Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 12, pp. 1829–1835, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00

Determination of residues of tetracycline antibiotics in animal tissues by high-performance liquid chromatography*

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Abstract: A method has been developed for the determination of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) in animal tissues. Tissues were macerated with a buffer solution and centrifuged. The supernatant solution was purified over a Sep-Pak C18 cartridge which had previously been deactivated by silylation. Tetracyclines were eluted with methanol, the solvent evaporated and the residue dissolved in the HPLC mobile phase. Compounds were separated on a Novapak Phenyl Radial-Pak cartridge with a Resolve CN guard column using gradient elution and UV detection. The method was tested on chicken, porcine and bovine muscle. Recoveries were determined for OTC, TC, CTC and DC added to porcine, bovine and chicken muscle at levels of 0.05 and 0.2 mg kg^{-1}. They varied from about 90% for OTC to about 68% for DC with coefficients of variation of 1.8–7.5%. The limit of determination is 5–10 μg kg^{-1}. The method was validated on tissues of laying-hens after intramuscular injection of OTC.

Keywords: *Tetracyclines; animal tissue analysis; reversed-phase HPLC; UV detection; method development; silylation of SPE column.*

Introduction

Tetracycline (TC) antibiotics are widely used in veterinary medicine and as feed additives. In the Netherlands oxytetracycline (OTC) is by far the most widely used TC in feeds for swine, chicken and ducks. Tetracyclines may remain as residues in animal tissues and therefore give rise to concern for public health authorities as well as for consumer organizations.

They have often been monitored by microbiological or fluorimetric methods [1–4], but discrimination between the individual TCs is not achieved. During the past decade a large number of liquid chromatographic methods have been described [5–13]. However, these methods are usually insufficiently sensitive to determine TC residue levels of 10 μg kg^{-1}. Only Ikai *et al.* [8] have reported detection limits in animal tissues of 10 μg kg^{-1}, but we could not reproduce their results.

*Presented at the "Third International Symposium on Drug Analysis", May 1989, Antwerp, Belgium.
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This paper describes the development of a method for the analysis of TC, OTC, chlortetracycline (CTC) and doxycycline (DC) in animal tissues. The method involves deactivation by silylation of the silica C18 cartridge used for the purification of sample extracts. This prevents the adsorption of TCs, especially at low levels, on residual silanol groups of the solid-phase extraction (SPE) column, and it improves the recoveries of the TCs.

Experimental

Chemicals and reagents

The hydrochlorides of OTC, TC, CTC and DC were purchased from Sigma Chemical (St. Louis, MO, USA); *epi*-OTC and *epi*-TC as hydrochloride from Janssen Chimica (Beerse, Belgium). HPLC-grade water was produced in a milli-Q water purification system (Millipore, Bedford, MA, USA) and used throughout this work. All other chemicals were of analytical reagent grade or HPLC grade.

Tetracycline standard solutions

A stock solution was prepared by dissolving the TCs in water (10 μg ml^{-1} each). For HPLC calibration the stock solution was diluted with mobile phase, acetonitrile–0.02 M oxalic acid–methanol (15:80:5, % v/v/v), to obtain standard working solutions of 0.500, 0.250, 0.125 and 0.0625 μg ml^{-1} respectively.

The stock solution was stored at −20°C and was stable for at least 6 months. The calibration standards were stored at 4°C and were stable for several days.

High-performance liquid chromatography

The HPLC apparatus consisted of a Bruker LC 21-B ternary chromatograph equipped with a Bruker LC 51 autosampler, a Kratos Spectroflow 783 variable wavelength UV detector operated at 355 nm with 0.004 AUFS and a Spectra-Physics integrator (SP 4290).

The separation was performed on a Novapak 4 μm Phenyl Radial-Pak cartridge, 100 × 8 mm I.D., in a Radial-Pak compression module RCM 8 × 10, with a Resolve CN Guard-Pak precolumn (Waters Assoc., Milford, MA, USA) at ambient temperature.

Elution was started with acetonitrile–0.02 M oxalic acid–methanol (15:80:5, % v/v/v) as mobile phase with a linear gradient to 27:60:13, % v/v/v in 23 min. The flow rate was 1.5 ml min^{-1}. The injection volume was 75 μl.

Silylation of Sep-Pak C18 cartridge

A 4% dimethyldichlorosilane (DMCS) solution (1 ml) in toluene was transferred onto the Sep-Pak C18 cartridge and drained by gravity. After 5 min the column was rinsed with 5 ml of methanol followed by 10 ml of water.

