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HPLC-DAD and LC-ESI-MS analysis of doxycycline and related impurities in doxipan mix, a medicated premix for incorporation in medicated feedstuff

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Abstract

HPLC-DAD and LC-ESI-MS methods have been developed for the analysis of doxycycline (DOX), including the identification of the related impurities metacycline (MTC) and 6-epidoxycycline (EDOX) and its determination in a medicated premix. The chromatographic separations have been performed on Luna C_{18} stationary phase and on Synergi (4 μm) Polar-RP 80A, using both acidic (pH 2.5) and basic (pH 8.0) mobile phases. The Synergi Polar-RP column, in combination with a mobile phase of oxalic acid (0.02 M; pH 2.5)–acetonitrile 82:18 (v/v), allowed the complete separation of MTC, EDOX and DOX. The same separation was also obtained using Luna C_{18} stationary phase with a pH 8 mobile phase. Application of a LC-ESI-MS system and MS/MS analysis, using both positive and negative polarity, allowed the peak identity to be confirmed. A method based on Luna C_{18} column and UV detection at 346 nm was validated for the determination of DOX in a medicated premix for incorporation in medicated feedstuff.
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Keywords: Doxycycline; 6-Epidoxycycline; Metacycline; High performance liquid chromatography; Tandem mass spectrometry; Medicated premix.

1. Introduction

Doxycycline (DOX) is a semisynthetic broad spectrum tetracycline antibiotic, widely used in veterinary medicine and as an animal feed supplement to prevent diseases. The synthetic pathway of DOX involve Metacycline (MTC) as an intermediate [1]; during this process 6-epidoxycycline (EDOX) can be formed as a side product (structures in Fig. 1). Therefore these compounds can be contained as impurities in the bulk samples of DOX used to prepare animal feeds and premixes. European Pharmacopoeia (Ph. Eur) [2] and USP [3] set the limit of 2% for MTC and EDOX and 0.5% for any other impurity. Both Ph. Eur and USP prescribe chromatographic (HPLC) methods for the analysis of DOX and related compounds, based on a styrene-divinylbenzene copolimer stationary phase.

Tetracyclines exhibit general poor stability [4] and upon storage in animal feeds and premixes may be subjected to extensive degradation (i.e. epimerization). The quality control of tetracyclines, including DOX, and their determination in feeds by HPLC proved to be rather difficult [5]. The chromatographic conditions are a critical part of the HPLC methods; a variety of stationary phases, such as polymeric reversed phase [2,3,5–10], RP-8 [5,11–13], RP-18 [5,14–16] and graphitic carbon [17] columns, have been proposed.

As an alternative, capillary electrophoresis (CE) has been also applied to separate doxycycline from its impurities [18].

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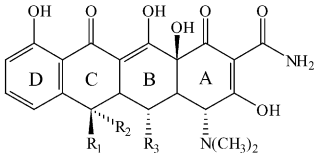

Compound	R1	R2	R3
Doxycycline	CH_3	H	OH
6-epidoxycycline	H	CH_3	OH
Metacycline	R1 + R2 = CH_2		OH
Tetracycline	CH_3	OH	H

Fig. 1. Structures of doxycycline, and related tetracyclines.

The present study was aimed to offer an useful contribution in the HPLC quality control of DOX and its determination in animal feeds; in particular, the following objectives are concerned: (a) development of HPLC methods based on a new polar stationary phase suitable to separate DOX from EDOX and MTC; (b) identification of the impurities (EDOX, MTC) by LC-ESI-MS approach using both positive and negative ionization of the analytes; (c) determination of DOX and related impurities in medicated premix for incorporation in medicated feedstuff.

2. Experimental

2.1. Materials

Doxycycline hydrochloride and tetracycline hydrochloride were obtained from Fluka Chemie (Buchs, Switzerland), doxycycline hyclate and medicated premix Doxipan MIX was furnished by TREI (Industria Italiana Integratori, Italy), metacycline and 6-epidoxycycline were supplied by Council of Europe (European Pharmacopeia). HPLC grade methanol and acetonitrile were from Romil Pure Chemistry (Cambridge, UK), reagent grade oxalic acid, ammonium acetate, acetic acid, trifluoroacetic acid (TFA) were obtained from Sigma–Aldrich (Steinheim, Germany).

