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Analysis of tetracyclines in raw urine by column-switching high-performance liquid chromatography and tandem mass spectrometry

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Abstract

The aim of the project was to develop a fast and reliable method for the quantification of the three tetracyclines: tetracycline, oxytetracycline and chlortetracycline in urine. The method is based on column-switching high-performance liquid chromatography with detection by MS–MS. Buffer is added to the sample before it is injected into the chromatographic system, and the first column which is an internal surface reversed-phase column separates the tetracyclines from the bulk of other compounds in urine. The tetracyclines are collected and concentrated on the analytical column before they are separated and eluted into the mass spectrometer in which the tetracycline are detected. The mass spectrometer is a triple quadrupole instrument and is equipped with an electrospray ion source. The MH^+ ions are selected in the first quadrupole and collisionally activated in the collision cell. Upon collision, activation all three tetracyclines form fragment ions which could be assigned as: $[M+H-H_2O-NH_3]^+$ which are selected in the sond mass filter. The detection limits for all three tetracyclines are about 10 ppb, and the calibration curves are linear from 10 to 1000 ppb.

Keywords: Tetracycline; Oxytetracycline; Chlortetracycline

1. Introduction

Tetracyclines, especially tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) are widely used in animal husbandry. Because of this and the increased awareness among the consumers, there is growing demand for faster and more sensitive methods for the determination of tetracyclines in biological matrices.

In recent years several methods for the determination of tetracyclines in various biological matrices have been based on high-performance liquid chromatography (HPLC). Some of these methods have been used for the determination of tetracyclines in urine [1–6]. The methods have all used reversed-phase separation with UV-detection. A few methods based on LC–MS determination of tetracyclines have been published but none of these have been used for the determination of tetracyclines in urine [7–12]. MS

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detection is often considered the preferred method of confirmation. As tetracyclines are not amenable to GC–MS [9], we here present an automated HPLC-column-switching MS–MS method for the determination of tetracyclines in urine.

2. Experimental

2.1. Chemicals

Tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) were supplied by Sigma. The remaining chemicals were supplied as follows: Trifluoro acetic acid (TFA) (Merck, for spectroscopy), potassium-di-hydrogen-phosphate (Riedel-de Haën) and acetonitrile (BDH HiPerSolv, for HPLC). Demineralized water was obtained from a Milli Q apparatus.

2.2. HPLC

The HPLC system is shown in Fig. 1. It consist of a CMA/200 refrigerated microsampler (CMA, Carnegie), two Rheodyne 7000 switching valves and two HPLCs: HPLC 1: A Shimadzu LC-10AD HPLC pump equipped with a degassing unit and a low-pressure gradient mixer and HPLC 2: An Applied Biosystems 130A syringe pump HPLC with an UV-detector. This instrument is equipped with one switching valve. The instrument was modified so that the pneumatic system can be used to drive two switching valves. In the standard system the position changes of the single valve is done by two pistons which actively drive the valve either clockwise or counter clockwise. When the two standard pistons are substituted by pistons where one of the position changes is done passively by a spring, two valves can be controlled by the system. Two HPLC columns were used: Column 1: a ChromSpher 5 BioMatrix (150×4.6 mm) column (Chrompack) and Column 2: a Brownlee RP-18, 5μ, spheri-5 (30×2.1 mm). It proved essential to condition the BioMatrix column with $(NH_4)_2EDTA$ prior to its use in the analysis. Otherwise the tetracyclines were not retained sufficiently. The mobile phases used were: Eluent 1A: 20 m*M* KH_2PO_4, eluent 2A: 0.1% TFA and eluent 2B: 80% Acetonitrile+0.1% TFA.

