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On the use of Kinetex™-C_{18} core-shell 2.6 μm stationary phase to the multiclass determination of antibiotics

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Fast and accurate analysis is a prerequisite in all analytical fields especially in food, biological, pharmaceutical, and environmental samples. The new trend of ultra performance liquid chromatography (LC) has the main drawback of expensive instrumentation, which can't be easily found in low-budget analytical laboratories. The evolution of core shell technology has contributed to this direction, since ultra high efficiency can be achieved on common LC instrument platforms.

Herein the novel core shell analytical column, KINETEX ™ 2.6 μm, (150 mm × 4.6 mm) was comparatively studied against two conventional reversed-phase silica-based and one monolithic column. Eight antimicrobial agents representing two different classes: penicillins and amphenicols, were separated using a typical 400 bar high performance liquid chromatography (HPLC) equipment.

Comparison of column performance was carried out by calculation of the number of theoretical plates N, the tailing factor T_f, the relative retention time RRT, the retention factor k, the resolution factor R_s, and the precision of the retention time and peak area.

Optimal chromatographic conditions were used to validate the method. Its applicability was proven by the analysis of veterinary drug formulations. The examined antibiotics were well resolved within 17 min. Limit of quantitation values were 25.9 ng for amoxicillin, 14.1 ng for ampicillin, 41.6 ng for thiamphenicol, 9.6 ng for oxacillin, 23.5 ng for florfenicol, 26.7 for cloxacillin, 23.5 ng for chloramphenicol and 42.3 ng for dicloxacillin for 20 μL injection volume. The developed method can be easily and readily transferred to any laboratory.

Keywords: core-shell particles; UHPLC; penicillins; amphenicols; veterinary drugs.

Introduction

Fast and accurate analysis is a prerequisite in all analytical fields especially in food, human and veterinary pharmaceuticals, biological, and environmental samples. Achieving reliable analysis by low-budget instrumentation is highly appreciated.

Lately column technology evolution has led to the manufacture of sub-2 μm particle size columns used for ultra high performance chromatography. However the use of these columns with conventional chromatographic systems (with a pressure limit of 400 bar) is restricted due to pressure limitations. The latter can be overcome by using new systems capable of withstanding higher pressures around 1000 bar, but the cost of these systems is high and sometimes restrictive for low-budget laboratories.

Monolithic columns exhibit an outstanding efficiency and a reduced analysis time, as low column backpressure allows high flow rates, while maintaining, or even increasing, resolution obtained with conventional particle packed columns in complex mixtures.[1]

New analytical columns, such as Kinetex™, are packed with 2.6 μm core shell particles which consist of a solid core (1.9 μm) and a porous shell of 0.35 μm and have attracted the scientific interest. This sorbent material is not fully porous. Using sol-gel processing techniques, that incorporate nano-structuring technology, a durable, homogenous porous shell is grown on a solid silica core. This process leads to the formation of nearly spherical, with a smooth external surface, and a uniform shell thickness, around the solid core particles, with a narrow particle size distribution, which reduces short-range interchannel velocity biases and thus dramatically reduces two major sources of peak dispersion – Eddy diffusion and resistance to mass transfer between the mobile and the stationary phase (A and C terms of the van Deemter equation). Compared with classic fully porous silica particles, analytes spend less time diffusing into and out of the pores as they travel through the column. This shorter diffusion path allows for faster mass transfer and in this way all major sources of band broadening are decreased, thus allowing for ultra-high performance on any liquid chromatography (LC) system. The very flat C term obtained thereby, allows for high flow rates, leading to fast separations without significant loss in efficiency.[2]

The performance of the KINETEX™ column packed with 2.6 μm shell particles for both small and large molecule separation under isocratic conditions has been investigated by Oláh *et al*., who have proved that it is comparable to sub-2 μm columns and other commercially available shell-type packings.[3]

Gritti *et al*. have demonstrated the competent performance of Kinetex-C_{18} under isocratic conditions, having already depicted a promising chromatographic performance under gradient elution. Their results show the advantage of using shell particles to separate large biomolecules.[4]

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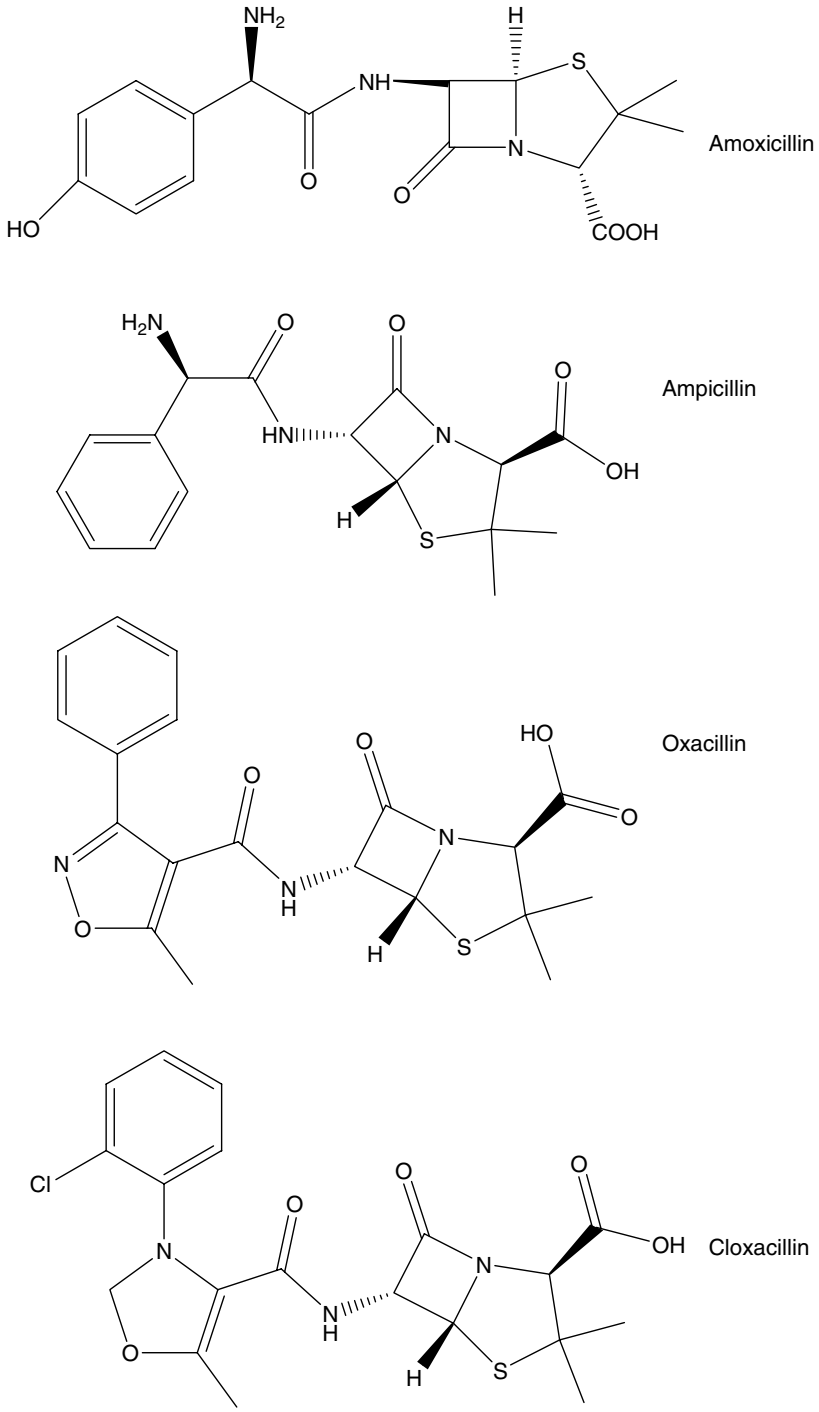
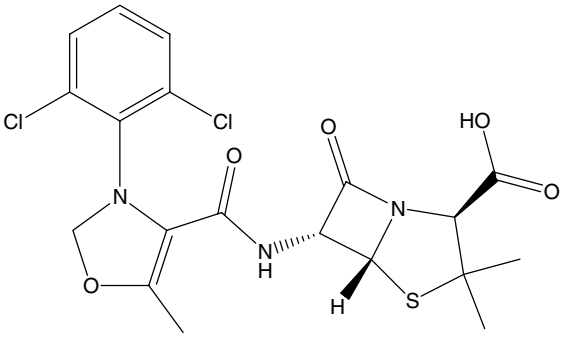