2.2. Standard solutions

Standard solutions of doxycycline hydrochloride, metacycline hydrochloride, 6-epidoxycycline hydrochloride and tetracycline hydrochloride (Fig. 1), were prepared in acidified water (0.01N HCl) at the concentration of 0.05, 0.025, 0.025, 0.025, 0.025 mg/ml, respectively. A mixture of doxycycline and its impurities was prepared in the same solvent; these solutions were then analysed by LC-MS techniques.

2.3. Apparatus and chromatographic conditions

Quantitative analysis were performed on a Hewlett Packard Ti series 1050 liquid chromatograph, equipped with a Rheodyne Model 7125 injector and connected to a photodiode array detector (DAD, HP Ti series 1050). Chromatographic analysis were performed on a Phenomenex Luna C_{18}, 3.5 μm (150 mm × 2.0 mm i.d.) column, using a mobile phase consisting of oxalic acid/NaOH (pH 2.5; 0.02 M)–acetonitrile–methanol 75:17:8 (v/v) (phase A), at a flow rate of 0.3 ml/min. A Phenomenex Synergi 4 μm Polar-RP 80A (150 mm × 2.00 mm i.d.) was also used with a mobile phase consisting of oxalic acid (0.02 M; pH 2.5)–acetonitrile 82:18 (phase B) (v/v) at a flow rate of 0.3 ml/min. The injection volume was 20 μl and UV detection at 346 nm was used.

LC-MS analysis were carried out on a Jasco PU-1585 Liquid Chromatograph (Jasco Corporation, Tokio, Japan) interfaced with LCQDuo Mass Spectrometer (Thermo Finnigan, San Jose, CA, USA), equipped with heated capillary interface and electrospray ionization (ESI) source, and operating with an Ion Trap analyser. ESI system employed a 4.5 kV (positive polarity) and 5.00 kV (negative polarity) spray voltage and a heated capillary temperature of 200 °C. The sheath gas and the auxiliary gas (nitrogen) flow rate was set to 0.75 and 1.2 l/min, respectively. Electrospray ionisation was optimised using doxycycline as reference compound. The mass chromatograms were acquired in total ion current (TIC) modality from 110 to 600 *m/z*, in MS/MS mode (relative collision energy 20%) on the ESI generated ions at *m/z* 445 (positive polarity) and 443 (negative polarity) for doxycycline, 6-epidoxycycline, tetracycline, at *m/z* 443 (positive polarity) and 441 (negative polarity) for metacycline. Chromatographic analysis were performed on a Phenomenex Luna C_{18}, 3.5 μm (150 mm × 2.0 mm i.d.) column, using two different mobile phases: 0.01% acetic acid (pH 3)–acetonitrile–methanol 75:10:15 (v/v) (phase C) for positive polarity, triethylamine (TEA) acetate buffer (20 mM; pH 8)–methanol 70:30 (v/v) (phase D) for negative polarity and on a Phenomenex Synergi 4 μm Polar-RP 80A (150 mm × 2.00 mm i.d.) using a mobile phase consisting of 0.1% TFA/NH_3 in water (pH 2.5)–acetonitrile 87:13 (phase E) (v/v) in positive polarity, at a flow rate of 0.3 ml/min. The injection volume was 20 μl.

2.4. Calibration graphs

Solutions of doxycycline hyclate in acidified water (0.01N HCl) were prepared in triplicate, obtaining the following final concentration 0.0125, 0.025, 0.05, 0.075 and 0.1 mg/ml of doxycycline base. These solutions were analysed by HPLC-DAD as described above (mobile phase A) and calibration graph was constructed by plotting the peak area versus the corresponding drug concentration. The linearity of the method was studied for doxycycline alone and for the reconstituted pharmaceutical formulation, obtained by spiking placebo DOXIPAN MIX with doxycycline at theoretical concentrations corresponding to 20, 50, 100, 150 and 200% of the pharmaceutical product concentration (0.0125, 0.025, 0.05, 0.075 and 0.1 mg/ml). The two linearity studies were then compared and accuracy was calculated.

2.5. Assay procedure

The sample solutions were obtained dissolving 250 g of Doxipan MIX, containing 10% of doxycycline as base form derived from doxycycline hyclate, in 250 ml of a mixture water–acetonitrile 80:20 acidified with 1.5 ml of 0.1N HCl. These solutions were heated at 45 °C and stirred for 30 min and, after previous filtration through 15 mm, 0.22 μm syringe filter (Gyro-Disc, Orange Scientific, Braine-l′Alleud, Belgium), were diluted 1:2 with water.

