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Separation and determination of synthetic impurities of difloxacin by reversed-phase high-performance liquid chromatography

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Abstract

A simple and rapid reversed-phase high-performance liquid chromatographic method for separation and determination of process-related impurities of difloxacin (DFL) was developed. The separation was achieved on a reversed-phase C_{18} column using methanol–water–acetic acid (78:21.9:0.1, v/v/v) as a mobile solvent at a flow rate of 1.0 ml/min at 28 °C using UV detection at 230 nm. It was linear over a range of 0.03×10^{-6} to 1.60×10^{-6} g for process related impurities and 0.05×10^{-6} to 2.40×10^{-6} g for difloxacin. The detection limits were 0.009 $\times 10^{-6}$ to 0.024×10^{-6} g for all the compounds examined. The recoveries were found to be in the range of 97.6–102.0% for impurities as well as difloxacin. The precision and robustness of the method were evaluated. It was used for not only quality assurance, but also monitoring the synthetic reactions involved in the process development work of difloxacin. The method was found to be specific, precise and reliable for the determination of unreacted levels of raw materials, intermediates in the reaction mixtures and the finished products of difloxacin.

Keywords: Antibiotics; Difloxacin; Synthetic impurities; Reversed-phase HPLC

1. Introduction

Difloxacin (DFL) [6-fluoro-1-(4-fluorophenyl)-1,4-dihydro-7-(4-methyl-1-piperazinyl)-4-oxo-3-quinoline carboxylic acid] (Fig. 1) is a new aryl fluoroquinoline antibiotic, used effectively against Gram positive and Gram negative bacteria. It has shown exceptional efficacy [1,2] against intracellular pathogens in experimental infections such as L. *Pneumophila and Salmenella typhimurium* [3]. It is generally synthesized by condensation of 7-chloro-6-fluoro-1-(4-fluorophenyl)-4-oxo-1,4-dihydro-3-quinoline carboxylic acid (CFQ) with *N*-methyl piperazine in a laboratory. During its synthesis not only the unreacted CFQ, but also its related analogues: (i) methyl 2-(2,4-dichloro-5-fluorobenzoyl)-3-(4-fluoroanilino)-(E)-2-propenoate (MFP), (ii) methyl 2-(2,4-dichloro-5-fluorobenzoyl)-3-(2,4-difluoroanilino)-(E)-2-propenoate (MDF) and (iii) 7-chloro-1-(2,4-difluorophenyl)-6-fluoro-4-oxo-1,4-dihydro-3-quinoline carboxylic acid (CDF) are usually carried over in small quantities in to the bulk of DFL. According to the USFDA such impurities present at the levels of >0.1% must be identified and quantified using validated analytical procedures. Therefore, the separation and determination of synthetic impurities of DFL is of great importance not only for quality assurance but also monitoring of reactions during process development and control.

Several HPLC methods for determination of DFL in biological matrices have been reported. Granneman et al. have proposed a procedure for the determination of DFL and its metabolites in plasma and urine [4] using a C_{18} Adsorbosphere-HS column (25 cm × 4.6 mm i.d., particle size 7 μm) at ambient temperature. The mobile phase used was 0.05 M phosphate, 0.2% sodium dodecyl sulfate and

