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Short communication

Validation of a high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline in bovine milk and muscle

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Abstract

High-performance liquid chromatography with diode-array detection (HPLC–DAD) was optimised and validated for the determination of tetracyclines in bovine milk and tissues. Milk and tissue samples were extracted and purified using a solid-phase extraction HLB Oasis cartridge and analysed using HPLC–DAD set at 365 nm. The analyses were carried out using the mobile phase of 0.01 *M* oxalic acid–acetonitrile–methanol (60:25:15, v/v/v) on a C_8 column (250×4.6 mm I.D., 5 μm). Recoveries of tetracyclines from spiked samples at the three concentrations (0.5, 1 and 1.5) of the maximum residues limits (corresponding to 100 μg/kg for milk and the muscle) were higher than 81.1% in milk and 83.2% in muscle. The method was successfully validated for bovine milk and muscle in compliance with requirements set by draft SANCO/1805/2000 European Decision. The decision limit (CCα) was in the range 113.2–127.2 μg/kg and 107.7–129.9 μg/kg for all compounds in milk and muscle, respectively. The detection capability (CCβ) was in the range 117.2–131.3 μg/kg and 114.9–133.1 μg/kg for all compounds in milk and muscle, respectively.

Keywords: Validation; Milk; Food analysis; Tetracyclines; Antibiotics

1. Introduction

Tetracyclines are used routinely in veterinary medicine for prevention and control of disease. They are antibiotics with a broad antibacterial spectrum and bacteriostatic activity, and have a good activity against acute disease caused by Gram-positive and Gram-negative bacteria, including the species *Spirochete*, *Actinomyces*, *Ricketsia* and *Mycoplasma*.

However, the use of these drugs has become a serious problem as regards infectious diseases, as they are substances that leave residues in milk or meat which can be directly toxic or else cause allergic reactions in some hypersensitive individuals. Even more important, low-level doses of antibiotic in foodstuffs consumed for long periods can lead to problems regarding the spread of drug-resistant micro-organisms [1]. To ensure human food safety, the European Union has set tolerance levels for many drugs in animal products. The maximum residue limits (MRLs) for tetracyclines in milk, meat and other foods were established by European regulation 2377/90 [2] and subsequent modifications. The MRLs for milk and muscle were set at 100 μg/kg for all species and the levels set for liver and kidney are 300 and 600 μg/kg, respectively [3].

To detect tetracycline residues in food, bioassay techniques [4] are widely used as screening methods,

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although these methods lack specificity and provide only semiquantitative measurements of residues detected and sometimes produce false positives. Nevertheless, they continue to be used because of their simplicity and cheapness. However to confirm the presence of drug residues in foods, these methods need to be supported by chemical methods. For quantitative determinations of tetracycline, high-performance liquid chromatography (HPLC) with diode-array (DAD) [5] and fluorimetric (FL) [6] detection has been used, but some chromatographic procedures are suitable for multiresidue analyses [7,8]. Some methods have involved mass spectrometry detection [9] but this technique is not suitable for routine use. The present paper describes a simple, rapid and specific method for the quantitative determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline in milk and tissue. To enhance the precision and accuracy of the method, the internal validation used was compliant with Draft SANCO/1805/2000 European Decision requirements [10].

2. Experimental

2.1. Chemicals and reagents

Methanol and Acetonitrile were HPLC-grade and were purchased from J.T. Baker (Deventer, Netherlands). Also oxalic acid, citric acid monohydrate, disodium hydrogenphosphate dihydrate, ethylenediaminetetraacetic acid disodium salt and trichloroacetic acid were reagent-grade and purchased from J.T. Baker. Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Solid-phase extraction (SPE) HLB Oasis cartridges (200 mg, 6 ml) were obtained from Waters (Milford, MA, USA). Standards of oxytetracycline, tetracycline, chlortetracycline and doxycycline were supplied by Sigma (St Louis, MO, USA).

2.2. Standard solutions

Stock standard solutions of oxytetracycline, tetracycline, chlortetracycline and doxycycline were prepared by dissolving 10 mg of each compound in 10 ml of methanol to obtain a final concentration of 1 mg/ml. Stock standard solutions were stored at −20 °C and were stable for at least 4 weeks. These solutions were diluted to give a series of working standard solutions that were prepared daily.