The repeatability and reproducibility of the quantitative method were evaluated by analysing the Doxipan MIX six times in three different days.

3. Results and discussion

3.1. Chromatographic conditions

This work was firstly aimed to develop a reversed-phase HPLC method suitable to separate DOX from its principal impurities EDOX and MTC and useful to monitor the stability of the drug in feeds. To this end two different stationary phases were used: Phenomenex Luna C_{18} and Phenomenex Synergi 4 μm Polar-RP, an ether-linked phenyl phase with polar endcapping.

Using Luna C_{18} column, with mobile phase A and UV (DAD) detector at 346 nm, separation of DOX (tr = 12 min) from its impurities (tr = 9.5 min for EDOX and MTC) was achieved (Fig. 2a). Interferences due to the matrix (sample Doxipan MIX) were not observed (Fig. 2b). Mobile phase A contained oxalic acid able to improve the separation and the peak symmetry [5]. Using the Synergi Polar-RP column, in combination with the mobile phase B, a baseline separation of the two impurities EDOX and MTC was achieved (Fig. 3) according to the Ph. Eur. requirements. Therefore this stationary phase appears to offer a useful alternative chromatographic condition in the field of the HPLC separation of tetracyclines.

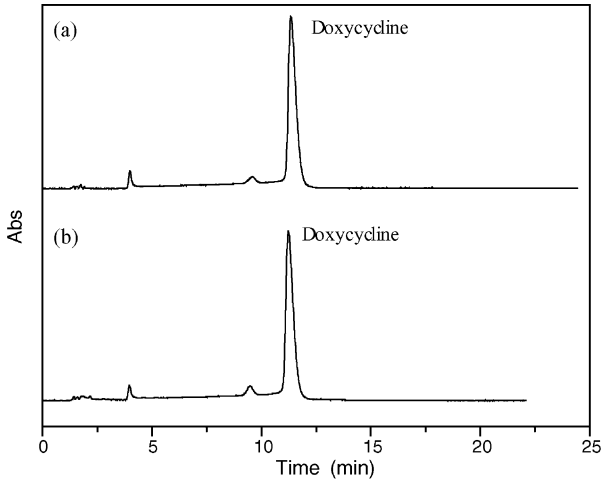

Fig. 2. HPLC-DAD chromatograms of doxycycline standard TREI (a) and Doxipan Mix (b). Chromatographic conditions: Luna C_{18} column, mobile phase consisting of oxalic acid/NaOH (pH 2.5; 0.02 M)–acetonitrile–metanolo 75:17:8 (v/v), UV detection at 346 nm.

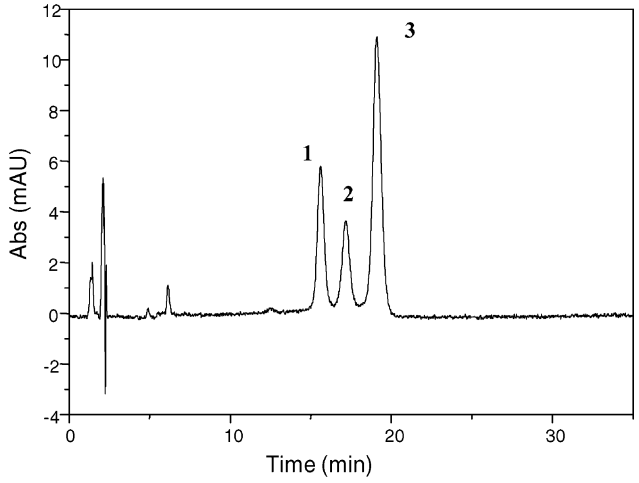

Fig. 3. HPLC-DAD chromatogram of the mixture of EDOX (1), MTC (2) and DOX (3). Chromatographic conditions: Synergi Polar-RP column, mobile phase consisting of oxalic acid (0.02 M; pH 2.5)–acetonitrile 82:18 (v/v), UV detection at 346 nm.

When LC-MS was applied, the use of oxalic acid was avoided and the mobile phase composition was adjusted. Using Luna RP-18 column and acidic mobile phase C, LC-ESI-MS and tandem mass analysis were performed in positive polarity. This approach has been widely used for the determination of impurities in tetracyclines [5,16] and tetracycline residues in feeds [5,10,12,13,15].

