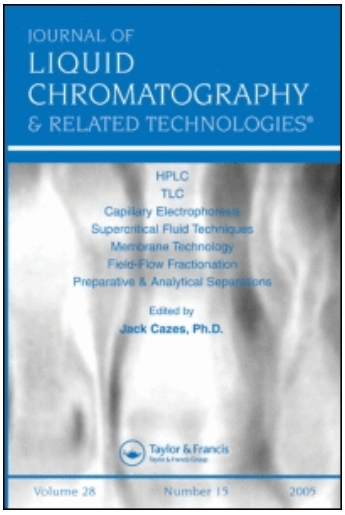

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MULTIRESIDUE ANALYSIS OF TETRACYCLINES IN KIDNEY BY HPLC AND PHOTODIODE ARRAY DETECTION

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ABSTRACT

The method describes the analysis of six tetracyclines at a low residue level by HPLC. The tetracyclines were extracted from kidney by a succinate buffer, for their purification Chelating Sepharose and Bond Elut C-18 columns were used successively. The samples were analyzed on a liquid chromatograph fitted with two Chromspher C-8 columns and a photodiode array detector.

The quantification limits are 10 or 30 μg/kg kidney depending on the kind of tetracyclines, the absolute detection limits are 4 or 12 ng respectively. The method has already been used for routine analysis. All positive samples were confirmed by comparing the spectrum of the unknown peak, supposed to be a tetracycline, with the spectrum of the corresponding standard.

INTRODUCTION

Tetracyclines are antibiotics with a broad antibacterial spectrum. They are frequently used as a feed additive or in drinking water to

maintain an optimal health for food-producing animals. Residues may remain in edible animal tissues and then affect human health.

The Council Directive 86/469/ECC (1) impose to each member state of the European Economic Community a surveillance program of residues in animals and their tissues which includes the research of residues of antibiotics. The temporary recommanded maximum residue limit (MRL) is fixed at 600 μg/kg for kidney as the sum of all residues of detected tetracyclines and is in force until the 1.1.1994 (2).

Two aims were therefore important: To have a multiresidue method to detect a great number of tetracyclines at once and to have a low detection limit which allows to use the method even if the MRL would be lower. As matrix we have chosen kidney since microbiological tests done in our laboratory indicated the presence of tetracyclines in this organ. Tetracyclines have a similar molecular structure which makes it difficult to separate them. A fluorometric method (3) and thin-layer chromatography (4) were developed for screening purposes. For quantitative determinations reversed-phase thin-layer chromatography (5), spectrophotometry (6), fluorometry (7) and principally high performance liquid chromatography (HPLC) were used (4,8-20), but only few methods were adapted to multiresidue analyses. A review of chromatographic methods for tetracycline analysis in food is given in (21). Concerning HPLC good separations were achieved by reversed-phase C-18 or C-8 columns and solvent systems composed of methanol, acetonitrile and oxalic acid, followed by UV detection. No method indicated a detection limit being satisfactory for us. A metal chelate affinity chromatography/HPLC attracted our attention (22). We applied the first step of the extraction and clean-up procedure but carried on with Bond-Elut C-18 columns which are much less solvent and time consuming than Amberlite resin used by the authors. Three more

tetracyclines could be included as well, so that six compounds can be determined at a low residue level.

EXPERIMENTAL

Apparatus

An ultrasonic bath Bransonic 5200 from Branson (Conn.,USA) was used to liberate the tetracyclines from the matrix. Centrifugations were achieved by a J2-HS centrifuge from Beckman (Calif.,USA) and the purification steps by use of a Baker-10 extraction system. Evaporation of the solvent mixture with nitrogen was carried out in a Reacti-Therm heating module from Pierce Chemical Company (Illinois, USA). HPLC analyses were performed with a 9010 liquid chromatograph from Varian (USA) and an automatic sample injector model 231 from Gilson (France). The tetracyclines were detected with a photodiode array detector 991 from Waters (Massachusetts, USA). The results were acquired by an APC IV personal computer from NEC corporation (Massachusetts, USA). Chromatograms and spectra were printed by a printer plotter 990 from Waters (USA).