2.3. Experimental procedure

Milk blank samples were collected from two daily milkings (8 a.m. and 8 p.m.) from a group of healthy untreated cows. Muscle samples were obtained from a group of healthy untreated calves at the same farm. All samples were stored at −20 °C until assayed.

2.4. Sample preparation procedure

An aliquot of 5 g of milk and muscle were homogenised, placed in a glass centrifuge tube and 2 ml of 20% trichloroacetic acid (TCA) added. The sample was shaken, 20 ml of McIlvaine buffer (11.8 g of citric acid monohydrate; 13.72 g of disodium hydrogenphosphate dihydrate; 33.62 g of ethylenediaminetetraacetic acid disodium salt diluted in 1 liter of water 0.01 *M*) added and the mixture centrifuged at 4000 rpm for 20 min. The supernatant was then applied to a SPE HLB Oasis cartridge, previously activated with 3 ml of methanol and 2 ml of water. After sample loading, the cartridge was washed with 2 ml of methanol 5% in water and finally tetracyclines were eluted with 3 ml of methanol. The solvent was removed under a nitrogen stream and the residue was dissolved in 1 ml of methanol and filtered with a 0.45-μm PTFE filter. An aliquot (20 μl) was injected into the HPLC–DAD system.

2.5. HPLC–DAD equipment and conditions

Analyses were carried out on a HPLC–DAD model HP 1100 system equipped with an autosampler (Agilent Technologies, Palo Alto, CA, USA). Separations were carried out under isocratic conditions using a Hypersil C_8 column (250×4.6 mm I.D., 5 μm) (ThermoQuest, Kleinosteim, Germany) coupled with a guard column and using a mobile phase of 0.01 *M* oxalic acid–methanol–acetonitrile (60:25:15, v/v/v). The flow-rate was 1 ml/min and the photodiode array detector was set at 365 nm.

2.6. Calibration curves

Linearity of the detector response was verified with oxytetracycline, tetracycline, chlortetracycline and doxycycline standard solutions over the range of 0.1–5 mg/l. Calibration curves were prepared daily and estimates of the amount of the analytes in samples were interpolated from these graphs.

3. Results and discussion

We were looking for a simple, rapid and specific method to determine tetracyclines in animal foods. A typical chromatogram of oxytetracycline, tetracycline, chlortetracycline and doxycycline standards monitored at 365 nm is shown in Fig. 1. The separation was obtained using a reversed-phase Hypersil C_8 column at 40 °C, with a mobile phase of oxalic acid–acetonitrile–methanol (60:25:15, v/v/v) at a flow-rate of 1 ml/min. Under the conditions adopted, the analytes were fully separated in 12 min with symmetrical peaks. Retention times were 5.5, 6.2, 9.2 and 11.1 min, respectively. Representative chromatograms of blank milk sample and of a sample spiked with 100 μg/kg of tetracyclines are reported in Fig. 2. Fig. 3 shows the chromatogram of blank muscle sample and of a sample spiked with 100 μg/kg of tetracyclines. The relative retention times for each analyte in both chromatograms, corresponded to that of the calibration standard within a tolerance of 2.5%. In order to verify the specificity of the method, 20 blank milk and muscle samples from different origins were analysed. No interferences were observed in the region of interest where the analytes were eluted as is shown in the blank sample chromatograms (Figs. 2 and 3). Quantification was carried out by comparison of the analyte peak areas versus an externally generated calibration curve. We used five calibration standards dissolved in methanol ranging from 0.1 to 5.0 ng/ml to generate external calibration curves. The correlation coefficient exceeded 0.998 for all the analyses carried out during the validation procedure of the analytical method. In compliance with the draft SANCO/1805/2000 revision, the validation procedure includes the determination of detection limit (CCα), detection capability (CCβ), and specificity for the quantitative confirmation method. CCα is defined as: ''the concentration above which it can be determined with a statistical certainty of $1-\alpha$ that the identified analyte content is truly above MRL''. CCα calculated by spiking 20 blank milk and 20 blank muscle samples at MRL (100 μg/kg) is 113.2 and 110.9 μg/kg for oxytetracycline, 114.9 and 107.7 μg/kg for tetracycline, 121.6 and 128.8 μg/kg for chlortetracycline and 127.2 and 129.9 μg/kg for doxycycline. respectively. CCβ is ''the concentration at which the method is able to detect MRL concentrations with a statistical certainty of $1-\beta$. The β error should be less than or equal to 5%. CCβ was calculated by analysing 20 blank spiked samples at corresponding CCα level for each analyte in both matrices. CCβ in milk is 117.2, 120.9, 126.5 and

