Turkish Journal of Chemistry

http://journals.tubitak.gov.tr/chem/

Research Article

Turk J Chem
(2019) 43: 846 – 856
© TÜBİTAK
doi:10.3906/kim-1901-37

Determination of N-acetylcysteine in the presence of ciprofloxacin or levofloxacin in microparticulate dry powder inhalers

Yağmur AKDAĞ[1], **Selma ŞAHİN**[1], **Emirhan NEMUTLU**[2], **Levent ÖNER**[1,*]
[1]Department of Pharmaceutical Technology, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey
[2]Department of Analytical Chemistry, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey

Received: 16.01.2019 • **Accepted/Published Online:** 04.03.2019 • **Final Version:** 11.06.2019

Abstract: A fast and easy method was validated for simultaneous determination of ciprofloxacin hydrochloride monohydrate (CP), levofloxacin hemihydrate (LV), and N-acetylcysteine (NAC) in samples. The analysis was performed on a C_{18} column (250 × 4.6 mm, 5 µm) (Inertsil ODS-3V) using an isocratic elution method with a mobile phase composed of 25 mM KH_2PO_4 (pH 3.0) and methanol (72:28, v/v) at a flow rate of 1 mL/min. UV detection was performed at 214 nm for NAC and 293 nm for CP and LV. The method was validated for linearity, accuracy, precision (repeatability and reproducibility), specificity, sensitivity, and stability. The calibration study using several media demonstrated that the calibration curves were linear for all compounds in all media (R^2 >0.9993). The limit of detection was 0.098 µg/mL for CP, 0.049 µg/mL for LV, and 0.487 µg/mL for NAC. The limit of quantification was 0.328 µg/mL for CP, 0.165 µg/mL for LV, and 1.624 µg/mL for NAC. Precision and accuracy values of the method fulfilled the required limits. All these outcomes demonstrate that the validated HPLC method is appropriate for simultaneous analysis of CP, LV, and NAC in samples for content uniformity of dry powder inhaler and permeability studies.

Key words: Ciprofloxacin, levofloxacin, N-acetylcysteine, HPLC, validation, dry powder inhaler

1. Introduction

Cystic fibrosis (CF) is a life-shortening autosomal recessive genetic disease that can be caused by mutations in the gene that results in abnormally viscous secretions in various exocrine tissues, including the respiratory tract [1–4].

In CF, the fluidity of the mucus layer decreases, which is crucial for the clearance of bacteria and other microorganisms from the airways. Moreover, lower mucus secretion and reduced pH of airway surface liquid may cause inhibition of antimicrobial activity [3,5]. Thus, recurrent bronchiectasis and infections, especially caused by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, are the major reason for morbidity and mortality in CF [4–6]. Although there are various antibiotics administered orally and intravenously to treat lung infections in CF patients, inhalation is the most efficient administration route to directly reach the infection site [7,8].

Fluoroquinolone antibiotics are broad-spectrum synthetic drugs widely used for the treatment of lung infections [9–11]. Ciprofloxacin and levofloxacin were selected for this study as fluoroquinolone antibiotics. A mucolytic was added to the formulations to increase the effectiveness of antibiotics in CF treatment. The most important problem in the treatment of CF-associated infections is the difficulty of antibiotic penetration into the

*Correspondence: loner@hacettepe.edu.tr

This work is licensed under a Creative Commons Attribution 4.0 International License.

dehydrated and viscous mucus layer in the lungs. Therefore, N-acetylcysteine (NAC) was chosen as a mucolytic agent to increase the penetration ability of ciprofloxacin and levofloxacin.

Content uniformity and permeability are some of the quality attributes considered for prepared dry powder inhalers (DPIs). To estimate the content uniformity and permeability values correctly, it is crucial to use a validated method with enough sensitivity, selectivity, accuracy, and precision for each compound in different matrixes. In this study, we aimed to validate an HPLC method for the simultaneous analysis of NAC in the presence of ciprofloxacin and levofloxacin in samples obtained from content uniformity and permeability studies.

Several HPLC methods are available in the literature for the determination of ciprofloxacin [12–14], levofloxacin [15–17], and NAC [18–20] individually in pharmaceutical formulations. There are studies in the literature where NAC was combined with ciprofloxacin hydrochloride monohydrate (CP) [21–24] or levofloxacin hemihydrate (LV) [25,26]. These studies were clinical or microbiological studies and unfortunately none of them included quantification of NAC or the fluoroquinolones. It is important to be able to detect not only fluoroquinolone antibiotics but also NAC since all of them are active pharmaceutical ingredients and have physiological effects on living organisms. Moreover, quantification of all active ingredients with a single method will be faster, efficient, and more cost-effective compared to separate analyses. For the analysis of ciprofloxacin, levofloxacin, and NAC, a short, sensitive, and fully validated analysis method is needed. In this study, simultaneous quantification of NAC and its combination with ciprofloxacin or levofloxacin was performed for the first time. The method was suitable for the analysis of ciprofloxacin, levofloxacin, and NAC in DPIs and permeability studies.