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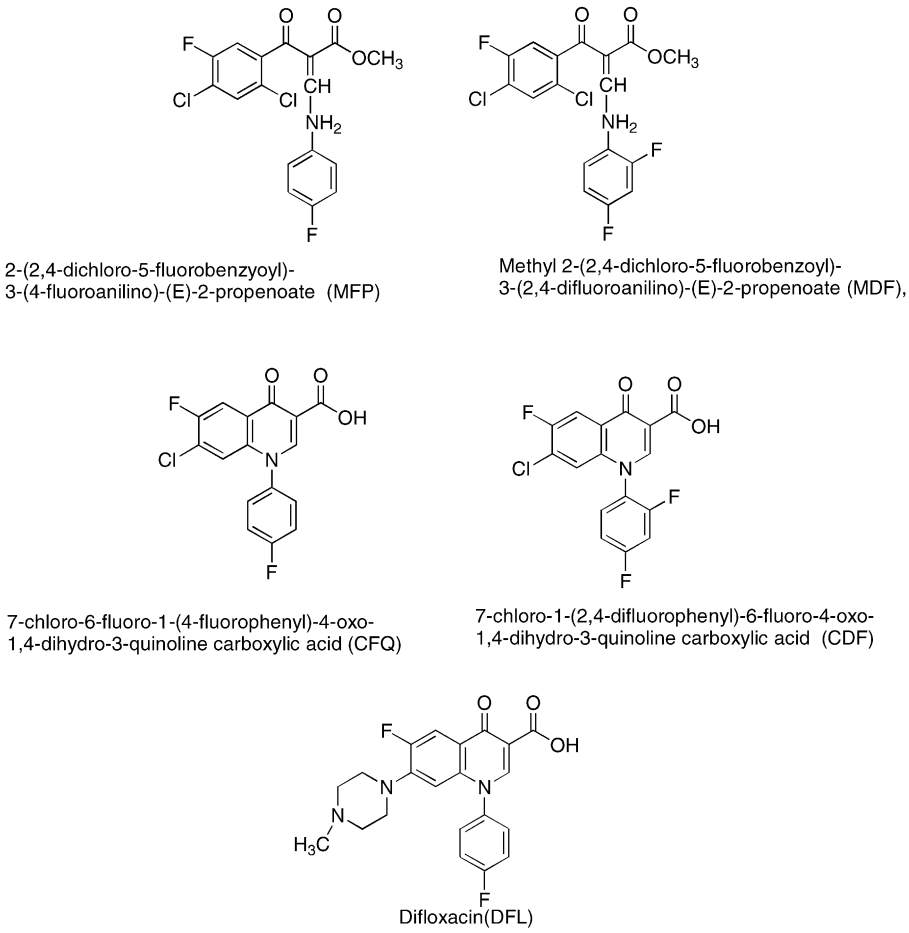

Fig. 1. Difloxacin and its synthetic impurities.

50% acetonitrile pumped at 1.5 ml/min and the detection was at 280 nm. Bauer et al. have determined temafloxacin, sarafloxacin and difloxacin in bulk drug substances and in a variety of dosage forms using HPLC [5]. The detection at 280 nm, provided a linear response of the subject compounds to at least 20 μg/ml. Same protocols are also applied for ciprofloxacin and norfloxacin. Simultaneous determination of benofloxacin, danofloxacin, difloxacin, enrofloxacin, ofloxacin and orbifloxacin in beef, pork and chicken was reported [6]. The drugs were extracted from meat with 0.3% meta phosphoric acid in acetonitrile and the supernatant was defatted with *n*-hexane and concentrated to dryness. The residue was dissolved in 5 ml of mobile phase and analyzed by HPLC. The HPLC analysis was carried out on a TSK-gel ODS 80 TS column (25 cm × 4.6 mm id) with 0.05 mol/l phosphate buffer (pH 2.5)–acetonitrile (4:1) containing 0.25 g/l sodium octane sulfonate as the mobile phase at a flow rate of 1.0 ml/min operating a spectrofluorimetric detector at Ex 290 nm and Em 460 nm. Posyniak et al. have reported a simple HPLC method for the determination of difloxacin, ciprofloxacin, enrofloxacin and sarafloxacin in animal tissues using Lichrospher 100 RP-8, 5 mm column and mobile phase (0.25 M *o*-phosphoric acid–acetonitrile 70:30, v/v) in an ion-pair mode [7]. Roybal et al. described a LC method with fluorescence detection for concurrent determination of difloxacin, ciprofloxacin, enrofloxacin, and sarafloxacin in catfish, shrimp and salmon. It involves an isocratic elution with acetonitrile–2% acetic acid(16:84) mobile phase and a PLRP-S polymer column with fluorescence detection, excitation 278 nm and emission 450 nm [8]. A liquid chromatographic method to separate a series of quinolone antibiotics used as veterinary drugs such as difloxacin, danofloxacin, enrofloxacin, marbofloxacin, norfloxacin and sarfloxacin was reported [9]. The separation was performed by using a high-purity silica-based C_8 column and a mobile phase consisting of acetonitrile–aqueous oxalic acid buffer. A three-factor Doehlert experimental design was applied to establish the optimum conditions for an isocratic separation. Gradient elution was applied to reduce analysis time. Figures of merit of the method proposed, with fluorimetric detection, were evaluated. Apart from HPLC, spectrophotometric [10,11], LC–MS [12] and capillary electrophoresis [13] methods were also reported. However, to the best of our knowledge, no single analytical method for the separation and determination of synthetic impurities of DFL is available in literature. In this paper, we describe a simple and rapid HPLC method for separation and determination of synthetic impurities of DFL in bulk drugs using a reversed-phase C_{18}

