

LC DETERMINATION OF ENROFLOXACIN IN NASAL SECRETIONS AND PLASMA OF HEALTHY PIGS USING RESTRICTED ACCESS MATERIAL FOR ON-LINE SAMPLE CLEAN-UP



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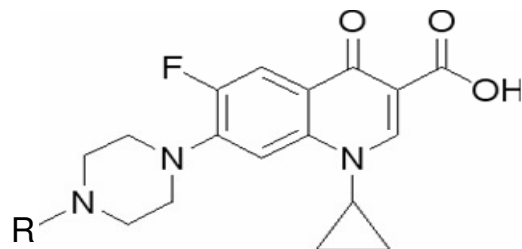
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OBJECTIVE

- To test restricted access material (RAM), namely LiChrospher ADS (Alkyl Diol Silica), for on-line sample clean-up coupled to liquid chromatography (LC) for the determination of enrofloxacin (ENRO) in plasma and nasal secretions of healthy pigs, using the column-switching technique.
- To optimize the most important parameters likely to influence the sample preparation method for the analysis of ENRO and ciprofloxacin (CIPRO), its active metabolite.
- To increase method detectability by selecting fluorescence detection.
- To validate the method developed and to apply it to the analysis of actual plasma and nasal secretion samples.

STRUCTURE



ENRO : R = CH₂CH₂ – CIPRO : R = H

RESULTS AND DISCUSSION

Initial chromatographic conditions

Although a pharmacopoeia method using UV detection for the analysis of CIPRO was tested successfully for the determination of ENRO in aqueous solutions, it could not be applied to nasal secretions of healthy pigs, due to very low volumes sampled (20 – 100µl). It is well known that antibiotics belonging to the group of fluoroquinolones can be detected by fluorescence. Table 1 summarizes the initial chromatographic conditions.

Table 1. Initial chromatographic conditions

Stationary phase	PURSUIT-C18 (5 µm) (150 x 4.6 mm;i.d)
Mobile phase	25mM phosphoric acid and triethylamine in water (pH 3.0) – acetonitrile (83:17;v/v)
Flow-rate	1.5 ml/min
Temperature	40°C
Detection	λ _{Exc.} : 278 nm / λ _{Em.} : 445 nm

Effect of the composition of the washing liquid, the flow-rate and the pre-column type on breakthrough time of ENRO and CIPRO

As can be seen in Table 2, ENRO and CIPRO were retained sufficiently on LiChrospher RP-18 ADS, when purified water or 25mM ammonium phosphate buffer of pH 7.4 were used as washing liquid. This buffer gave rise to less carryover from late-eluting components and was selected. The flow-rate was set to 0.8 ml/min.

Table 2. Influence of the pH and the composition of the washing liquid, the flow-rate and the pre-column type on the breakthrough time of ENRO and CIPRO

Washing liquid (25mM)	pH	Flow-rate (ml/min)	Pre-column type	Breakthrough time (min)	
				CIPRO (0.5µg/ml)	ENRO (0.5µg/ml)
phosphate buffer / triethylamine	3.0	1.0	ADS-RP 8	< 1	~ 1.5
phosphate buffer / triethylamine	3.0	1.0	ADS-RP 8	< 1	~ 3.0
Purified water	ND	1.0	ADS-RP 8	~ 4	~ 9
sodium phosphate buffer	3.0	1.0	ADS-RP 8	< 1	~ 6
sodium phosphate buffer	7.4	1.0	ADS-RP 8	~ 1.5	~ 7
potassium phosphate buffer	7.4	1.0	ADS-RP 8	~ 2.0	~ 7
ammonium phosphate buffer	7.4	1.0	ADS-RP 8	~ 4	~ 8
ammonium phosphate buffer	7.4	0.8	ADS-RP 8	~ 6	~ 13
phosphate buffer / triethylamine	3.0	0.8	ADS-RP 18	~ 1.5	~ 7
Purified water	ND	0.8	ADS-RP 18	> 20	> 20
sodium phosphate buffer	7.4	0.8	ADS-RP 18	~ 7	~ 13
potassium phosphate buffer	7.4	0.8	ADS-RP 18	~ 9	~ 15
ammonium phosphate buffer	7.4	0.8	ADS-RP 18	> 20	> 20
ammonium phosphate buffer 98% / MeOH 2%	7.4	0.8	ADS-RP 18	~ 6	~ 13

