Sys Rev Pharm 2020; 11(5): 458 – 463
A multifaceted review journal in the field of pharmacy
E-ISSN 0976-2779 P-ISSN 0975-8453

Identification and Quantitation of Phospholipid Binding Sites in Lipid Drug Conjugate Pharmacosomes using Model Drug

Abdullah Q. Khudhur[1*], Nidhal K. Maraie[2], Ayad M.R. Raauf[3]
[1*]Department of Pharmaceutics, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq
[2]Department of Pharmaceutics, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq
[3]Department of Pharmaceutical Chemistry, College of Pharmacy, Mustansiriyah University, Baghdad, Iraq
E-mail: qusayabdulla1994@gmail.com
Pharm.dr.nidhal.khazaal@uomustansiriyah.edu.iq
pharm.dr.ayad@uomustansiriyah.edu.iq

Article History: *Submitted: 26.02.2020* *Revised: 16.04.2020* *Accepted: 21.05.2020*

ABSTRACT

Background: Different types of lipids can be conjugated with drugs by covalent or non- covalent association to overcome some problems of drug delivery. The aim of this study is to identify and quantify binding sites of phosphatidylcholine in its conjugate with the model drug mefenamic acid (MA-PC) from which pharmacosomes were prepared and evaluated in comparison with conventional liposomes.

Method: Solvent evaporation method was used to prepare MA-PC conjugate and the conjugation sites were confirmed using DSC, P-XRD, ^{1}H-NMR, ^{13}C-NMR, ^{31}P-NMR, FT-IR, and SEM. Physical properties and dissolution study for the drug, conjugate, and the prepared liposomes were studied.

Results: Conjugation was confirmed between the drug and phosphatidylcholine (PC) through mainly hydrogen bond between –COOH group of mefenamic acid (MA) and phosphate polar head of PC in a ratio of 1:1 mainly. The solubility and partition coefficient of MA in its conjugate were significantly higher than pure MA. Pharmacosomal dispersion was prepared using MA-PC conjugate and evaluated in comparison to conventional liposomes prepared with the same lipid ratio, where it showed higher entrapment efficiency (% EE) than conventional liposomes with more rigid morphology. The sequential in-vitro dissolution profile revealed significantly higher dissolution for pharmacosomes than conventional liposomes and much higher than marketed MA suspension.

Conclusion: The phosphate group in PC is the predominant binding site for its conjugation with MA in 1:1 ratio, conjugation improved the drug physical properties in its conjugate and pharmacosomal dispersion in a significant way than the conventionally prepared liposomes in the same ratio.

Keywords: Binding sites, conjugate, liposomes, mefenamic acid, pharmacosomes, phosphatidylcholine.

Correspondence:
Abdullah Q. Khudhur
Department of Pharmaceutics, College of Pharmacy, Mustansiriyah University
Baghdad, Iraq
E-mail: qusayabdulla1994@gmail.com

DOI: 10.31838/srp.2020.5.63

INTRODUCTION

Lipids are essentially fatty (waxy) materials which are soluble in non-polar solvents and not soluble in organic solvents of high polarity. [1] A promising drug delivery nano carriers were prepared using lipids. The excellent biodegradation (as their origin are natural sources) and high ability to pass the gastrointestinal tract barrier are typical properties of lipids that made it one of the preferable excipients. [2] Different types of lipids such as glyceride, fatty acids, sterols, and phospholipids can form Lipid Drug Conjugates (LDC) or complexes by covalent or non- covalent association with drugs. The development of LDC was to overcome some problems of drug delivery such as drug toxicity, drug targeting, and bioavailability. [3] The conjugation of drugs to lipids can occur through different strategies and chemical linkers, this depends on chemical structure of both the drug and lipid. [4]

Phospholipids (PLs) are amphipathic molecules, they have a polar and a non-polar part in their structures. [5] The superior biocompatibility and amphiphilicity are unique properties of PLs which made them applied in a variety of drug delivery systems. Additionally, PLs act as solubility enhancer, permeation enhancer, and surfactants. [6]

