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Validation of a simple and rapid HPLC-UV method for simultaneous analysis of co-delivered doxorubicin and verapamil and its application to characterization of PLGA nanoparticles

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Abstract

To overcome developing drug resistance in cancer treatment, combination therapy could be an attractive strategy. It has been shown that doxorubicin anti-cancer properties are improved by P-glycoprotein inhibitors such as verapamil. Polymeric nanoparticles (NPs) of poly lactic-co-glycolic acid (PLGA) can simultaneously deliver verapamil and doxorubicin and provide an effective anti-cancer drug delivery system. The present study aimed to develop an efficient high performance liquid chromatography (HPLC) method for the simultaneous determination of doxorubicin and verapamil encapsulated in PLGA nanoparticles (NPs). Quantification of doxorubicin and verapamil was performed by the HPLC method. The method was developed by evaluating combination of different solvents ratios as mobile phase and modification of the mobile phase rate. A series of doxorubicin and verapamil solutions at concentrations of "6.25, 12.5, 25, 50, and 100 µg/ml" and "0.625, 1.25, 2.5 and 5 µg/ml" were prepared, respectively. The method was validated by calculating selectivity, linearity, accuracy, intra- and inter-day precision. The validated method was used to characterize prepared doxorubicin-verapamil PLGA NPs by determination of drug loading, encapsulation efficiency% and in vitro release. Results indicated that analysis method was selective with notable separation efficiency and acceptable limit of detection and limit of quantification which shows the sensitivity of the method. The linear standard curve with suitable accuracy and precision confirms the validation of method for simultaneous analysis of doxorubicin and verapamil in NPs.

Keywords: PLGA nanoparticles, Doxorubicin, Verapamil, HPLC, Validation.

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1. Introduction

Cancer is abnormal and uncontrollable cell proliferation which can disseminate to other healthy tissues. This dissemination is called metastasis which is the main reason for deaths caused by cancer. Worldwide epidemiologic studies have notified “cancer” with more than 10 million new cases annually, as the second most frequent reason of death (1). The financial burden of cancer for patients and their families are an increasingly major issue of concern for health care policy managers, clinicians, and society overall (2).

Over the last decades, considerable successful progress in the understanding cancer etiology and discovery of newer efficient diagnostic and therapeutic approaches have been achieved, nevertheless, cancer-related mortality is expected to rise to ~13.1 million deaths in the world by the year 2030 (3). Current treatment strategies for cancer include chemotherapy, surgery, and radiation therapy, or a combination of them (4);

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however, chemotherapy seems to be the most reliable and effective treatment method (1). Over the past few decades, drug resistance against cytotoxic agents and chemotherapy is the most important reason for cancer recurrence that leads to decreasing the rate of complete and successful treatment of cancer patients (5).There is increasing evidence that the multidrug resistance (MDR) mechanism underlies chemo-resistance in some tumors (6). MDR, is the cancer cells resistance against cytotoxic drugs with varying action mechanisms and structural compositions (7, 8) and its development is affected by different intrinsic important factors (9), including the activation of pro-survival signaling pathways (10), overexpression of anti-apoptotic protein Bcl-2 (11), and up-regulation of p-glycoprotein (P-gp) (12). Among these critical mechanisms, the overexpression of P-gp which is encoded by the ABCB1 gene is the most frequently encountered mechanism in the drug-resistant process. P-gp transporter with a considerable binding capacity, pump and transport cytotoxic agents from cell cytoplasm to extracellular space, thus leading to the lower concentration of drugs in the intracellular environment and inducing drug resistance (12).

In order to overcome drug resistance in cancer, combination therapy may be an attractive therapeutic strategy, since it utilizes different drugs with various mechanisms. Combination therapy of cytotoxic drugs with different pharmacological performance may synergistically increase cytotoxicity, but these chemical drugs are different in pharmacokinetics behavior, solubility, hydrophobicity, and the maintenance of the in vivo molecular ratio of the combined drugs in the cancerous cells is difficult. Administration of P-gp inhibitors with chemotherapeutics agents were utilized to overcome MDR and providing the chance of reducing dose of the cytotoxic drug and adverse effects (13, 14)

Verapamil (VER), a derivative of papaverine, has been shown to act as P-gp inhibitor in vitro (15) while doxorubicin (DOX) has been found to stabilize the topoisomerase II (TOP II) cleavage complex after it has broken the DNA chain for replication, and thus inhibiting the process of DNA replication. It has been shown that verapamil and doxorubicin combinations provide better anti-cancer properties than doxorubicin alone (16).