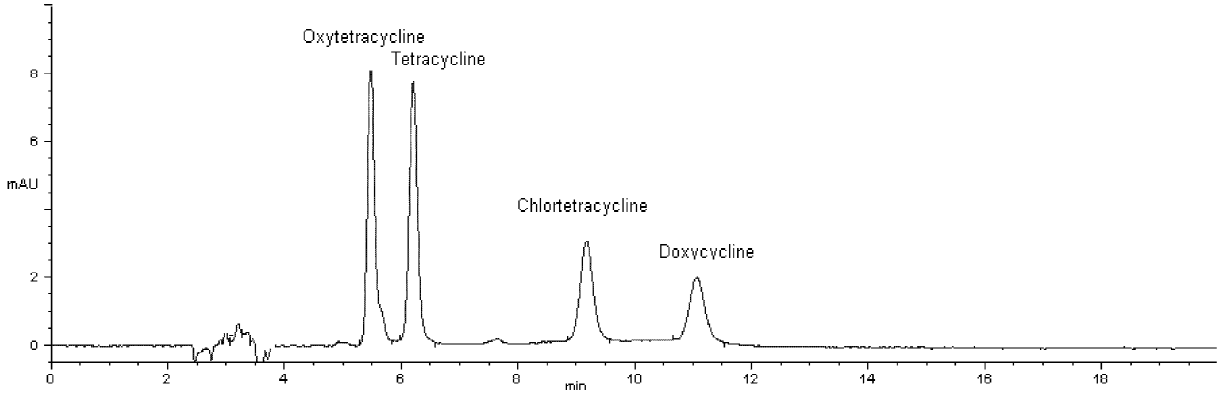

Fig. 1. Standard solution of oxytetracycline, tetracycline, chlortetracycline and doxycycline.

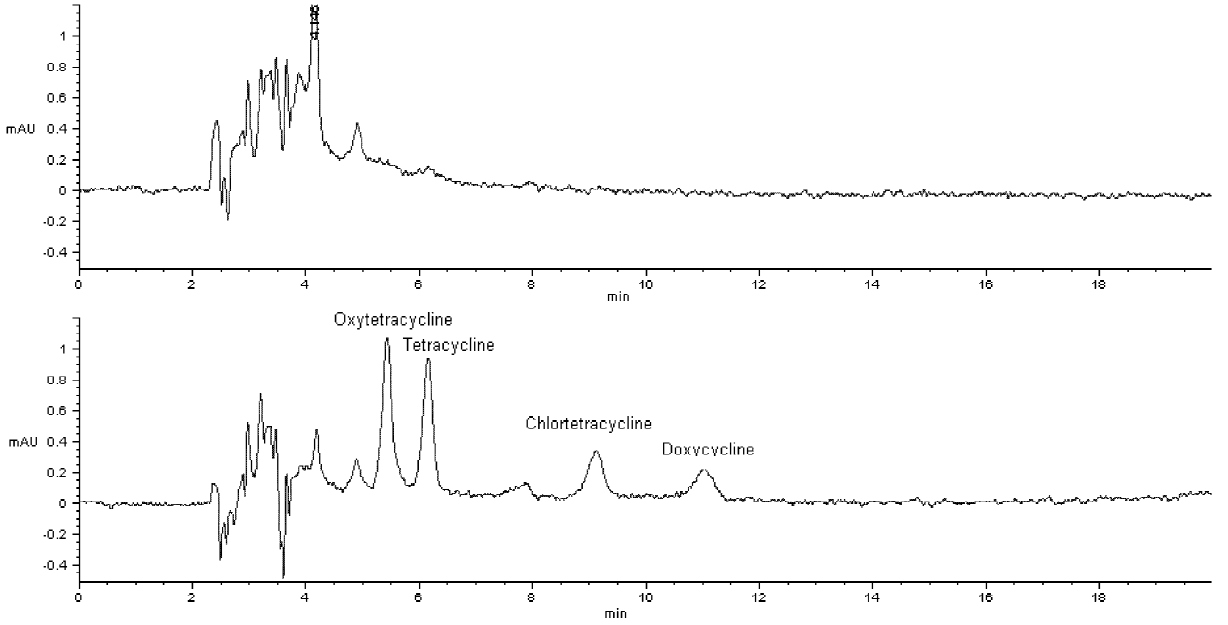

Fig. 2. Chromatograms of blank milk sample and of a sample spiked with 100 μg/kg of tetracyclines.