Drugs that have an active hydrogen atom (-COOH, -OH, -NH2, etc.) can interact with phospholipid molecules through van der Waals forces and/or hydrogen bond between the two molecules. [5, 7] Phospholipid-drug complex can be used to form liposomes (pharmacosomes) and to increase the drug loading into lipid-based delivery systems. [5, 8] Pharmacosomes are colloidal vesicular dispersion containing drugs linked with lipids which they assemble into one or more layer to form vesicles. [9] Mefenamic acid (MA); the non-steroidal anti-inflammatory drug was chosen as a model drug since it contains –COOH group in its structure in addition to its poor solubility and bioavailability. [10] The structure of mefenamic acid and phosphatidylcholine are shown in Figure 1.

The aim of this study is to identify and quantify binding sites of phosphatidylcholine in its conjugate with the model drug mefenamic acid (MA-PC) from which pharmacosomes were prepared and evaluated in comparison with conventional liposomes.

MATERIALS AND METHODS

Materials

Reagents and solvents used are analytical grade and commercially available, J.T Baker, Biosolve and Romil. Lipoid S 100 (Phosphatidylcholine from soybean 94%) was provided by Lipoid GmbH, Germany and mefenamic acid purchased from Sigma-Aldrich, Germany.

Preparation of mefenamic acid-phosphatidylcholine conjugate

The solvent evaporation method was used to prepare mefenamic acid-phosphatidylcholine conjugate (MA-PC). Mefenamic acid (MA) and highly purified (94%) soybean phosphatidylcholine (PC) were put in a round bottom flask in a molar ratio of 1:1 (50 mg MA and 163 mg PC) and dissolved in dichloromethane (27 mL). The mixture was put

on a magnetic stirrer for 3 hours at 35 °C. A rotary evaporator was used to remove the solvent under reduced pressure. The resultant lipid film placed in a desiccator overnight. [11]

Effect of the drug: lipid ratio on the formation of drug-lipid conjugate

The stoichiometric ratio for drug and phosphatidylcholine complexation was determined by continuous variation method. [12] Briefly, the dried lipid film (expected to contain MA-PC) was prepared in different molar ratios of drug: lipid (1:1, 1:2, and 2:1) by applying the previously mentioned method. Each of the dried lipid film (for each ratio) was dissolved in dichloromethane. The absorbance of MA in the conjugate was determined by using a UV-visible spectrophotometer at the estimated λmax (280 nm) and subtracted from the absorbance of the total MA added. The higher the absorbance difference the more MA-PC prepared and the best drug: lipid ratio was chosen accordingly. [12]

Characterization of MA-PC

Differential scanning calorimetry (DSC)

Thermal habits of pure MA, PC, MA-PC, physical mixture of MA and PC were carried out with a linseis DSC (STA PT-1000 linseis, Germany). Samples were loaded separately into aluminum pans with a heating rate of 10 °C/min under a nitrogen atmosphere from 25 to 300 °C. [12]

Powder X-Ray diffraction (P-XRD)

A powder X-ray diffractometer (XRD-6000 Shimadzu, Japan) was applied for MA, PC, MA-PC, physical mixture of MA and PC at room temperature. The X-ray generator operated with radiation source of Cu Ka at 45 kV and 30 mA. [12]

Fourier Transform Infrared spectroscopy (FT-IR)

The FT-IR analysis of pure MA, PC, MA-PC, physical mixture of MA and PC were carried out with an FTIR-600 Biotech, UK. Samples were compressed with KBr into pellets and scanned from 400-4000 cm^{-1}.

Nuclear magnetic resonance (NMR) analysis

Proton NMR (^{1}H-NMR) and carbon NMR (^{13}C-NMR) for MA, PC, MA-PC were recorded by Varian NMR (USA) with frequency 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR (USA), deuterated chloroform was used as a solvent and tetramethylsilane (TMS) used as an internal standard. Phosphorous NMR (^{31}P-NMR) for PC and MA-PC were carried out using Bruker NMR (USA) with frequency 200 MHz, acetone was used as a solvent and TMS as an internal standard.