Interestingly, nanotechnology can provide an effective drug delivery system for anticancer therapy by simultaneous loading and releasing of different cytotoxic agents. Therapeutic nanoparticles (NPs) for cancer chemotherapy can be manufactured by efficient biomaterial carriers such as poly lactic-co-glycolic acid (PLGA) -an FDA-approved synthetic polymer- (17), which is the most common and successful polymer for drug delivery due to its biodegradable and sustainable characteristics with low systemic toxicity (18-20). Thus, we aimed at designing doxorubicin and verapamil co-encapsulated PLGA NPs with the goal of overcoming drug resistance. In this regard, an efficient analysis method was required to simultaneously determine doxorubicin and verapamil in samples taken. To calculate drug loading efficiency and drug release studies, a selective high-performance liquid chromatography (HPLC) method was developed and validated to analyze doxorubicin and verapamil amount encapsulated in PLGA-NPs and released.

2. Material and methods

2.1. Materials

Doxorubicin was purchased from EBEWE pharmagen (Austria), Verapamil was a gift from Sobhan Darou (Iran), PLGA (50:50) was purchased from Boehringer (Germany), polyvinylalcohol (PVA) was purchased from Fluka (Germany). Acetic acid, acetonitrile, methanol, mannitol, KH2PO4, NaOH and dichloromethane were purchased from Merck (Germany).

2.2. Methods

2.2.1. Doxorubicin and verapamil analysis

Doxorubicin and verapamil analysis was performed simultaneously by High-performance liquid chromatography (HPLC) method employing an ultraviolet (UV) detector. The HPLC system consisted of a 515 intelligent solvent delivery pump, a computerized system controller, and a Waters 2487 UV detector. To determine the most suitable method, different combinations of stationary and mobile phase were examined. A specific concentration of DOX was injected directly to the HPLC and the peak and the retention time of DOX

was detected, then specific concentration of VER was injected to the HPLC and the peak and retention time of VER was identified, finally the equal volume and the same concentrations of each drug admixed with each other and mixed drugs were injected directly to the HPLC and parameters of the method were optimized.

Chromatographic separation was performed using a C18 column (Eurosphere 100-5 C18, 250 mm×4.6 mm with pre-column) at room temperature as the stationary phase. The mobile phase was KH2PO4 solution and acetonitrile (1:1) at pH 4 which was eluted at a flow rate of 1 ml/min. The detection wavelength was set to 280 nm. The injection volume was 50 µl.

2.2.2. Standard curve construction

To construct the standard curve, doxorubicin, and verapamil standard solutions at concentrations of "6.25, 12.5, 25, 50, and 100 µg/ml" and "0.625, 1.25, 2.5, and 5 µg/ml" were prepared by serial dilution, respectively. Equal volumes (50 µl) of the specified concentrations of each drug were mixed and 50µl of each mixed standard solution was injected. Thus, the standard calibration curve was constructed by plotting peak area of doxorubicin and verapamil versus different concentrations.

2.2.3. Standard curve validation

The developed method and constructed standard curve was validated for selectivity, linearity, accuracy, precision and sensitivity according to the US Food and Drug Administration (FDA) guidelines (21).

2.2.3.1. Selectivity

The selectivity of the analysis method was evaluated by individual injections of doxorubicin and verapamil separately and simultaneously to show the power of the method in separating two peaks apart from each other with no interaction.
In order to calculate selectivity of the method, analyzing DOX and VER was done with less interference by other ingredients (22). Following formula was used for calculating the resolution factor (Rs) as demonstrated in (Eq.1):

$$Rs = 2\left(t_2 - t_1\right) \ / \ W_1 + W_2 \qquad \text{(Eq. 1)}$$

Where t2 and t1 are the retention times of the two components, W2 and W1 are the widths at the bases of the peaks.

2.2.3.2. Linearity

To determine method linearity, the regression line of average areas of doxorubicin and verapamil peak versus their concentrations was plotted and the coefficient of correlation r, slope, y–intercept and residual sum of squares were determined.

2.2.3.3. Accuracy

The accuracy of the method indicates the closeness of the theoretical and practical value of different concentrations. In other word, the accuracy may be defined by determination of the ratio of obtained concentration from the regression equation to the real concentration. For this purpose, two concentrations of 8 and 40 µg/ml were selected.