2. Materials and methods

2.1. Chemicals

CP, LV, and NAC were kindly provided by Neuland Labs. (Hyderabad, India). Methanol and potassium dihydrogen phosphate were purchased from Sigma Aldrich (St. Louis, MO, USA). Water was purified using a Milli-Q system (Millipore). All other chemicals were of analytical grade.

2.2. Instrumentation and chromatographic conditions

The HPLC system used for analysis was Agilent 1200 series (Germany). CP, LV, and NAC were separated using an Inertsil ODS-3V C_{18} column (250 × 4.6 mm 5 µm; Japan). The HPLC system was operated at 40 °C using a mobile phase consisting of 25 mM KH_2PO_4 (pH 3.0) and methanol (72:28, v/v). The mobile phase was filtered through a 0.45-µm membrane filter, degassed before use, and delivered to the HPLC system at a flow rate of 1 mL/min. The detection was performed at 293 nm for CP and LV and at 214 nm for NAC. The total run time for the analysis was 15 min and the injection volume was 5 µL.

2.2.1. Preparation of standard solutions

Standard stock solutions of CP, LV, and NAC (1000 µg/mL) were prepared in the mobile phase. These stock solutions were then diluted with the mobile phase to obtain solutions within the concentration range of 5–400 µg/mL for CP, LV, and NAC.

2.3. Method validation

The HPLC method was validated as to specificity, linearity, sensitivity (limit of detection (LOD) and limit of quantitation (LOQ)), accuracy, precision (repeatability and reproducibility), and stability according to the ICH guidelines [27].

2.3.1. Specificity

The specificity of the analytical method was assessed by injecting a drug-free mobile phase and Hank's Balanced Salt Solution (HBSS) (10 mM HEPES) (transport medium) into the HPLC system. HBSS (10 mM HEPES) is often used as a transport buffer for cell cultures because it provides a buffering system and maintains the physiological pH range and osmotic balance of the culture medium. It is also a source of water, inorganic ions, and energy for cells.

2.3.2. Linearity

The linearity of the method was evaluated by spiking at least 6 different concentrations within the concentration range of 10–400 µg/mL for CP, LV, and NAC (six different series were prepared). Calibration curves for CP, LV, and NAC were constructed by plotting the peak areas of each compound against the corresponding nominal concentrations. Linearity of the analytical method was demonstrated by the calibration equation obtained from the calibration curve and the equation was characterized by intercept, slope, and determination coefficient.

2.3.3. Sensitivity

By determining the LODs and LOQs, the sensitivity of the method was assessed. LOD is described as the lowest amount of analyte in a sample that can be detected, and LOQ as the lowest amount of analyte in a sample that can be quantitatively determined with suitable accuracy and precision. The signal to noise ratios of 3:1 and 10:1 were taken as the LOD and LOQ, respectively.

2.3.4. Precision

Intra- and interday precision studies were carried out for assessment of the assay precision. Three different concentrations of compounds (10, 100, and 400 µg/mL for CP and LV; 10, 100, and 300 µg/mL for NAC) within the calibration range were analyzed 6 consecutive days (interday) and 6 times within the same day (intraday) to determine the precision of the method. The relative standard deviations (RSDs) of intra- and interday studies were calculated for assessment of precision of the method.

2.3.5. Accuracy

Accuracy studies were carried out using 3 different concentrations of compounds (10, 100, and 400 µg/mL for CP and LV; 10, 100, and 300 µg/mL for NAC). Accuracies of the method were calculated as percentage of bias and percentage of recovery.

2.3.6. Stability

Stability studies of samples containing all compounds (CP, LV, and NAC) were performed for 24 h. All samples were stored at room temperature and then analyzed using the developed HPLC method. The results were compared with the initial values of the compounds.

2.4. Production of microparticulate DPIs

The details of the production and characterization of the microparticulate DPIs were published in our previous paper [27]. Briefly, LV solutions and CP suspensions (prepared using ball milling and high pressure homogenization methods) were obtained. The resulting suspensions or solutions were then spray-dried, either alone or with the addition of NAC solution. At the end of production, formulations containing CP were in crystalline form, whereas the formulations containing LV were amorphous. Aerodynamic parameters of manufactured formulations were evaluated by Andersen Cascade Impactor. Fine particle dose (< 5 µm), fine particle fraction (%) (< 5 µm), emitted dose, and mass median aerodynamic diameter results were 11.25–12.14 mg, 74.02%–82.17%, 53.7%–58.26%, and 2.60–3.06 µm for CP-containing formulations and 12.59–14.61 mg, 65.82%–85.18%, 61.95%–68.18%, and 2.29–3.20 µm for LV-containing formulations, respectively [28].