column and methanol–water–acetic acid (78:21.9:0.1, v/v/v) as eluent at 28 °C temperature.

2. Experimental

2.1. Materials and reagents

All reagents were of analytical reagent-grade unless stated otherwise. HPLC-grade methanol, acetic acid obtained from Qualigens, Mumbai, India, were used. Glass-distilled and deionized water (Nanopure, Barnsted, USA) was used throughout the study. DFL and its synthetic impurities were gifted by Metropolitan Overseas Limited, Hirehalli, Tumkur, Karnataka, India.

2.2. Apparatus

The HPLC system was composed of two LC-10 AT VP pumps, an SPD-M 10 A VP diode array detector, an SIL-10AD VP auto injector, a DGU 12 A degasser and an SCL-10 VP system controller (all from Shimadzu, Kyoto, Japan). A reversed-phase Symmetry C_{18} (Waters, Milford, USA) column (25 cm × 4.6 mm i.d., particle size 5 μm) was used for separation. The chromatographic and integrated data were recorded using an HP-Vectra (Hewlett Packard, Waldron, Germany) computer system.

2.3. Chromatographic conditions

The mobile phase was methanol–water–acetic acid (78:21.9:0.1, v/v/v); before delivering in to the system it was filtered through 0.45 μm PTFE filter and degassed using a vacuum. The analysis was carried out under isocratic conditions using a flow rate of 1.0 ml/min at 28 °C temperature. Chromatograms were recorded at 230 nm using an SPD-M 10 A VP diode array detector.

2.4. Analytical procedure

Standards of DFL, CFQ, CDF, MFP and MDF (10 mg) were accurately weighed and transferred in to 100 ml volumetric flasks and dissolved in methanol:dichloromethane (9:1, v/v). After dissolving, the volume was made up to the mark with methanol:dichloromethane (9:1, v/v). Synthetic mixtures containing DFL, CFQ, CDF, MFP and MDF were prepared and a 20 μl volume of each sample was injected and chromatographed under the above conditions. Samples of bulk drugs, standards of DFL and its related substances were prepared at a concentration of 2 mg/ml. For determination of LOD and LOQ a 2 mg/ml solution was used. The amounts of impurities were calculated from their respective peak areas. The peak areas were normalized against DFL and response factors thus obtained were used in calculating the content of impurities.