Sample : aqueous solution of CIPRO and ENRO (0.5µg/ml)

Selection of final operating conditions

The time for a complete elimination of the sample matrix being 3.5 min, it was decided to maintain sample clean-up for 7 min, which corresponded to the first time for the rotation of the switching valve. The period of time needed to transfer the analyte quantitatively from the pre-column to the analytical column with the LC mobile phase in the back-flush mode was 5 min under isocratic conditions. As shown in Figure 1, a dramatic peak tailing associated to a poor resolution between ENRO and CIPRO was observed. A gradient elution mode was then tested. Figure 1 illustrates the improvement of peak shape under the final chromatographic conditions summarized in Table 3. Seventeen minutes after sample application, the switching valve returned to its initial position, allowing the pre-column to be re-equilibrated with the washing liquid.

Figure 1 shows typical chromatograms obtained after on-line coupling of a pre-column packed with RP-18 ADS to the analytical column and elution under isocratic conditions (A – LC mobile phase : 25mM phosphate buffer of pH 3.0 / acetonitrile (83:17; v/v) and by using a gradient program (B) as given in Table 3. The washing liquid was composed of 25mM ammonium phosphate buffer of pH 7.4.

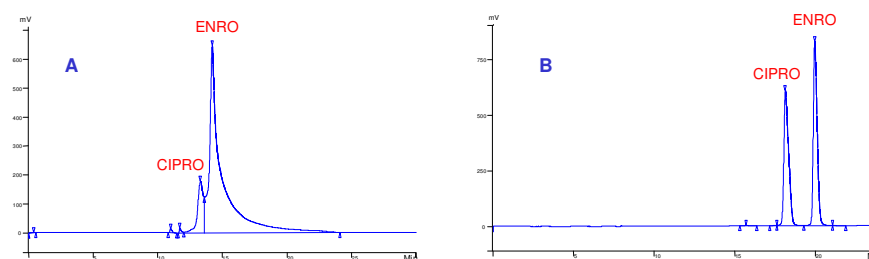


Table 3. Final chromatographic conditions

Stationary phase	PURSUIT – C18 (5 µm) (150 x 4.6 mm;i.d)
Mobile phase	25mM phosphoric acid and triethylamine (pH 3.0) - acetonitrile Gradient program: 0 to 7min: 95:5 (v/v) - 7 to 15min: 95:5 to 75:25 (v/v) - 15 to 17min: 20:80 (v/v) - 17 to 20min: 95:5 (v/v).
Flow-rate	1.5 ml/min
Temperature	40°C
Detection	λ _{Exc.} : 278 nm / λ _{Em.} : 445 nm

Validation

An original strategy based on total measurement error and accuracy profiles as a decision tool was used for validation [1-2]. As can be seen in Table 4 and Figure 2, the method was successfully validated. The selectivity is illustrated in Figure 3.

Table 4. Results for method validation

Validation criterion	Enrofloxacin in Plasma	Enrofloxacin in nasal secretions	
Response function (k=3; n=2)	Weighted (1/X) quadratic regression Calibration range (m=6): 30-15000 ng/ml		
	Series 1	Series 2	
	Slope	204.5	204.4
	Intercept	-1999	-2361
	Weight	1/X	1/X
	r ²	0.9997	0.9999
Trueness (k=3; n=3)	Weighted (1/X) quadratic regression Calibration range (m=6): 30-15000 ng/ml		
	Series 1	Series 2	
	Relative bias (%)	30 ng/ml	13
		90 ng/ml	-7.7
		150 ng/ml	-
		300 ng/ml	5.2
	7500 ng/ml	-8.3	
	15000 ng/ml	0.8	
Precision (k=3; n=3)	Repeatability/Intermediate precision (R.S.D.%)		
	30 ng/ml	4.6	
	90 ng/ml	0.8	
	150 ng/ml	-	
	300 ng/ml	2.2	
	7500 ng/ml	1.8	
Accuracy (k=3; n=3)	relative β - expectation lower and upper tolerance limits (%)		
	30 ng/ml	[-0.4 ; 26]	
	90 ng/ml	[-9.5 ; -6.0]	
	150 ng/ml	[13 ; 22]	
	300 ng/ml	[-1.6 ; 12]	
	7500 ng/ml	[-15 ; -2.0]	
Linearity (k=3; n=3)	Range (ng/ml)		
	30 - 15000	90 - 15000	
	Slope	1.01	
	Intercept	9.98	
	r ²	0.9992	
	LOD (ng/ml)	9.3	
LOQ (ng/ml)	30.5		

