Department of Pharmaceutical Technology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey

Development and validation of a LC-FL method for the simultaneous determination of doxorubicin and celecoxib in nanoparticulate fixed dose combination (NanoFDC)

S. Kozlu, A. Sahin, S. Calis, Y. Capan*

Received April 29, 2017, accepted July 7, 2017

**Corresponding author: Yilmaz Capan, Department of Pharmaceutical Technology, Faculty of Pharmacy, Hacettepe University, 06100 Ankara, Turkey*
ycapan@hacettepe.edu.tr

Pharmazie 72: 568–570 (2017) *doi: 10.1691/ph.2017.7077*

An isocratic reversed phase HPLC method for the simultaneous determination of doxorubicine (DOX) and celecoxib (CXB) out of a nanoparticulate fixed dose combination (NanoFDC) was developed and validated. Linearity of the results was demonstrated from 1-11 µg/mL for both components. Lower limits of detection were determined as 7 ng/mL for DOX and 13 ng/mL for CXB. Total run time was approximately 15 min.

1. Introduction

Doxorubicin (DOX) is a potent cytotoxic drug, indicated for various cancers; but it also shows important side effects. To overcome these side effects, many different approaches were studied (Ramanlal Chaudhari et al. 2012; Swami et al. 2014). On the other hand most of the preclinical studies and clinical trials showed that non-steroidal anti-inflammatory drugs (NSAID) and specifically cyclooxygenase-2 (COX-2) enzyme inhibitors such as celecoxib (CXB) increase the effect of chemotherapeutic drugs (Chan 2002). In ongoing studies, it was demonstrated that CXB has synergistic activity with DOX against cancer but usage of these combination is limited due to cardiotoxic side effects of these drugs (Groen et al. 2011).

Nanoparticulate drug delivery systems provide many opportunities such as targeting drug delivery and encapsulating more than one drug into a nanocarrier particle (Allen 2002). This combined drug delivery approach recently named as Nanoparticulate Fixed Dosage Combinations (NanoFDC) (Arora et al. 2015). Improved chemotherapy and decreased side effects could be possible by targeted NanoFDC formulations. Although there are numerous studies for the combination effect of these molecules, there is not any combined dosage form or combined analytical method, published in the literature yet. Both of two molecules have its monograph in the United States Pharmacopeia (USP) and European Pharmacopeia (EP). Besides the analytical methods for DOX and CXB suggested by major pharmacopoeias, there are also many other methods for the analysis of both molecules separately. For DOX analysis, these include fluorescence spectrophotometry (Subedi et al. 2009), UV–visible spectrophotometry (Tewes et al. 2007), HPLC with fluorescence detection (Alhareth et al. 2012; Reddy et al. 2005), and HPLC with UV detection (Dharmalingam et al. 2014). For CXB analysis, spectrofluorometry (Damiani et al. 2003), HPLC-UV (Saha et al. 2002; Rose et al. 2000) and HPLC-mass (Dongari et al. 2014) and UV spectrometry methods are published. These methods are suitable for dosage forms with a single active pharmaceutical ingredient. In this study, an isocratic reversed phase HPLC method for the simultaneous determination of DOX and CXB was developed and validated using fluorescent detector at gradient excitation and emission wavelengths according to the European Pharmacopoeia and International Conference on Harmonization (ICH) requirements.

2. Investigations and results

A RP-HPLC system was selected to analyze two compounds with an efficient separation and acceptable peak symmetries. The experiments were performed with a C18 column by trying various mobile phase conditions systematically. After the initial experiments, the optimum conditions were found to be the mobile phase of acetic acid solution (pH 3.0, 1 %): acetonitrile (40:60 v/v) mixture pumped at 1 mL min/min flow rate. Under the optimum conditions, due to distinct differences of polarity of two compounds, DOX and CXB were eluted at 2.0 min and 12.9 min, respectively.

After the suitability test applied to the chromatograms, the system was found suitable. Resolution was more than 2 and column efficiency was more than 2000 for both compounds. The relative standard deviations of the repeatability of peak areas for both compounds were less than 1 %. The peak symmetries measured at a point 5 % of the peak height from the baseline for both compounds were less than 1.1, whereas the capacity factors were more than 2.0. The total run time was 15 min.