2.4.1. Content uniformity

In this study, four different DPI formulations (two of them containing only quinolones, and the other two containing combinations of the antibiotics with NAC) were developed and their content uniformities were tested using the developed HPLC method. The DPIs were dissolved in 25 mL of mobile phase for CP-containing formulations (CP DPI or CP-NAC DPI) and LV-containing formulations (LV DPI or LV-NAC DPI). The solutions were filtered and analyzed using the HPLC method to determine the amount of CP, LV, and NAC in DPIs.

2.4.2. Permeability

The Calu-3 cell line (human airway epithelial cells) was used to measure the permeability of the produced inhaler formulations as described in our previous work [28]. To evaluate permeabilities, cells were grown as liquid-covered culture (LCC) and air-interfaced culture (AIC). Seeding to ThinCert (0.4 µm, 1.13 cm^2) was performed in such a way that the density of the cells was 5×10^5 cells/cm^2. The growth medium was changed every other day for 21 days to obtain the LCC. Growth medium at the apical side was removed 10 days after seeding to obtain the AIC, and then only the media at the basolateral sides were changed for another 10 days. Raw CP, raw LV, and manufactured DPIs were applied as dry powders (formulations containing 3.7 mg CP (and 0.244 mg NAC) or 3.7 mg LV (and 0.211 mg NAC)) and as solutions in HBSS (10 mM HEPES) (formulations containing 0.65 mM CP (and 0.118 mM NAC) or 1.161 mM LV (and 0.184 mM NAC)). Samples were taken from basolateral sides after 2 h of incubation. Concentrations of CP, LV, and NAC in samples were detected using the developed method. Permeability (P_{app}; cm/s) data were obtained using Eq. (1).

$$P_{app} = \text{Rate of transport} / (\text{surface area} \times \text{initial donor concentration}) \quad (1)$$

2.5. Statistical analysis

The data were analyzed using the Mann–Whitney U test. For this purpose, GraphPad Prism 6 was utilized. The difference between the results was considered significant at $P < 0.05$.

3. Results and discussion

3.1. Optimization of chromatographic conditions

An HPLC method was validated for simultaneous determination of CP, LV, and NAC for content uniformity and permeability studies. To determine the amount of NAC and antibiotics in the obtained samples, the

method developed by Chamseddin and Jira [12] was adapted. The published method was only tested for chromatographic behavior of three fluoroquinolone antibiotics and was not applied for any pharmaceutical or biological samples. They tested different stationary phases and mobile phase variables such as methanol content, pH, and buffer concentration. Finally, they suggested a C18 (100 × 4.6 mm, 2.4 µm) HPLC column and an isocratic mobile phase consisting of MeOH/25 mM phosphate buffer 28/72 (v/v) at pH 3 and flow rate 1 mL/min. There was no validation study for the method, and the selectivity of the method was not tested. However, in our study, NAC was analyzed in addition to CP and LV. Since CP, LV, and NAC, as active pharmaceutical substances, are frequently formulated together, the ability to analyze these three substances at the same time with a single method is an important innovation that accelerates pharmaceutical studies, and also their monitoring in pharmaceutical formulation during stability studies, which is an obligatory regulatory requirement. In addition, another difference and advantage of our study is that the method of analysis has been tested in DPI and the transport media where permeability studies were carried out by Caco-2 cell culture method. The initial optimization studies started with the chromatographic conditions suggested by Chamseddin and Jira, except for the length and particle size of the column, which were different from the original method. In our study, an Inertsil ODS-3V C18 column (250 × 4.6 mm 5 µm; Japan) was used for the separation of NAC and antibiotics. Therefore, the system suitability and selectivity of the method were initially tested for NAC and antibiotics. Moreover, the detection of active pharmaceutical ingredients was performed at 214 nm for NAC and at 293 nm for CP and LV in order to increase the sensitivity of the method. Additionally, column temperature was set to 40 °C to avoid any related temperature changes. Because of its simplicity, stable baseline, and unvarying response factor, isocratic elution of the mobile phase was used for analysis. Increasing flow rate decreases retention times, but also brings closer peaks of substances. Therefore, 1 mL/min flow rate was chosen for the analysis. An injection volume of 5 µL was adequate to analyze the compounds.