Extraction and clean-up

To 5 g of tissue in a glass tube of 50 ml, 2 ml dichloromethane was added. The sample was macerated three times with 20 ml of 0.1 M Na_2EDTA-McIlvaine buffer (pH 4), using an Ultra Turrax and centrifuged at 1400 *g* for 10 min. The supernatant was filtered through a fluted filter paper and the filtrate made up to 100 ml.

A 50 ml aliquot of the filtrate was applied on a previously silylated Sep-Pak C18

cartridge using a Sep-Pak cartridge rack and vacuum pump. The cartridge was washed with 20 ml of water and air-dried by aspiration for 5 min. The TCs were eluted with 30 ml of methanol.

The eluate was concentrated under reduced pressure at 30°C to a small volume, 5 ml of ethanol was added and the solution further evaporated to dryness. The residue was dissolved in 0.5 ml of the HPLC mobile phase solution acetonitrile–0.02 M oxalic acid–methanol (15:80:5, % v/v/v).

Results and Discussion

Solid-phase extraction columns

Solid-phase extraction columns are widely being used in analytical chemistry. To develop a method for the determination of TCs in animal tissues we compared Baker 10 C18 columns with Sep-Pak C18 cartridges. TCs were added to porcine muscle extracts and applied on both cartridges. They were eluted with a methanol–0.01 M oxalic acid solution as described by Oka *et al.* [7]. However, the recoveries of the TCs determined by HPLC were poor, namely about 40% for the Baker column and 75% for the Sep-Pak cartridge, much lower than described by Oka. Moreover, the Baker 10 C18 columns tended to clog soon after application of the extract, so that the elution rate was very low.

To detect the sources of these losses, we first examined the evaporation step of the methanol–oxalic acid eluate and further the elution of TC working standard solutions from the cartridges. Evaporation of standard solutions in methanol–oxalic acid on a rotary evaporator showed losses of up to 60% for TC, probably due to the pure oxalic acid solution remaining at the end of the evaporation. This phenomenon has also been reported by Ikai *et al.* [8]. We ascertained that lowering the temperature of the water-bath to about 5°C could prevent these losses. However, this is not an attractive procedure because of the great prolongation of evaporation time, and hence not suitable when a lot of samples have to be handled. Evaporation of standard solutions of TCs in pure methanol at 30°C, as is done in our method, did not lead to losses, in contrast to findings of Onji *et al.* [13] who have reported losses of TCs of 30–40%. Nevertheless, the concentration step could only be partially responsible for the losses of TCs in the whole procedure.

For this reason we also investigated the performance of the solid-phase extraction column. Further experiments were carried out with Sep-Pak cartridges only, chiefly because we had much experience with this type of clean-up column in our laboratory. Oka *et al.* [7] prefer the use of Baker 10 C18 to Sep-Pak C18 because of the greater adsorption power of the former, but the differences are small. The break-through point of TCs on Sep-Pak should correspond to about 10,000 mg TC per kg tissue in the analytical procedure used, so the slight differences are of minor significance. Furthermore, as already stated, the Baker 10 C18 columns gave slow elution rates.

Irreversible adsorption of the TCs to the free silanol groups of the octadecylsilylated silica cartridge might play a predominant part and therefore provide an explanation of the poor recoveries. In the past we have encountered other adsorption phenomena of this kind. For the analysis of deoxynivalenol, a trichothecene mycotoxin in cereals, we have solved this problem by deactivation of the glass wall through silylation with DMCS [14]. Likewise, we tried to deactivate the residual silanol groups of the Sep-Pak C18 cartridge with a solution of 4% DMCS in toluene prior to the treatment with methanol and water. The result was a significant increase in recoveries for the TCs from standard

Table 1
Recoveries (%) of standard solutions of tetracyclines from non-silylated and silylated Sep-Pak C18 cartridges, means of duplicate determinations

Tetracycline	Amount (mg)	Non-silylated	Silylated
OTC	0.25	84	98
	0.125	84	102
TC	0.25	71	87
	0.125	73	93
CTC	0.25	69	86
	0.125	73	95
DC	0.25	80	87
	0.125	80	93

solutions from about 84 to 100% for OTC, 80 to 90% for DC and 70 to 90% for TC and CTC (Table 1). Consequently, comparing the figures for the non-silylated and silylated cartridges, a considerable change in the ratio of TCs recovered and not recovered can be observed in favour of the silylated cartridges.

High-performance liquid chromatography

Most HPLC systems employed for the determination of TCs use reversed-phase columns, e.g. LiChrosorb RP8 and μBondapak C18. Although good separations can be obtained on LiChrosorb RP8 columns, the lifetime of the column and the reproducibility of results were poor in our hands, probably due to the low pH of the mobile phase.