3. Results and discussion

3.1. Method development

Sample preparation largely influences the success of any chromatographic experiment. In the present study, all the samples were dissolved in methanol:dichloromethane (9:1, v/v) diluent due to higher solubility of the compounds investigated. Reversed-phase liquid chromatography method has been chosen for the analysis of these compounds. In order to study the effect of methanol–dichloromethane mixture (9:1, v/v) in an aqueous mobile phase, a 20 μl of the mixture was injected into the chromatograph and found that it is eluting at about 1 min resulting no interference from DFL and its impurities. In the present investigation different columns such as Symmetry C_{18}, Spherisorb C_{18}, Inertsil ODS and Inertsil CN were used for analysis. Initially a mixture of acetonitrile (50–90%) and water was used as a mobile phase for the separation. Poor selectivity was observed on Inersil CN column and was not considered for further analysis. DFL, MFP and MDF were eluted at different retention times while CDF and CFQ were co-eluted as a single peak using Symmetry C_{18}, Spherisorb C_{18}, Inertsil ODS columns. The peak shapes were not good in all the three columns. Later, acetonitrile was replaced with methanol, which is a significantly stronger competitor for intra molecular selector-solute hydrogen bonding due to its more pronounced H-donor and H-acceptor properties. When analyzing DFL and its impurities using Spherisorb C_{18} and Inertsil ODS, these compounds strongly interact with polar ends of HPLC column packing materials causing severe peak asymmetry and low separation efficiencies. Consequently for method development Symmetry C_{18} column was used. This packing was selected because it has one of the lowest hydrophobicity and silanol activity as seen in commercial catalogues. On Symmetry C_{18} column all the compounds were eluted using methanol and water mixtures but baseline separation was not observed between CDF and CFQ. Acetic acid (0.1%) was used to improve the separation between CDF and CFQ when methanol concentration was at 78%. All these substances were subjected to separation by reversed-phase HPLC using methanol–water–acetic acid (78:21.9:0.1, v/v/v) and Symmetry C_{18} column. A typical chromatogram of a synthetic mixture containing DFL,

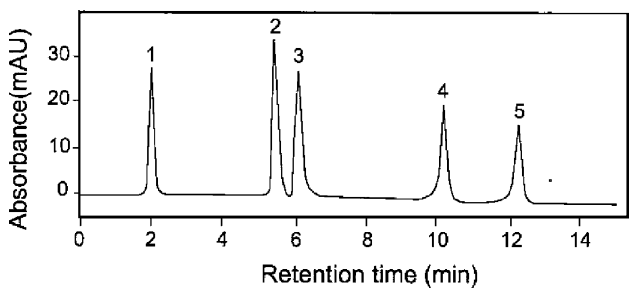

Fig. 2. Typical chromatogram of a synthetic mixture containing 0.2 μg of each of (1) DFL (2) CFQ (3) CDF (4) MFP and (5) MDF.

Table 1
Accuracy data for synthetic mixtures containing DFL, CFQ, CDF, MFP and MDF (n = 3)

Compound	Taken ($\times 10^{-6}$ g)	Found ($\times 10^{-6}$ g)	Recovery (%)
DFL	0.51	0.52 ± 0.01	102.0
	1.02	1.05 ± 0.02	102.9
	2.04	2.09 ± 0.04	102.5
CFQ	0.42	0.41 ± 0.01	97.6
	0.84	0.86 ± 0.02	102.4
	1.21	1.23 ± 0.02	101.7
CDF	0.50	0.51 ± 0.01	102.0
	1.03	1.05 ± 0.02	101.9
	1.54	1.58 ± 0.03	102.6
MFP	0.52	0.53 ± 0.01	101.9
	1.02	1.04 ± 0.02	102.0
	1.51	1.55 ± 0.04	102.7
MDF	0.54	0.55 ± 0.01	101.9
	1.00	1.02 ± 0.01	102.0
	1.52	1.54 ± 0.02	101.3

CFQ, CDF, MFP and MDF is shown in Fig. 2. The peaks were identified by injecting and comparing the retention times with those of authentic standards. Reproducible peak shapes were obtained under the optimum conditions. The wavelength of maximum absorption (λ_{max}) of DFL, CFQ, MFP and MDF were found to be 231, 234, 229, 226 and 236 nm, respectively, with an average absorption of 231 ± 5 nm. The UV detector was set at 230 nm for both detection and quantification. This was selected based on the observations that the detector response was high when compared to the determinations made at other wavelengths for all the compounds.

