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Research article

A FAST AND ACCURATE METHOD FOR THE QUANTIFICATION OF DOXYCYCLINE IN GOAT PLASMA AND MILK BY HPLC USING A FLUORESCENCE DETECTOR

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Doxycycline is an antimicrobial agent used in veterinary medicine to treat a variety of bacterial infections. To date, no analytical technique utilising HPLC with fluorescence detection has been documented for the quantification of doxycycline concentrations in goat plasma or milk. Consequently, the objective of the present study was to propose a rapid HPLC assay with fluorescence detection for the quantification of doxycycline in the aforementioned samples, thereby facilitating the conduct of pharmacokinetic studies and the detection of residues in diverse goat tissues. Proteins were precipitated with methanol and trifluoroacetic acid in a single step. Doxycycline was separated on a XBRIDGE C18 column using an isocratic method. Sample volume injected into the HPLC system was 50 µl. Fluorescence detection was conducted with an excitation wavelength of 380 nm and an emission wavelength of 520 nm. The retention times of doxycycline and danofloxacin (internal standard) were determined to be 8.0 and 5.5 minutes, respectively. The calibration curves for plasma and milk exhibited linearity over the concentration range of 0.1 to 2 µg/mL. The limit of detection was 0.065 µg/mL, while the limit of quantification was 0.1 µg/mL in both matrices. The accuracy and precision of the method were consistently within the limits of 10.9% for plasma and 10.5% for milk. The findings of this study may be employed in the quantification of doxycycline in goat plasma and milk, thus facilitating the conduct of pharmacokinetic studies.

Keywords: Doxycycline, fluorescence, HPLC, milk, plasma

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INTRODUCTION

Doxycycline is an antimicrobial agent with a broad spectrum of bacteriostatic activity [1]. This antimicrobial acts by inhibiting protein synthesis in a time-dependent manner by reversibly binding to the 30S ribosomal subunit [2]. Doxycycline is active against a wide range of aerobic and anaerobic Gram-positive and Gram-negative bacteria, including those such as *Pasteurella, Escherichia coli, Chlamydia spp, Mycoplasma spp, Rickettsia spp* and protozoa [3-5]. In veterinary practice, this antibiotic is used to treat a variety of bacterial diseases in different species [4].

Doxycycline has a high lipophilicity compared to other tetracyclines, which gives it a good oral absorption, a wide volume of distribution and a long half-life [5]. These pharmacokinetic properties permit the administration of a single daily dose, which is of significant interest in the veterinary field. Furthermore, the drug demonstrates excellent tissue penetration, with levels reaching the therapeutic range in a multitude of organs and tissues, including the lungs, kidneys, prostate, gastrointestinal tract, myocardium, tonsils, and others [6]. Nevertheless, prior to administering the substance to different species, it is of the utmost importance to quantify it in various fluids and tissues, such as milk, in order to conduct pharmacokinetic studies and optimise the dose and dosing regimen for each species. This will also help to prevent the occurrence of drug residues in the food chain.

Initially, several microbiological methods have been published for the quantification of doxycycline concentrations in goat plasma, urine, milk and other biological fluids [6-9]. Microbiological assays do not rely on the use of specialised equipment or toxic solvents. However, high-performance liquid chromatography (HPLC) methods offer a more accurate, reproducible and precise technique than the aforementioned methods, making them suitable for pharmacokinetic studies. To date, a number of studies have employed liquid chromatography/mass spectrometry (HPLC/MS) techniques for the quantification of doxycycline in plasma from a range of species, including chicken and human [10-12]. Although these methods offer high sensitivity and low detection limits, they require solid-phase extraction (SPE) sample preparation, which is relatively expensive due to the large number of samples required in pharmacokinetic studies. Furthermore, this equipment is not widely available in most laboratories.

A number of studies have employed high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection to quantify doxycycline concentrations in plasma and urine from a variety of species [13-16]. However, only one study has been conducted in goat plasma [17]. The latter method involved extracting plasma samples with buffer/EDTA and perchloric acid, achieving an LQ of 40 μg/L and a high recovery rate. However, no method is described for the quantification of doxycycline in milk samples. In recent years, the fluorescence detection method has been employed for the detection of tetracyclines due to its advantageous characteristics, including ease of use, low cost, rapid response and high sensitivity. [18]. To date, no analytical HPLC technique employing fluorescence detection for the quantification of doxycycline

concentrations in goat plasma or milk has been published. Published fluorescence-detector HPLC methods have been used to quantify doxycycline concentrations in a variety of tissues from chickens, turkeys, pigs and fish, as well as in human saliva and gingival crevicular fluid. [19-22]. A single manuscript has quantified the concentration of this antimicrobial in milk, yet fails to specify the species in question. Furthermore, the sample processing is lengthy and comprises a number of complex steps. [23].