2.2.3.4. Precision

The precision of an analytical method indicates the degree of proximity of data obtained from repeated concentrations. Thus, the coefficient of variation (CV%) within and between different days were calculated. For intra-day precision (within-day), each concentration was prepared three times in a day and for inter-day precision (between days), different concentrations were prepared and injected into the system in three separate days.

2.2.3.5. Sensitivity

Sensitivity of a method can be determined by its limit of detection (LOD) and limit of quantification (LOQ). Limit of detection (LOD) was measured as the drug concentration with signal/noise ratio of 3. The limit of quantification (LOQ) was determined as the lowest drug concentration which can be quantitated with acceptable accuracy and precision (23).

2.2.4. Preparation of doxorubicin-verapamil PLGA NPs

To prepare sample doxorubicin-verapamil PLGA NPs (DOX-VER-NPs), two different amounts of doxorubicin and a known amount of verapamil were dissolved in water while two

Table 1. Composition of doxorubicin and verapamil nanoparticles (DOX-VER-NPs).

Formulation	PLGA (mg)	PVA%	Doxorubicin(mg)	Verapamil(mg)
F1	20	0.5	8	4
F2	10	0.5	4	4

different amounts of PLGA was dissolved in dichloromethane (DCM) to form the organic phase. A certain amount of drug solutions as aqueous phase was added to the organic phase and sonicated for 60 seconds and was slowly dropped into a PVA aqueous solution (0.5%) while sonicated. Afterward, the emulsion was stirred gently at room temperature for several hours to evaporate DCM. The NPs suspension obtained was centrifuged for 30 min at 15,000 rpm and freeze-dried (24). DOX-VER-NPs composition are shown in Table 1.

2.2.5. Drug loading and encapsulation efficiency

One ml DCM was added to 3 mg of DOX-VER-NPs and was stirred to dissolve PLGA in DCM completely. Then, 3 ml distilled water was added to dissolve doxorubicin and verapamil. Then, DCM was evaporated by nitrogen gas and the sample was centrifuged at 18000 rpm for 15 min. Considering the validated analysis method, the drug loading percentage (LA%) and encapsulation efficiency (EE%) were calculated using the following equations:

$$\textit{Drug loading amount}\,(LA\%) = \frac{\text{Encapsulated drug weight}}{\text{NP weight}} \times 100$$

$$\text{Encapsulated efficiency}\,(EE\%) = \frac{\text{Drug loaded in particle}}{\text{Drug used for encapsulation}} \times 100$$

2.2.6. In vitro release of drugs

In order to determine the release profile, 3 mg DOX-VER-NPs was dispersed in 10 ml phosphate buffer (pH 7.4) and placed in a shaker incubator at 18 rpm (37 ±1 °C for 24h). Samples were taken at 1, 2, 4, 6, 8, and 24 h and were centrifuged at 18000 rpm for 10 min. The amount of doxorubicin and verapamil was determined at different times using validated analysis method and the release profile was plotted based on the released amount versus time.

2.2.7. Statistical analysis

The data obtained were analyzed by SPSS software (version 2010). Descriptive data including mean and standard deviation were calculated. Analysis of variance (ANOVA) was used to evaluate the difference between encapsulation efficiency, drug loading and release. P-values of 0.05 or less were considered statistically significant.

3. Results and discussion

The main concern of the present study was to develop and validate an HPLC method for simultaneous determination of verapamil and doxorubicin in co-encapsulated DOX-VER-NPs.

3.1. Doxorubicin and verapamil analysis

In order to simultaneously assay doxorubicin and verapamil, a rapid and reliable HPLC method was developed and validated. As it is shown in Figure 1, the obtained retention time for doxorubicin and verapamil was 3 and 7 min, respectively.

There are very few studies that developed method for simultaneous analysis of DOX and VER. A study used UV spectrometer at 480 nm for determining the amount of released doxorubicin, while the amount of verapamil was measured by HPLC method using a C18 column (4.1 mm×300 mm, 10 µm particle size) with the flow rate of 1 ml/min and isocratic elution of the mobile phase of methanol/20mM phosphate buffer and triethylamine (pH 4.0) (45:55:1 V/V) at 210 nm (25). Another research showed determination of DOX and VER in nanoparticles by the spectrometer at 480 and 280 nm, respectively (26). Our method is unique for simultaneous analysis of these two drugs by a simple and sensitive HPLC system without need to change in the system condition.