3.2. Accuracy and precision

Standard mixtures containing known amounts of DFL, CFQ, CDF, MFP and MDF were prepared and analyzed by HPLC. The accuracy of the method was checked for three different concentration levels by standard addition technique. Small quantities of impurities (0.4×10^{-6} to 1.5×10^{-6} g) were added to the sample and chromatographed. It was found that these additions were accurately reflected in their peak areas. All estimations were repeated (n = 3) and standard deviations (S.D.) were calculated (Table 1). The precision of the method was determined (R.S.D. 1.1%) on five replicate injections of a standard solution of DFL and reported.

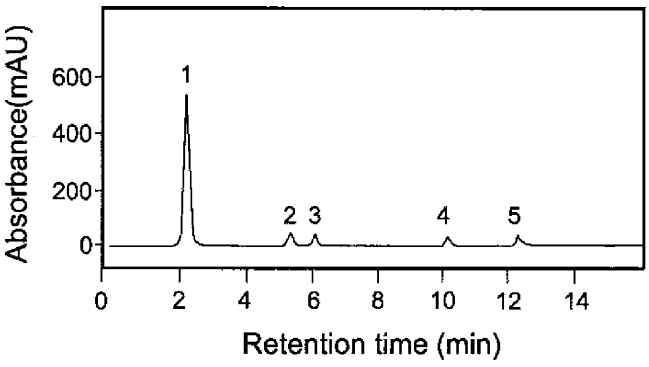

Fig. 3. Typical chromatogram of (1) DFL (250 μg), spiked with low-level impurities containing (2) CFQ (1 μg) (3) CDF (1 μg) (4) MFP (1 μg) and (5) MDF (1 μg).

3.3. Specificity

To demonstrate the specificity of the method a sample of DFL bulk drug was spiked with known quantities of potential impurities and chromatographed. All the impurities were clearly separated and are found to be not interfering with others (Fig. 3). This gives an indication that the method is specific for the separation and determination of DFL and its synthetic impurities in bulk drugs.

3.4. Linearity

Calibration graphs (concentration versus peak area) were constructed at six different concentrations for DFL (0.05×10^{-6} to 2.4×10^{-6} g), CFQ (0.03×10^{-6} to 1.2×10^{-6} g), CDF (0.04×10^{-6} to 1.60×10^{-6} g), MFP (0.06×10^{-6} to 1.50×10^{-6} g) and MDF (0.08×10^{-6} to -1.50×10^{-6} g). Three independent determinations were carried out at each concentration and good linearity was found between the mass integral responses for each of the compounds examined. Table 2 gives linear equation, mass range and correlation coefficients for all compounds.

3.5. Limits of detection and quantification (LOD and LOQ)

The LOD and LOQ values were calculated for DFL and its impurities based on the three and ten times of noise level, respectively, and the values are given in Table 2.

Table 2
Linear-regression data for DFL and its impurities

Compound	Mass range ($\times 10^{-6}$ g)	Linear regression	Correlation coefficient (r)	LOD ($\times 10^{-6}$ g)	LOQ ($\times 10^{-6}$ g)	$S_{y,x}$
DFL	0.05–2.40	$1038410x + 8031$	0.9998	0.016	0.048	8678
CFQ	0.03–1.20	$2429776x - 1907$	0.9991	0.009	0.028	18110
CDF	0.04–1.60	$1989291x + 5415$	0.9999	0.012	0.037	6905
MFP	0.06–1.50	$1885603x + 10571$	0.9996	0.020	0.061	10570
MDF	0.08–1.50	$1790532x + 7798$	0.9998	0.024	0.074	7798

Table 3
Robustness data for DFL and its impurities (n = 3)