Figure 1. Chemical structures of examined antibiotics.

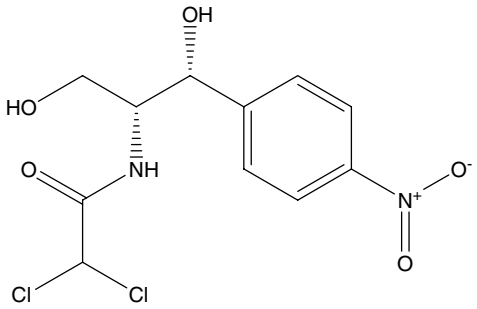
Our primary target was to compare the performance of a core shell column towards columns which are typically used for the analysis of a group of analytes of wide interest; for example, antibiotics that belong to different classes with varying chemical structures and properties. The current trend in their analysis is the multiclass, as well as the multiresidue determination of different agents.[5]

Penicillins from β-lactams group and amphenicols with a phenylpropanoid structure have been chosen as model target antibiotics. Their use in food animals is illegal in most countries.[6,7]

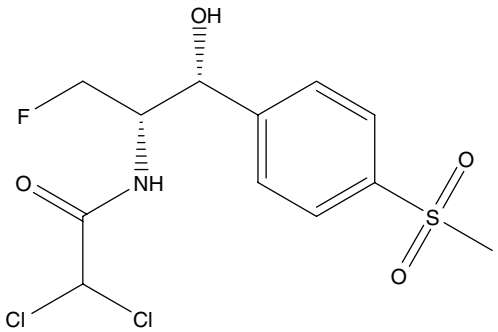
Penicillins' determination in pharmaceutical formulations and biological fluids is based mainly on reversed-phase high performance liquid chromatography (HPLC) using bonded silica analytical columns. From the currently available methods 90% use C_{18} columns, 7% use C_8 and 3% use other types of analytical columns, such as CN, SCX, MAX, MIP, cyclodextrin, etc.[8] The same conclusion can also be drawn for food samples (fish, milk), in cattle, pig, and chicken tissues (muscle, kidney, liver), animal feed.[9–12]



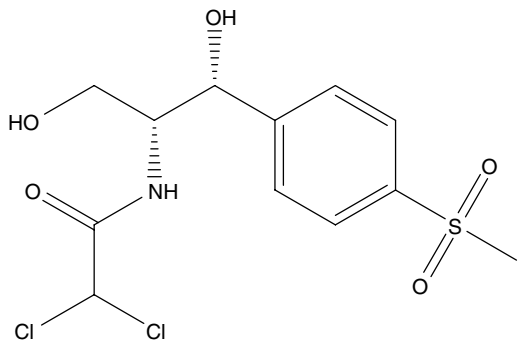
Dicloxacillin



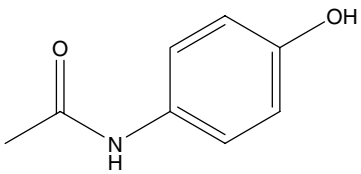
Chloramphenicol



Florfenicol



Thiamphenicol



IS: Paracetamol

Figure 1. (*Continued*).

In the authors' laboratory, antibiotics have been already investigated as target analytes in various matrices during the past five years with conventional analytical columns.[13–15]

Only a limited number of multi-analyte methods can be found in the literature and these methods involve the use of conventional analytical columns. A Vydac C_{18} column has been applied for the isocratic determination of four penicillins within 8 min in milk analysis.[16] A Luna™ C_{18} column (150 mm × 4.6 mm, 5 µm) has been used for the determination of amoxicillin and penicillin V within 14 min, in swine feeding stuffs,[17] for beta-lactam

antibiotics within 23 min, in wastewater;[18] as well as for ten β-lactam antibiotics of human and veterinary use in milk, chicken meat, and environmental water samples within 16 min.[19]