Therefore, the objective of the present investigation was to validate an easy and rapid HPLC method with fluorescence detection for the quantification of doxycycline in goat plasma and milk samples. This method was developed with the intention of facilitating the undertaking of pharmacokinetic studies and the assessment of the occurrence of residues in different goat tissues.

MATERIAL AND METHODS

Chemicals, solvents and reagents

Doxycycline and danofloxacin (internal standard) were purchased from Cymit Química (Barcelona, Spain). All solvents were of HPLC quality. Acetonitrile (ACN), methanol and water were provided by Merck Life Science (Madrid, Spain). PanReac AppliChem (Barcelona, Spain) provided magnesium chloride, ammonium acetate, Na_2EDTA, triethylamine and trifluoroacetic acid.

Collection of samples

Plasma and milk samples were obtained from eight clinically healthy lactating female Murciano-Granadina goats with no history of drug administration in the previous two months. The University of Murcia Ethics Committee (CEEA 758/2021) approved the protocol for the animal study. Blood samples were collected from the jugular vein using heparinised tubes and centrifuged at 600 g for 10 min. Milk samples were collected by manually milking the mammary glands until they were empty. Plasma and milk samples were collected at 10:00 am and stored at – 40 °C until analysed.

Instrumentation and chromatographic conditions

The HPLC equipment employed was the same as that utilised for the quantification of delafloxacin in human plasma. [24].

A XBRIDGE, C18 column (100mm, 4.6mm, 3.5µm) supplied by WATERS CROMATOGRAFÍA (Barcelona, Spain), was used for the chromatographic separation. The isocratic elution was carried out with a mobile phase consisting of: (A) an aqueous phase containing 50 mM ammonium acetate, 50 mM magnesium chloride and 1mM Na_2EDTA, buffered to pH 7.5 with ammonium hydroxide. Finally, 1 ml of triethylamine was added to each 500 ml of mobile phase A; (B) acetonitrile. A

15:85 volume ratio of aqueous phase A and acetonitrile B was used. At a flow rate of 1 mL/min, 50 μL of sample was injected. Fluorescence detection was performed at a $\lambda_{excitation}$ = 380 nm and $\lambda_{emission}$= 520 at 20 ºC. The total analysis run time was 12 minutes.

Standard solutions

Stock solutions (100 μg/mL) of danofloxacin (internal standard: IS) and doxycycline were prepared in water. Working solutions of 0.1, 1, 10, 20 and 50 μg/mL of doxycycline were prepared by diluting the stock solution with water, and they were stored at – 80ºC.

Preparation of quality controls and calibration curve

Calibration curve and quality control (QC) samples were spiked by the addition of 20 μL of the appropriate working solution to 180 μL of unmedicated plasma or milk. Seven concentration levels from 0.1 to 2.5 μg/mL were used by diluting the appropriate working solutions. Four concentration levels were prepared for QCs (0.1, 0.5, 1 and 2 μg/mL) from plasma and milk.

Sample preparation

10 μL of IS solution (10 μg/mL) was added to 200 μL of goat plasma or milk. Plasma and milk proteins were precipitated by adding a mixture of 100 μL of methanol and 100 μL of a 1:2 solution of trifluoroacetic acid and methanol. This sample was then vortexed for 10 seconds and sonicated for 5 minutes. The sample was centrifuged at 14000 rpm for 10 minutes. The supernatant was injected into the HPLC system at a rate of 50 μL per sample.

Method Validation

Method validation was performed according to the FDA Guidance for the Validation of Bioanalytical Methods [25]. The parameters evaluated for goat plasma and milk were: linearity, lower limit of detection (LLOD), lower limit of quantification (LLOQ), accuracy, precision, recovery, selectivity and carry-over. The protocol followed to validate each of the above parameters, as well as the coefficients of variation that were deemed acceptable are outlined in a previous publication by our research group [24].

