Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 271 (2004) 115–123
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Co-encapsulation of isoniazid and rifampicin in liposomes and characterization of liposomes by derivative spectroscopy

Ayla Gürsoy [a,*], Ece Kut [a], Sumru Özkırımlı [b]

[a] *Department of Pharmaceutical Technology, Faculty of Pharmacy, Marmara University, 34668 Haydarpaşa, Istanbul, Turkey*
[b] *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Istanbul University, Beyazıt, Istanbul, Turkey*

Received 17 January 2003; received in revised form 27 October 2003; accepted 30 October 2003

Abstract

Taking into consideration the benefits of the combined therapy of isoniazid (INH) and rifampicin (RIF), this study focused on co-encapsulation of INH and RIF in the same liposome formulation. INH was incorporated in the aqueous phase and RIF in the lipid layer. Liposomes containing either INH or RIF were also prepared. All liposome formulations were compared for their loading capacity, encapsulation percentage and release properties. Drug amounts in the liposomes were estimated using peak-to-peak first-order derivative UV spectroscopy. Among the liposome formulations DPPC:chol liposomes showed the highest loading capacity (106.70 ± 0.12 for INH and 18.17 ± 0.06 ($\times 10^{-3}$) for RIF) and encapsulation percentage (73.84 ± 0.78 for INH and 81.53 ± 2.06 for RIF) compared to EPC:chol liposomes (loading capacity 93.36 ± 0.58 for INH and 17.87 ± 0.11 ($\times 10^{-3}$) for RIF; encapsulation percentage 64.61 ± 0.51 for INH and 74.45 ± 0.48 for RIF).

Co-encapsulation of INH and RIF increased their individual encapsulation percentage and extended drug release compared to the formulations containing drug alone (Table 2). Results of this study support the conclusion that lipid and water soluble drugs can be successfully co-encapsulated in the same liposome formulation and also show that derivative UV spectroscopy is a sensitive method for direct and accurate quantification of these co-encapsulated drugs.

Keywords: Isoniazid; Rifampicin; Liposome; Drug co-encapsulation in liposomes; Drug release; Derivative spectrophotometry

1. Introduction

Isoniazid (INH) and rifampicin (RIF) are effective drugs for the treatment of tuberculosis. Although the mechanism of action of INH is not clearly known there is evidence that it inhibits the synthesis of mycolic acid, an essential components of the bacterial cell wall, and also combines with an enzyme that is uniquely found in INH-sensitive strains of mycobacteria. Resistance to INH can occur due to reduced intracellular penetration of the drug. On the other hand, RIF acts by binding to, and inhibiting, DNA dependent RNA polymerase. It enters phagocytic cells and can kill intracellular microorganisms including the tubercule bacillus. However, resistance to RIF can develop rapidly (Rang et al., 1999). Since resistance occurs in one drug therapy, treatment with combination of these drugs is recommended. According to Chambers and Jawetz (1998) an INH–RIF combination administered for 9 months will cure 95–98% of cases of tuberculosis caused by susceptible strains. However, more effective strategies are now available for the treatment of intracellular bacterial infections. The concept of using submicron carrier systems for the delivery of antibiotics (Gürsoy, 2000) and tuberculostatic drugs

* Corresponding author.

0378-5173/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2003.10.033

has gained increasing interest in recent years. INH or RIF stealth liposomes with enhanced affinity towards lung tissue were prepared by modifying the surface of stealth liposomes by tagging *O*-stearylamylopectin thus resulting in an increase in the affinity of these liposomes towards the lung tissue of mice (Deol and Khuller, 1997). Multiple emulsion of RIF also gave a sustained release profile, and coating these emulsions with polysaccharide was found to reduce toxicity compared to the free drug (Khopade et al., 1996).

Several techniques have been used to estimate the drug content of liposomes. Of these methods, derivative UV spectrophotometry is a reliable technique to solve the problem of liposomal turbidity and spectral overlap. It also yields reproducible results in a short time. In a study on ciprofloxacin liposomes derivative UV spectroscopy was found to be an easy and sensitive method for direct estimation of drug amount and gave useful information about changes in the physical state of the drug (Gürsoy and Senyücel, 1997). Hence derivative UV spectrophotometry was used in this study to evaluate INH and RIF in liposome formulations.