A Novapak Phenyl cartridge in a Radial-Pak compression module was a good alternative. Gradient elution was used not only for a better separation of the compounds, but also for improving the sensitivity and hence the detection limits of the later eluting compounds. Hundreds of injections have now been carried out, and although the column has been kept for months under methanol outside the system, no significant deterioration was observed.

The separation of a standard solution of the four TCs and of *epi*-OTC and *epi*-TC obtained on a Novapak Phenyl cartridge in a Radial-Pak compression module with gradient elution is shown in Fig. 1. As can be seen from the chromatogram, this system enables the simultaneous determination of OTC and TC as well as their metabolites *epi*-OTC and *epi*-TC.

Validation

The calibration graphs prepared by the peak height method showed linear relationships between 2.5 and 30 ng;

OTC: $y = 5.536x + 1.429$, $r = 0.9999$;
TC: $y = 3.817x + 0.905$, $r = 0.9998$;
CTC: $y = 1.566x + 0.126$, $r = 1.0000$;
DC: $y = 3.077x - 0.093$, $r = 0.9998$.

The minimum detection limit (3 × signal/noise ratio) for the TCs is in the range 1–3 ng.

Recoveries and relative standard deviations of individual TCs added to bovine, porcine and chicken muscle, determined according to the procedure described, are

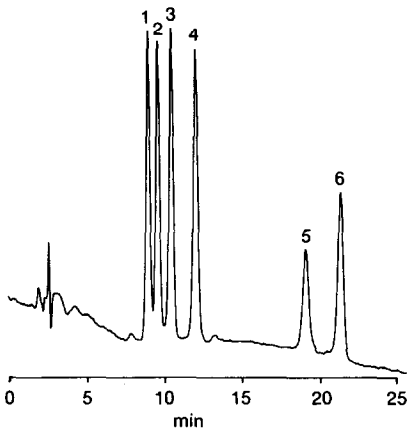

Figure 1
Chromatogram of a standard solution of tetracyclines (35 ng each) separated on a Novapak Phenyl cartridge with gradient elution.
1 = 4-epioxytetracycline, 2 = oxytetracycline, 3 = 4-epitetracycline, 4 = tetracycline, 5 =chlortetracycline, 6 = doxycycline.
UV detection at 355 nm with 0.004 AUFS. For experimental conditions, see text.

Table 2
Recoveries of tetracyclines from spiked animal tissues

Sample	Number of replicates	Added (mg kg^{-1})	Recovery % (RSD %)			
			OTC	TC	CTC	DC
Bovine muscle	5	0.05	89(2.6)	85(3.8)	77(6.5)	66(5.4)
	5	0.2	88(3.4)	77(2.3)	71(3.8)	76(6.4)
Porcine muscle	5	0.05	90(1.8)	78(1.8)	65(3.8)	68(7.4)
	5	0.2	90(3.0)	77(2.8)	71(2.0)	77(5.8)
Chicken muscle	2	0.02	79(1.9)	82(0.94)	69(6.2)	68(2.0)
	5	0.05	80(2.4)	76(4.0)	67(6.8)	67(3.1)
	5	0.2	82(4.4)	74(2.1)	68(2.4)	69(2.5)

summarized in Table 2. Recoveries of TCs obtained from chicken muscle spiked at the low level of 0.02 mg kg^{-1} did not differ from those obtained at higher levels.

Chromatograms of blank samples and samples spiked with TCs at a level of 0.05 mg kg^{-1} (bovine and porcine muscle) or 0.02 mg kg^{-1} (chicken muscle) are given in Fig. 2. The chromatograms of the blanks of bovine, porcine and chicken muscle show no interfering peaks with the same retention time as OTC, TC, CTC or DC, indicating a satisfactory clean-up of the extracts. *Epi*-OTC and *epi*-TC can be determined as well, although for chicken muscle *epi*-TC is probably not completely resolved from an interfering compound. The limit of determination for each individual TC is 5–10 μg kg^{-1}.

Application to chicken tissues

The method was tested on tissues of laying-hens slaughtered at several periods of time after intramuscular injection of 10 mg OTC–HCl per kg body weight. Homogenates of both muscle tissue and the injection site were investigated with the procedure described. Blood serum was extracted according to the procedure of Reeuwijk and Tjaden [9] and OTC determined with the HPLC system described. The results are summarized in Table 3.