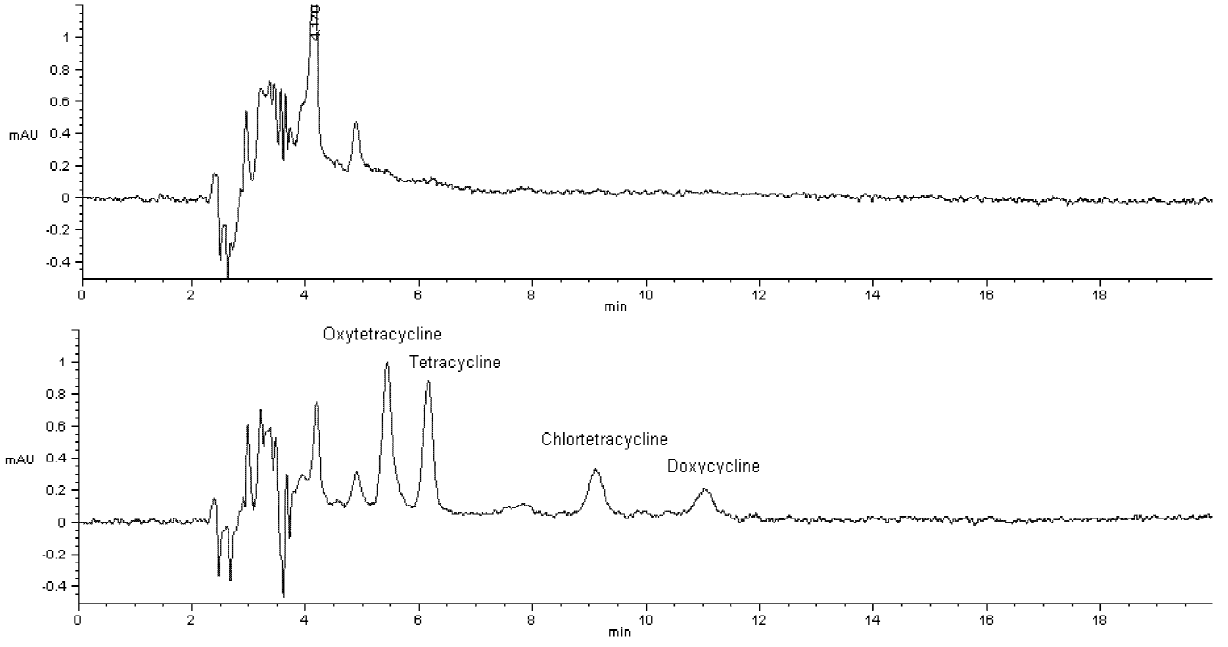

Fig. 3. Chromatogram of blank muscle sample and of a sample spiked with 100 μg/kg of tetracycline.

131.3 μg/kg while CCβ in muscle is 114.9, 112.5, 132.4 and 133.1 μg/kg for oxytetracycline, tetracycline, chlortetracycline and doxycycline, respectively. In order to evaluate the precision, accuracy and recoveries of the analytical method in milk and muscle bovine samples, the samples were prepared at three different concentrations (50, 100 and 150 μg/kg) and then analysed. Inter-day precision and accuracy data on three different days for tetracyclines in bovine milk and muscle samples are shown in Tables 1 and 2, respectively. The precision of the method was determined by calculating the relative standard deviation (RSD, %) for the repeated measurements. The accuracy of the method (RE, %) was determined by assessing the agreement between the measured and nominal concentrations of analysed samples. For inter-day data, the overall precision ranged from 5.04 to 1.70 and from 7.01 to 1.65 for the milk and muscle samples, respectively, while the accuracy ranged from −19.68 to −6.7 and from −18.16 to −7.96 for the milk and muscle samples, respectively. These values were considered satisfactory, on account of the complexity of the biological matrices. Recoveries are shown in Table 3. The recoveries were obtained by spiking milk and muscle samples at three different concentrations (50, 100 and 150 μg/kg) and then by analysing the samples six times on three different days ($n=18$). Good recoveries were observed for the analytes under investigation at all fortification levels and average recoveries were higher than 81% for milk samples and 83.2% for muscle samples with the RSD for each analyte less than 5.8%.