Parameter	DFL		CFQ		CDF			MFP		MDF	
	RRT	k'	RRT	k'	R_s	RRT	k'	RRT	k'	RRT	k'
Mobile phase composition (MeOH, %)											
73	0.98	3.20	3.39	13.50	2.54	4.07	16.40	8.58	35.72	11.12	46.60
78	1.00	3.28	2.48	9.60	1.67	2.83	11.10	4.77	19.40	5.79	23.76
83	1.04	3.44	2.08	7.90	1.30	2.29	8.82	3.41	13.60	3.96	15.94
Mean ± S.D.	1.01 ± 0.02	3.31 ± 0.02	2.65 ± 0.04	10.33 ± 0.03	1.84 ± 0.03	3.06 ± 0.02	10.36 ± 0.04	5.58 ± 0.03	22.90 ± 0.02	6.96 ± 0.03	28.76 ± 0.04
Flow rate (ml/min)											
0.9	1.00	3.30	2.33	8.96	1.67	2.70	10.54	4.60	18.68	5.50	22.54
1.0	1.00	3.28	2.48	9.60	1.67	2.83	11.10	4.77	19.40	5.79	23.76
1.1	0.99	3.24	2.66	10.38	1.67	3.00	11.84	5.10	20.82	6.10	25.10
Mean ± S.D.	1.00± 0.01	3.27± 0.02	2.49 ± 0.03	9.65 ± 0.03	1.67 ± 0.01	2.84 ± 0.04	11.16 ± 0.04	4.82 ± 0.02	19.62 ± 0.03	5.80 ± 0.02	23.80 ± 0.03
Temperature (°C)											
25	1.00	3.28	2.50	9.70	1.71	2.87	11.28	4.84	19.72	5.88	24.18
28	1.00	3.28	2.48	9.60	1.67	2.83	11.10	4.77	19.40	5.79	23.76
31	1.00	3.28	2.44	9.46	1.52	2.79	10.92	4.68	19.04	5.65	23.20
Mean ± S.D.	1.00 ± 0.01	3.28 ± 0.01	2.47 ± 0.02	9.59 ± 0.03	1.63 ± 0.03	2.83 ± 0.03	11.11 ± 0.04	4.76 ± 0.02	19.39 ± 0.02	5.77 ± 0.03	23.71 ± 0.03

3.6. Robustness

In order to evaluate the robustness of the method the influence of small and deliberate variation of analytical parameters on the retention times of DFL and its impurities was studied. The parameters selected were mobile phase composition, flow rate and temperature. Only one parameter was changed while the others were kept constant. The results are recorded in Table 3. It could be seen from Table 3 that the there is a significant change in the retention of impurities namely CFQ, CDF, MFP and MDF in case of change of concentration of methanol by ±5%. However, no significant change was noticed by deliberate change of flow rate and temperature. Further the retention of DFL was unaffected, when methanol concentration was changed. It could be further observed from the results recorded in Table 3 that the separation between the impurities has not been collapsed by varying the concentration of methanol. On the other hand, the separation was enhanced significantly and without affecting the accuracy of the quantitative determination of impurities. Table 3 results were presented in visual graphs (Fig. 4) and in addition to RRT and k', the resolution of CFQ and CDF used to investigate the robustness of the method. Thus, it was considered that the method is robust and suitable for separation and determination of impurities in bulk drugs of DFL.

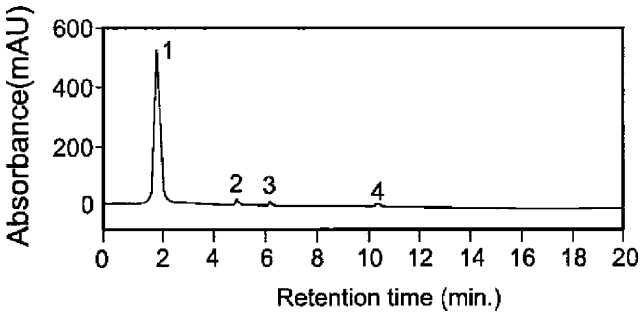

Fig. 4. Typical chromatogram of bulk drug of DFL (for peak identification see Fig. 3).