The positive MS/MS chromatograms relative to standard doxycycline hydrochloride (Fluka), Doxipan MIX, 6-epidoxycycline hydrochloride, metacycline hydrochloride and tetracycline hydrochloride are reported in Fig. 4. As shown, these chromatographic conditions did not allow to separate the two impurities, EDOX and MTC. However, using MS/MS modality, the separation of DOX from the impurities and their identity was confirmed.

On the contrary the resolution between the analytes DOX (tr = 26.5 min), MTC (23.4 min) and EDOX (tr = 21.2 min) was obtained on the column RP-POLAR with the mobile phase E. Molecular mass, reactant and product ions *m/z*, and relative fragment ion intensities are listed in Table 1. These results are in agreement with the literature data [10,12,13,15] Fragmentation of doxycycline, 6-epidoxycycline, peaks X and Y (Fig. 4), produced a single product ion corresponding to $[M+H\text{-}NH_3]^+$ at *m/z* = 428 due to the loss of ammonia which occurs from the carboxyamide moiety in the A ring of the molecule. Similarly, fragmentation of metacycline produced the product ion at *m/z* = 426. By comparison between the retention times and the MS/MS spectra, the peak Y can be ascribed to 6-epidoxycycline. Differently the comparison between the MS/MS spectra of impurity X and tetracycline (Table 1), having the same retention time, shows different fragmentation profiles, with the additional loss of H_2O (*m/z*

Table 1
Molecular mass, reactant and product ions *m/z*, and relative fragment ion intensities for the compounds investigated

Compound	Nominal molecular mass (Da)	Reactant ion (*m/z*)	Product ions	Product ion efficiency (%)
Doxycycline	444	445	$[M + H\text{-}NH_3]^+$ *m/z* 428	100
6-Epidoxycycline	444	445	$[M + H\text{-}NH_3]^+$ *m/z* 428	100
X	444	445	$[M + H\text{-}NH_3]^+$ *m/z* 428	100
Y	444	445	$[M + H\text{-}NH_3]^+$ *m/z* 428	100
Metacycline	442	443	$[M + H\text{-}NH_3]^+$ *m/z* 426	100
Tetracycline	444	445	$[M + H\text{-}NH_3]^+$ *m/z* 428	15
			$[M + H\text{-}H_2O]^+$ *m/z* 427	70
			$[M + H\text{-}NH_3\text{-}H_2O]^+$ *m/z* 410	15

427) and the formation of the tetracycline fragment $[M + H\text{-}NH_3\text{-}H_2O]^+$ at *m/z* 410 due to the loss of an hydroxy group in the C ring, excluding the same identity. On the basis of the retention time [14] and tandem mass spectrum [10], the peak X could be ascribed to 4-epidoxycycline.

The positive MS/MS chromatogram (precursor ion at *m/z* 443) of Doxipan MIX (Fig. 4c) shows the presence of metacycline as impurity in trace level.

The resolution between the two impurities was obtained on the C_{18} column by using the mobile phase at pH 8 (phase D) and the LC-MS analyses were carried out in negative polarity. The negative MS/MS chromatograms of doxycycline, metacycline and 6-epidoxycycline and the relative MS/MS spectra are reported in Figs. 5 and 6, respectively. As shown fragmentation in negative polarity produced other product ions in addition to the single product ion at *m/z* 428 obtained in positive polarity (Table 1). Moreover the fragmentation of doxycycline, impurity Y and 6-epidoxycycline presented the same product ions but in different relative abundance. In particular, the formation of the ion at *m/z* 358 is higher for fragmentation of Y and 6-epidoxycycline, respect to that of doxycycline, confirming the Y identity as EDOX. Therefore, the fragmentation in negative polarity can offer further information useful for confirmatory purposes.

