from American Type Culture Collection (ATCC). Results are reported as mean ± standard error.

1.1 Radiolabeling Studies

^{99m}Tc-DOX was prepared using previously published method by İlem-Özdemir *et al.* (13,14). ^{99m}Tc-DOX was prepared from lyophilized cold kit, which is included; 1 mg DOX, 30 µg stannous tartarate and 0.1 mg ascorbic acid. 1 mL of 0.9% sodium chloride was injected into the kit to dissolve the dried powder. Then the content was radiolabeled with (370 MBq) ^{99m}Tc. The vial was shaken and incubated at room temperature for 15 min. After incubation the reaction product was filtered through a 0.22 µm pore size filter for sterilization. Radiochemical analysis was performed with RTLC studies.

RTLC Procedure

The radiochemical purity was determined by instant thin layer chromatography. One drop of reaction product was spotted on two (ITLC) chromatographic paper strips (each of 1x8 cm). First strip was developed in acetone while the other one is developed in Acetonitrile/Water/Trifluoroacetic acid (ACN/W/TFA; 50/25/1.5) solvent system. After complete the development both strips were dried and scanned in a TLC scanner (Bioscan AR 2000), % radiochemical purity of ^{99m}Tc-DOX was calculated from the following equation by subtracting from 100 the sum of measured impurities percentages (11,12).

RP (%) = 100 – (Free ^{99m}Tc (%) + R/H ^{99m}Tc (%))

Equation 1

(Radiochemical purity (RP), Reduced/Hydrolyzed ^{99m}Tc (R/H ^{99m}Tc))

1.2 Preparation of ^{99m}Tc-DOX-S

Two milligrams of DOX included ^{99m}Tc-DOX-S was prepared as prescribed above. Then ^{99m}Tc-DOX-S filtered through 0.22 mm membrane sterile filter (Minisart, Sartorius) into a sterile vial under laminar airflow Class II cabinet (EsCo Class 2, Selangor, Malaysia).

1.3 Preparation, Characterization and Stability of ^{99m}Tc-DOX-M

For a phase diagram at a specific surfactant (S)/co-surfactant (CoS) weight ratio, the ratios of oil to the mixture of S/CoS were varied as 1:0.5, 1:1, 2:1, 3:1, 4:1 and 5:1 (w/w). The composition of soybean oil (oil), tween 80 (S), colliphor EL (S) and transcutol (CoS) was mixed and then each mixture was dispersed with 0.9% sodium chloride solution. After the identification of microemulsion region in the phase diagrams, the microemulsion formulation was selected at desired component ratios. ^{99m}Tc-DOX was added to the microemulsion as water phase and formulation filtered through 0.22 µm pore sized membrane sterile filter (Minisart, Sartorius) into a sterile vial under laminar airflow Class II cabinet (EsCo Class 2 ACIIG34M, Selangor, Malaysia).

The microemulsions were analyzed for various physicochemical properties. The droplet size of microemulsion was measured using a Zeta Sizer (3000 HSA, Malvern Instruments, Worcestershire, UK). The viscosity of the microemulsion was measured by using a Brookfield digital viscometer-III rheometer V 3.3 HB (Middleboro, MA). The refractive index and electric conductivity of the microemulsion were measured by using refractometer (Atago RX-7000 CX, Japan) and monitored quantitatively by using conductometer (Jenway 4071, UK), respectively. The pH of microemulsion was determined using a pH meter (Jenway 3040 Ion Analyze, Mettler-Toledo, Switzerland).

Microemulsion was placed in stability cabins (NUVE, Cabinet ID 300, Ankara, Turkey) for stability test. Stability studies at 25 ± 2 °C temperature and 60 ± 5 % humidity and 40 ± 2 °C temperature and 75 ± 5 % were conducted according to ICH guidelines. Sampling humidity was done at specified intervals over a period of 6 months for short-term and accelerated conditions. The physical stability of formulations was determined the clarity and droplet size studies.

1.4. *In Vitro* Stability Studies of ^{99m}Tc-DOX

To test *in vitro* stability of ^{99m}Tc-DOX during cell culture studies, 0.1 mL ^{99m}Tc-DOX was added 0.9 mL cell media. The mixture was incubated during studies and radiochemical purity of the labeled complex was calculated by RTLC studies up to 2 hours.