The specificity of the method was confirmed by absence of any interfering peaks with DOX and CXB peak in the obtained chromatograms. Neither solution of PLGA nanoparticles, nor dilution solvent (mobile phase) have a fluorophore group and do not generate florescent signal. The method was found specific for DOX and CXB analyses and linear in the concentration range of 1.0-11.0 µg/mL for DOX and CXB analysis. A sample chromatogram of doxorubicin and celecoxib analysed from PLGA nanoparticles is presented in the Fig..

According to the DOX linear regression analyses results, the coefficient of determination of the regression line (R^2) was 0.9996, the % y-intercept values were less than 2.0 % for all points except 1 ppm. The regression equation was $y = 77214\,x + 2072.2$, where y is the observed peak area and x is the concentration of the solution. Considering the linearity of CXB, coefficient of determination of the regression line (R^2) was found to be 0.9998, the % y-intercept values were less than 2.0 % for all points except 1 ppm. The calculated linear regression equation was $y = 57855x + 2190$. Detailed linearity statistics are presented in Table 1.

Table 1: Linearity data of the developed method (n=6)

	DOX	CXB
Regression equation	$y = 77214x + 2072.2$	$y = 57855x + 2190$
Standard error of intercept	4579.8	2378.1
Standard error of slope	711.5	369.5
Coefficient of determination	0.9996	0.9998

	DOX	CXB
Linearity range (µg/mL)	1-11	1-11
Number of data points	7	7
LOD (ng/mL)	7	13
LOQ (ng/mL)	14	19

The RSD% values were less than 2 % and the recovery value percentages were 98-102 % for three of the concentrations for both compounds as presented in detail in Table 2. The LODs were 7 ng/mL for doxorubicin and 13 ng/mL for celecoxib. The quantitation limits were 14 ng/mL for doxorubicin and 19 ng/mL for celecoxib.

3. Experimental

3.1. Chemicals

Celecoxib was purchased from Santa Cruz Biotech (Santa Cruz, USA) and Doxorubicin HCl manufactured by Hisun Pharmaceuticals (Zhejiang, China) was kindly gifted by Deva (Istanbul, Turkey) Pharmaceuticals. Acetonitrile, glacial acetic acid was HPLC grade, triethylamine (TEA) was synthesis grade and they were all purchased from Sigma-Aldrich. PLGA RG502H, acetone and poly (vinyl alcohol) (PVA) (87–90% hydrolyzed, average molecular weight 30,000–70,000) that were used for nanoparticle preparation were obtained from Sigma-Aldrich. Water was used for the preparation of buffers and aqueous solutions as obtained by a Millipore Simplicity® UV system (Bedford, USA) (18.2 Ω.cm).

3.2. Sample preparation and solutions

Stock solutions of DOX and CXB were prepared by dissolving 5.0 mg of doxorubicin HCl and 5.0 mg of celecoxib in 100 mL of mobile phase in amber colored volumetric flask separately. Standard solutions used for the calibration were prepared by an appropriate dilution process using mobile phase. Acetic acid solution (1 %) was prepared by addition of 10 mL glacial acetic acid into deionized water and completed to a volume of 1 L and pH was adjusted to 3.0 with triethylamine. Blank PLGA nanoparticles were prepared with nanoprecipitation as described previously (Sahin et al. 2017). Matrix effect was evaluated according to the literature (Yerlikaya et al. 2010). Lyophilised dried nanoparticles were dissolved in mobile phase and mixed with DOX and CXB standard and vortexed for 1 min.

3.3. Instrumentation

The HPLC system, was a modular Shimadzu Prominence Series consisted of a degasser (DGU 20A), a quaternary pump (LC 20AT), an auto-sampler (SIL 20A), a thermostated column compartment (CTO 10AS) and a fluorescent detector (RF-20A) was used for separation and quantification of two molecules. The column was a GL Sciences Inertsil ODS-3 C_{18} HPLC column (250 mm x 4.6 mm, 5 µm) (Japan) thermostated at 25 °C. The mobile phase was composed of 1% acetic acid solution (pH 3.0) and acetonitrile (60:40, *v:v*). The flow rate of mobile phase was set to 1 mL/min and injection volume was 20 µL. Detector was set with a gradient program; first 5 min excitation at 480 nm and emission at 590 nm; after then excitation was set at 240 nm and emission at 380 nm.