Solvents and reagents

Acetonitrile and methanol were supplied by Lab-Scan (Ireland), oxalic acid G.R., succinic anhydride, copper (II) sulfate 5-hydrate G.R. and ethylenedinitrilo tetraacetic acid, disodium salt dihydrate (Titriplex III G.R.) by Merck (Germany). All reagents were of analytical grade. For the extraction of the tetracyclines a succinate buffer 0.05 M pH 4 was prepared. To use this buffer for the elution of the tetracyclines from the affinity-columns, 3.7 % Titriplex III was added. A solution of 0.5 %

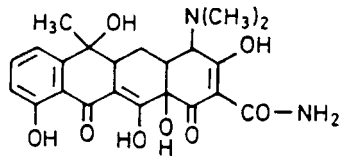
copper (II) sulfate was necessary for the conditioning of the affinity-columns as well as a mixture of ethanol/water (20 + 80,v/v). For the purification step a solution of methanol/acetonitrile (50 + 50,v/v) was prepared. The mobile phase used for HPLC was a mixture of 0.01 M oxalic acid pH 2 and acetonitrile (80 + 20,v/v). Buffer and acetonitrile were filtered and degassed by helium before use. The extracts were filtered through ashless filter paper, Black ribbon 589 from Schleicher and Schuell (Germany).

Columns

For the purification Chelating Sepharose from Pharmacia (Sweden) and Analytichem Bond Elut C-18 columns from Varian (USA) were used.
Conditioning of the affinity-columns:
10-ml Econocol columns fitted with two-way stop-cocks from Bio-Rad (Nazareth, Belgium) were filled with 5 ml Chelating Sepharose, allowed to settle and then conditioned with 20 ml of the 0.5 % copper (II) sulfate solution. Air bubbles were eliminated by agitation and the columns rinced with 15 ml succinate buffer 0.05 M pH 4.
The Bond Elut C-18 columns were conditioned with 10 ml methanol followed by 10 ml water. The affinity-columns as well as the Bond Elut C-18 columns may never be dry.

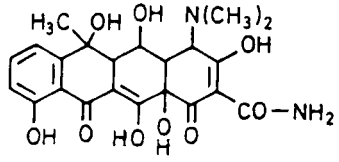
Standards and standard solutions

Tetracycline (TC)
4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2- naphthacenecarboxamide



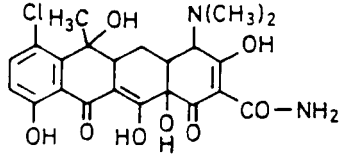
Oxytetracycline (OTC)

4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide



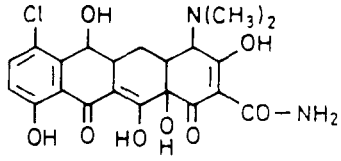
Chlortetracycline (CTC)

7-Chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11,-dioxo-2-naphthacenecarboxamide



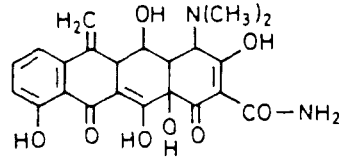
Demethylchlortetracycline (DMTC)

7-Chloro-4-dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-1,11,-dioxo-2-naphthacenecarboxamide



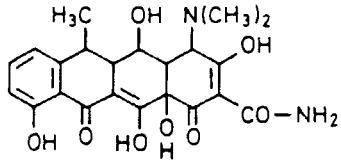
Methacycline (MC)

4-Dimethylamino-1,4,4a,5,5a,6,11,12a-octahydro- 3,5,10,12,12a-pentahydroxy-6-methylene-1,11-dioxo-2- naphthacenecarboxamide



Doxycycline (DC)

4aS-(Dimethylamino)-1,4,4$a\alpha$,5,5$a\alpha$,6,11,12a-octahydro-3,5a,10,12,12$a\alpha$-pentahydroxy-6a-methyl-1,11-dioxo-2-naphthacenecarboxamide



Tetracycline, oxytetracycline, chlortetracycline, methacycline and doxycycline were purchased from Sigma Chemical Co (St. Louis, MO,

USA), demethylchlortetracycline from the National Institute for Medical Research (London, England).