3.2. Quantitative aspects

The quantitative analyses were restricted to the determination of doxycycline for the quality control of premix. For

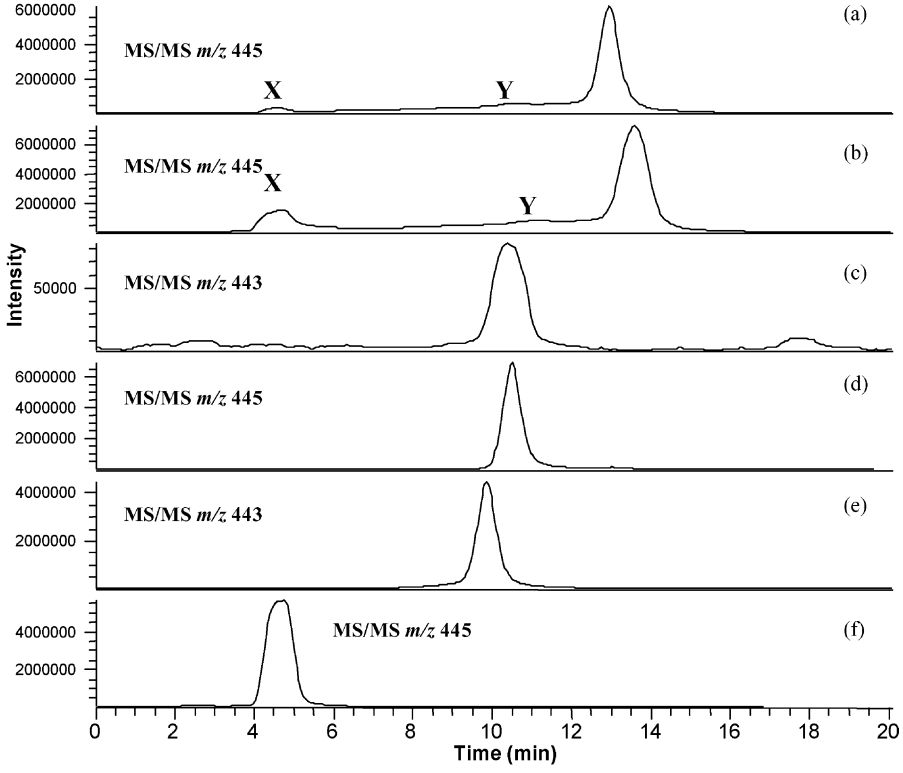

Fig. 4. Positive MS/MS chromatograms relative to standard doxycycline hydrochloride (Fluka) (a), Doxipan MIX (b and c), 6-epidoxycycline hydrochloride (d), metacycline hydrochloride (e) and tetracycline hydrochloride (f). Chromatographic conditions: Luna C_{18} column, mobile phase consisting of 0.01% acetic acid (pH 3)–acetonitrile–metanolo 75:10:15 (v/v).