Figure 2 : Accuracy profiles for the quantification of ENRO in nasal secretion and plasma of healthy pigs using a simple linear regression model (A1 : plasma; A2 : nasal secretions) and a weighted (1/X) quadratic regression model (B1 : plasma; B2 : nasal secretions). Relative bias (—) - Acceptance limits (•••••) - Beta expectation tolerance limits (---) - Relative back-calculated concentrations (•).

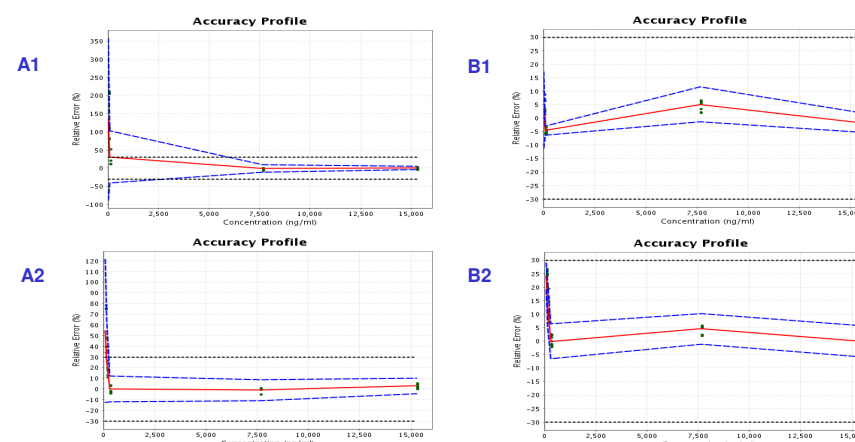
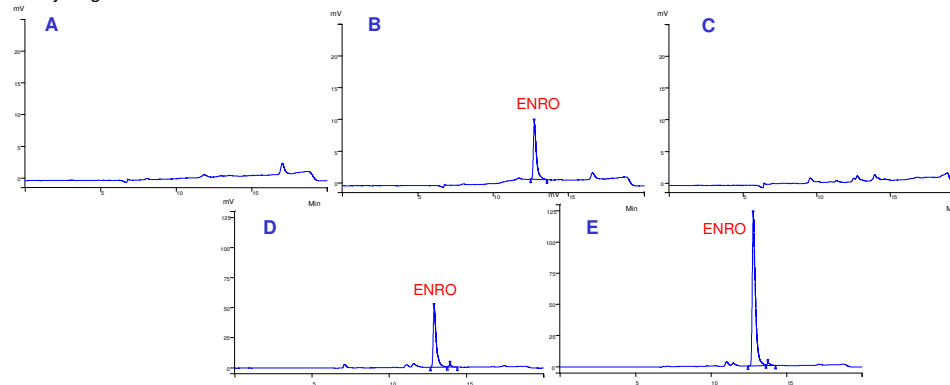


Figure 3 : A. Chromatogram of a blank plasma sample – B. Chromatogram of a plasma sample spiked with ENRO (15 ng/ml) – C. Chromatogram of a blank nasal secretion - Chromatogram of a plasma sample (D.) and of a nasal secretion sample (E.) from a healthy pig treated by an intra-muscular injection of ENRO at 30mg/kg bodyweight and collected after 24h.



[1] Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap et al, STP Pharma Pratiques 13 (2003) 101.
 [2] Ph. Hubert, J.-J. Nguyen-Huu, B. Boulanger, E. Chapuzet, P. Chiap et al, J. Pharm. Biomed. Anal. 36 (2004) 579.