RESULTS

Peaks were obtained at 8.0 and 5.5 min corresponding to doxycycline and IS, respectively, in goat plasma (Figure 1) and milk (Figure 2). The calibration curves demonstrated linearity within the concentration range of 0.1 μg/ml to 2.5 μg/ml in both goat plasma and milk samples. The curves were highly linear ($r^2 \geq 0.997$ and

0.991 for plasma and milk, respectively). The linear regression equations for both matrices (plasma and milk) were $y=6.0\cdot10^{-7}\cdot X$, and the LOD and LOQ values were 0.065 and 0.1 μg/mL, respectively. These findings indicate the potential suitability of the proposed method for the quantification of doxycycline in milk and plasma using HPLC with fluorescence detection.

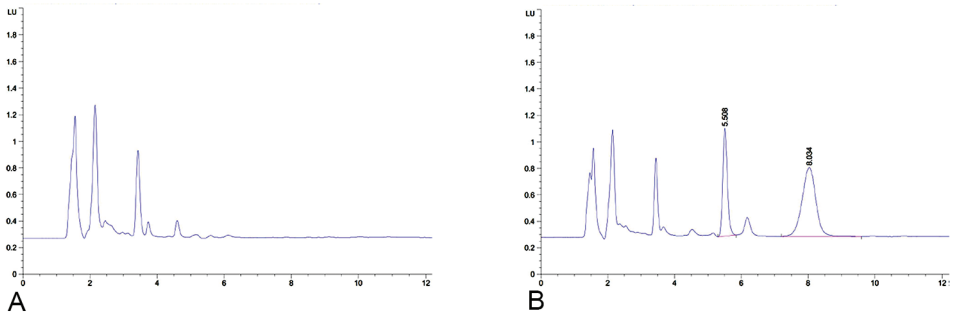

Figure 1. Chromatograms of doxycycline in plasma by HPLC: blank plasma **(A)**; and blank plasma spiked with doxycycline (0.5 μg/mL) and danofloxacin as internal standard **(B)**.

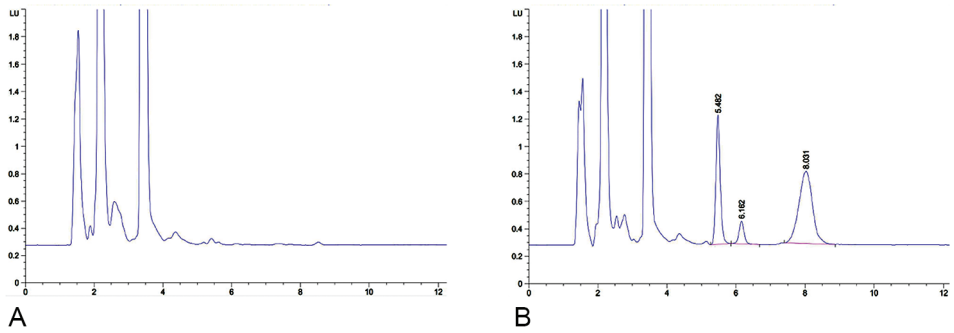

Figure 2. Chromatograms of doxycycline in milk by HPLC: blank milk **(A)**; and blank milk spiked with doxycycline (0.5 μg/mL) and danofloxacin as internal standard **(B)**.

Table 1 for plasma and Table 2 for milk show the accuracy and precision data results. The intra – and interday accuracy and precision of the method were evaluated at four quality control concentrations. The concentrations were 0.1, 0.5, 1 and 2 μg/mL in both matrices. The CV precision values for plasma samples were <6.7% and <6.9% for within-day and between-day precision, respectively, and for milk samples were <8.7% and <8.3%, respectively. Accuracy ranged from – 6.0% to 10.9% for plasma samples and from – 4.8% to 10.5% for milk samples. The results obtained were consistent, indicating that the method is reliable for quantitative determination in goat milk and plasma samples.

Table 1. Intra-day and inter-day precision and accuracy of doxycycline in goat plasma samples.

Intra-day	Nominal concentration (µg/mL)	Mean concentration ± SD (µg/mL)	CV (%)	Accuracy (%)
	0.1	0.099 ± 0.006	5.7	-1.5
	0.5	0.470 ± 0.030	6.7	-6.0
	1	1.076 ± 0.022	2.0	7.1
	2	2.119 ± 0.082	3.9	6.0
Inter-day				
	0.1	0.097 ± 0.006	6.3	-4.8
	0.5	0.489 ± 0.016	3.7	-2.4
	1	1.086 ± 0.075	6.9	8.6
	2	2.220 ± 0.146	6.6	10.9

Table 2. Intra-day and inter-day precision and accuracy of doxycycline in goat milk samples.