On the other hand, there is limited data on co-encapsulation of two different drugs in one liposome formulation (Kut et al., 1998; Rodrigues et al., 2003). Taking into consideration the well established combined therapy of INH and RIF, this study focused on co-encapsulation of INH and RIF in the same liposome formulation. INH was incorporated in the aqueous phase and RIF in the lipid layer. Liposomes containing either INH or RIF were also prepared. All liposome formulations were compared for loading capacity, encapsulation percentage and release properties.

2. Materials and methods

2.1. Materials

Egg yolk phosphatidylcholine type XI-E (EPC), dipalmitoylphosphatidylcholine (DPPC) were obtained from Sigma Chemical Co., St. Louis, MO, USA. Cholesterol (chol) was obtained from Merck, Darmstadt, Germany. The phospholipid (PL) test system employed was the enzymatic colorimetric test obtained from Boehringer Mannheim, Germany. INH, RIF and their USP grade RS samples were gifts from Koçak İlaç A.Ş., Istanbul, Turkey. All other chemicals were analytical reagent grade.

2.2. Preparation of liposomes

Multilamellar neutral liposomes (MLV) of (EPC:chol), (DPPC:chol) were prepared in a molar ratio (10:5) by the conventional thin-film hydration method. Briefly, the lipids were dissolved in chloroform and evaporated to dryness under vacuum. The resultant dry lipid film was hydrated by vortexing with saline solution until all the lipids were dispersed. Then the dispersion was stirred for 10 min at 14,000 rpm. After being stored at 4 °C over night, the liposomal dispersion was centrifuged at 30,000 rpm for 5 min to remove unencapsulated drugs. The liposomal pellet was resuspended in hydration medium and recentrifuged two additional times until no detectable amounts of drugs were found in the last supernatant. Liposomal suspensions were extruded through Millipore membrane filters (Type RA, Millipore Corp., Bedford, USA) with a pore size of 1.2, 0.8, 0.47 and 0.22 μm, respectively. Liposomes between 0.8 and 0.47 μm were used through out the study. In all formulations during agitation and extrusion, the temperature was kept above the transition temperature (T_g) of the lipids.

For the preparation of INH and RIF co-encapsulated EPC:chol or DPPC:chol liposomes; RIF was dissolved in chloroform (24×10^{-3} μmol RIF/μmol EPC or 24×10^{-3} μmol RIF/μmol DPPC) and INH in saline solution (145.5 μmol INH/μmol EPC or 145.5 μmol INH/μmol DPPC). On the other hand liposomes containing only INH or RIF and empty liposomes were also prepared and spectra of all the samples against saline were then recorded. In all formulations PL determinations were made using enzymatic assay (Takayama et al., 1977).

2.3. First-order derivative UV spectrophotometric analysis

The apparatus used was a Shimadzu-2100 (Japan) double-beam UV–Vis spectrophotometer with 1 cm quartz cell. Suitable settings were: Slit width 2 nm, Δλ 20 nm for first-order derivation, Δλ 10 nm for smoothing functions and scan speed 200 nm min^{-1}.

2.3.1. Standard curves of INH or RIF encapsulated in EPC:chol or DPPC:chol liposomes

Standard solutions of INH spiked with a constant amount of RIF (5 μg ml^{-1}) and those of RIF spiked with a constant amount of INH (5 μg ml^{-1}) in saline were prepared. The first derivative spectra of the mixtures against saline were then recorded.

Standard solutions of INH or RIF were spiked with 40 μl of empty EPC:chol or DPPC:chol liposome suspension and analyzed by first-order derivative mode measuring the peak-to-peak amplitudes between + 249.2 and −280.1 nm for INH and +317.5 and −348.5 nm for RIF. Calibration curves were prepared plotting peak to peak amplitudes against concentrations.

Forty microliter aliquots of INH or RIF loaded liposome suspension were diluted to 3 ml with saline and subjected to first-order derivative spectrophotometric analysis against saline. Drug amounts in the liposomes were calculated using corresponding linear regression equations of the relevant drugs.

2.3.2. Standard curve of INH or RIF in the INH and RIF co-encapsulated EPC:chol or DPPC:chol liposomes

Forty microliter of liposome suspension was added to 3 ml of standard solution of INH (2–30 μg ml^{-1}) containing constant amount (5 μg ml^{-1}) of RIF or standard solution of RIF (1–20 μg ml^{-1}) containing constant amount (5 μg ml^{-1}) of INH. Samples were subjected to first-order derivative spectrophotometric analysis against saline as described above. The calibration curve of each drug was prepared by plotting peak to peak amplitudes against concentrations and linear regression equations were estimated.