1.5. Cell Culture Studies

Caco-2 was obtained from ATCC and cells were grown from passage number 50 to passage number 77. Cell culture was maintained at 37°C under 90% humidity and 5% CO_2. Caco-2 cell monolayers were prepared by seeding $1x10^5$ cells/well on six wells with a transwell insert filter with a collagen-coated polycarbonate membrane with a pore size of 0.4 µm and a surface area of 4.7 cm^2 in cluster. Permeability studies were performed from apical to basolateral (A→B) and basolateral to apical (B→A) directions. 0.2 mCi ^{99m}Tc-DOX-M or ^{99m}Tc-DOX-S was incubated with cells for 120 min at 37°C. Samples were collected at different time intervals (30, 60, 90 and 120 min). Radioactivity of collected samples was counted in a gamma counter (Sesa Uniscaller). The amount of permeably DOX was calculated as the percentage of the activity counted in the samples relative to the total activity incubated in ^{99m}Tc-DOX-M or ^{99m}Tc-DOX-S.

1.6. Statistical Analysis

Statistical analyses were performed with SPSS software, V.19. The P values were calculated, and statistical significance was accepted within 95% confidence limits. All results were reported as means. The comparisons at the different time points in the same group and the comparisons among groups at the same time point were subjected to the Bonferroni test. SPSS 19.0 software (SPSS, Chicago, IL, United States) was used for analysis and $P < 0.05$ was considered statistically significant. Sentence was added to the statistical analysis part of the manuscript.

2. Results and Discussion

2.1. Radiolabeling Studies

An instant cold kit was prepared contained DOX, stannous tartrate and ascorbic acid. DOX was labeled with ^{99m}Tc by a direct labeling method. Labeling efficiency of the ^{99m}Tc-DOX was assessed by RTLC studies. Two solvent systems were used to distinguish and quantify the amounts of radioactive contaminants.

In RTLC, using acetone as the solvent, free ^{99m}Tc moved with the solvent front, while ^{99m}Tc-DOX and R/H ^{99m}Tc remained at the spotting point. R/H ^{99m}Tc was determined by using ACN/W/TFA (50/25/1.5) as the mobile phase where the R/H ^{99m}Tc remained at the point of spotting while free ^{99m}Tc and ^{99m}Tc-DOX moved with the solvent front.

Previously İlem-Özdemir *et al.* Standardized and developed a new, simple and ready to use kit of DOX for radiolabeling with ^{99m}Tc (13,14). Labeling studies were performed by

changing the selected parameters one by one and optimum labeling conditions were determined. After observing the conditions for maximum labeling efficiency and stability, lyophilized freeze dry kits were prepared accordingly. Since the researchers found the labeling efficiency of ^{99m}Tc-DOX higher than 95%, in this study we aimed to label DOX with ^{99m}Tc by using ready to use lyophilized kit.

In this study, the radiochemical purity of ^{99m}Tc-DOX was found greater than 95%, acquired via RTLC studies. Over a period the resulting complex was quite stable and radiochemical purity was found >90 % up to 6 hours without any significant change ($p>0.05$) (Figure 1). So our results are found to be in compliance with the results of the reference methods (13,14).

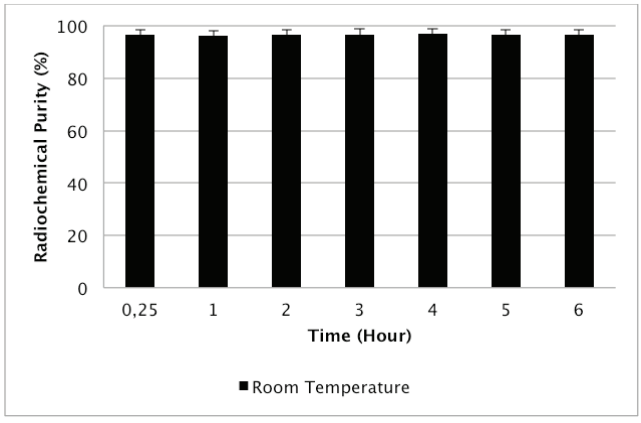

Figure 1. Radiolabeling efficiency and stability of ^{99m}Tc-DOX.