Intra-day	Nominal concentration	Mean concentration ± SD	CV	Accuracy
	(µg/mL)	(µg/mL)	(%)	(%)
	0.1	0.096 ± 0.001	1.2	-4.2
	0.5	0.490 ± 0.004	8.7	-2.1
	1	1.075 ± 0.013	1.4	7.5
	2	2.135 ± 0.110	5.1	6.7
Inter-day				
	0.1	0.097 ± 0.005	5.5	-4.1
	0.5	0.476 ± 0.033	7.0	-4.8
	1	1.102 ± 0.023	2.2	10.5
	2	2.097 ± 0.158	8.3	7.9

At low, medium and high QC levels (0.1, 0.5 and 2 µg/ml) recoveries were measured in plasma and milk. The results are presented in Table 3 for plasma and Table 4 for milk. The mean recoveries at these three concentrations ranged from 80.6±9.6% to 60.6±2.9% in plasma and from 81.4±9.8% to 60.7±3.9% in milk. The efficiency of the method was demonstrated by the good recoveries obtained.

Table 3. Mean ± SD recovery of doxycycline in goat plasma samples.

Nominal Concentration (µg/L)	Recovery (%) (Mean ± SD)
0.1	80.6 ± 9.6
0.5	60.6 ± 2.9
2	62.2 ± 2.1

Table 4. Mean ± SD recovery of doxycycline in goat milk samples.

Nominal Concentration (µg/L)	Recovery (%) (Mean ± SD)
0.1	81.4 ± 9.8
0.5	63.1 ± 2.7
2	60.7 ± 3.9

Six blank plasma and milk samples were analysed. The results demonstrated that there was no endogenous interference with the retention times of doxycycline and IS, which remained consistent throughout the experiment (Figures 1 and 2). Well resolved peaks for doxycycline and IS were observed in both matrices. The results were adequate, indicating the high selectivity of the method. Finally, no peaks at the same doxycycline retention time were observed in six blank plasma and milk samples after running a set of plasma and milk samples with high concentrations of doxycycline (n = 6), indicating that no carryover effects were observed.

DISCUSSION

The objective of this investigation was to develop a fast and accurate analytical method for the quantification of doxycycline in goat plasma and milk by HPLC with fluorescence detection. A number of methods have been published for the quantification of doxycycline in porcine, goat and chicken plasma using HPLC with a UV detector [13,16-17], but none have been published using HPLC with fluorescence detection. For the quantification of doxycycline in tissues other than plasma, analytical methods have been published for the quantification of this antimicrobial in chicken, turkey, fish and pork meat [19,20,22]. However, in the case of milk, there is only one published analytical method using fluorescence detection [23], The method is complex, as it is based on a fluorescent sensor comprising a zirconium-based metal-organic framework, which must be prepared and incubated with the milk samples. Other authors have developed ultra-high performance liquid chromatography-tandem mass spectrometry techniques for quantifying doxycycline in milk, although none have been developed specifically for goat's milk [26-27]. Nevertheless, this sophisticated and costly instrumentation is not commonly accessible in most laboratories. Furthermore,

liquid chromatography–mass spectrometry (LC/MS) is susceptible to a high matrix effect, which significantly impacts its accuracy, precision, and sensitivity [28], all of which are crucial for effective analysis.

Method development

The processing of plasma samples was found to vary according to the species in which the pharmacokinetic study was performed. For porcine plasma [13], trifluoroacetic acid was used to precipitate proteins, but the amount of plasma used was large (500 μl). In the case of goat plasma [17], the use of buffer/EDTA and perchloric acid was observed. Despite the smaller plasma volume (200 μl) in this study compared to the aforementioned, the samples were filtered, which resulted in a more time-consuming and costly sample processing method. For plasma from different birds [16,29-31], buffer/EDTA and formic acid was employed, however, the volume of plasma utilised was considerable (500 μl), and solid phase extraction (SPE) was also utilised, which also renders the analysis considerably more costly. A simple method has been optimised for the analysis of doxycycline in goat plasma and milk using only 200 μl of milk or plasma and a mixture of trifluoacetic acid/methanol for precipitation of proteins and extraction of doxycycline. The quantity of plasma represents a significant challenge, particularly in smaller species where pharmacokinetic studies are unable to extract a sufficient volume of blood per sample due to the inherent risk of significantly altering the total blood volume of the animal. In terms of mobile phase conditions, the use of buffer solutions was the best option. The disadvantage of this mobile phase is that it takes longer to prepare and increases the possibility of salt precipitation and clogging within the chromatographic system. The potential of gradient and isocratic elution was also explored. The rationale behind the selection of isocratic elution was to achieve the shortest possible run times.