Forty microliter suspension of EPC:chol or DPPC:chol liposomes containing co-encapsulated drugs was diluted to 3 ml with saline and a first-order derivative spectrum was recorded against saline. INH or RIF amount were estimated according to their corresponding linear regression equation.

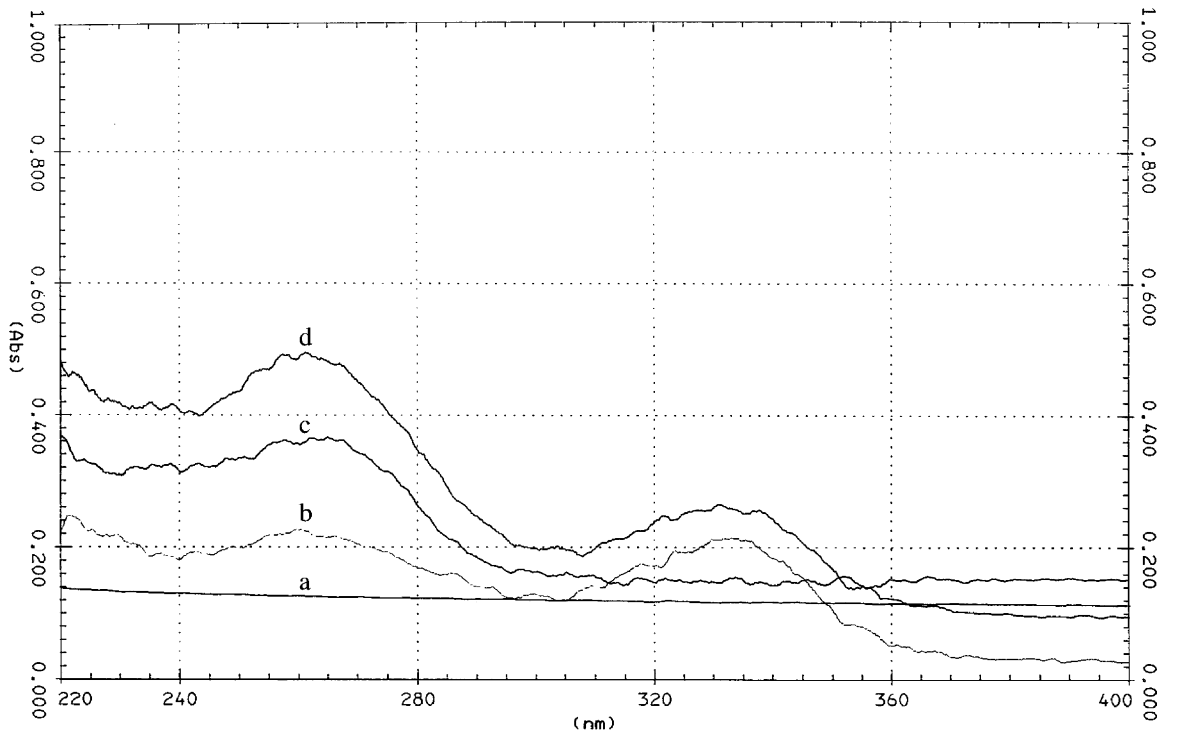

Fig. 1. UV spectra of empty liposomes (a), RIF containing liposomes (16.52 μmol RIF/μmol PL) (b), INH containing liposomes (86.88 μmol INH/μmol PL) (c), INH and RIF co-encapsulated liposomes (92.94 μmol INH/μmol PL, and 17.77 μmol RIF/μmol PL) (d).

2.4. Loading capacity and encapsulation (%)

The loading capacity of liposome formulations containing INH or RIF alone or their co-encapsulated form was expressed as μmol of INH or RIF per μmol of PL. Encapsulation percentage was also calculated based on the ratio of μmol INH or RIF per μmol PL between the final and initial liposome formulations containing drug alone or in combination.

2.5. Drug release

Liposome suspension, whose final drug and PL amounts had been determined was placed within a dialysis bag (cellulose membrane, Sigma Chemical Co.) and immersed into a vessel containing 100 ml saline. Release studies were carried out at a temperature of 37±0.5 °C under mechanical stirring at 50 rpm. At fixed time intervals samples were withdrawn from the solution and INH or RIF content was determined by first-order derivative spectrophotometry.

3. Results and discussion

INH and RIF were successfully co-encapsulated in the same liposome formulations by loading RIF in the lipid layer and INH in the aqueous phase.