System suitability parameters were tested to show that the system was working accurately during the analysis. The fact that the capacity factor (k) is between 1 and 10 indicates that the method has optimum separation ability [27]. Determined capacity factor (k) values were 4.41, 3.10, and 3.02 for CP, LV, and NAC, respectively, indicating that these values were within the acceptable limits. The theoretical plate numbers were 5196, 6728, and 6471 for CP, LV, and NAC, respectively, which were in the acceptable limit of $N \geq 2000$. The tailing factors, which were also in the acceptable limit ($T \leq 2$), were obtained as 1.08, 0.92, and 1.10 for CP, LV, and NAC, respectively. Overall, these results indicate that the validated method fulfilled the recommended system suitability parameters for the simultaneous analysis of CP with NAC and of LV with NAC.

3.2. Method validation

3.2.1. Selectivity

The ability to differentiate and measure the target analyte in the presence of other components in the sample indicates the selectivity of the analytical method [27]. Following separate injection of 25 mM KH_2PO_4 (pH 3.0) and methanol (72:28, v/v) (mobile phase) and HBSS (transport medium) into the HPLC column, there was no interfering peak at the retention times of CP (13.516), LV (10.253), and NAC (4.022) (Figures 1 and 2). Based on the obtained chromatograms, the validated method was considered to be selective.

3.2.2. Linearity

The high correlation between the concentrations of an analyte and the representative peak areas shows the linearity of an analytical method. Calibration curves of the compounds were formed by plotting the peak

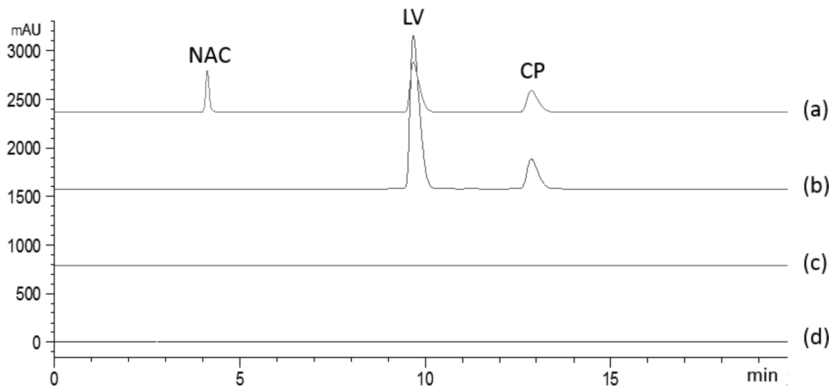

Figure 1. HPLC chromatograms of 250 µg/mL CP, LV, and NAC at a) 214 nm and b) 293 nm, in mobile phase (25 mM KH_2PO_4 (pH 3.0) and methanol (72:28, v/v)). Chromatograms of drug-free mobile phase at c) 214 nm and d) 293 nm.

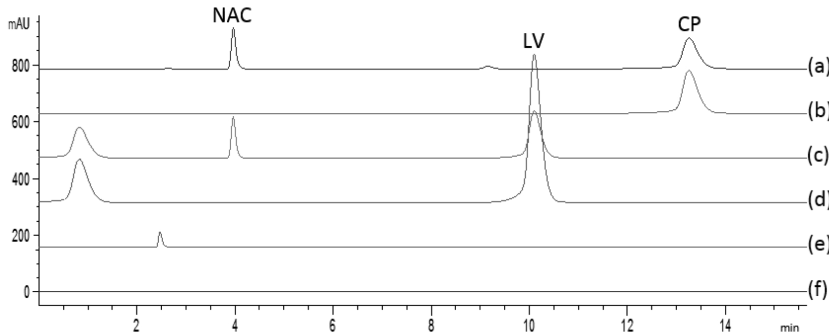

Figure 2. HPLC chromatograms of CP and NAC at a) 214 nm and b) 293 nm; LV and NAC at c) 214 nm and d) 293 nm in HBSS (10 mM HEPES). Chromatograms of drug-free HBSS (10 mM HEPES) at e) 214 nm and f) 293 nm.

areas of each compound against the corresponding concentrations. Regression analysis of the calibration curves showed linearity between peak areas and the corresponding CP, LV, and NAC concentrations. Although there was no interference from the transport medium (Figures 1 and 2), the calibration curves were obtained for the mobile phase and transport medium (HBSS). Since there was no significant difference between the calibration curves, only linearity data in the mobile phase for all compounds were demonstrated (Table 1).

Table 1. The linearity data of the validated method (n = 6).

Parameter	CP	LV	NAC
Regression equation*	y = 9.167x – 32.493	y = 22.815x –8.927	y = 2.421x + 3.755
Standard error of slope	0.017	0.020	0.061
Standard error of intercept	1.798	5.400	5.134
Determination coefficient (r^2)	0.9999	0.9999	0.9999
Linearity range (µg/mL)	10–400	10–400	10–400
LOD (µg/mL)	0.098	0.049	0.487
LOQ (µg/mL)	0.328	0.165	1.624

*The regression equation is expressed as y = ax + b, where x is the concentration (mg/mL) and y is the peak area.