A Gemini C_{18} (150 mm × 4.6 mm I.D., 5 µm) column has been used for the separation of 11 antibiotics belonging in sulfonamides, tetracyclines, penicillins, and amphenicols and some main metabolites in human urine within 34 min.[20] A Waters X-bridge C_{18} column (30 × 4.6 mm, 2.5 µm) was used for the determination of 12 penicillin, cephalosporin and carbapenem antibiotics within 26 min, in human plasma.[21] An Econosphere C_{18} 5 µm column has been applied to the analysis of 13 antibiotics, including penicillins, cephalosporins, metronidazole, ofloxacin, and sulfamethoxazole and 4 protease inhibitors.[22] An octadecyl X Terra®C_{18} hybrid column has been used for some tetracyclines, sulfonamides and chloramphenicol in milk within 30 min.[23] A Varian Pursuit-C_{18} column (5 µm, 4.6 mm × 250 mm) was used for the determination of chloramphenicol and thiamphenicol in honey;[24] a Cosmosil 5C_{18}-MS-II 4.6 mm × 250 mm (5 µm) for tetracyclines and chloramphenicol in animal feed[25] and a Zorbax Eclipse XDB-C_{18} column (3.0 × 150 mm, 3.5 µm) for sulfonamides quinolones and chloramphenicol in wastewater samples.[26] Finally three different C_{18} columns were used for the determination of the structurally different antibiotics cefazoline, cefotiame, cefuroxime, chloramphenicol, ciprofloxacin, ofloxacin, sulfamethoxazole and trimethoprim from environmental and biological monitoring in three detection modes.[27]

Apparently measuring the level of antibiotic residues in various samples can be time- and labour-consuming work, since the analysis time is long and usually the number of samples is large. Herein the new core shell KINETEX™ column was used for the first time to the simultaneous determination of members of two structurally different antibiotic classes. The chemical structures of targets are illustrated in Figure 1. The developed method was further validated and applied to the analysis of veterinary drugs.

Materials and Methods

Instrumentation

A Shimadzu (Kyoto, Japan) quaternary low-pressure gradient system was used for the chromatographic determination of the examined analytes. The solvent lines were mixed in an FCV-10ALVP mixer. An LC-10ADVP pump equipped with an SCL-10ALVP System Controller, permitting fully automated operation, was used to deliver the mobile phase to the analytical column. Sample injection was performed via a Rheodyne 7725i injection valve (Rheodyne, Cotati, CA, USA) with a 20 µL loop. Detection was achieved by an SPD-M10AVP Photodiode Array Detector, supplied with data acquisition software LabSolutions-LC solutions by Shimadzu. Degassing of the mobile phase was achieved by helium sparging in the solvent reservoirs by a DGU-10B degassing unit.

Two conventional analytical columns, an Inertsil ODS-3 5 µm, 250 × 4 mm, and an Orbit 100 C_{18} 5 µm, 250 × 4 mm (both purchased from MZ-Analysentechnik, Mainz, Germany), a monolithic one, Chromolith RP-18e 100 × 4.6 mm, purchased from Merck (Darmstadt, Germany) and a core shell KINETEX™ 150 × 4.6 mm, 2.6 µm by Phenomenex (Torrance, CA, USA) were used comparatively.

A glass vacuum filtration apparatus obtained from Alltech Associates was employed for the filtration of ammonium acetate, using Whatman cellulose nitrate 0.2 mm membrane filters (Whatman Laboratory Division, Maidstone, England). An Ultrasonic bath Transonic 460/H (Elma, Germany) were employed for sample pretreatment.

Table 1. Gradient programs applied to different columns

t (min)	A: CH_3COONH_4 (0.05M)	B: ACN	Flow rate* (mL/min)	Observed Inlet pressure (bar)
		Inertsil		
0	90	10	0.9	138-152
17	60	40		
25	60	40		
		Orbit		
0	90	10	0.8	180-195
23	75	25		
35	75	25		
40	60	40		
		Chromolith		
0	95	5	1.0	85-96
5	95	5		
30	60	40		
40	40	60		
		Kinetex		
0	95	5	0.9	222-243
17	60	40		
20	60	40		

* The higher flow rates that yielded optimum resolution.

Chemicals and reagents

Thiamphenicol (THI), florfenicol (FLO), cloxacillin sodium salt (CLO), dicloxacillin sodium salt (DICLO), oxacillin sodium salt (OXA), amoxicillin (AMO), and ampicillin sodium salt (AMP) were purchased from Sigma-Aldrich (St Louis, MO, USA). Chloramphenicol (CHL) was purchased from Alfa-Aesar (Karlsruhe, Germany) and paracetamol (IS) was purchased from Merck (Darmstadt, Germany). HPLC grade methanol and acetonitrile were obtained from Fisher Scientific (Steinheim, UK). Ammonium acetate was supplied by Merck. High purity water, obtained by a Milli-Q purification system (Millipore, Bedford, MA, USA), was used throughout this study.

In order to study the applicability of the proposed method, florfenicol was determined in NUFLOR injectable solution (labeled concentration 300 mg/mL) supplied by Schering-Plough Animal Health USA, while ampicillin and dicloxacillin were determined in Cloxalene Plus injectable solution (labeled concentration 11g/100 mL for AMP and 5g/100 mL for DICLO, supplied by FATRO S.p.A. Bologna, Italy. Both pharmaceuticals are intended for veterinary use.

Preparation of standards

Aqueous stock standard solutions were prepared at a concentration of 100 ng/µL. When stored at 4 °C, stock solutions were stable for one month. Working aqueous standards were prepared by further dilution at various concentrations covering the entire linear range. These were freshly prepared every three days.

All working standards contained paracetamol as internal standard at a concentration of 2 ng/µL. Aliquots of 20 µL were injected onto the column and quantitative analysis was based on peak area measurements as ratios versus peak area of internal standard.