3.1. First-order derivative UV spectrophotometric analysis

First-order derivative UV spectrophotometry was used because of its simplicity, and it is a sensitive method for estimating drug content in liposomes without disrupting their bilayer structure and it also solves the problem of liposomal turbidity (Di Giulio et al., 1991; Gürsoy and Senyücel, 1997). Hence INH and RIF amounts were determined using the first-order derivative spectroscopic method.

It was confirmed that first-order derivative spectra of EPC:chol and DPPC:chol empty liposome formulations were zero. There was no interference due to lipid composition of liposome bilayer.

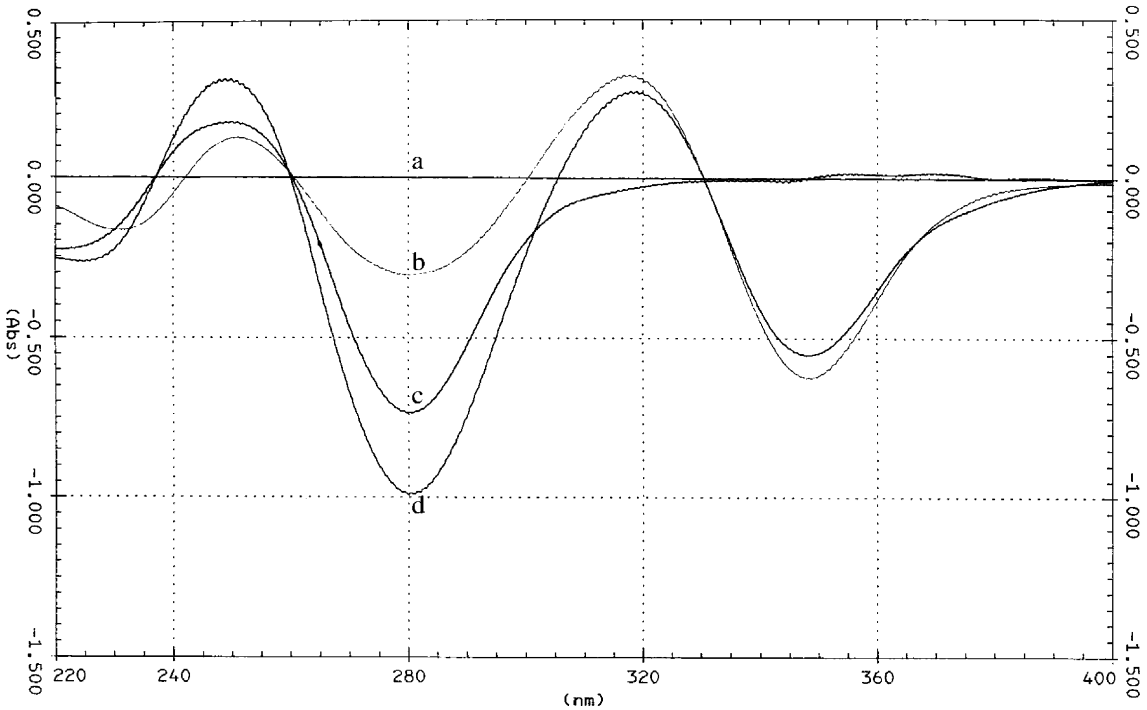

Fig. 2. First-order derivative spectra of empty liposomes (a), RIF containing liposomes (16.52 μmol RIF/μmol PL) (b), INH containing liposomes (86.88 μmol INH/μmol PL) (c), INH and RIF co-encapsulated liposomes (92.94 μmol INH/μmol PL, and 17.77 μmol RIF/μmol PL) (d).