3.2. Standard curve construction

Two standard curves were constructed in different concentrations of doxorubicin and verapamil mixtures. The curves were plotted based on area under curve (AUC) of each drug versus concentration. All solutions were prepared in three different days by serial dilution and each concen-

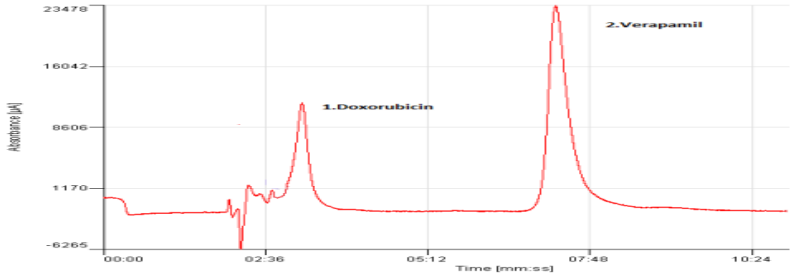

Figure 1. HPLC Chromatogram of doxorubicin (50 µg/ml) and verapamil (100 µg/ml).

tration was examined 5 times. The standard curve of doxorubicin and verapamil are shown in Figure 2.

3.3. Method validation

The developed analysis method was validated by characterization of different parameters including selectivity, linearity, accuracy, and precision.

3.3.1. Selectivity

The selectivity of an analysis method is defined by the ability to the separate analytes without any interaction. To determine the selectivity of developed method, doxorubicin and verapamil were analyzed separately and simultaneously. As it is seen in Figure 1, doxorubicin and verapamil were separated and there was no interference in the chromatogram. Resolution factor (Rs) displays the extent of peak separation and values greater than 2 indicates the suitable level of selectivity. Consequently, the calculated Rs, 3.2, is very good for selectivity.

3.3.2. Linearity

To determine the standard curve linearity, the line regression was calculated after plotting curve based on the AUC of doxorubicin and verapamil versus concentration. The standard curve of doxorubicin and verapamil were shown in Figure 2. The correlation coefficients were greater than 0.999 which demonstrated that there is a linear correlation between doxorubicin (6.25 to 100 µg/ml) and verapamil (0.625 to 5 µg/ml) AUC versus concentration in the mentioned range. Table 2 shows the accuracy (mean of the two concentrations tested) and linearity of the method for determination of doxorubicin and verapamil.

3.3.3. Accuracy

Accuracy of the developed method was between 80% to 120% and was acceptable based on the related guidelines (27). The mean accuracy for doxorubicin and verapamil was calculated to be 96.03±3.6 % and 98.19±2.02 %, respectively.

3.3.4. Precision

The precision of developed method was in the acceptable range. Precision was reported as coefficient of variation percentage (CV %) for inter-day and intra-day experiments. The mean inter-day and intra-day CV % for doxorubicin was 0.87 and 2.24 % and for verapamil was 1.71 and 3.22 %, respectively. The results were shown in Table 3.

3.3.5. Sensitivity

The developed analysis method sensitiv-

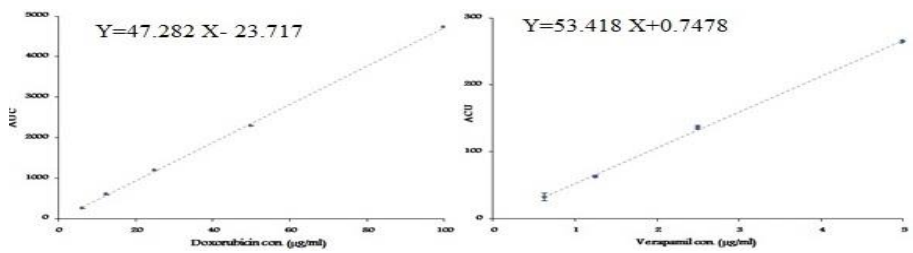

Figure 2. Standard curves of Doxorubicin and Verapamil.

Table 2. Results of the validation of the standard curve.