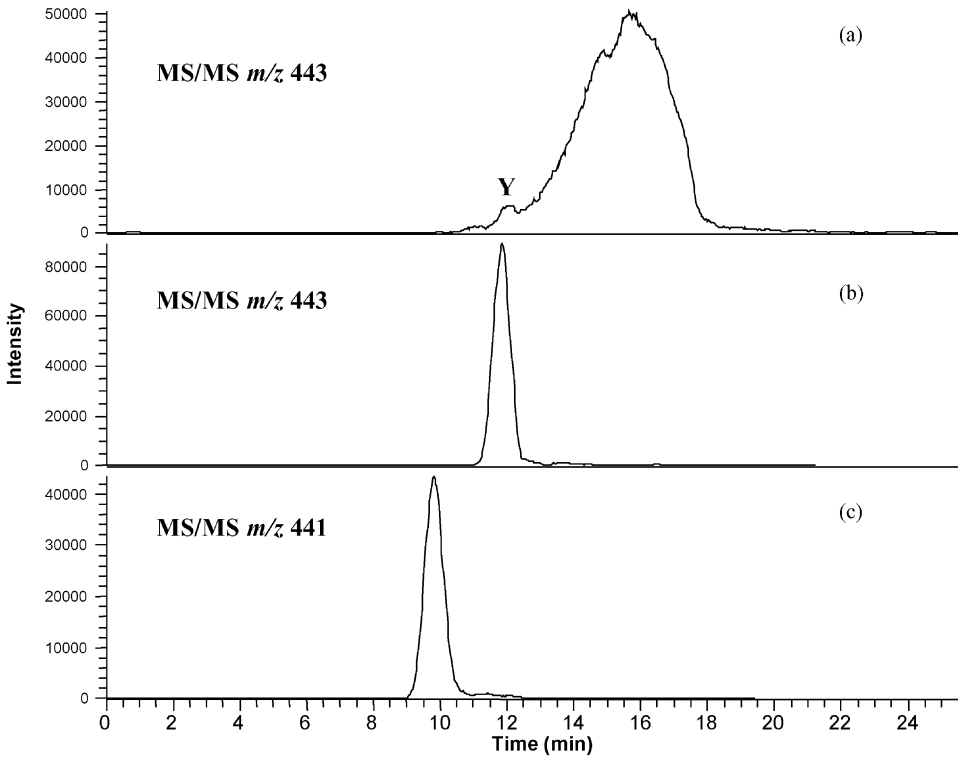

Fig. 5. Negative MS/MS chromatograms relative to standard DOX (Fluka) (a), EDOX hydrochloride (b) and MTC hydrochloride (c). Chromatographic conditions: Luna C_{18} column, mobile phase consisting of TEA acetate buffer (20 mM; pH 8)–methanol 70:30 (v/v).

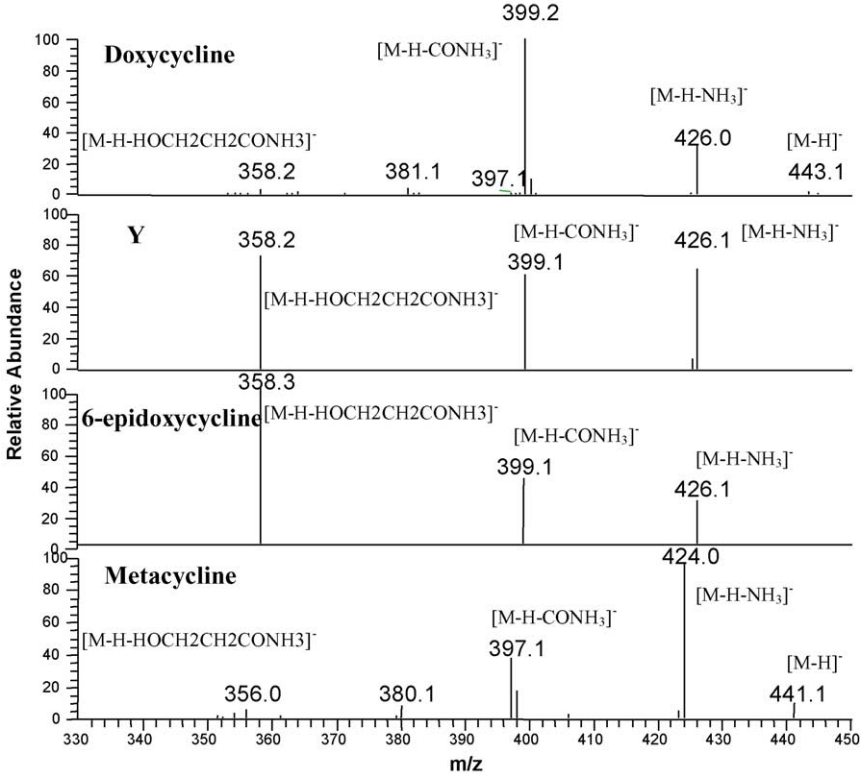

Fig. 6. Negative ESI tandem mass spectra of the investigated compounds under ESI-LC-MS-MS conditions, as in Fig. 5.

this application Luna C_{18} column, with mobile phase A, was used to develop a validated method.

3.2.1. Linearity

A linear relationship between peaks area of the drug (y) and the corresponding concentration (x) was found. The following data for doxycycline alone and the reconstituted pharmaceutical formulation were obtained:

- Doxycycline: y = 116,000(± 1643) x + 167.7(± 102.10) r^2 = 0.9974.
  The y-intercept of the regression curve is not significantly different from 0: t = 1.642 < t(0.05, 13) = 2.160. The slope is significant: F = 4988 > $F^1{}_{13}$ (0.05) = 4.667.
- Reconstituted pharmaceutical formulation: y = 113,400(± 661.6) x + 85.12(± 41.31) r^2 = 0.9996.

The y-intercept of the regression curve is not significantly different from 0: t = 2.061 < t(0.05, 13) = 2.160. The slope is significant: F =29360 > $F^1{}_{13}$ (0.05) = 4.667.

The slopes and the y-intercepts of the two regression curves previously obtained were compared using the t-test and the following results were found: the slopes of the regression curves are not significantly different; calculated t = 1.468 < t(0.05; 26) = 2.056. The y-intercepts of the regression curves are not significantly different; calculated t = 0.552 < t(0.05; 26) = 2.056. The two regression curves are superimposable. The Doxipan MIX 100 excipients have no effect on the assay of doxycycline.