2.2. Stability of ^{99m}Tc-DOX in Cell Media

During incubation in cell media, compound was found stable as determined by RTLC. According to studies, percentage of ^{99m}Tc-DOX was decreased from 87.34 ± 0.62 to 79.50 ± 0.41 within 2 hours.

2.3. Preparation, Characterization and Stability of DOX Microemulsion

In this study, microemulsion was developed with water titration method. The microemulsion system contained soybean oil as oil phase and the mixtures of tween 80-colliphor EL/Transcutol as S/CoS. The Tween 80/Colliphor EL ratio was 1:1 (w/w) and the mixture ratio of surfactants was 1:19 (w/w). According to pseudo-ternary phase diagram results (Figure 2), the ratio of oil, surfactants and co-surfactant of chosen microemulsion were determined as; Soybean oil (7.75 %), Tween 80 (28.50 %), Colliphor EL (1.50 %), Transcutol (30.00 %) and Saline (32.75 %). In addition, when DOX was added to microemulsion system, microemulsion had no change in its transparency.

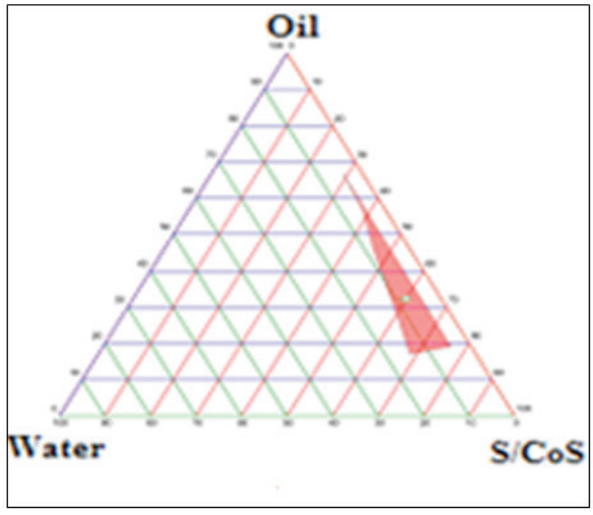

Figure 2. The pseudo-ternary phase diagram of the ideal microemulsion system.

The particle size analysis showed that the mean droplet size of microemulsion was below 100 nm. DOX microemulsion was transparent colloidal dispersions with average diameter of 39.046±0.808 nm. Polydispersity index of both formulations was found less than 0.5. The viscosity of microemulsion was found to be 53.460±0.152. The refractive index of microemulsion was found to be 1.436±0.003. The pH of the microemulsion was found to be around 5.4.

During storage period, the microemulsion was still clear and transparent without any phase separation. The droplet size of microemulsion was not changed significantly at 25±2°C temperature and 60±5% humidity and 40±2°C temperature and 75±5% humidity. It was suggested that microemulsion was stable under these storage time period (Table 1).

Table 1. Stability of optimum microemulsion containing at 25 ± 2 °C temperature and 60 ± 5 % humidity and 40 ± 2 °C and 75 ± 5 % humidity ($p>0.05$)

Month	Droplet size (25 ± 2 °C) (nm) ± ss	Droplet size (40 ± 2 °C) (nm) ± ss
0	39.046 ± 0.808	39.046 ± 0.808
3	39.270 ± 1.121	39.916 ± 1.181
6	39.083 ± 1.104	37.826 ± 0.938

2.4. Permeability Studies for DOX Microemulsion and Solution

The permeability of DOX from microemulsion and solution was evaluated with radioactive studies by using a gamma counter. The permeable amount of ^{99m}Tc-DOX from apical to basolateral direction (A→B) and basolateral to apical direction (B→A) for microemulsion and solution was shown in Figure 2 and Figure 3 respectively.