3.2.3. Sensitivity

The LOD values were 0.098 µg/mL for CP, 0.049 µg/mL for LV, and 0.487 µg/mL for NAC. The LOQ values were 0.328 µg/mL for CP, 0.165 µg/mL for LV, and 1.624 µg/mL for NAC (Table 1). All these results imply that this analytical method is sensitive enough for determination of CP, LV, and NAC in content uniformity and permeability samples.

3.2.4. Precision and accuracy

Precision represents the degree of proximity between successive measurements under the same analytical conditions.

Table 2 demonstrates the intraday and interday precision data determined at 3 different concentrations (low, medium, and high). The RSD values estimated for the intraday and interday precision of the assay were lower than 2%, demonstrating that the precision of the validated HPLC method was acceptable.

Table 2. Precision (repeatability and reproducibility) and accuracy data of CP, LV, and NAC (n = 6).

Compounds	Added (µg/mL)	Intraday				Interday			
		Found[a] (µg/mL)	RSD[b] (%)	Accuracy[c] (bias %)	Recovery (%)	Found[a] (µg/mL)	RSD[b] (%)	Accuracy[c] (bias %)	Recovery (%)
CP	10	10.04 ± 0.06	0.60	0.40	100.40 ± 0.61	10.06 ± 0.14	1.39	0.60	100.60 ± 1.44
	100	99.26 ± 0.88	0.89	–0.74	99.26 ± 0.88	100.91 ± 1.13	1.12	0.91	100.91 ± 1.13
	400	402.95 ± 5.15	1.28	0.74	100.74 ± 1.29	401.68 ± 5.15	1.28	0.42	100.42 ± 1.29
LV	10	9.93 ± 0.05	0.50	–0.70	99.30 ± 0.54	10.03 ± 0.10	1.00	0.30	100.30 ± 1.04
	100	99.93 ± 0.34	0.34	–0.07	99.93 ± 0.34	99.65 ± 1.21	1.21	–0.35	99.65 ± 1.21
	400	396.94 ± 0.63	0.16	–0.77	99.24 ± 0.16	397.83 ± 1.35	0.34	–0.54	99.46 ± 0.34
NAC	10	9.97 ± 0.16	1.60	–0.30	99.70 ± 1.62	10.07 ± 0.13	1.29	0.70	100.70 ± 1.33
	100	99.75 ± 0.89	0.89	–0.25	99.75 ± 0.89	99.31 ± 0.77	0.78	–0.69	99.31 ± 0.77
	300	300.41 ± 1.66	0.55	0.14	100.14 ± 0.55	298.99 ± 1.21	0.40	–0.34	99.66 ± 0.40

[a]Mean ± SD, [b]RSD, [c]Bias % = ((Found – Added)/Added) × 100.

Accuracy is the measure of how close the experimental value is to the true value. For all substances and concentrations, recovery (%) values were between 99% and 101%, indicating that the experimental values are close enough to actual values (Table 2).

3.2.5. Stability

To demonstrate the stability of the drugs during analysis, CP, LV, and NAC solutions prepared at certain concentrations were examined. The stability was evaluated for 24 h since the analysis time did not exceed 24 h. The obtained data showed that the concentration changes in CP, LV, and NAC solutions were statistically insignificant during this period ($P > 0.05$). These results indicate that our substances were stable during the time analysis period (Table 3).

3.3. Content uniformity

The manufactured formulations were evaluated for CP, LV, and NAC contents using the validated HPLC method. The content uniformity results are given in Table 4 and HPLC chromatograms of CP-NAC- and LV-NAC-containing DPIs are shown in Figure 3.

Table 3. The stability results in mobile phase and HBSS (transport medium; mean ± SD; n = 3).

Compound	Remained %					
	Mobile phase			HBSS (transport medium)		
	4 h	12 h	24 h	4 h	12 h	24 h
CP	100.50 ± 0.39	100.53 ± 1.25	100.65 ± 1.26	101.04 ± 0.31	101.37 ± 0.52	100.55 ± 1.13
LV	99.03 ± 0.34	98.93 ± 0.75	98.73 ± 0.94	100.91 ± 0.49	99.33 ± 0.81	99.76 ± 0.76
NAC	100.01 ± 0.52	99.41 ± 0.41	99.74 ± 0.56	99.81 ± 0.27	99.98 ± 1.03	99.41 ± 0.85

Table 4. Content uniformity results of produced DPIs (mean ± SD, n = 3).