3.4. System suitability

A system suitability test was performed to check chromatographic parameters suitable with the directions of European Pharmacopeia using 5.0 µg/mL of DOX and CXB standard solution.

3.5. Analytical method validation

The parameters stated in recommendation of ICH Guideline Q2(R1) were investigated (ICH 2005). Dissolved PLGA, mobile phase and standard solution of DOX and CXB were injected and the chromatograms were visually compared to assess the specificity of the analytical method. A linearity study was performed to show the linearity of the capability of the analysis in the range of 1.0-11.0 µg/mL. For this, a regression analysis was performed and evaluated (n = 3). Standard solutions were prepared in three concentrations (1.0, 5.0 and 11.0 µg/mL) triplicate for the accuracy and repeatability studies. Intermediate precision of the method was evaluated by repeating the same analysis in two consecutive days. The intermediate precision (inter-day) was shown by repeating the same procedure for two consecutive days. Recovery percentage was used to point out the accuracy and RSD% to point out the precision. The detection and quantitation limits (LOQ, LOD) are not a requirement for assay studies, thus this parameters were calculated using signal to noise ratio (*S/N)* method to represent the sensitivity of the method.

Conflicts of interest: None declared.

References

Alhareth K, Vauthier C, Gueutin C, Ponchel G, Moussa F (2012) Hplc quantification of doxorubicin in plasma and tissues of rats treated with doxorubicin loaded poly(alkylcyanoacrylate) nanoparticles. J Chromatogr B Analyt Technol Biomed Life Sci 887-888: 128-132.

Allen TM (2002) Ligand-targeted therapeutics in anticancer therapy. Nat Rev Cancer 2: 750-763.

Arora A, Shafiq N, Jain S, Khuller G K, Sharma S, Malhotra S (2015) Development of sustained release "nanofdc (fixed dose combination)" for hypertension – an experimental study. PLoS One 10: e0128208.

Chan TA (2002) Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention. Lancet Oncol 3: 166-174.

Damiani P, Bearzotti M, Cabezon MA (2003) A validated spectrofluorometric method for the determination of celecoxib in capsules. Anal Bioanal Chem 376: 1141-1146.

Table 2: Accuracy and precision data for DOX and CXB analyses (n = 3)

	Theoretical concentration (µg/mL)	Intra-assay				Inter-assay			
		Observed Mean (µg /mL)	Recovery %	RSD %	95 % Confidence interval	Observed Mean (µg /mL)	Recovery %	RSD %	95 % Confidence interval
DOX	1	1.013	101.27	1.652	0.994-1.032	0.987	98.68	1.542	0.970-1.004
	5	4.934	98.68	1.127	4.871-4.997	4.978	99.56	0.532	4.948-5.008
	11	11.067	100.61	0.365	11.021-11.112	11.122	101.11	0.124	11.106-11.137
CXB	1	0.992	99.20	1.453	0.976-1.008	1.012	101.23	1.212	0.998-1.026
	5	5.090	101.79	0.956	5.034-5.145	4.922	98.44	1.012	4.866-4.978
	11	10.879	98.90	1.021	10.753-11.005	11.212	101.93	0.345	11.168-11.256