Accuracy and precision of the method were in compliance with draft SANCO/1805/2000; recovery data are in the range 80–110% and the RSD is less than 15%.

4. Conclusions

The goal of this work was to develop and to validate a specific, rapid and simple multiresidue method for determining oxytetracycline, tetracycline,

Table 1
Inter-day precision and accuracy for tetracyclines in bovine milk samples

Drug	Parameter	Validation sample level (ng/ml)		
		50	100	150
Oxytetracycline	Average (ng/ml)	46.86	91.93	138.01
	SD (ng/ml)	1.78	4.32	3.25
	Precision (RSD, %)	3.81	4.70	2.35
	Trueness	−6.70	−8.07	−7.99
	n	18	18	18
Tetracycline	Average (ng/ml)	40.16	85.51	126.14
	SD (ng/ml)	1.95	1.91	3.83
	Precision (RSD, %)	4.85	2.23	3.04
	Trueness	−19.68	−14.49	−15.91
	n	18	18	18
Chlortetracycline	Average (ng/ml)	44.05	84.88	126.01
	SD (ng/ml)	2.22	1.88	2.14
	Precision (RSD, %)	5.04	2.21	1.70
	Trueness	−11.9	−15.12	−15.99
	n	18	18	18
Doxycycline	Average (ng/ml)	43.47	86.12	127.95
	SD (ng/ml)	2.25	4.5	3.60
	Precision (RSD, %)	3.17	5.22	2.81
	Trueness	−13.06	−13.88	−14.7
	n	18	18	18

Table 2
Inter-day precision and accuracy for tetracyclines in bovine muscle samples

Drug	Parameter	Validation sample level (ng/ml)		
		50	100	150
Oxytetracycline	Average (ng/ml)	46.02	90.34	137.97
	SD (ng/ml)	2.11	1.78	3.70
	Precision (RSD, %)	4.58	2.0	2.68
	Trueness	−7.96	−9.66	−8.02
	n	18	18	18
Tetracycline	Average (ng/ml)	40.92	83.32	126.86
	SD (ng/ml)	1.32	4.97	8.90
	Precision (RSD, %)	3.22	5.96	7.01
	Trueness	−18.16	−16.18	−15.43
	n	18	18	18
Chlortetracycline	Average (ng/ml)	44.75	85.23	124.69
	SD (ng/ml)	2.28	3.47	3.26
	Precision (RSD, %)	5.09	4.07	2.61
	Trueness	−10.5	−14.77	−16.87
	n	18	18	18
Doxycycline	Average (ng/ml)	42.44	88.34	126.28
	SD (ng/ml)	2.03	1.47	2.09
	Precision (RSD, %)	4.78	1.66	1.65
	Trueness	−15.12	−11.66	−15.81
	n	18	18	18

Table 3
Recoveries for tetracyclines in bovine milk and muscle samples

Drug	Spiked level (μg/kg)	Milk samples			Muscle samples		
		Average recovery (*n*=18)	SD	RSD (%)	Average recovery (*n*=18)	SD	RSD (%)
Oxytetracycline	50	93.3	3.6	3.9	84.9	4.1	4.8
	100	91.9	4.2	4.6	84.3	1.5	1.7
	150	92.2	2.2	2.4	84.5	0.7	0.8
Tetracycline	50	80.3	3.9	4.8	81.8	2.6	3.2
	100	83.5	1.9	2.3	83.3	5.0	6.0
	150	84.1	2.6	3.1	84.6	5.9	7.0
Chlortetracycline	50	88.0	4.4	5.0	89.5	4.6	5.1
	100	84.9	1.9	2.2	85.2	3.5	4.1
	150	84.0	1.4	1.7	83.1	2.2	2.6
Doxycycline	50	86.9	4.5	5.2	84.9	4.1	4.8
	100	86.1	4.5	5.2	84.3	1.5	1.7
	150	85.3	2.4	2.8	84.2	1.4	1.7

chlortetracycline and doxycycline in milk and muscle samples. In future, kidney and liver bovine samples will be validated in compliance with draft SANCO 1805/2000. This method is also used routinely to analyse milk and muscle samples collected as a part of a National Plan of Residues coming from all regions of Italy and from European countries.

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