The standard stock solutions contained 1 mg/ml MeOH and have to be stored in a refrigerator. The working solution used as standard and to spike the samples, contained 1 μg of each standard/ml water.

Tetracyclines are sensitive to light. Standard solutions as well as extracts have to be protected.

Sample preparation

Extraction procedure of tetracyclines from kidney

4 g minced kidney were homogenized with 40 ml succinate buffer in a plastic centrifuge tube. To extract the tetracyclines an ultrasonic bath was used in which the plastic tubes were immersed during 10 min. The mixture was then centrifuged during 10 min at 9000 rpm and the supernatant filtered through filter paper. The extraction was repeated once and the two fractions were rassembled.

For positive samples containing a high concentration of one of the six tetracyclines, the extraction has to be repeated with less extraction material.

Purification procedure of tetracyclines from kidney

The purification included two steps which follow each other. The extracts were applied to the preprepared affinity-columns and allowed to pass through them with a flow rate of 5 to 7 ml/min. The columns were rinced with 10 ml water, 30 ml methanol and again with 20 ml water. After that, the tetracyclines were eluted with 50 ml succinate buffer +

Titriplex III and immediately applied to the preconditioned Bond Elut C-18 columns with a flow rate of 5 to 7 ml/min also. The columns were rinced with 10 ml water and dried by air aspiration during 10 min. The tetracyclines were then eluted with 5 ml of a mixture methanol/acetonitrile (10 + 10,v/v). After the evaporation of the solvent mixture with nitrogen at 40 °C the residue was dissolved in 0.5 ml mobile phase.

After the elution of the tetracyclines, the affinity-columns were rinced with 20 ml water followed by 20 ml ethanol/water (20 + 80,v/v). At this stage the columns may be stored in a refrigerator at 4°C. In case of immediate reuse of the columns they have to be rinced with 20 ml water and reconditioned with 20 ml 0.5 % copper (II) sulfate solution followed by 15 ml succinate buffer.

Chromatography

For the analyses of the extracts two Chromspher C-8 columns (100x300mm,5μm, cat.no.:28262) from Chrompack (USA) and a precolumn containing Perisorb RP-8 from Merck (Germany) were joined together and conditioned at room temperature with the mobile phase (0.01 M oxalic acid pH 2 and acetonitrile (80 + 20,v/v). The flow rate was 0.8 ml/min. The tetracyclines were detected at 365 nm.

RESULTS AND DISCUSSION

Kidney is known as a target organ to accumulate residues. Preliminary microbiological tests indicated the presence of tetracyclines in kidney, but lacked specificity. The method described in this article allowed us to identify and to quantify individual tetracyclines. Their identity was confirmed by comparing the spectrum of an unknown peak,

TABLE 1

Correlation Coefficients of Tetracyclines

Added drug	Correlation coefficient
OTC	0.9972
TC	0.9954
DMTC	0.9944
CTC	0.9931
MC	0.9696
DC	0.9898

supposed to be a tetracycline, with the spectrum of the corresponding standard. The purification procedure is a combination of metal chelate affinity chromatography (MCAC) and the application of the extracts on Bond Elut C-18 columns. Sep-Pak C-18 and Baker C-18 columns were tried as well, but with less success. The columns filled with Chelating Sepharose may be used up to 15 times. It is important to protect the extracts and the standard solutions against light to avoid the degradation of the tetracyclines.

The calibration curves were obtained in the range of 0.030 μg/ml to 3 μg of each tetracycline/ml working solution. All plots were linear. The correlation coefficients are given in table 1. Table 2 summarizes the recoveries, standard deviations and coefficients of variation for blank samples spiked with concentrations at five different levels, the lowest being 37.5 μg of each tetracycline/kg kidney, the highest 500 μg/kg. The average value for each level is the mean of five different extractions. The recoveries, the standard deviations and the coefficients of variation are satisfactory. Higher values of coefficients of variation were registered for low concentrations.