Scanning electron microscopy (SEM)

The surface morphology of MA, PC, MA-PC, physical mixture of MA and PC were observed by using Tescan scanning electron microscope (Tescan, Czech).

Solubility study

The saturated solubility of pure MA and MA-PC in 0.1N hydrochloric acid (HCl) solution pH 1.2, phosphate buffer (PB) solution pH (6.8 and 7.4), and deionized water were determined by adding an excess quantity of MA and MA-PC (each one separately) into volumetric flask containing 25 mL from each medium. The volumetric flask was placed in a magnetic stirrer at a temperature of 25 °C for 24 hours and then sonicated for 10 min in the bath sonicator (where a sufficient stress condition and time to produce the saturated solubility can be provided by this procedure. [13] The solution was purified through a 0.45μm syringe filter, after proper dilution, the absorbance was determined through UV spectrophotometer at the selected λmax and converted into concentrations by using MA calibration curves.

n-Octanol/water partition coefficient study

To determine the oil/water partition coefficient for pure MA and the prepared MA-PC; an excess amount of MA and MA-PC (each one separately) were placed in an equal volume of n-octanol/water system (12.5:12.5 mL) with stirring at 25 °C for 24 hours. The two layers were separated after standing and MA concentration in each layer was determined spectrophotometrically using UV spectrophotometer at the selected λ max. [12]

Preparation of mefenamic acid vesicular systems

Preparation of mefenamic acid pharmacosomes

The resultant dried lipid film containing MA-PC conjugates (prepared as mentioned previously) was hydrated by a phosphate buffer saline solution (PBS, pH 7.4) at a temperature of 55±2 °C. Upon hydration, the lipid film peeled off from the round bottom flask wall and swell to get pharmacosomal dispersion. [14]

Preparation of mefenamic acid conventional liposomes

Lipid film formation technique was used to prepare conventional liposomes of mefenamic acid. Highly purified (94%) soybean PC was dissolved in dichloromethane in a round bottom flask to which MA was incorporated in a ratio of 1:1. The solvent was evaporated using a rotary evaporator and a thin film was formed. The film was hydrated by phosphate buffer saline solution (PBS, pH 7.4) at a temperature of 55±2 °C to get conventional liposomal dispersion. [14]

Characterization of the prepared mefenamic acid vesicular systems

Entrapment efficiency determination

The entrapment efficiency of MA in the prepared pharmacosomes and conventional liposomes was determined by using the centrifugation method. The pharmacosomal and liposomal dispersion (each one separately) were centrifuged at 10000 rpm for 30 minutes using a cooling centrifuge at a temperature of 4 °C. [15] The supernatant layer was separated, diluted appropriately and the absorbance was estimated using a UV visible spectrophotometer at λmax 286 nm. The calibration curve of mefenamic acid was used to calculate drug concentration, then the percent of entrapment efficiency (% EE) was

calculated using the following equation: {%EE = (total drug - free drug) / total drug × 100}

Examination using transmission electron microscopy (TEM)

Pharmacosomal and liposomal dispersions were examined by a transmission electron microscope (Philips, Netherland). A drop from each dispersion was located on carbon-coated copper grids then stained by using 1% phosphotungstic acid. The grid was left to dry and examined. [14]

Zeta potential, Particle size and Polydispersity index determination

The pharmacosomal and liposomal dispersion is milky-white. The sample (from each dispersion separately) was diluted with phosphate buffer saline at pH 7.4, Then the particle size (mean diameter), and polydispersity index (size range of particles) were measured using a dynamic light scattering (DLS) instrument. Zeta potential (particle surface charge) was measured by the same instrument using a cell provided with two electrodes. [16]

In-vitro dissolution study

Dissolution of pure MA, pharmacosomal and liposomal dispersion of MA in comparison to marketed MA suspension (containing 250 mg of MA in each) were carried out using rotating paddle dissolution apparatus type II with 100 rpm. [17] In vitro dissolution study was performed in 900 mL (containing 1% w/v sodium lauryl sulfate) 0.1N HCl pH 1.2 and PB with sequential pH values (5.8, 6.8, and 7.4) for suitable transit time to mimic the gastrointestinal tract (GIT) [18], as shown in Table 1. The medium was preconditioned and retained at a 37 °C. Five mL aliquots were drawn at a suitable time interval for each transit time (Table 1) and filtered using a 0.45 µm filter syringe then replenished with a fresh dissolution medium. The solution drug content was determined spectrophotometrically using a UV-Vis spectrophotometer at the selected λmax.