Validation

The LLOQ obtained was 0.1 μg/mL for plasma and milk. No comparative data are available for HPLC with fluorescence detection. A comparison of this value with the data obtained with HPLC/UV on plasma of different species [13,16-17] revealed that the LLOQ obtained in this study was higher. However, the technique employed in this study demonstrated several advantages over the aforementioned techniques, including greater speed and lower cost than those mentioned above. The findings demonstrated that the proposed methodology was appropriate and sufficiently sensitive to ascertain the concentration of doxycycline in goat plasma and milk.

Good recoveries were obtained, ranging from 80.6 ± 9.6 to 60.6 ± 2.9 % in plasma and from 81.4 ± 9.8 to 60.7 ± 3.9 % in milk. The recovery rate was observed to decline in proportion to the concentration of doxycycline present in the sample. This may be attributed to the saturation of the liquid employed for the extraction of doxycycline, which can be addressed by increasing the quantity of this liquid. Nevertheless, this

procedure would result in an increase in the LLOQ. Alternatively, to circumvent this, the liquid obtained following centrifugation would have to be dried and then redissolved, which would extend the processing time for each sample and increase solvent consumption. For the intra – and inter-day coefficients obtained for plasma and milk, the LLOQ was < 20% and the low, medium and high QCs were < 15%. Therefore, our results indicate that this method was efficient and reproducible. Six blank plasma and milk samples were analysed and showed no endogenous interference with doxycycline and danofloxacin retention times (Figures 1 and 2). Spiked samples were used to compare these chromatograms. The doxycycline and danofloxacin peaks were well resolved. The high selectivity of the method was demonstrated by satisfactory results. Finally, there were no carry-over effects.

CONCLUSION

A simple, rapid, and reproducible fluorescence-detected HPLC method was developed for the quantification of doxycycline in goat plasma and milk. Furthermore, this method may be employed for routine analysis, kinetic disposition studies and clinical trials with a minimal run time.

Authors' contributions

JM, EB, VH, PM, MTY, JS, and EE participated in conceptualization, methodology, validation, investigation, original draft, writing, and review of the manuscript. MTY and JM participated in the acquisition of data and analysis. JM and PM participated in the interpretation of data. PM, EB, MTY and EE made supervision, reviewed and editing of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy of the integrity of any part of the work are appropriately investigated and resolved.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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BRZA I PRECIZNA METODA ZA KVANTIFIKACIJU DOKSICIKLINA U PLAZMI I MLEKU KOZA PRIMENOM HPLC-a I DETEKTORA FLUORESCENCE

José MARTÍNEZ, Verónica HERNANDIS, Elena BADILLO, Elisa ESCUDERO, María Teresa YUSTE, Juan Sebastián GALECIO, Pedro MARÍN

Doksiciklin je antimikrobni agens koji se koristi u veterinarskoj medicini za lečenje raznih bakterijskih infekcija. Do danas nije dokumentovana nijedna analitička tehnika koja koristi HPLC sa detekcijom fluorescence za kvantifikaciju koncentracija doksiciklina u plazmi ili mleku kod koza. Shodno tome, cilj ove studije bio je da se predloži brzi HPLC test sa detekcijom fluorescence za kvantifikaciju doksiciklina u prethodno navedenim uzorcima, čime se olakšava sprovođenje farmakokinetičkih studija i detekcija ostataka u različitim tkivima koza. Proteini su precipitirani metanolom i trifluorosirćetnom kiselinom u jednom koraku. Doksiciklin je odvojen na KSBRIDGE C18 koloni korišćenjem izokratske metode. Zapremina uzorka ubrizganog u HPLC sistem bila je 50 µl. Detekcija fluorescence je sprovedena sa talasnom dužinom ekscitacije od 380 nm i talasnom dužinom emisije od 520 nm. Vreme zadržavanja doksiciklina i danofloksacina (interni standard) je određeno na 8,0 i 5,5 minuta. Kalibracione krive za plazmu i mleko su pokazale linearnost u opsegu koncentracija od 0,1 do 2 µg/mL. Granica detekcije bila je 0,065 µg/mL, dok je granica kvantifikacije bila 0,1 µg/mL u obe matrice. Tačnost i preciznost metode bile su dosledno u granicama od 10,9% za plazmu i 10,5% za mleko. Nalazi ove studije mogu se koristiti za kvantifikaciju doksiciklina u plazmi i mleku kod koza, čime se olakšava sprovođenje farmakokinetičkih studija.