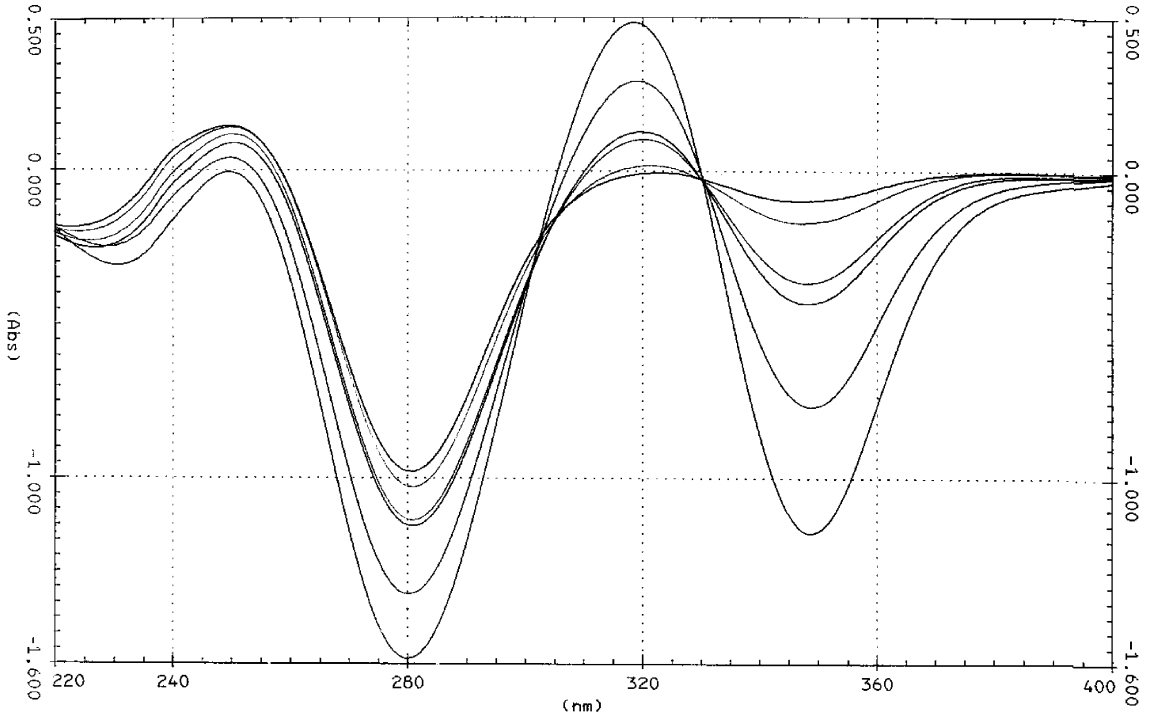

Fig. 3. First-order derivative spectra of different concentrations of RIF in saline solution. Each concentration of RIF contains empty EPC:chol liposomes and INH (5 μg ml^{-1}).

As shown in Fig. 1, there was interference between the zero-order spectra of empty liposomes, liposomes containing either INH (86.88 μmol INH/μmol PL) or RIF (16.52 μmol RIF/μmol PL) and INH and RIF co-encapsulated liposomes (92.94 μmol INH/μmol PL, and 17.77 μmol RIF/μmol PL) in saline solution. Hence, it was not possible to estimate the amount of INH or RIF in liposomes by this spectra. However, when the first-order derivative spectra of these liposomes were investigated, no interference was observed and the amount of INH or RIF either alone or in a co-encapsulated formulation was easily determined (Figs. 2–4).

No significant difference was found between the slope of the working curves of the two mixtures of RIF/empty liposomes, and RIF/constant amount of INH/empty liposomes ($P > 0.005$) (Table 1) (Fig. 3). Similar results were also obtained with working curves of the mixtures of INH/empty liposomes and INH/constant amount of RIF/empty liposomes ($P > 0.005$) (Table 1) (Fig. 4). These results were not affected by the lipid composition of the bilayers.

3.2. Loading capacity and encapsulation (%)

When EPC:chol and DPPC:chol liposomes were compared for their loading capacity and encapsulation (%), DPPC:chol liposomes showed a statistically higher encapsulation (%) than EPC:chol liposomes for both INH and RIF values alone and even in the case of co-encapsulated form ($P < 0.005$), suggesting that chain length of phosholipid was an important factor (Table 2). Increasing chain length of phospholipids increased the encapsulation percentage of drugs. Similar results have been reported with respect to chain lengths of phospholipids (Puglisi et al., 1995; Al-Angary et al., 1996).

On the other hand, a significant encapsulation (%) difference was observed between INH and RIF both in the liposome formulations containing these drugs alone, and in the co-encapsulated form. RIF gave a higher encapsulation (%) value than INH ($P < 0.005$) (Table 2). However, in some studies encapsulation (%) of hydrophobic drugs was found higher than hydrophilic drugs (Betageri and Parsons,