RESULTS AND DISCUSSION

Preparation of mefenamic acid-phosphatidylcholine conjugate

In this study, phosphatidylcholine complex of mefenamic acid was prepared by the solvent evaporation method and the effect of drug:lipid ratio on the formation of the conjugate (complex) was studied by a continuous variation method. [12] The MA-PC conjugate was prepared in different drug:lipid mole ratios (1:1, 1:2, and 2:1) and exposed to UV absorbance measurement. Figure 2 shows the relation between the drug:lipid molar ratios against the net difference in the absorbance (ΔA). It was found that the absorbance difference (ΔA) between total MA added and MA in the conjugate at the drug:lipid mole ratio of 1:1 was higher than other ratios (1:2, and 2:1), indicating more drug conjugated with phosphatidylcholine at the 1:1 ratio, similar to results observed with probucol. [12] Therefore drug:lipid (1:1) ratio was chosen for further work.

Characterization of (MA-PC) conjugate

The differential scanning calorimetry (DSC) overlay thermogram of pure MA, PC, MA-PC, physical mixture between MA and PC are shown in Figure 3. Pure MA showed two sharp melting endotherms at about 279.2 °C and 232.8 °C which indicates its crystalline state, while PC didn't show any sharp melting event ostensibly due to the amorphous nature of it. Furthermore, the physical mixture showed a peak at 129.2 °C (lower melting point than the individual components); due to the complex that formed by partial interaction between MA and PC in the presence of increasing temperature similar to that observed with rifampicin. [19] Instead, the DSC curve of MA-PC revealed that the original peaks of MA had disappeared indicating an interaction (complex) between MA and PC may be mainly through the development of hydrogen bonds and van der Waals forces. [19]

The powder X-ray diffraction patterns of MA, PC, MA-PC, physical mixture between MA and PC are shown in Figure 4. MA presented intense diffraction peaks which suggest the crystalline behaviour of the drug material while PC showed a broad peak due to its amorphous nature. The MA-PC showed that the crystalline peaks of MA vanished which suggested complete drug amorphization and/or conjugation, while the physical mixture showed the same diffraction peaks of MA (with less intensity due to the presence of PC) indicating that there is no interaction in the physical mixture between MA and PC. [20]

The FT-IR spectra of MA, PC, MA-PC, physical mixture between MA and PC are shown in Figure 5. The spectrum of the physical mixture showed approximately no differences from that of pure MA and PC where the characteristic absorption peaks of MA and PC were still present. On the other hand, in the spectrum of MA-PC, there were some significant changes, the absorption peak of OH stretching band of MA (3300-2500 cm^{-1}) was significantly less intense, OH bending band (1446 cm^{-1}) and C=O stretching band (1651 cm^{-1}) of MA was shifted to higher wavenumber (1452 cm^{-1}) and (1657 cm^{-1}) respectively, while absorption peak of C=O stretching of MA (1329 cm^{-1}) shifted to lower wavenumber (1325 cm^{-1}) with less intensity. Moreover, the absorption peak of the P=O stretching band of PC (1244 cm^{-1}) has been shifted and combined with the (1255 cm^{-1}) absorption band of MA to give a broad peak at (1254 cm^{-1}). These results suggested that there is an interaction between the (COOH) group of MA and the phosphate polar part of the PC which is similarly observed with kaempferol-phospholipid complex. [21]