For OTC and TC the quantification limit is 10 μg/kg kidney, the absolute detection limit is 4 ng. DMTC, CTC, MC and DC *have a*

TABLE 2

Recovery Data of Tetracyclines in Kidney by HPLC and Photodiode Array Detection

Added drug	Added quantity (μg/kg)	Mean (n = 5) (μg/kg)	Standard deviation	Coefficient of variation (%)	Recovery (%)
OTC	37.5	22.08	2.46	11.15	58.88
TC	37.5	16.39	2.25	13.71	43.70
DMTC	37.5	20.13	2.83	14.04	53.68
CTC	37.5	21.17	2.79	13.16	56.46
MC	37.5	19.60	2.68	13.68	52.27
DC	37.5	17.08	2.35	13.20	47.48
OTC	75.0	47.07	3.32	7.05	62.70
TC	75.0	33.05	3.88	11.74	44.07
DMTC	75.0	38.95	1.46	3.75	51.94
CTC	75.0	49.64	7.83	15.77	66.18
MC	75.0	44.79	6.23	13.92	59.72
DC	75.0	38.66	2.96	7.65	51.54
OTC	125.0	82.44	5.70	6.91	65.95
TC	125.0	55.06	7.68	13.95	44.05
DMTC	125.0	62.18	6.27	10.08	49.75
CTC	125.0	73.54	4.04	5.49	58.83
MC	125.0	62.45	5.14	8.24	49.97
DC	125.0	72.82	10.28	14.11	58.26
OTC	250.0	179.55	3.34	1.86	71.82
TC	250.0	132.57	4.38	3.30	53.03
DMTC	250.0	163.57	5.25	3.21	65.43
CTC	250.0	163.82	5.29	3.23	65.53
MC	250.0	141.67	4.04	2.85	56.67
DC	250.0	171.67	4.08	2.38	68.67
OTC	500.0	365.25	15.40	4.22	73.05
TC	500.0	230.66	15.48	6.71	46.13
DMTC	500.0	244.86	10.19	4.16	48.97
CTC	500.0	334.49	12.54	3.75	66.90
MC	500.0	355.56	21.70	6.10	71.11
DC	500.0	386.61	26.39	6.83	77.32