Table 2. Performance characteristics of evaluated columns

Analyte	t_R (min)	N	RRT	T_f	k	Rs	RSD[1]	RSD[2]
				INERTSIL ODS-3				
AMO	4.390	1003	0.62	1.50	0.30	AMO-IS:6.6	2.21	1.76
IS	7.129	8100	–	1.00	1.15	IS-AMP:5.5	1.12	1.98
AMP	9.064	13 091	1.27	1.50	1.69	AMP-THI:6.0	0.65	0.97
THI	11.928	22 678	1.67	1.00	2.54	THI-OXA:13.2	1.03	1.54
OXA	16.324	42 470	2.29	0.75	3.84	OXA-FLO:4.7	0.34	0.23
FLO	17.260	47 480	2.42	1.00	4.12	FLO-CLO:2.2	0.53	0.74
CLO	17.690	49 874	2.48	1.00	4.25	CLO-CHL:1.6	0.87	0.98
CHL	18.091	52 163	2.54	1.50	4.37	CHL-DICLO:4.4	1.02	1.59
DICLO	19.648	61 526	2.76	1.00	4.83		0.95	1.54
				ORBIT 100 C_{18}				
AMO	5.891	2590	0.60	1.00	2.15	AMO-IS:5.2	0.34	1.24
IS	9.878	7281	–	1.00	2.49	IS-AMP:6.4	0.76	1.67
AMP	14.581	15 867	1.48	1.00	2.93	AMP-THI:5.7	0.98	3.02
THI	20.011	29 888	2.03	0.75	3.04	THI-OXA:12.0	1.09	2.09
OXA	32.025	43 052	3.24	1.50	3.56	OXA-FLO:1.5	0.54	1.44
FLO	33.831	21 175	3.43	1.00	4.21	FLO-CLO:3.1	0.46	1.03
CLO	36.647	24 844	3.71	2.00	4.43	CLO-CHL:4.5	0.67	0.78
CHL	41.447	128 219	4.20	0.75	5.61	CHL-DICLO:2.8	0.87	1.07
DICLO	42.546	135 103	4.31	1.00	7.84		0.62	0.98
				CHROMOLITH RP-18e				
AMO	3.078	340	0.76	1.50	0.82	AMO-IS:2.2	2.32	2.76
IS	4.063	592	–	1.50	1.93	IS-AMP:7.8	2.42	3.98
AMP	11.693	2188	2.88	1.25	2.28	AMP-THI:-	3.02	3.87
THI	12.803	3304	3.15	1.50	2.54	THI-OXA:-	2.67	2.66
OXA	16.475	5473	4.12	1.50	2.82	OXA-FLO:4.8	1.76	2.54
FLO	18.736	7077	4.61	1.50	2.95	FLO-CLO:4.5	1.23	1.59
CLO	20.562	8524	5.06	2.00	3.21	CLO-CHL:3.5	1.65	1.94
CHL	22.286	13 079	5.49	2.00	3.99	CHL-DICLO:4.5	1.55	1.23
DICLO	24.786	16 179	6.10	1.25	5.10		1.33	2.12
				KINETEX C_{18}				
AMO	3.889	3600	0.69	1.00	1.00	AMO-IS:3.6	0.23	0.67
IS	5.675	13 626	–	1.50	1.93	IS-AMP:6.3	0.12	0.34
AMP	7.628	25 659	1.34	1.00	2.93	AMP-THI:7.7	0.76	0.99
THI	9.566	38 722	1.69	1.50	3.93	THI-OXA:9.1	0.34	0.53
OXA	13.375	75 698	2.36	1.00	5.90	OXA-FLO:3.1	0.69	0.87
FLO	14.137	84 568	2.49	0.75	6.29	FLO-CLO:1.9	0.87	1.02
CLO	14.533	89 328	2.56	1.00	6.50	CLO-CHL:8.2	0.45	0.76
CHL	15.352	56 096	2.71	1.50	6.92	CHL-DICLO:1.8	0.I5	1.21
DICLO	16.999	122 277	3.00	1.00	7.76		0.99	1.34

t_R: Retention time. Theoretical plates: $N = 16(t_R/t_W)$.[2] Tailing factor: $T_f = (A + B)_{5\%}/2A_{5\%}$. RRT: Relative retention time (with reference to IS Paracetamol): $RRT = t_R/t_{Ris}$. Retention factor: $k = (t_R - t_0)/t_0$. Resolution: $Rs = (t_2 - t_1)/0.5(t_{w1} + t_{w2})$. RSD[1]: RSD of retention times n=10 chromatograms, RSD[2]: RSD of peak areas n=10 chromatograms.
Columns Inertsil ODS-3: pore size100 Å, pore volume 1.05 cm^3/g surface area (BET) 450 m^2/g silica purity 99.999%, endcapped, carbon content C_{18}: 15%C.
Orbit 100 C_{18}: pore size 100 Å, pore volume 0.9 cm^3/g surface area (BET) 340 m^2/g silica purity >99.999%, endcapped, carbon content C_{18}: 19%C.
Chromolith RP-18e: Macropore Size: 2 mm, Micropore Size: 13 nm, pore volume 1 mL/g surface area (BET)300 m^2/g silica purity High Purity end, carbon content C_{18}: 18%C.
Kinetex C_{18}: total particle size (µm): 2.6, porous shell (µm): 0.35, solid core (µm): 1.9, pore size (Å): 100, effective surface area (m^2/g): 200, effective carbon load %: 12, pH Stability: 1.5–10, pressure stability: 600 bar.

Chromatography

Target analytes were separated by gradient elution. Different gradient programs were applied depending on the analytical column used, all consisted of the same solvents, but in different volume ratios, so that each column revealed optimal performance. Table 1 summarizes all gradient programs used. Monitoring of analytes was performed at 240 nm, while quantitation was performed at the wavelength of maximum absorbance for each analyte, as follows: AMO, AMP and CLO at 226 nm, THI, OXA, FLO and DICLO at 240 nm and CHL at 280 nm.

Peak identification was performed both by retention times and by spectral information provided by the diode array detector.