DPI formulations*	Found amount (antibiotic), mg	Found percent (antibiotic), %	Found amount (mucolytic), mg	Found percent (mucolytic), %
CP	9.80 ± 0.07	97.99 ± 0.66	NA	NA
CP-NAC	9.10 ± 0.12	90.99 ± 1.15	0.66 ± 0.03	6.6 ± 0.33
LV	4.78 ± 0.07	95.53± 1.32	NA	NA
LV-NAC	4.17 ± 0.11	83.36 ± 2.18	0.29 ± 0.03	5.7 ± 0.67

*CP DPI: CP-containing dry powder inhaler formulation.

CP-NAC DPI: CP- and NAC-containing dry powder inhaler formulation.

LV DPI: LV-containing dry powder inhaler formulation.

LV-NAC DPI: LV- and NAC-containing dry powder inhaler formulation.

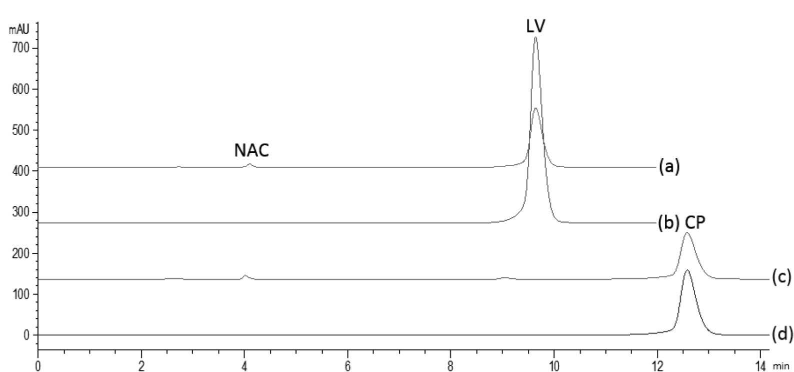

Figure 3. HPLC chromatograms of LV-NAC-containing DPI at a) 214 nm b) 293 nm, and CP-NAC-containing DPI at c) 214 nm, d) 293 nm obtained from content uniformity tests (25 mM KH_2PO_4 (pH 3.0) and methanol (72:28, v/v)).

3.4. Permeability

To investigate the permeability behavior of antibiotics from DPIs, Calu-3 cells (lung adenocarcinoma cell line) were used. As a result of the permeability study, it was observed that with dry powder application, lower and more variable permeability values were obtained than those of the solution application. The only condition in which the presence of the mucolytic changed the permeability of the antibiotic was when the formulations were applied as powder under LCC conditions. Moreover, as expected, CP had lower permeability than LV. Permeability data are shown in Table 5. HPLC chromatograms of CP-NAC- and LV-NAC-containing DPIs are shown in Figure 4.

Table 5. Permeability values of antibiotics from the manufactured DPIs (n = 3).

		Permeability, (cm/s) × 10^{-6}			
		LCC*		AIC*	
		Antibiotic	NAC	Antibiotic	NAC
Solution	CP	4.33 ± 0.06	NA**	5.14 ± 0.13	NA
	CP-NAC	4.26 ± 0.04	44.51 ± 0.84	5.28 ± 0.11	108.95 ± 2.53
	LV	6.02 ± 0.11	NA	8.57 ± 0.68	NA
	LV-NAC	6.25 ± 0.27	39.25 ± 0.36	9.71 ± 0.14	78.39 ± 0.36
Dry powder	CP	0.42 ± 0.01	NA	2.76 ± 0.01	NA
	CP-NAC	0.82 ± 0.02	3.22 ± 0.50	2.18 ± 0.15	13.35 ± 0.57
	LV	1.30 ± 0.22	NA	3.48 ± 0.67	NA
	LV-NAC	2.76 ± 0.10	3.52 ± 0.15	5.46 ± 0.64	2.34 ± 0.98

*LCC: liquid-covered culture, AIC: air-interfaced culture.

**NA: Not applicable.

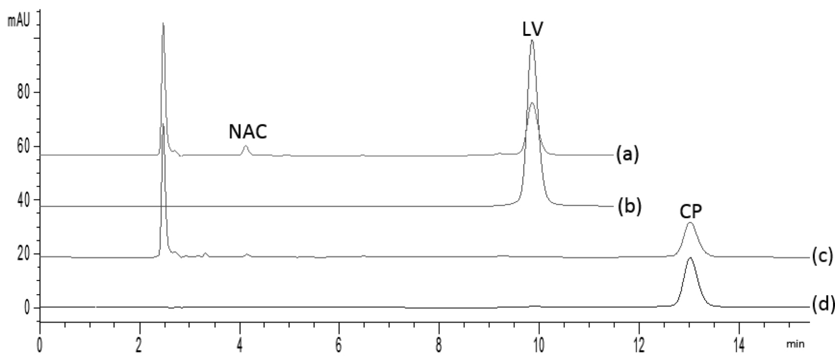

Figure 4. HPLC chromatograms of LV-NAC-containing DPI at a) 214 nm and b) 293 nm, CP-NAC-containing DPI at c) 214 nm and d) 293 nm obtained from permeability samples (HBSS (10 mM HEPES)).