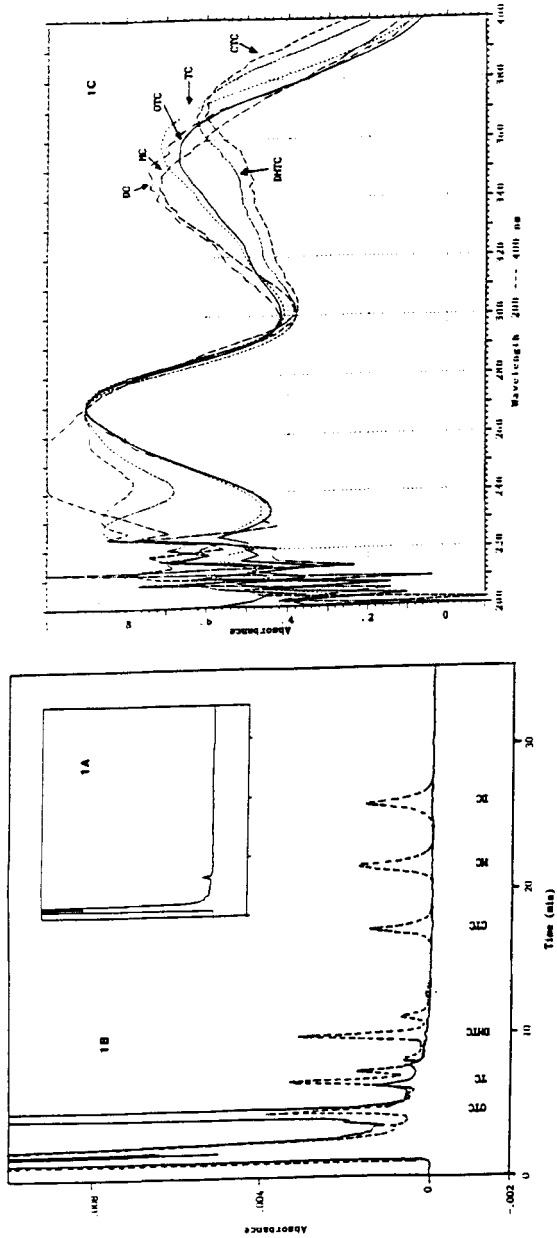


FIGURE 1 -A. Chromatogram of a Blank Kidney Sample. -B.(—) Kidney Sample containing nearly 4 mg OTC/kg. (----) Spiked Kidney Sample containing 250 μg of each Tetracycline/kg. -C. Ultraviolet Spectra of the corresponding Tetracyclines.

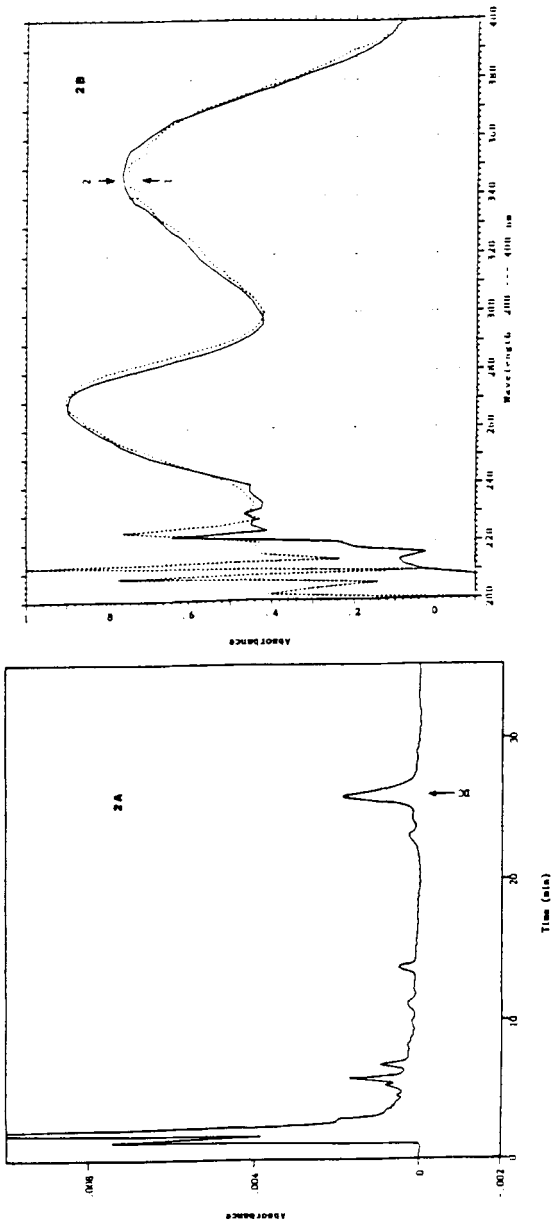


FIGURE 2 -A. Chromatogram of a Kidney Sample containing 340 µg DC/kg. -B. Ultraviolet Spectra of DC. 1. DC Standard (100ng/100µl Eluent); 2. Positive Kidney Sample (120ng DC/100µl Eluent).

quantification limit of 30 μg/kg kidney, their absolute detection limit is 12 ng. The quantification limits still allow the confirmation of an unknown peak at the retention time of one of the six tetracycline standards. This procedure is used to exclude false positive results arising from eventual interfering peaks.