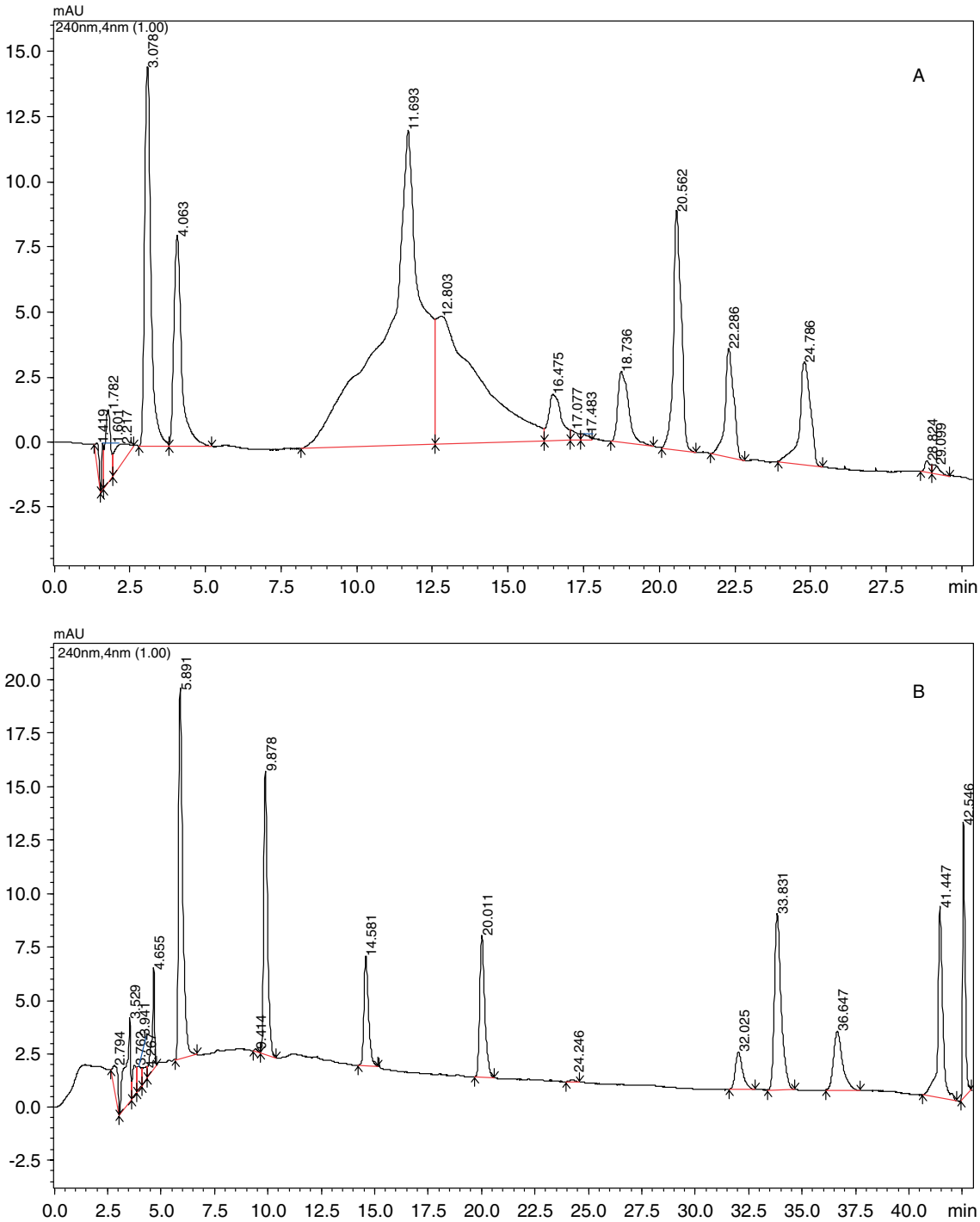


Figure 2. Typical HPLC chromatograms of standard solution (5 ng/μL) of five penicillins and three amphenicols with paracetamol as Internal Standard (IS 2 ng/μL). **A. Chromolith.**: Peaks: AMO (3.078 min), IS (4.063 min), AMP (11.693 min), THI (12.803 min), OXA (16.475 min), FLO (18.736 min), CLO (20.562 min), CHL (22.286 min) and DICLO (24.786 min). **B. ORBIT.**: Peaks: AMO (5.891 min), IS (9.878 min), AMP (14.581 min), THI (20.011 min), OXA (32.025 min), FLO (33.831 min), CLO (36.647 min), CHL (41.447 min) and DICLO (42.546 min). **C. Inertsil.**: Peaks: AMO (4.390 min), IS (7.129 min), AMP (9.064 min), THI (11.928 min), OXA (16.324 min), FLO (17.260 min), CLO (17.690 min), CHL (18.091 min) and DICLO (19.648 min). **D. KINETEX.**: Peaks: AMO (3.889 min), IS (5.675 min), AMP (7.628 min), THI (9.566 min), OXA (13.375 min), FLO (14.137 min), CLO (14.533 min), CHL (15.352 min) and DICLO (16.999 min).

Comparison of column performance

Column performance was evaluated by calculation of number of theoretical plates $N=16\ (t_R/t_w)^2$, the tailing factor $T_f = (A+B)_{5\%}/2A_{5\%}$, the relative retention time $R = t_R/t_{IS}$, the retention factor $k' = (t_R-t_0)/t_0$, the resolution factor $R_s = (t_2-t_1)/0.5(t_{w1}+t_{w2})$, and the relative standard deviation of the retention time and of the area of each peak. Peak capacity was also calculated according to the formula: $P=1 + t_g/w$, where t_g is the gradient time and w is the baseline peak width. The technical specifications of the examined columns are included in Table 2.