4. Conclusions

NAC is a frequently used mucolytic agent in formulations in combination with CP and LV antibiotics. In this study, the quantification of these 3 drugs was carried out by an HPLC method that was selective, accurate, sensitive, reliable, and reproducible. With the validated HPLC method, simultaneous CP, LV, and NAC determination was successfully performed in the produced microparticulate DPIs. The method was successfully applied to content uniformity and permeability samples to determine all of these compounds. The validated HPLC method can be used not only for DPI dosage forms but also in formulations containing CP, LV, and NAC in different dosage forms.

Acknowledgment

This project was supported by a grant from the Scientific and Technological Research Council of Turkey (TÜBİTAK) (SBAG-213S043).

References

1. Lay-Son G, Puga A, Astudillo P, Repetto GM; Collaborative Group of the Chilean National Cystic Fibrosis Program. Cystic fibrosis in Chilean patients: analysis of 36 common CFTR gene mutations. Journal of Cystic Fibrosis 2011; 10 (1): 66-70. doi: 10.1016/j.jcf.2010.10.002
2. van Gool K, Norman R, Delatycki MB, Hall J, Massie J. Understanding the costs of care for cystic fibrosis: an analysis by age and health state. Value in Health 2013; 16 (2): 345-55. doi: 10.1016/j.jval.2012.12.003
3. Garland AL, Walton WG, Coakley RD, Tan CD, Gilmore RC et al. Molecular basis for pH-dependent mucosal dehydration in cystic fibrosis airways. Proceedings of the National Academy of Sciences of the United States of America 2013; 110 (40): 15973-15978. doi: 10.1073/pnas.1311999110
4. Cohen-Cymberknoh M, Shoseyov D, Kerem E. Managing cystic fibrosis: strategies that increase life expectancy and improve quality of life. American Journal of Respiratory and Critical Care Medicine 2011; 183 (11): 1463-1471. doi: 10.1164/rccm.201009-1478CI
5. Pezzulo AA, Tang XX, Hoegger MJ, Alaiwa MH, Ramachandran S et al. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. Nature 2012; 487 (7405): 109-113. doi: 10.1038/nature11130
6. Elborn JS, Henig NR. Optimal airway antimicrobial therapy for cystic fibrosis: the role of inhaled aztreonam lysine. Expert Opinion on Pharmacotherapy 2010; 11 (8): 1373-1385. doi: 10.1517/14656566.2010.482102
7. Cipolla D, Chan HK. Inhaled antibiotics to treat lung infection. Pharmaceutical Patent Analyst 2013; 2 (5): 647-663. doi: 10.4155/ppa.13.47
8. Buttini F, Rossi I, Di Cuia M, Rossi A, Colombo G et al. Combinations of colistin solutions and nebulisers for lung infection management in cystic fibrosis patients. International Journal of Pharmaceutics 2016; 502 (1-2): 242-248. doi: 10.1016/j.ijpharm.2016.02.005
9. Hodson ME, Butland RJA, Roberts CM, Smith MJ, Batten JC. Oral ciprofloxacin compared with conventional intravenous treatment for pseudomonas-aeruginosa infection in adults with cystic fibrosis. Lancet 1987; 1 (8527): 235-237. doi: 10.1016/S0140-6736(87)90062-6
10. Bosso JA, Black PG, Matsen JM. Ciprofloxacin versus tobramycin plus azlocillin in pulmonary exacerbations in adult patients with cystic-fibrosis. Association of Professors of Medicine 1987; 82 (4A): 180-184.
11. Geller DE, Flume PA, Staab D, Fischer R, Loutit JS et al. Levofloxacin inhalation solution (MP-376) in patients with cystic fibrosis with *Pseudomonas aeruginosa*. American Journal of Respiratory and Critical Care Medicine 2011; 183 (11): 1510-1516. doi: 10.1164/rccm.201008-1293OC
12. Chamseddin C, Jira TH. Comparison of the chromatographic behavior of levofloxacin, ciprofloxacin and moxifloxacin on various HPLC phases. Die Pharmazie 2011; 66 (4): 244-248. doi: 10.1691/ph.2011.0314
13. Samanidou V, Demetriou C, Papadoyannis I. Direct determination of four fluoroquinolones, enoxacin, norfloxacin, ofloxacin, and ciprofloxacin, in pharmaceuticals and blood serum by HPLC. Analytical and Bioanalytical Chemistry 2003; 375 (5): 623-629. doi: 10.1007/s00216-003-1749-9
14. Espinosa-Mansilla A, Peña AMdl, Gómez DG, Salinas F. HPLC determination of enoxacin, ciprofloxacin, norfloxacin and ofloxacin with photoinduced fluorimetric (PIF) detection and multiemission scanning: application to urine and serum. Journal of Chromatography B 2005; 822 (1): 185-193. doi: 10.1016/j.jchromb.2005.05.045
15. Czyrski A, Szałek E. An HPLC method for levofloxacin determination and its application in biomedical analysis. Journal of Analytical Chemistry 2016; 71 (8): 840-843. doi: 10.1134/S1061934816080049
16. Siewert S. Validation of a levofloxacin HPLC assay in plasma and dialysate for pharmacokinetic studies. Journal of Pharmaceutical and Biomedical Analysis 2006; 41 (4): 1360-1362. doi: 10.1016/j.jpba.2006.02.010