3.2.2. Accuracy

Accuracy was determined from the data recorded during the linearity study of the reconstituted pharmaceutical formulation. Accuracy is calculated from the recovery between the measured concentration of doxycycline and the theoretical concentration, taking the regression curve obtained for the doxycycline alone as reference. The individual recoveries (Table 2) were calculated. Once it has been established that the variances were homogeneous (Cochran's test: C calculated < C table (0.05, 5, 2) = 0.684) and that the means were valid (t-test: t(P = 0.05; (n−1) = 14) 2.145), the mean recovery level and its confidence limits (C.F.) were calculated: mean recovery = 100.365%; C.F. = 100.365 ± 0.572. In conclusion the method for the assay of doxycycline in Doxipan MIX is accurate.

Table 2
Individual recovery of the reconstituted pharmaceutical formulation Doxipan Mix, calculated from the recovery between the measured concentration of doxycycline and the theoretical concentration

Individual recoveries					
Groups	1	2	3	4	5
Concentration (%)	25	50	100	150	200
	101.62	98.26	99.30	101.25	100.62
	97.67	100.40	102.49	97.98	100.33
	100.76	103.43	100.96	99.77	102.64
Mean recovery	100.02	100.70	100.25	99.67	101.20
nj	3	3	3	3	3
Variance S^2j	4.315	6.75	7.112	2.681	1.583

Table 3
Quantitative analysis results of Doxipan Mix expressed as grams of doxycycline/1000 g Doxipan Mix

Day	D1	D2	D3
Assay no.			
1	101.72	100.33	101.48
2	104.31	100.24	103.87
3	104.76	104.12	99.83
4	103.27	100.60	99.10
5	102.88	102.56	100.46
6	101.75	99.19	100.29
Mean	103.12	101.17	100.84
Variance	1.605	3.290	2.820

3.2.3. Precision

Precision, as repeatability and reproducibility, of the quantitative method were evaluated by analysing the Doxipan MIX six times in three different days. The results in grams of doxycycline/1000 g of Doxipan MIX and the statistical data are presented in Tables 3 and 4, respectively. As the variances are homogeneous, the calculation of the two coefficients of variation (repeatability and reproducibility) is valid. These coefficients show that the method is repeatable and reproducible.

The detection limit (LOD), based on the standard deviation of the response and the slope of the doxycycline standard calibration curve (EMEA, CVMP/VICH/591/98-FINAL) may be expressed as: DL = 3.3σ/S = 3.3 × 102.1/116,000 = 0.00290 mg/ml, where σ is the standard deviation of the response, which is the standard deviation of the y-intercepts of regression line, and S is the slope of the calibration curve.

The quantitation limit (LOQ), based on the standard deviation of the response and the slope of the doxycycline standard calibration curve (EMEA, CVMP/VICH/591/98-FINAL) may be expressed as: QL = 10σ/S = 10 × 102.1/116,000 = 0.00880 mg/ml, where σ is the standard deviation of the response, which is the standard deviation of the y-intercepts of regression line, and S is the slope of the calibration curve.

Table 4
Precision statistical data of the quantitative method

Homogeneity of the linked variances	
Result of Cochran's test	
C calculated = maximal variance/sum of variances	0.208
Critical C	0.707
The variances are homogeneous	
Mean of the means	101.71
Repeatability variance	2.57
Repeatability coefficient of variation	1.56
Between-group variance	2.223
Reproducibility variance	3.428
Reproducibility coefficient of variation	1.70

C Calculated < C table (0.05, 3, 5) = 0.707.

4. Conclusion

The obtained results showed that the Phenomenex Synergi (4 μm) Polar-RP 80A column can be regarded as a useful stationary phase for the analyses of tetracyclines; in this application the separation of MTC, EDOX and DOX was achieved under simple chromatographic conditions.

A conventional RP-18 stationary phase can be used when the separation of DOX from its impurities (MTC and EDOX) is the main objective; using this stationary phase a HPLC method was validated for the determination (UV detector) of DOX in a medicated premix. LC-ESI-MS and MS/MS analysis, in both positive and negative polarity, offer the opportunity of an effective integrated confirmatory test for the presence of related compounds in doxycycline.

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