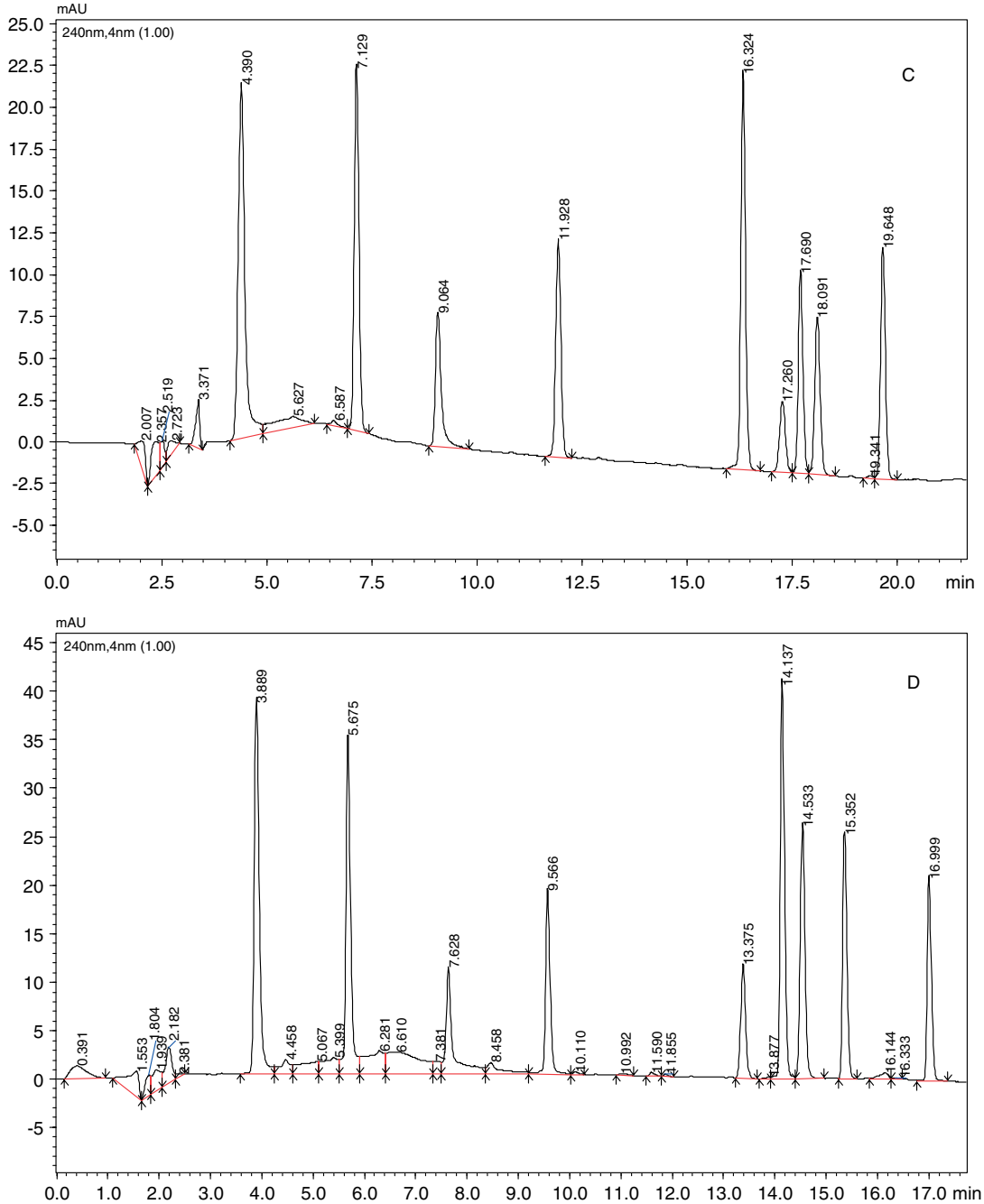


Figure 2. (*Continued*).

Method validation

The proposed analytical method under optimal chromatographic conditions was fully validated in terms of linearity, sensitivity, accuracy, within-day, and between-day precision and selectivity.

Linearity was studied by constructing the calibration curves by analysing mixtures of standard solutions covering the entire working range (0.5–20 ng/μL). Calibration lines were constructed from peak area ratios of the analyte versus internal standard at nominal drug concentration. The calculation of the slope, the intercept and the coefficient of determination of each calibration line was achieved through linear regression analysis. The calculations for the limits of quantitation (LOQs) were based on the standard deviation of y-intercepts of regression analysis (σ) and the slope (S), using the following equation $LOQ = 10\sigma/S$[28]

Accuracy was studied by analysing three concentration levels (2, 5 and 10 ng/μL). The relative error of the three measurements was calculated with Equation 1:

$$\text{Relative Error}(\%) = \frac{[\text{Mean determined value} - \text{added amount}]}{\text{added amount}} \times 100 \quad (1)$$

In order to evaluate within-day and between-day precision, standard solutions at three concentration levels (2, 5 and 10 ng/μL)

Table 3. Calibration and sensitivity data of the penicillins and amphenicols using two C_{18} analytical columns: Inertsil and Kinetex

Compound	Regression equation by KINETEX	Regression equation by INERTSIL	LOQ (ng) by Kinetex	LOQ (ng) by Inertsil	Upper Limit (ng)
AMO	Y=(0.010304 ± 0.00023)X − (0.02226 ± 0.026712)	Y=(0.009524 ± 0.000476)X + (0.080902 ± 0.048632)	25.9	51.1	400
	r^2 = 0.9985	r^2 = 0.9925			
AMP	Y=(0.002937 ± 0.0000754)X + (0.004837 ± 0.004147)	Y=(0.004144 ± 0.000218)X − (0.04639 ± 0.025734)	14.1	62.1	400
	r=0.9987	r^2 = 0.9918			
THI	Y=(0.00673 ± 0.000273)X-(0.02016 ± 0.027984)	Y=(0.005032 ± 0.000175)x + (0.010594 ± 0.019863)	41.6	39.5	200
	r^2 = 0.9967	r^2 = 0.9952			
OXA	Y=(0.002944 ± 0.000276)X + (0.00305 ± 0.002827)	Y=(0.006967 ± 0.000175)X − (0.00551 ± 0.024769)	9.6	35.6	400
	r^2 = 0.9998	r^2 = 0.9961			
FLO	Y=(0.008884 ± 0.000204)X + (0.026973 ± 0.020891)	Y=(0.00607 ± 0.000261)X + (0.083482 ± 0.026645)	23.5	43.9	200
	r^2 = 0.9990	r^2 = 0.9945			
CLO	Y=(0.005815 ± 0.000152)X + (0.071728 ± 0.015528)	Y=(0.004877 ± 0.000243)X + (0.09372 ± 0.028275)	26.7	58.0	400
	r^2 = 0.9979	r^2 = 0.9926			
CHL	Y=(0.019588 ± 0.00045)X + (0.054253 ± 0.045982)	Y=(0.020597 ± 0.000665)X − (0.1436 ± 0.075712)	23.5	36.8	200
	r^2 = 0.9984	r^2 = 0.9958			
DICLO	Y=(0.004529 ± 0.000154)X + (0.018578 ± 0.019163)	Y=(0.006325 ± 0.000308)X − (0.055796 ± 0.031452)	42.3	49.7	400
	r^2 = 0.9965	r^2 = 0.9929			

Y= peak area ratio of analyte versus internal standard, X= ng.

were analysed. Each concentration was measured four times within a day and three times for four consecutive days, respectively.

Veterinary drugs

Two injectable suspensions both intended for veterinary use were analysed. Nuflor containing florfenicol was simply diluted in water to prepare a 100 ng/μL solution. This was used to prepare dilute solutions at three different concentration levels: 2, 5 and 10 ng/μL.