	Equation	r2	Accuracy%	Recovery%	LOD (µg/ml)	LOQ(µg/ml)
Doxorubicin	Y=47.282x-23.717	0.9996	96.03±3.6	99.3±0.8	1.95	5.90
Verapamil	Y=53.418x+0.7478	0.9992	98.19±2.02	103.8±1.4	0.20	0.61

ity was calculated by LOD and LOQ which were obtained for doxorubicin 1.95 and 5.9 µg/ml and for verapamil 0.2 and 0.61 µg/ml, respectively.

3.4. Drug loading and encapsulation efficiency

Tow optimized formulations of dual loaded DOX-VER-PLGA-NPs were prepared and their drug loading and encapsulation efficiency (EE%) were determined using the validated HPLC analysis method that are presented in Table 4.

This result was predictable due to the equal amount of verapamil in both formulations. However, as it was observed for doxorubicin, several previous studies have reported that the method of preparing NPs, chemical and physical properties of polymers, and increased particle size may affect the loading% (28). Both drugs are hydrophilic in nature and their loading in a hydrophobic polymer is very problematic. Therefore, high values of loading and encapsulation could not be easily achieved.

3.5. In vitro drug release

Doxorubicin and verapamil release profiles of F1 and F2 formulations were studied using the validated HPLC analysis method. As seen in Figure 3, there is a significant difference ($P<0.05$) between release profiles of doxorubicin and verapamil. F2 formulation released both drugs faster than F1 formulation, which may be related to the lower amount of PLGA polymer that causes the higher chance for surface adsorption of doxorubicin and verapamil and may lead to the initial burst release. The rapid release of DOX from NPs indicates that the drug was unable to enter the NPs core and was instead adsorbed onto the NPs surface, which is logical considering the drug's hydrophilicity and the lipophilicity of the PLGA polymer (24, 29). Consistent with these results, other studies also indicated that the amount of released doxorubicin is high 1h after NPs injection, which is influenced by NPs type, drug combination, and drug amount as well (30).

4. Conclusion

In summary, the current study developed an isocratic HPLC analysis method for simultaneous determination of doxorubicin and verapamil dually loaded in PLGA NPs. Results indicated a validated HPLC method with notable sensitivity for both drugs. The selectivity of the method was completely proved by distinct doxorubicin and verapamil peaks in chromatograms with no interference. The developed HPLC method showed

Table 3. Results of precision of the method.

Drug	Concentration (µg/ml)	AUC 1st day			AUC 2nd day	AUC 3rd day	CV% (Intra-day)	CV% (Inter-day)
Doxorubicin	6.25	248	244	248	238	236	0.9	2.3
	12.5	607	609	628	552	580	1.9	5.0
	25	1191	1211	1199	1167	1201	0.8	1.4
	50	2270	2279	2267	2339	2309	0.3	1.3
	100	4750	4740	4780	4639	4680	0.4	1.2
Verapamil	0.625	32	35	34	32	30	4.5	6.0
	1.25	62	63	63	65	64	0.9	1.8
	2.5	139	140	139	135	131	0.4	2.8
		261	266	264	275	259	1.0	2.3

AUC: Area under the curve, CV%: coefficient of variation%.

Table 4. Doxorubicin/Verapamil loading and encapsulation efficiency %.

	Loading%		Encapsulation efficiency%	
	Doxorubicin	Verapamil	Doxorubicin	Verapamil
F1	10.21±0.51	0.44±0.01	44.65±2.25	3.86±0441
F2	4.35±0.04	0.45±0.01	31.27±0.26	3.23±0.06

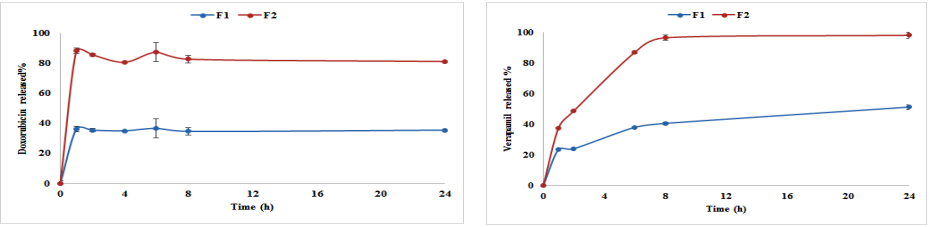

Figure 3. *In vitro* release profiles of doxorubicin and verapamil from F1 and F2 formulations (n=3).

considerable linearity with r2 more than 0.999 and considerable accuracy and inter- and intra-day precision. Two prepared dual loaded DOX-VER-PLGA NPs were characterized using the validated HPLC analysis method for drug loading, encapsulation efficiency and in vitro release. This rapid and simple HPLC analysis method is efficient for simultaneous assay of these drugs in different experiments.