3.7. Stability

To determine the stability of DFL, the drug was stored in methanol:dichloromethane (9:1, v/v) for 24 h and chromatographed on the following day. The solutions were found to be stable for 24 h and observed that there is no degradation/increase in the percentage of impurities and also no significant change were observed. Replicate injections of (n = 5) of DFL solutions were performed and the relative standard deviation of peak area was determined with 1.25–1.72%.

Table 4
Determination of process impurities in bulk drugs of difloxacin (n = 3)

Manufacturer	CFQ (%, w/w)	R.S.D. (%)	CDF (%, w/w)	R.S.D. (%)	MFP (%, w/w)	R.S.D. (%)	MDF (%, w/w)	R.S.D. (%)
A	0.14	1.92	0.03	1.09	0.08	1.53	<LOD	
B	0.20	1.43	0.06	1.73	0.05	2.41	0.04	1.85
C	0.13	1.75	0.05	2.05	0.08	2.28	0.05	2.45
D	0.18	1.29	0.07	2.22	0.06	2.53	0.03	1.92
E	0.15	1.63	0.07	1.83	0.05	2.18	0.06	2.34

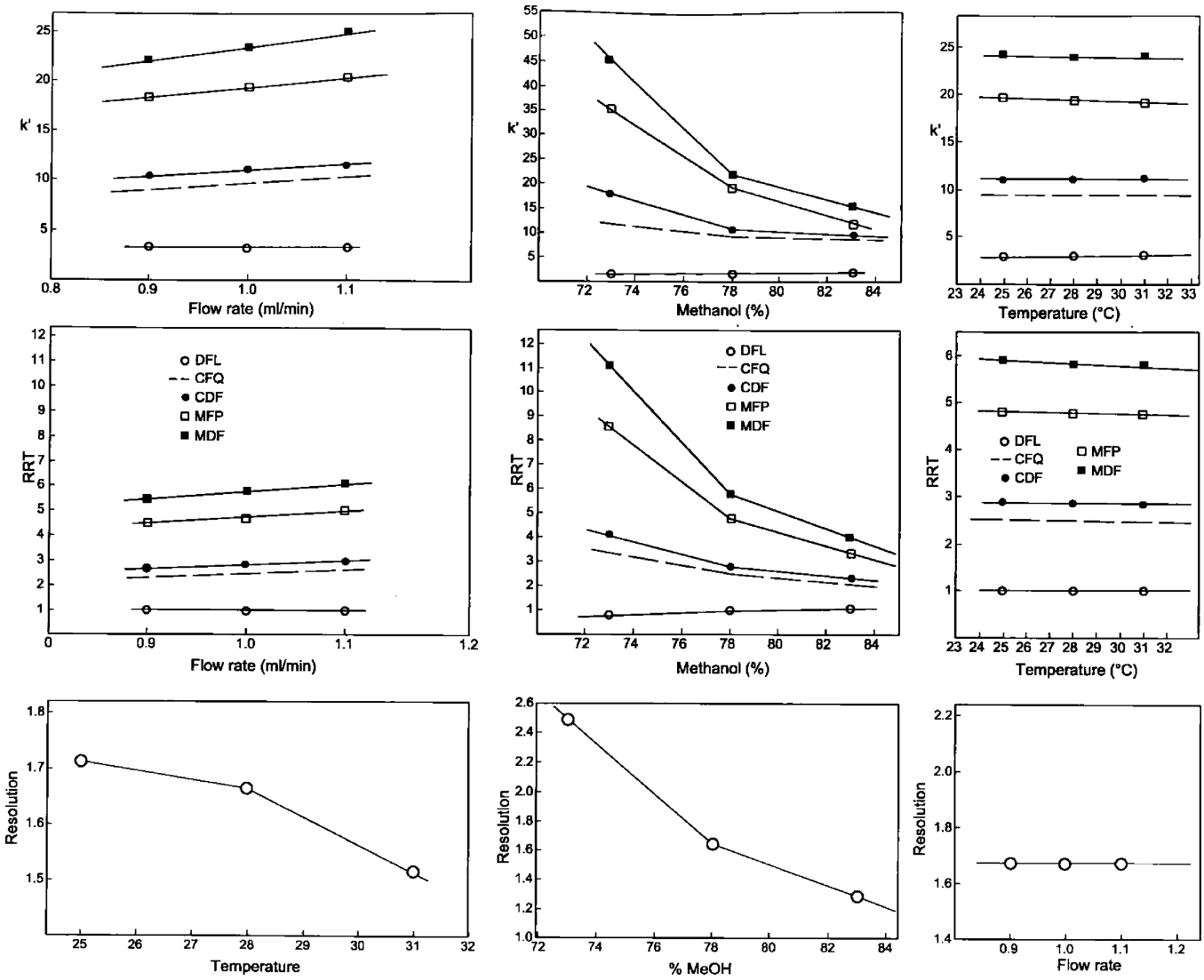

Fig. 5. Graphical representation of interactions between retention parameters of DFL and its impurities.

3.8. Analysis of bulk drugs

The quality of DFL in several batches of bulk drug samples obtained from different manufacturers was checked thoroughly by HPLC. A typical chromatogram of a bulk drug of DFL is shown in Fig. 5. The levels of various impurities were determined and the purity of DFL was evaluated. The results are given in Table 4.

4. Conclusions