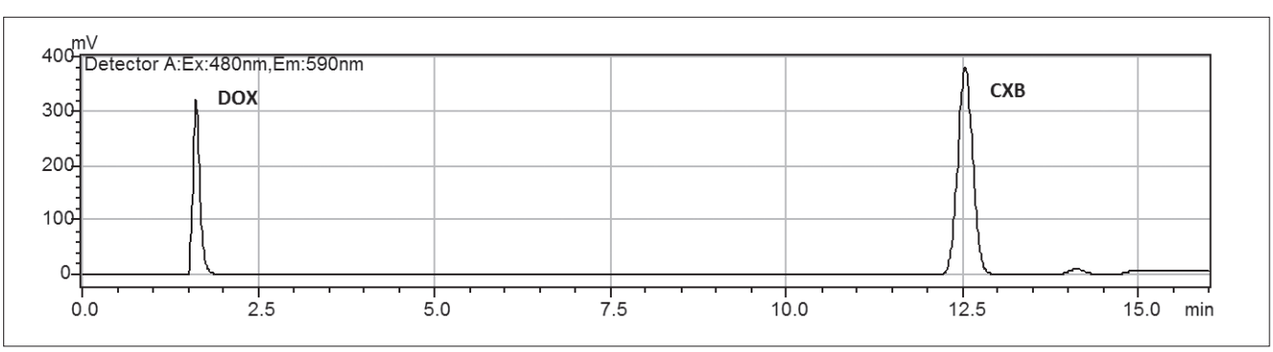

Fig. 1: Representative chromatogram of DOX and CXB from PLGA nanoparticles

Dharmalingam SR, Madhappan R, Ramamurthy S, Chidambaram K, Srikanth M V, Shanmugham S, Senthil Kumar KL (2014) Investigation on antidiarrhoeal activity of aristolochia indica linn. Root extracts in mice. Afr J Tradit Complement Altern Med 11: 292-294.

Dongari N, Sauter ER, Tande BM, Kubatova A (2014) Determination of celecoxib in human plasma using liquid chromatography with high resolution time of flight-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 955-956: 86-92.

Groen H J, Sietsma H, Vincent A, Hochstenbag M M, Van Putten J W, Van Den Berg A, Dalesio O, Biesma B, Smit H J, Termeer A, Hiltermann T J, Van Den Borne B E, Schramel F M (2011) Randomized, placebo-controlled phase iii study of docetaxel plus carboplatin with celecoxib and cyclooxygenase-2 expression as a biomarker for patients with advanced non-small-cell lung cancer: The nvalt-4 study. J Clin Oncol 29: 4320-4326.

ICH (2005) Validation of analytical procedures: Text and methodology q2(r1).

Ramanlal Chaudhari K, Kumar A, Megraj Khandelwal V K, Ukawala M, Manjappa A S, Mishra A K, Monkkonen J, Ramachandra Murthy R S (2012) Bone metastasis targeting: A novel approach to reach bone using zoledronate anchored plga nanoparticle as carrier system loaded with docetaxel. J Control Release 158: 470-478.

Reddy LH, Meda N, Murthy RR (2005) Rapid and sensitive HPLC method for the estimation of doxorubicin in dog blood--the silver nitrate artifact. Acta Pharm 55: 81-91.

Rose MJ, Woolf EJ, Matuszewski BK (2000) Determination of celecoxib in human plasma by normal-phase high-performance liquid chromatography with column switching and ultraviolet absorbance detection. J Chromatogr B Biomed Sci Appl 738: 377-385.

Saha RN, Sajeev C, Jadhav PR, Patil SP, Srinivasan N (2002) Determination of celecoxib in pharmaceutical formulations using uv spectrophotometry and liquid chromatography. J Pharm Biomed Anal 28: 741-751.

Sahin A, Esendagli G, Yerlikaya F, Caban-Toktas S, Yoyen-Ermis D, Horzum U, Aktas Y, Khan M, Couvreur P, Capan Y (2017) A small variation in average particle size of plga nanoparticles prepared by nanoprecipitation leads to considerable change in nanoparticles' characteristics and efficacy of intracellular delivery. Artif Cells Nanomed Biotechnol: 1-11.

Subedi RK, Kang KW, Choi HK (2009) Preparation and characterization of solid lipid nanoparticles loaded with doxorubicin. Eur J Pharm Sci 37: 508-513.

Swami A, Reagan M R, Basto P, Mishima Y, Kamaly N, Glavey S, Zhang S, Moschetta M, Seevaratnam D, Zhang Y, Liu J, Memarzadeh M, Wu J, Manier S, Shi J, Bertrand N, Lu Z N, Nagano K, Baron R, Sacco A, Roccaro A M, Farokhzad O C, Ghobrial I M (2014) Engineered nanomedicine for myeloma and bone microenvironment targeting. Proc Natl Acad Sci USA 111: 10287-10292.

Tewes F, Munnier E, Antoon B, Ngaboni Okassa L, Cohen-Jonathan S, Marchais H, Douziech-Eyrolles L, Souce M, Dubois P, Chourpa I (2007) Comparative study of doxorubicin-loaded poly(lactide-co-glycolide) nanoparticles prepared by single and double emulsion methods. Eur J Pharm Biopharm 66: 488-492.

Yerlikaya F, Aktas Y, Capan Y (2010) Lc-uv determination of melatonin from chitosan nanoparticles. Chromatographia 71: 967-970.