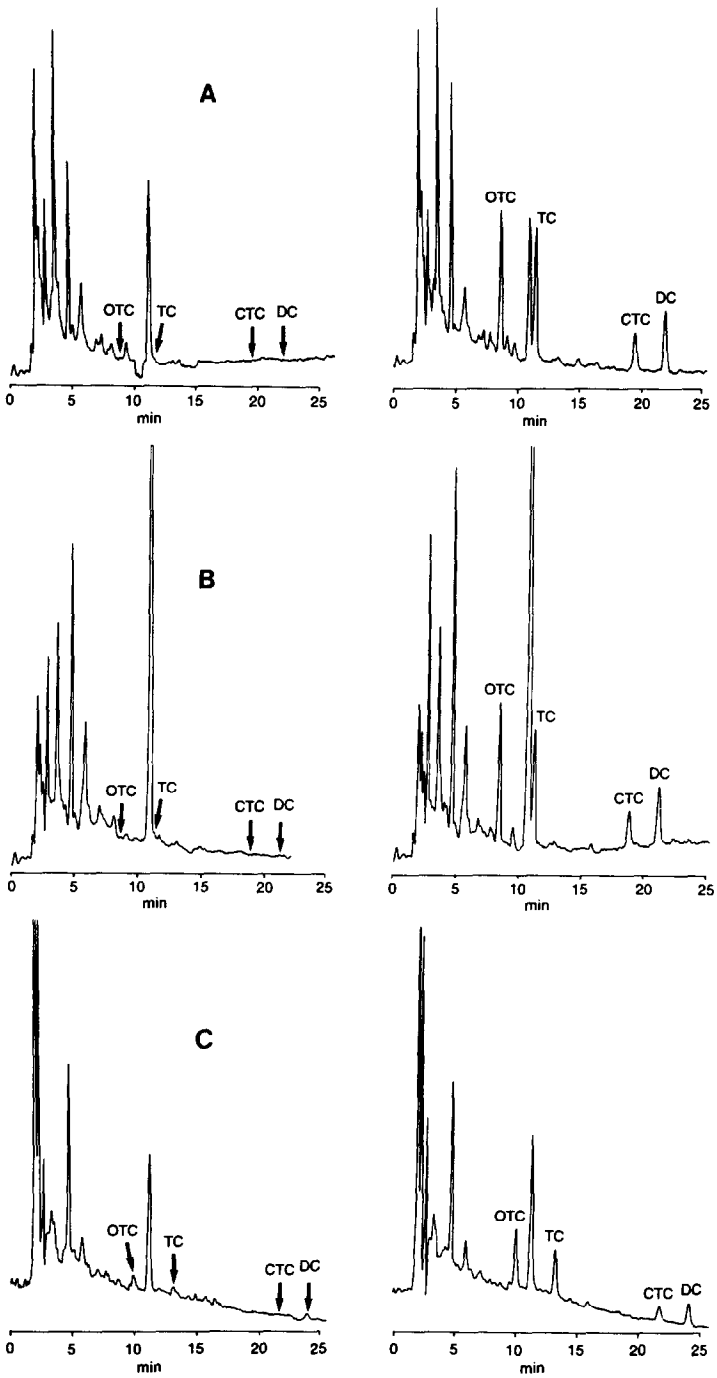

Figure 2
Chromatograms of blank (left) and spiked (right) muscle samples. A = bovine, B = porcine, C = chicken. Spiking level of each tetracycline: A and B 0.05 mg kg^{-1}; C 0.02 mg kg^{-1}. UV detection at 355 nm with 0.004 AUFS.

Table 3
Amounts of oxytetracycline* in tissues of laying-hens after intramuscular injection

Tissue	Time passed afer injection (h)			
	24 (control)	6	24	96
Injection site	—	48	11	0.04
Muscle	<0.005	2.7	1.0	0.03
Blood serum	<0.02	5.8	0.80	0.03

*Concentrations in the injection site and in muscle are expressed in mg kg^{-1}, those in blood serum in mg l^{-1}.

Conclusion

It can be concluded from this study that when determining TCs in animal tissues, deactivation of the Sep-Pak C18 cartridge by silylation leads to better results. The Novapak Phenyl cartridge with gradient elution allows the simultaneous determination of OTC, TC, CTC and DC, as well as *epi*-OTC and *epi*-TC, with a limit of determination of 5–10 μg kg^{-1}.

Acknowledgement — The authors are grateful to E. C. Rietveld of the TNO Institute of Animal Nutrition and Physiology for carrying out the animal experiments with laying-hens.

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[*Received for review 16 May 1989; revised manuscript received 14 June 1989*]