^{1}H-NMR and ^{13}C-NMR were done for MA, PC, and MA-PC, while ^{31}P-NMR was done for PC and MA-PC. The spectra of ^{1}H-NMR, ^{13}C-NMR, and ^{31}P-NMR are shown in Figures 6 to 8. In ^{1}H-NMR spectra of MA-PC; proton peak at δ 11.21 that represent MA (OH) proton was missing which suggests the involvement of (COOH) group of MA in interaction with PC. NH proton peak of MA (δ 9.15) was shifted downfield to δ 9.39 which can be assigned to the break of an intramolecular hydrogen bond that occurs in MA molecule between the amino and carboxyl groups. [22] In ^{13}C-NMR spectra of MA-PC, carbon peak at δ 173.02 that represent MA (COOH) carbon was shifted upfield to δ 171.24 which confirms the

suggestion of the (COOH) involvement of MA in the interaction. In ^{31}P-NMR spectra of MA-PC, phosphate peak at δ 0.38 that represents phosphorus of PC was shifted to δ -0.14 which indicates the involvement of this group from PC in the interaction with (COOH) of MA. Same results observed with gemcitabine phospholipid complex. [23] The suggested MA-PC structure is shown in Figure 9.

Figure 10 showed the surface morphology of MA, PC, MA-PC, and physical mixture between MA and PC. The surface morphology of MA showed crystals appearance as well as the physical mixture, while the MA-PC showed lack of crystalline appearance of the drug which can be assigned to complexation with PC that masks the crystallinity of the drug and causes polymorphic changes. [20]

Solubility study

Solubility study was performed for MA-PC in comparison with the pure MA, the solubility was investigated in various media including 0.1N HCl solution pH 1.2, PB solution pH (6.8 and 7.4), and deionized water as shown in Table 2. It was found that the solubility of MA-PC was significantly ($p < 0.05$) higher than MA in all the media, where the solubility of MA-PC was approximately 16 times higher than MA in the solution of 0.1N HCl solution pH 1.2 and 5 times higher in PB solution pH (6.8 and 7.4), and deionized water. The improvement in solubility can be explained by the amorphous character of the MA-PC and the amphiphilic nature of PC. The same results observed with naringenin in its phospholipid complex. [25, 26]

n-Octanol/water partition coefficient study

An oil/water partition coefficient (C_o/C_w) study which is used as a measure for lipophilicity. The study was done for pure MA and the prepared MA-PC. As shown in Table 3, the MA-PC had significantly ($p < 0.05$) higher log C_o/C_w (log P) value than MA. The improvement in lipophilicity may be attributed to the masked hydrophilic groups of MA by the interaction (conjugation) between MA and PC. Same results observed with dabigatran etexilate phospholipid complex. [11]

Preparation of mefenamic acid vesicular systems

Two types of vesicular systems for MA were prepared including pharmacosomes (drug-lipid conjugate liposomes) using PC and liposomes (prepared using the conventional thin-film method) using PC also in the same ratio. Characterization of the prepared pharmacosomes was carried out in comparison with the conventionally prepared liposomes to detect the efficacy of each type.

Characterization of the prepared mefenamic acid vesicular systems

Entrapment efficiency (% EE)

Mefenamic acid pharmacosomal and conventional liposomal dispersions show different Entrapment efficiency (% EE) (Table 4), the higher entrapment efficiency in pharmacosomes indicates that the applied technique in the preparation of drug-lipid conjugate is applicable and reliable, while for conventional liposomes the possibility of drug leakage may lead to lower entrapment efficiency. Same results observed with silymarin phytosomes and its corresponding liposomes. [28, 29]

Transmission electron microscopy (TEM)

The mefenamic acid pharmacosomal and conventional liposomal dispersions were examined by TEM and the photographs are shown in Figures 11. The TEM images revealed spherical structures with an inner core surrounded by lighter ridges of phospholipid, indicating vesicle formation. Same results observed with erlotinib conjugated vesicles using phospholipids. [30]

Zeta potential, Particle size and Polydispersity index determination

A laser light scanning instrument was used to analyze the particle size, polydispersity index (PDI), and surface zeta potential of mefenamic acid pharmacosomal and conventional liposomal dispersions, the results are shown in Table 5.