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Conflict of Interest

None declared.

References

1. Gupta P, Jani KA, Yang DH, Sadoqi M, Squillante E, Chen ZS. Revisiting the role of nanoparticles as modulators of drug resistance and metabolism in cancer. *Expert Opin Drug Metab Toxicol.* 2016;12(3):281-9. doi: 10.1517/17425255.2016.1145655. Epub 2016 Feb 15. PMID: 26799671.
2. Yabroff KR, Lund J, Kepka D, Mariotto A. Economic burden of cancer in the United States: estimates, projections, and future research. *Cancer Epidemiol Biomarkers Prev.* 2011 Oct;20(10):2006-14. doi: 10.1158/1055-9965. EPI-11-0650.
3. Boyle P, Levin B. World cancer report 2008: IARC Press, International Agency for Research on Cancer; 2008. 21-3 p.
4. Senapati S, Mahanta AK, Kumar S, Maiti P. Controlled drug delivery vehicles for cancer treatment and their performance. *Signal Transduct Target Ther.* 2018 Mar 16;3:7. doi: 10.1038/s41392-017-0004-3. PMID: 29560283; PMCID: PMC5854578.
5. Majidinia M, Mirza-Aghazadeh-Attari M, Rahimi M, Mihanfar A, Karimian A, Safa A, et al. Overcoming multidrug resistance in cancer: Recent progress in nanotechnology and new horizons. *IUBMB Life.* 2020 May;72(5):855-871. doi: 10.1002/iub.2215.
6. Bissett D, Kerr DJ, Cassidy J, Meredith P, Traugott U, Kaye SB. Phase I and pharmacokinetic study of D-verapamil and doxorubicin. *Br J Cancer.* 1991 Dec;64(6):1168-71. doi: 10.1038/bjc.1991.484.
7. Kathawala RJ, Gupta P, Ashby CR Jr, Chen ZS. The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. *Drug Resist Updat.* 2015 Jan;18:1-17. doi: 10.1016/j.drup.2014.11.002.
8. Zhang YK, Wang YJ, Gupta P, Chen ZS. Multidrug Resistance Proteins (MRPs) and Cancer Therapy. *AAPS J.* 2015 Jul;17(4):802-12. doi: 10.1208/s12248-015-9757-1.
9. Wang H, Gao Z, Liu X, Agarwal P, Zhao