Cloxalene plus, containing ampicillin and dicloxacillin, was diluted in methanol to prepare a solution at 45.4 ng/μL for DICLO and 100 ng/μL for AMP. Further dilution was made in water at three different concentration levels (2, 5 and 10 ng/μL for AMP and 0.9, 2.3 and 4.5 ng/μL for DICLO). All dilute solutions were containing the internal standard. Each solution was analysed in triplicate.

Results and Discussion

Chromatography

The multistep gradient elution programs yielded optimum separation of the eight target antibiotics and the internal standard within ca 20 min using the Inertsil analytical column, 43 min the Orbit, 25 min the Chromolith and 17 min the KINETEX™ column. The typical chromatograms of standard solutions for each analytical column are shown in Figure 2. Under the conditions described above, the studied penicillins and amphenicols as well as the internal standard were baseline resolved with all four columns. Any attempt to reduce retention times by increasing the flow rate, led to a significant loss of resolution.

As already mentioned, KINETEX™ is packed with a core-shell particle sorbent with unique characteristics. In order to establish its applicability to multi-residue analysis, column validation was performed by means of several parameters. These include the number of theoretical plates N, the tailing factor T_f, the relative retention time RRT, the retention factor k′, the resolution factor R_s and the relative standard deviation of the retention time and of the area of each peak. The results are summarised in Table 2. Number of theoretical plates is higher by KINETEX™ and the respective height of theoretical plate (data not shown) is lower, leading to greater efficiency. KINETEX™ also achieves the fastest separation, while Inertsil the second faster one. Chromolith column did not perform well with regard to peak shape and baseline stability. Using KINETEX™, precision in retention time and peak area was much better for most analytes. It also provided lower LOQs for most of the examined compounds (Table 3). Peak capacity of KINETEX™ was 74, towards 69 for Inertsil, by shorter column length and in shorter gradient time. For all these reasons further method development and validation was conducted on this column.

Method validation

Full method validation was performed using the KINETEX™ column according to ICH Guidance for Industry, Q2(R1) Validation of Analytical Procedures: Text and Methodology.[28] Figures of merit are described in the following paragraphs.

Linearity and sensitivity were studied comparatively with Inertsil, since the latter also provided fast separation. Calibration curves were constructed with working standards obtained by least-squares linear regression analysis of the peak area ratio of analyte to the internal standard versus analyte injected amount.

Table 4. Within- (n=4) and between-day (n=3×4) precision and accuracy data on Kinetex analytical column

Compound	Added (ng)	Within-day (n=4)			Between-day (n= 3 × 4)		
		Found ± SD (ng)	RSD	RE (%)	Found ± SD (ng)	RSD	RE (%)
AMO	40	36.2 ± 3.6	9.9	−10.6	37.3 ± 1.6	4.3	−6.7
	100	98.8 ± 5.2	5.9	−1.2	108.7 ± 7.2	6.6	8.7
	200	195.0 ± 5.0	2.6	−2.5	201.8 ± 5.7	2.8	0.9
AMP	40	36.1 ± 2.4	6.8	−9.8	36.1 ± 1.1	2.9	−9.8
	100	101.9 ± 2.3	2.2	1.9	109.1 ± 2.6	2.4	9.1
	200	189.3 ± 5.2	2.7	−5.6	197.1 ± 3.1	1.6	−1.4
THI	40	37.8 ± 3.7	11.5	−5.5	37.7 ± 1.2	3.4	−5.8
	100	93.4 ± 6.6	7.1	−6.4	98.3 ± 4.2	4.3	−1.7
	200	211.7 ± 8.0	3.8	5.8	200.5 ± 4.1	2.0	0.3
OXA	40	43.6 ± 2.5	5.8	8.9	43.2 ± 1.0	2.2	8.2
	100	106.6 ± 8.4	7.8	6.6	106.5 ± 2.3	2.2	6.5
	200	194.5 ± 5.8	3.0	−2.8	199.4 ± 3.3	1.6	−0.3
FLO	40	38.2 ± 2.0	5.3	8.9	38.9 ± 1.1	2.8	−2.9
	100	106.6 ± 8.4	7.8	6.6	112.4 ± 2.8	2.5	12.4
	200	194.5 ± 5.8	3.0	−2.8	199.4 ± 3.3	1.6	−0.3
CLO	40	38.2 ± 1.8	4.8	−4.4	38.21 ± 1.2	3.2	−4.5
	100	98.1 ± 5.5	5.6	−2.0	99.1 ± 2.3	2.3	−0.9
	200	203.5 ± 8.5	4.2	1.7	198.7 ± 4.7	2.4	−0.7
CHL	40	37.3 ± 1.4	3.7	−6.4	38.8 ± 1.4	3.6	−2.9
	100	94.3 ± 5.5	5.8	−5.7	95.4 ± 2.1	2.2	−4.6
	200	189.6 ± 6.7	3.5	−5.2	199.4 ± 2.0	1.0	−0.3
DICLO	40	38.9 ± 2.2	5.6	−2.6	38.4 ± 0.5	1.4	−3.9
	100	105.7 ± 3.1	2.9	5.7	98.9 ± 1.0	1.0	−1.1
	200	202.8 ± 3.2	1.6	1.4	203.6 ± 3.1	1.5	1.8

Table 5. Precision and Accuracy (Relative Error RE) data of penicillins (ampicillin and dicloxacillin) and amphenicols (florfenicol) in veterinary drug formulations

Veterinary Drug	Nominal amount (ng)	Found[a] ± SD (ng)	RE%	RSD
AMP (injectable solution)	40	38.3 ± 1.0	−4.3	2.5
	100	98.5 ± 5.7	−1.5	5.8
	200	188.1 ± 9.6	−5.9	5.1
Labeled Concentration: 11g/100 mL. Found Concentration[b]: 10.8 ± 0.2g/100 mL.				
FLO (injectable solution)	40	35.9 ± 2.8	−10.3	8.0
	100	94.9 ± 5.8	−5.1	6.1
	200	192.4 ± 6.8	−3.8	3.5
Labeled Concentration: 300 mg/mL. Found Concentration[b]: 273.9 ± 0.6 mg/mL.				
DICLO (injectable solution)	18	17.1 ± 1.1	−5	6.3
	46	42.0 ± 2.8	−8.7	6.8
	90	90.4 ± 5.0	+0.4	5.6
Labeled Concentration: 5g/100 mL. Found Concentration[b]: 4.7 ± 0.3g/100 mL.				