The results showed a difference in average vesicle size between pharmacosomes and conventional liposomes of MA. This can be explained that the conjugation between MA and the lipid leads to larger molecular weight molecules; therefore, generating larger liposomal size upon assembly in comparison to the encapsulation of the pure MA in the conventional liposomes. Same results observed with boswellic acid conjugated vesicles using phospholipids. [14]

It is reported that the Polydispersity index (PDI) value of 0.08-0.7 is considered preferable for homogeneous distribution of particles, while PDI value (> 0.7 to < 1) is assumed to have a wide-ranging of particle size. [31] Accordingly, both dispersions have a uniform distribution but pharmacosomal dispersion gives bell shape peak indicating better uniform distribution.

The zeta potential of pharmacosomal and conventional liposomal dispersion was -26.31 ± 0.11 mV and -32.2 ± 0.7 mV respectively, this difference may be due to the difference in the molecular organization of pharmacosomes and conventional liposomes. The negative charge is mainly due to the phosphotidylcholine which is generally made the surface charge of the particle negative, this help in decreasing the accumulation of particles and hence give a stable dispersion. [26]

In-vitro dissolution study

The dissolution profiles of pure MA, MA pharmacosomal dispersion, MA conventional liposomal dispersion in comparison to the marketed suspension of MA in 0.1N HCl solution pH 1.2 and PB solution pH (5.8, 6.8, and 7.4 sequentially) are shown in Figure 12. Pharmacosomal and liposomal dispersion of MA exhibited higher dissolution in HCl pH 1.2 medium than the pure MA, this dissolution differences could be attributed to the vesicular structure of both dispersions where the PC polar head groups play an essential part in decreasing the surface interfacial tension which leads to rapid and enhanced dissolution rate as compared to the pure drug, same results observed with furosemide-phospholipid complex. [32] In addition, drug conjugation with phosphatidylcholine (PC) in pharmacosomes resulted in an amorphous form of the drug

which improved its dissolution significantly ($p < 0.05$) higher than conventional liposomes. [21]

While the drug release from the marketed MA suspension in pH 1.2 is significantly ($p < 0.05$) high due to the presence of suspending agents usually used in the formulation of drug suspension that contributes to the high drug release. [33]

For the sequential dissolution of the drug in PB pH (5.8-7.4), the dissolution of the drug for MA pharmacosomal dispersion, MA conventional liposomal dispersion, pure MA, and MA suspension is non-significantly different, although pharmacosomal dispersion showed higher dissolution than others. Pharmacosomal and conventional liposomal dispersion of MA showed higher dissolution profile than pure MA and MA suspension due to presence of crystalline form of the drug in pure MA and MA suspension, which lead to reduce the exposed surface area for dissolution media. [34] But pharmacosomal dispersion showed higher dissolution profile than all other types due to the amorphous form of the drug in the drug-lipid complex in addition to the presence of the polar head of PC which gives further support to the enhancement of the solubility of the drug. [21]

CONCLUSION

This study proved that the main binding site for phosphatidylecholine is phosphorous group that combine with a model drug having one carboxyl group (MA) in 1:1 ratio with much less extent in 1:2 and 2:1, and such complex lead to transfer of MA from crystalline to amorphous form leading to significant improvement in its solubility and partition coefficient. The liposomes prepared from the conjugate (pharmacosomes) showed higher entrapment efficiency and significant impact on the drug release than the conventionally prepared liposomes (without conjugation) containing the same content and ratio with much rigid morphology. The pharmacosomal dispersion gave much higher drug release in different sequential pHs than the marketed MA suspension which can be a good alternative to enhance drug bioavailability, lowering the dose, less side effect and improves patient compliance.

ACKNOWLEDGEMENTS

The authors are grateful to Mustansiriyah University (www.uomustansiriyh.edu.iq) Baghdad-Iraq for supporting the present work.

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