S, Conroy DW, et al. Targeted production of reactive oxygen species in mitochondria to overcome cancer drug resistance. *Nat Commun.* 2018 Feb 8;9(1):562. doi: 10.1038/s41467-018-02915-8.
10. Jiang J, Wang K, Chen Y, Chen H, Nice EC, Huang C. Redox regulation in tumor cell epithelial-mesenchymal transition: molecular basis and therapeutic strategy. *Signal Transduct Target Ther.* 2017 Aug 18;2:17036. doi: 10.1038/sigtrans.2017.36.
11. Saatci Ö, Borgoni S, Akbulut Ö, Durmuş S, Raza U, Eyüpoğlu E, et al. Targeting PLK1 overcomes T-DM1 resistance via CDK1-dependent phosphorylation and inactivation of Bcl-2/xL in HER2-positive breast cancer. *Oncogene*. 2018 Apr;37(17):2251-2269. doi: 10.1038/s41388-017-0108-9. Epub 2018 Feb 2. PMID: 29391599.
12. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, Gottesman MM. P-glycoprotein: from genomics to mechanism. *Oncogene*. 2003 Oct 20;22(47):7468-85. doi: 10.1038/sj.onc.1206948.
13. Szakács G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. *Nat Rev Drug Discov.* 2006 Mar;5(3):219-34. doi: 10.1038/nrd1984.
14. Zhang M, Liu E, Cui Y, Huang Y. Nanotechnology-based combination therapy for overcoming multidrug-resistant cancer. *Cancer Biol Med.* 2017 Aug;14(3):212-227. doi: 10.20892/j.issn.2095-3941.2017.0054.
15. Bergman AM, Pinedo HM, Talianidis I, Veerman G, Loves WJ, van der Wilt CL, et al. Increased sensitivity to gemcitabine of P-glycoprotein and multidrug resistance-associated protein-overexpressing human cancer cell lines. *Br J Cancer*. 2003 Jun 16;88(12):1963-70. doi: 10.1038/sj.bjc.6601011.
16. Zheng W, Li M, Lin Y, Zhan X. Encapsulation of verapamil and doxorubicin by MPEG-PLA to reverse drug resistance in ovarian cancer. *Biomed Pharmacother.* 2018 Dec;108:565-573. doi: 10.1016/j.biopha.2018.09.039.
17. Mir M, Ahmed N, Rehman AU. Recent applications of PLGA based nanostructures in drug delivery. *Colloids Surf B Biointerfaces.* 2017 Nov 1;159:217-231. doi: 10.1016/j.colsurfb.2017.07.038.
18. Risnayanti C, Jang YS, Lee J, Ahn HJ. PLGA nanoparticles co-delivering MDR1 and BCL2 siRNA for overcoming resistance of paclitaxel and cisplatin in recurrent or advanced ovarian cancer. *Sci Rep.* 2018 May 14;8(1):7498. doi: 10.1038/s41598-018-25930-7.
19. Danhier F, Ansorena E, Silva JM, Coco R, Le Breton A, Préat V. PLGA-based nanoparticles: an overview of biomedical applications. *J Control Release.* 2012 Jul 20;161(2):505-22. doi: 10.1016/j.jconrel.2012.01.043.
20. Kamaly N, Yameen B, Wu J, Farokhzad OC. Degradable Controlled-Release Polymers and Polymeric Nanoparticles: Mechanisms of Controlling Drug Release. *Chem Rev.* 2016 Feb 24;116(4):2602-63. doi: 10.1021/acs.chemrev.5b00346.
21. Imre S, Vlase L, Muntean DL. Bioanalytical method validation. *Rev Romana de Medicina de Lab*. 2008;Vol 10(1):4-5.
22. Song D, Wang J. Modified resolution factor for asymmetrical peaks in chromatographic separation. *J Pharm Biomed Anal.* 2003 Aug 21;32(6):1105-12. doi: 10.1016/s0731-7085(03)00026-8. PMID: 12907253.
23. Emami J, Ghassami N, Ahmadi F. Development and validation of a new HPLC method for determination of lamotrigine and related compounds in tablet formulations. *J Pharm Biomed Anal.* 2006 Mar 3;40(4):999-1005. doi: 10.1016/j.jpba.2005.07.045.
24. Ahmadi F, Bahmyari M, Akbarizadeh A, Alipour S. Doxorubicin-verapamil dual loaded PLGA nanoparticles for overcoming P-glycoprotein mediated resistance in cancer: Effect of verapamil concentration. *J Drug Deliv Sci Technol.* 2019;53:101206.
25. Liu Y, Li LL, Qi GB, Chen XG, Wang H. Dynamic disordering of liposomal cocktails and the spatio-temporal favorable release of cargoes to circumvent drug resistance. *Biomaterials*. 2014 Mar;35(10):3406-15. doi: 10.1016/j.biomaterials.2013.12.089.
26. Qin M, Lee YE, Ray A, Kopelman R. Overcoming cancer multidrug resistance by codelivery of doxorubicin and verapamil with hydrogel nanoparticles. *Macromol Biosci*. 2014 Aug;14(8):1106-15. doi: 10.1002/mabi.201400035.
27. Breaux J, Jones K, Boulas P. Analytical methods development and validation. *Pharm Technol.* 2003;1:6-13.
28. Takka S, Ocak OH, Acartürk F. Formula-

tion and investigation of nicardipine HCl-alginate gel beads with factorial design-based studies. Eur *J Pharm Sci*. 1998 Jul;6(3):241-6. doi: 10.1016/s0928-0987(97)10005-7. PMID: 9795073.

29. Amjadi I, Rabiee M, Hosseini MS. Anticancer Activity of Nanoparticles Based on PLGA and its Co-polymer: In-vitro Evaluation. *Iran J Pharm Res.* 2013 Fall;12(4):623-34.

30. Liu SQ, Tong YW, Yang YY. Incorporation and in vitro release of doxorubicin in thermally sensitive micelles made from poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide)-b-poly(D,L-lactide-co-glycolide) with varying compositions. *Biomaterials*. 2005 Aug;26(24):5064-74. doi: 10.1016/j.biomaterials.2005.01.030.