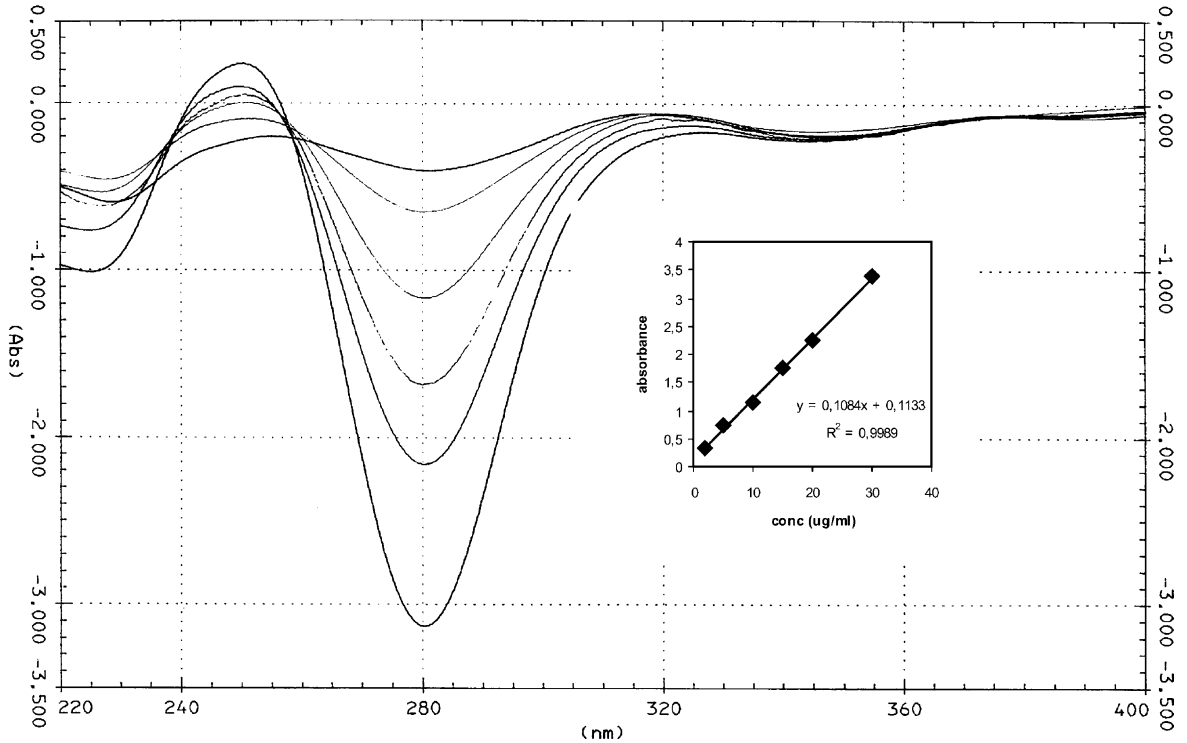

Fig. 4. First- order derivative spectra of different concentrations of INH in saline solution. Each concentration of INH contains empty EPC:chol liposomes and RIF (5 μg ml^{-1}). The inset shows corresponding regression line.

1992; Di Giulio et al., 1993). On the other hand, in one study (Rodrigues et al., 2003), INH and RIF were co-encapsulated in LUV liposomes prepared from DMPG or DMPC and NMR and fluorimetric studies of these liposomes revealed that INH was located at the membrane surface whereas RIF was buried deep inside the lipid bilayers. The results of the article in BBA, about the INH location, differed from ours. This is because the lipids used to prepare liposomes, the formulation of liposomes, the type and the drug loading methods of liposomes differed between these two studies. These differences might be responsible for the observed variation in INH location. Another point to support the location of INH in the aqueous phase of the liposomes was the similar release rates of INH and RIF. If INH was located at the membrane

Table 1
Statistical analysis for the determination of INH and RIF encapsulated or co-encapsulated in liposomes by first-order derivative spectroscopy

Liposome formulation	EPC:chol				DPPC:chol			
Drug	INH	INH + RIF[a]	RIF	RIF + INH[b]	INH	INH + RIF[a]	RIF	RIF + INH[b]
Slope (b)[c]	0.1067	0.1084	0.1136	0.1080	0.1125	0.1110	0.1228	0.1200
Intercept (a)[c]	0.1283	0.1134	−0.0389	−0.0660	0.1736	0.1376	−0.0489	−0.0459
Correlation coefficient (r^2)	0.9919	0.9989	0.9989	0.9993	0.9910	0.9998	0.9958	0.9917

[a] Varying amount (2–30 μg ml^{-1}) of INH and constant amount (5 μg ml^{-1}) of RIF.
[b] Varying amount (1–20 μg ml^{-1}) of RIF and constant amount (5 μg ml^{-1}) of INH.
[c] $Y = a + bC$, where C is concentration of drug in μg ml^{-1} and Y is peak to peak amplitude.