17. Böttcher S, von Baum H, Hoppe-Tichy T, Benz C, Sonntag HG. An HPLC assay and a microbiological assay to determine levofloxacin in soft tissue, bone, bile and serum. Journal of Pharmaceutical and Biomedical Analysis 2001; 25 (2): 197-203. doi: 10.1016/S0731-7085(00)00478-7
18. Sana S, Rajania A, Sumedha N, Pravin P, Shripad N. Development and validation of RP-HPLC method for the estimation of N-acetylcysteine in wet cough syrup. International Journal of Drug Development and Research 2012; 4 (2): 284-293.
19. Tsikas D, Sandmann J, Ikic M, Fauler J, Stichtenoth DO et al. Analysis of cysteine and N-acetylcysteine in human plasma by high-performance liquid chromatography at the basal state and after oral administration of N-acetylcysteine. Journal of Chromatography B: Biomedical Sciences and Applications 1998; 708 (1): 55-60. doi: 10.1016/S0378-4347(97)00670-1
20. Ercal N, Oztezcan S, Hammond TC, Matthews RH, Spitz DR. High-performance liquid chromatography assay for N-acetylcysteine in biological samples following derivatization with N-(1-pyrenyl)maleimide. Journal of Chromatography B: Biomedical Sciences and Applications 1996; 685 (2): 329-334. doi: 10.1016/S0378-4347(96)00196-X
21. El-Feky MA, El-Rehewy MS, Hassan MA, Abolella HA, Abd El-Baky RM et al. Effect of ciprofloxacin and N-acetylcysteine on bacterial adherence and biofilm formation on ureteral stent surfaces. Polish Journal of Microbiology 2009; 58 (3): 261-267.
22. Ozdil B, Kece C, Cosar A, Akkiz H, Sandikci M. Potential benefits of combined N-acetylcysteine and ciprofloxacin therapy in partial biliary obstruction. Journal of Clinical Pharmacology 2010; 50 (12): 1414-1419. doi: 10.1177/0091270010361257
23. Moon JH, Jang EY, Shim KS, Lee JY. In vitro effects of N-acetyl cysteine alone and in combination with antibiotics on *Prevotella intermedia*. Journal of Microbiology 2015; 53 (5): 321-329. doi: 10.1007/s12275-015-4500-2
24. Goswami M, Jawali N. N-Acetylcysteine-mediated modulation of bacterial antibiotic susceptibility. Antimicrobial Agents and Chemotherapy 2010; 54 (8): 3529-3530. doi: 10.1128/AAC.00710-10
25. Landini G, Di Maggio T, Sergio F, Docquier JD, Rossolini GM et al. Effect of high N-acetylcysteine concentrations on antibiotic activity against a large collection of respiratory pathogens. Antimicrobial Agents and Chemotherapy 2016; 60 (12): 7513-7517. doi: 10.1128/AAC.01334-16
26. del Prado G, Ruiz V, Naves P, Rodríguez-Cerrato V, Soriano F et al. Biofilm formation by *Streptococcus pneumoniae* strains and effects of human serum albumin, ibuprofen, N-acetyl-l-cysteine, amoxicillin, erythromycin, and levofloxacin. Diagnostic Microbiology and Infectious Disease 2010; 67 (4): 311-318. doi: 10.1016/j.diagmicrobio.2010.03.016
27. ICH. ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2 (R1). Geneva, Switzerland: International Conference on Harmonization, 2005.
28. Akdag Cayli Y, Sahin S, Buttini F, Balducci AG, Montanari S et al. Dry powders for the inhalation of ciprofloxacin or levofloxacin combined with a mucolytic agent for cystic fibrosis patients. Drug Development and Industrial Pharmacy 2017; 43 (8): 1378-1389. doi: 10.1080/03639045.2017.1318902