100 porc kidneys provided by slaughterhouses were analyzed and the method proved to be reliable. Detected tetracyclines were OTC and DC. DC is known as one of the most widely used in broad spectrum antibiotic therapy. In both cases quantitative determinations were done without interferences. Seven kidneys were positive for OTC. Thirty three kidneys were positive for DC. All positive samples were confirmed by comparing the spectrum of the unknown peak with the spectrum of the corresponding standard. Figure 1A shows a chomatogram of a blank kidney sample. Figure 1B shows one of the samples supplied by a slaughterhouse containing nearly 4 mg OTC/kg kidney. This chromatogram was superposed with a chromatogram of a blank kidney sample fortified with the six tetracycline standards. The tetracyclines are very well separated. The retention times are situated between 4.5 min for OTC and 26 min for DC, the last eluted tetracycline. Figure 1C shows the corresponding ultraviolet spectra. Tetracyclines are similar compounds and their spectra are similar as well, differing by a small shift of their respective absorption maxima. A positive sample containing 340 μg DC/kg kidney is illustrated in figure 2A. Figure 2B shows the corresponding spectrum superposed with the spectrum of the DC standard.

The described method is reliable and suitable for routine analysis at a low residue level of tetracyclines (10 and 30 μg/kg kidney) and will be extended soon to the analysis of tetracyclines in fish.

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REFERENCES

1. Council Directive of 16 september 1986 on the determination of residues in animals and in fresh meat. Off. J. of the Eur. Comm. N°L 275/36 of 26.09.1986

2. Reglementation N°675/92 (ECC) of 18 mars 1992. Off.J. of the Eur. Comm. N°L 73/8 of 19.3.1992

3. N. Haagsma, M.J.B. Mengelers, Z.Lebensm. Unters. Forsch., 188: 227-231 (1989)

4. C.D.C. Salisbury, W. Chan, J.R. Paterson, J.D. Mac Neil, C.A. Kranendonk, Food Addit. Contam., 7:369-373 (1990)

5. Hisao Oka, Keiichi Uno, Ken-Ichi Harada, Makoto Suzuki, J. Chromatogr., 284:227-234 (1984)

6. A.A. Alwarthan, S.A. Al-Tamrah, S.M. Sultan, Analyst, 116:183-186 (1991)

7. C. van den Bogert, A.M. Kroon, J. Pharm. Sc., 70:186-189 (1981)

8. Hisao Oka, Keiichi Uno, Ken-Ichi Harada, Keiko Yasaka, Makoto Suzuki, J. Chromatogr., 298:435-443 (1984)

9. Hoshino Youji, Horie Masakazu, Nose Norihide, Iwasaki Hisao, Shokuhin Eiseigaku Zasshi, 25:430-435 (1984)

10. Hisao Oka, Hiroshi Matsumoto, Keiichi Uno, Ken-Ichi Harada, Satsuki Kadowaki, Makoto Suzuki, J. Chromatogr. 325:265-274 (1985)

11. Hisao Oka, Yoshitomo Ikai, Norihisa Kawamura, Junko Hayakawa, J. Assoc. Off. Anal. Chem., 74:894-896 (1991)

12. R.B. Ashworth, J. Assoc. Off. Anal. Chem. 68:1013-1018 (1985)

13. A. Aszalos, Chromatographia, 20:313-322 (1985)

14. W.A. Moats, J. Chromatogr., 358:253-259 (1986)

15. H.J.E.M. Reeuwijk, U.R. Tjaden, J. Chromatogr. 353:339-350 (1986)

16. G.O. Korsrud, J.D. Mac Neil, Food Addit. Contam., 5:149-153 (1987)

17. Fusao Kondo, Seiji Morikawa, Susumu Tateyama, J. of Food Prot., 52:41-44 (1988)

18. Von M. Kühne, A. Kobe, R. Fries, A. Ebrecht, Arch. Lebensmittelhyg., 43:49-72 (1992)

19. H.J. Nelis, J. Vandenbranden, A. De Kruif, F. Belpaire, A.P. De Leenheer, J. Pharm. Sc. 81:1216-1218 (1992)

20. J.R. Walsh, L.V. Walker, J.J. Webber, J. Chromatogr. 596:211-216 (1992)

21. S.A. Baker, C.C. Walker, J. Chromatogr. 624:195-209 (1992)

22. W.H.H. Farrington, J. Tarbin, J. Bygrave, G. Shearer, Food Addit. Contam., 8:55-64 (1991).

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