Table 2
INH or RIF loading capacity and encapsulation (%) of (EPC:chol) and (DPPC:chol) liposomes containing drug alone or together (mean±S.D.)

Liposome formulation	INH		RIF	
	Loading capacity[a]	Encapsulation (%)	Loading capacity[a]	Encapsulation (%)
INH + (EPC:chol)	88.91 ± 2.18	61.53 ± 1.63	–	–
INH + (DPPC:chol)	99.46 ± 1.53	68.83 ± 0.08	–	–
RIF + (EPC:chol)	–	–	16.96 ± 0.30 ($\times 10^{-3}$)	70.68 ± 0.30
RIF + (DPPC:chol)	–	–	19.57 ± 0.49 ($\times 10^{-3}$)	75.69 ± 0.24
INH+RIF + (EPC:chol)	93.36 ± 0.58	64.61 ± 0.51	17.87 ± 0.11 ($\times 10^{-3}$)	74.45 ± 0.48
INH + RIF + (DPPC:chol)	106.70 ± 0.12	73.84 ± 0.78	18.17 ± 0.06 ($\times 10^{-3}$)	81.53 ± 2.06

[a] μmol drug/μmol PL.

surface then the release of INH would be faster than that of RIF.

According to Table 2, co-encapsulation of drugs in DPPC:chol or EPC:chol liposome formulations increased the encapsulation (%) of both drugs compared to the formulations containing INH or RIF alone ($P < 0.005$). For example, in INH loaded DPPC:chol liposomes, encapsulation (%) of INH was 68.83 ± 0.08 whereas in INH-RIF co-encapsulated liposomes this value increased to 73.84 ± 0.78. A similar increase in the encapsulation (%) of RIF was observed: 75.69 ± 0.24 and 81.53 ± 2.06 were found for the liposomes containing RIF alone and when co-encapsulated with INH, respectively. This was probably due to the enhanced solubility of drugs in their mixtures. The solubility of INH alone was 23.71 mg ml^{-1} in saline solution but it increased to 31.15 mg ml^{-1} in the RIF solution. Similar increased solubility values were observed for RIF. The solubility of RIF in saline solution was 1.04 mg ml^{-1} and increased to 10.77 mg ml^{-1} in INH saline solution.

3.3. Release studies

Release of INH and RIF from liposomes either loaded alone or in co-encapsulated form gave a sustained release profile.

As shown in Fig. 5, liposomes containing INH or RIF alone showed a fast release profile compared to the liposomes which co-encapsulated both drugs ($P < 0.005$). Although the molecular weight and solubility of INH and RIF were different no release difference between the two drugs was observed in either liposome formulations containing drugs alone or in co-encapsulated form ($P > 0.05$) (Fig. 5). However, a release difference was observed between liposome formulations. In DPPC:chol liposome formulations the release of both drugs either alone or in co-encapsulated form was significantly slower than in the EPC:chol liposomes ($P < 0.005$) (Fig. 6).This difference was due to the T_g values of the phospholipids used to prepare liposomes. Since DPPC has a higher T_g value than EPC the drug release from DPPC:chol liposomes was slower than from EPC:chol liposomes. These results were in close agreement with the results of the other studies (Al-Angary et al., 1996; Gürsoy and Senyücel, 1997).

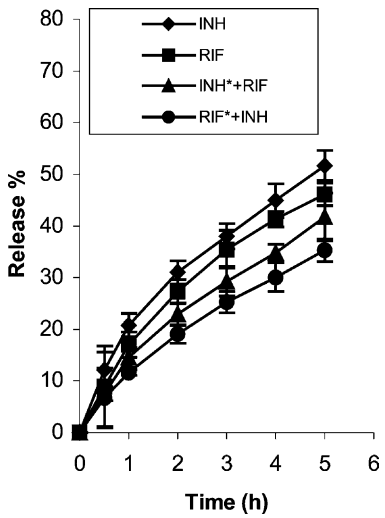

Fig. 5. INH or RIF release from DPPC:chol liposomes containing drug alone or co-encapsulated form (*, released drug).