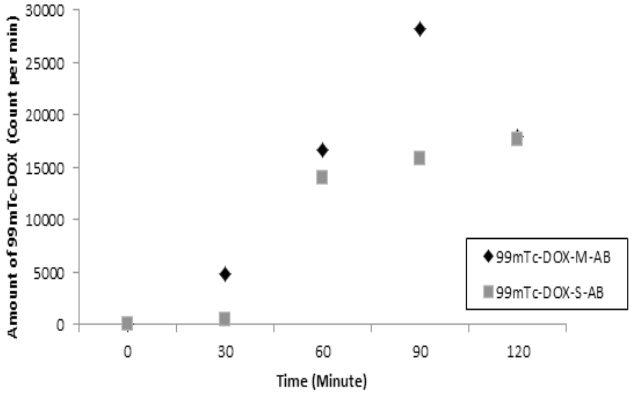

Figure 3. The permeability amount of ^{99m}Tc-DOX from apical to basolateral direction for microemulsion and solution form.

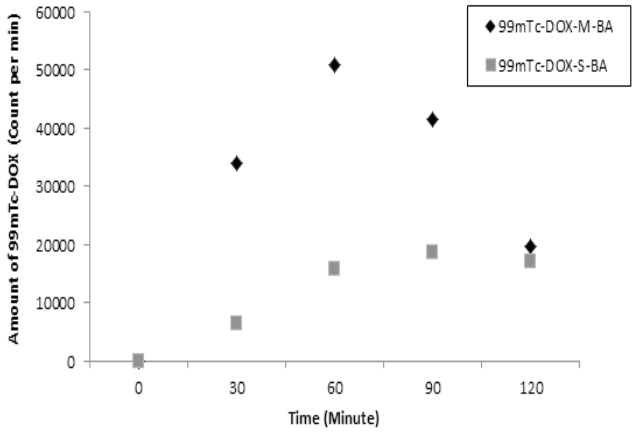

Figure 4. The permeability amount of ^{99m}Tc-DOX from apical to basolateral direction for microemulsion and solution form.

The radioactivity of ^{99m}Tc-DOX-M for the apical to basolateral direction (P_{app} (A→B)) and basolateral to apical direction (P_{app} (B→A)) were found higher than ^{99m}Tc-DOX-S (Figure 3 and 4). According to these results, the use of microemulsion for DOX can be leaded to an enhancement of permeated drug concentration. A similar result concerning the release of a submicron lipid emulsion formulation was also observed (15,16).

Doksisiklin Hiklat'ın mikroemülsiyon ve çözelti dozaj şekillerinden radyoaktif permeabilite çalışmaları

ÖZ

Tetrasiklin grubu üyesi olan Doksisiklin Hiklat'ın (DOX) absorpsiyonu süt ile birlikte alındığında yaklaşık %20 oranında azalmaktadır. Bu nedenle DOX için ilaç besin etkileşimini azaltacak ve biyoyararlanımını arttıracak yeni dozaj şekillerinin geliştirilmesi önerilebilir. Bu çalışmanın amacı, DOX'ın mikroemülsiyon formülasyonunu hazırlayarak, ilaç besin etkileşimini azaltıp, oral biyoyaralanımını arttırmaktır. Bu amaç ile, DOX ^{99m}Tc ile radyoişaretlendi. ^{99m}Tc-DOX kompleksinin radyokimyasal saflığı radyoaktif ince tabaka kromatografisi çalışmaları ile belirlendi. DOX'ın permeabilitiesi, ^{99m}Tc-DOX çözeltisi (^{99m}Tc-DOX-S) ve ^{99m}Tc-DOX yüklü mikroemülsiyon (^{99m}Tc-DOX-M) uygulanan insan kolonik adenokarsinoma (Caco-2) hücre hatları ile yapılan *in vitro* hücre kültürü çalışmaları ile belirlendi. ^{99m}Tc-DOX-M formülasyonu uygulanan hücrelerde apikal yönden bazolateral yöne (Papp (A→B)) ve bazolateral yönden apikal yöne (Papp (B→A)) geçiş ^{99m}Tc-DOX-S uygulanan hücrelerden daha yüksek bulunduğundan, mikroemülsiyon formülasyonunun DOX'ın oral uygulamasında umut vaad edeceği kanaatindeyiz.

Anahtar kelimeler: Doksisiklin Hiklat, Permeabilite, Caco-2, Mikroemülsiyon, ^{99m}Tc.

3. Conclusion

DOX was radiolabeled with high radiochemical purity. The labeled compound was found to be stable in room temperature up to 6 hours and in cell media during the experiments. Based on the *in vitro* cell culture studies, this dosage form is a promising formulation as an alternative for oral drug delivery of DOX.

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