A rapid, robust and sensitive HPLC procedure has been developed for the separation and determination of DFL and its synthetic impurities CFQ, CDF, MFP and MDF. The developed HPLC method is suitable not only for the separation and determination of process impurities, but also for monitoring the synthetic procedures of DFL. The method is thus suitable for process development and quality assurance of DFL and related substances.

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References

[1] P.B. Fernandes, N. Shipkowtiz, R.R. Bower, K.P. Jarvis, J. Weisz, D.T.W. Chu, J. Antimicrob. Chemother. 18 (1986) 693–701.

[2] P.B. Fernandes, in: P.B. Fernandes, J.R. Prous (Eds.), Proceedings of the International Telesymposium on Quinolones, Barcelona, Spain, 1989, pp. 1–8.
[3] D.T.W. Chu, G.R. Granneman, P.B. Fernandes, Drugs Future 10 (1985) 543–548.
[4] G.R. Granneman, L.T. Sennello, J. Chromatogr. 413 (1987) 199–206.
[5] J.F. Bauer, L. Elrod Jr., J.R. Fornnarino, D.E. Heathcote, S.K. Linton, B.J. Norris, J.E. Quick, Pharm. Res. 7 (1990) 1177–1180.
[6] M. Nagao, T. Tsukahara, S. Jaroenpoj, C. Ardsoongnearn, Shokuhin Eiseigaku Zasshi 39 (1998) 329–332.
[7] A. Posyniak, J. Zmudzki, S. Semeniuk, J. Niedzielska, R. Ellis, Biomed. Chromatogr. 13 (1999) 279–285.
[8] J.E. Roybal, C.C. Walker, A.P. Pfenning, S.B. Turnipseed, J.M. Storey, S.A. Gonzales, J.A. Hurlbut, J. AOAC Int. 85 (2002) 1293–1301.
[9] J.A. Hernandez-Arteseros, I. Boronat, R. Compano, M.D. Prat, Chromatographia 52 (2000) 295–300.
[10] G.A. Saleh, Bull. Pharm. Sci. Assiut Univ. 20 (1997) 27–36.
[11] H.F. Askal, Bull. Pharm. Sci. Assiut Univ. 20 (1997) 75–85.
[12] B. Delepine, D. Hurtaud-Pessel, P. Sanders, Analyst 123 (1998) 2743–2747.
[13] D. Barron, E. Jimenez-Lozano, S. Bailac, J. Barbosa, J. Chromatogr. B 767 (2002) 313–319.