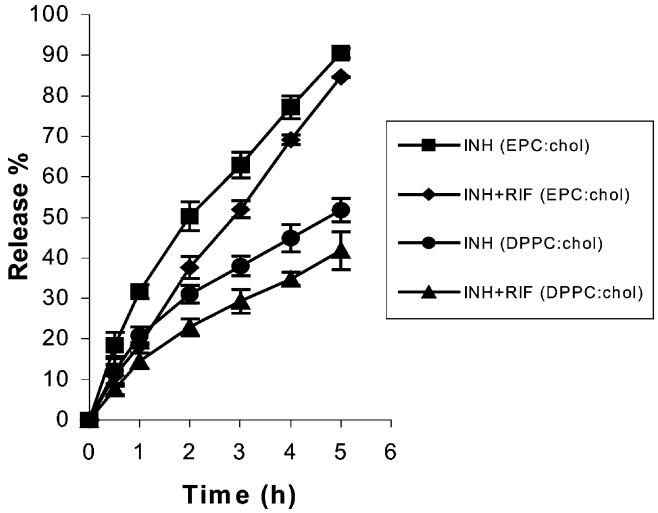

Fig. 6. INH release from EPC:chol or DPPC:chol liposomes containing INH alone or co-encapsulated with RIF.

4. Conclusion

In this study the derivative UV spectrophotometry was found to be a reliable technique for the estimation of two different drug amounts in the same liposome formulation.

Another interesting out come of this study was the possibility of co-encapsulation of two different drugs (one lipid soluble and the other water soluble) in the same liposome formulation. This could be a good alternative for combined therapy in clinical setting. In addition co-encapsulation of two different drugs in the same liposome formulation can also extend the release rate compared to that of liposomes containing only one drug.

Acknowledgements

The first author would like to thank the Fund of Marmara University for financial support of this project (SB-DYD-80).

References

Al-Angary, A.A., Meshal, M.A., Bayomi, M.A., Khidr, S.H., 1996. Evaluation of liposomal formulations containing antimalarial agent. Arteether. Int. J. Pharm. 128, 163–168.

Betageri, G.V., Parsons, P.L., 1992. Drug encapsulation and release from multilamellar and unilamellar liposomes. Int. J. Pharm. 81., 235–241.

Chambers, H.F., Jawetz, E., 1998. Antimycobacterial drugs. In: Katzung, B.G. (Ed.), Basic and Clinical Pharmacology. Appleton and Lange, Connecticut, pp. 770–779.

Deol, P., Khuller, G.K., 1997. Lung specific stealth liposomes: stability, biodistribution and toxicity of liposomal antitubercular drugs in mice. Biochim. Biophys. Acta 1334, 161–172.

Di Giulio, A., Maurizi, G., Odoardi, P., Saletti, M.A., Amicosante, G., Oratore, A., 1991. Encapsulation of ampicillin in reverse-plane evaporation liposomes: a direct evaluation by derivative spectrophotometry. Int. J. Pharm. 74, 159–1164.

Di Giulio, A., Saletti, M.A., Impagnatielb, A., Lucarelli, M., Storm, R., Oratore, A., 1993. Encapsulation and efflux of lactone and hydroxy acid forms of, simvastatin in reverse-phase evaporation vesicles. Int. J. Pharm. 89, 133–138.

Gürsoy, A., Senyücel, B., 1997. Characterization of ciprofloxacin liposomes: derivative ultraviolet spectrophotometric determinations. J. Microencapsulation 14, 769–776.

Gürsoy, A., 2000. Liposome-encapsulated antibiotics: physico-chemical and antibacterial properties, a review. S.T.P. Pharm. Sci. 10, 285–291.

Khopade, A.J., Mahadik, K.R., Jain, N.K., 1996. Targeting of multiple emulsions to the lung. Pharmazie 51, 558–561.

Kut, E., Gürsoy, A., Özkırımlı S., 1998. Evaluation of isoniazid and rifampicin entrapped liposomes by derivative UV spectrophotometry. In: Proceeding of International Pharmaceutical Technology Symposium. Ankara, 7–9 September, pp. 83–84.

Puglisi, G., Fresta, M., Mazzone, G., Furneri, P.M., Tempera, G., 1995. Formulation parameters of fluoroquinolone-loaded liposomes and in vitro antimicrobial activity. Int. J. Pharm. 118, 65–76.

Rang, H.P., Dale, M.M., Rither, J.M., 1999. Drugs used to treat tuberculosis. In: Pharmacology, fourth ed. Churchill Livingstone, London, pp. 703–707.

Rodrigues, C., Gameiro, P., Prieto, M., de Castro, B., 2003. Interaction of rifampicin and isoniazid with large unilamellar liposomes: spectroscopic location studies. Biochim. Biophys. Acta 1620, 151–159.

Takayama, M., Itah, S., Nagasaki, T., Tanimizu, I., 1977. A new enzymatic method for determination of serum choline-containing phospholipids. Clin. Chim. Acta 79, 93–98.