[(a)] : Mean values of 3 measurements.
[(b)] : Mean values of 3 × 3 = measurements.

The analytical method was linear up to 20 ng μL^{-1} for penicillins and up to 10 ng μL^{-1} for amphenicols. LOQ values ranged from 9.6 ng to 42.3 ng for 20 µL injected volume. Lower LOQ values were observed by KINETEX™ as well as better linearity. All calibration and sensitivity data are presented in Table 3. Regression equations revealed good coefficients of determination ranging between 0.9982 and 0.9999 over the examined range.

Within-day repeatability by four replicated and between-day precision by three measurements within four consecutive days revealed RSD values lower than 12%. Accuracy in both assays was in the range from −10.6% to 12.4%. Precision and accuracy results are summarized in Table 4.

Analysis of veterinary drug

Commercially available veterinary formulations, one containing florfenicol and one containing ampicillin and dicloxacillin, were analyzed at three different concentrations. Results of accuracy tests are given in Table 5. These are mean values from three concentration levels and three measurements for each level.

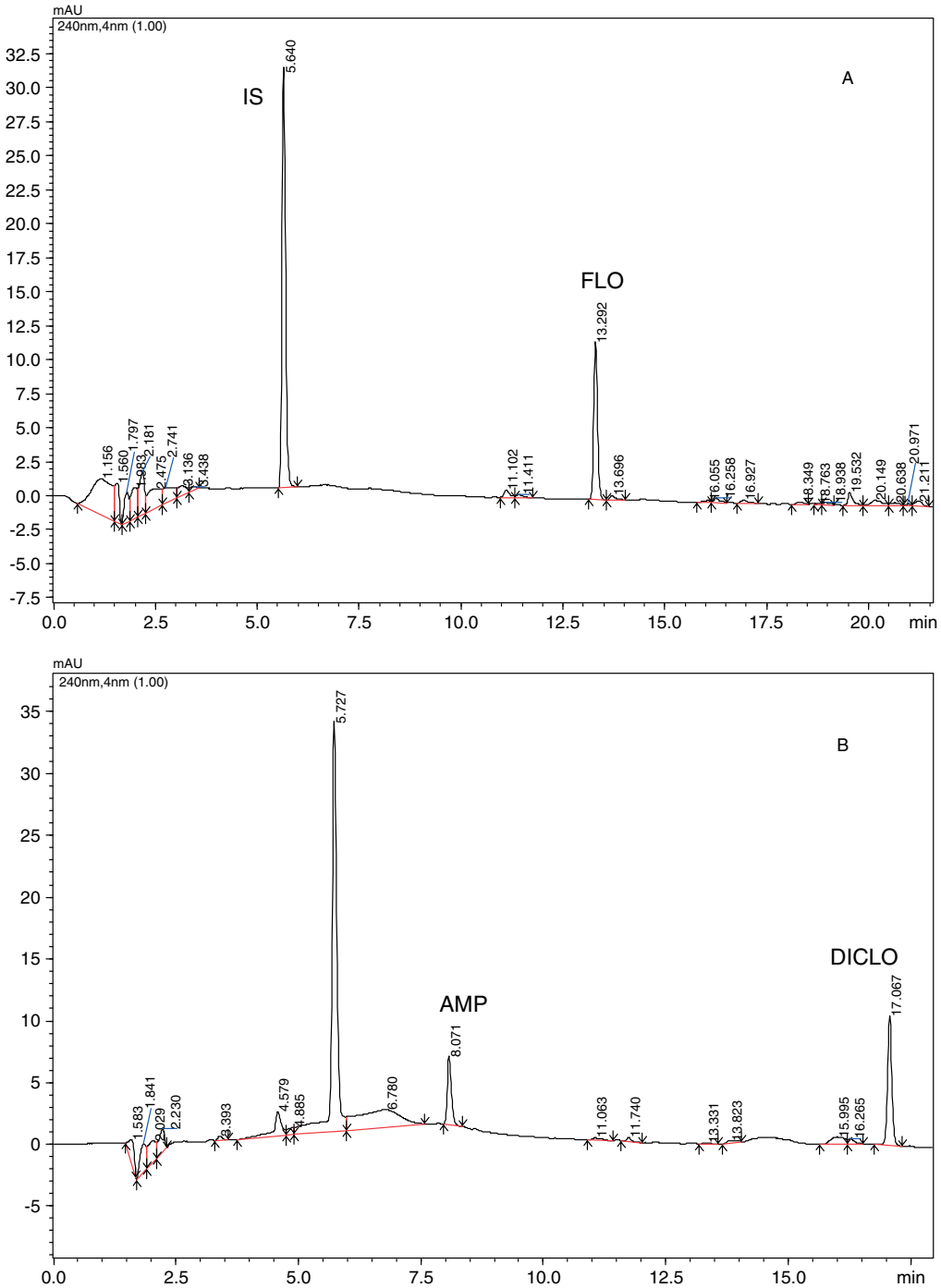


Figure 3. High performance liquid chromatograms of veterinary drugs. A: Florfenicol (5 ng/µL) in Nuflor, IS: 5.640 min, FLO 13.292 min, B: AMP (5 ng/µL) and DICLO (2.3 ng/µL) in Cloxallen, IS: 5.127 min, AMP: 8.071 min, DICLO 17.067 min.

Excellent accuracy can be observed for both drug formulations. Typical chromatograms are given in Figure 3.

Conclusions

The primary goal of this work was to evaluate the new core-shell C_{18} KINETEX™ analytical column in the determination of penicillins and amphenicols compared with a monolithic and two conventional C_{18} columns, which are commonly used for such analyses.

The expected better chromatographic performance due to its innovative unique characteristics was proven by application to the multiclass analysis of five penicillins and three amphenicols.

The analysis was completed within 17 min. The method was validated and proved to be suitable to monitor the concentration of the studied antibiotics in drug formulations.

Narrower peaks were observed with higher sensitivity and better precision in peak area and retention time, thus ultra-high performance is achieved by using a typical 400 bar HPLC instrument commonly found in any analytical laboratory.

This is the first reported method for the application of the new core shell KINETEX™ column to the simultaneous determination of antibiotics.

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