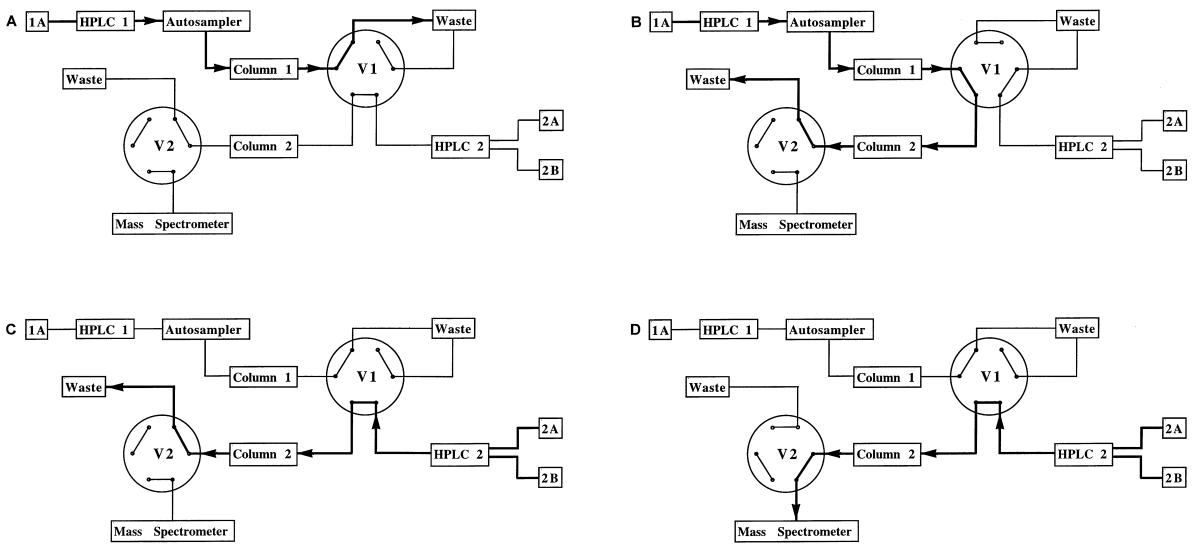

Fig. 1. Schematics of experimental set-up. (A) Separation on preparative column (column 1); (B) transfer of sample from preparative to analytical column (column 2); (C) elution from analytical column to waste; (D) elution of tetracyclines from analytical column into the mass spectrometer.

2.3. Mass spectrometry

All mass spectrometric measurements were performed on a Finnigan MAT TSQ 700 triple stage mass spectrometer. The instrument was equipped with an atmospheric pressure ion source which was used in electrospray mode. The electrospray conditions were as follows: temperature of the heated capillary 210°C; spray voltage 4.5 kV and sheath gas pressure at 80 p.s.i. The auxiliary gas was turned off. The potentials on the interface tubelens and heated capillary were manually tuned by optimising the signals obtained from injection of a mixture of the three tetracyclines. For the collision activation the collision gas was atmospheric air, the pressure in the collision cell was 2.0 mTorr and the off-set potential was −35 V. In the MS/MS analyses the MH^+ ions at m/z 479 (CTC), m/z 461 (OTC) and m/z 445 (TC) were selected by the first mass filter and the second mass filter was set to pass the fragment ions at m/z 444 (CTC), m/z 426 (OTC) and m/z 410 (TC). The scanwidth and scantime were ±0.5 a.m.u. and 0.5 s. The resolution in both mass filters was set to 0.7u.

2.4. Sample preparation

One hundred μl 0.2 *M* KH_2PO_4 is added to 900 μl urine and the sample is mixed thoroughly on a Whirley mixer, after which the sample is transferred to an autosampler vial and placed in the autosampler. Two hundred and fifty μl is injected. The automated analysis follows the timetable shown in the table below which is controlled by the microprocessor in the Applied Biosystems 130A HPLC controller.

Time	Instrument	Event	Fig. 1
	Autosampler	Sample injection	
0	HPLC 1	Flow 1 ml/min, eluent 1A	A
	HPLC 2	Flow 300 μl/min, eluent 2A	
3.00	Valve 1	Elute from column 1 to column 2	B
17.00	Valve 1	Start elution from column 2	C
17.50	HPLC 2	Flow 400 μl/min, eluent 2A	
21.00	HPLC 2	Start gradient from 100% 2A to 100% 2B	
22.00	Mass Spectrometer	Turning on Sheath gas and multiplier, start recording	
22.10	Valve 2	Elute from column 2 to mass spectrometer	D
25.00	Mass Spectrometer	Turning off Sheath gas and multiplier, stop recording	
25.10	Valve 2	Elute from column 2 to waste	C
26.00	HPLC 2	Stop gradient, 100% 2B	
28.00	HPLC 2	End of program	

3. Results and discussion

3.1. HPLC

The aim of the present work was to develop a method for quantification of tetracyclines in urine which required a minimum of sample preparation. This requires a very robust column for the initial separation by which the tetracyclines are separated from the majority of other compounds in the urine. Internal surface reversed-phase columns meet this condition. In this type of column the particle surfaces are hydrophilic, whereas the pores are hydrophobic; and small hydrophobic compounds are consequently retained in the particle pores whereas big hydrophobic molecules which cannot get access to the pores are not retained. The non-volatile buffer used in the preparative column is incompatible with effective ionization in the ion source, and optimisation of the preparative column was consequently done by monitoring the eluate by UV. It was attempted to find an eluent with which the tetracyclines would be immobilised on the column, but especially for oxytetracycline (the most hydrophilic of the three tetracyclines) it turned out to be difficult to find conditions under which it will be retained on the column. With most solvents oxytetracycline elutes close to the solvent front. However with eluents such as 0.020 *M* KH_2PO_4, 0.020 *M* ammonium acetate and 0.010 *M* $(NH_4)_2EDTA$, all three

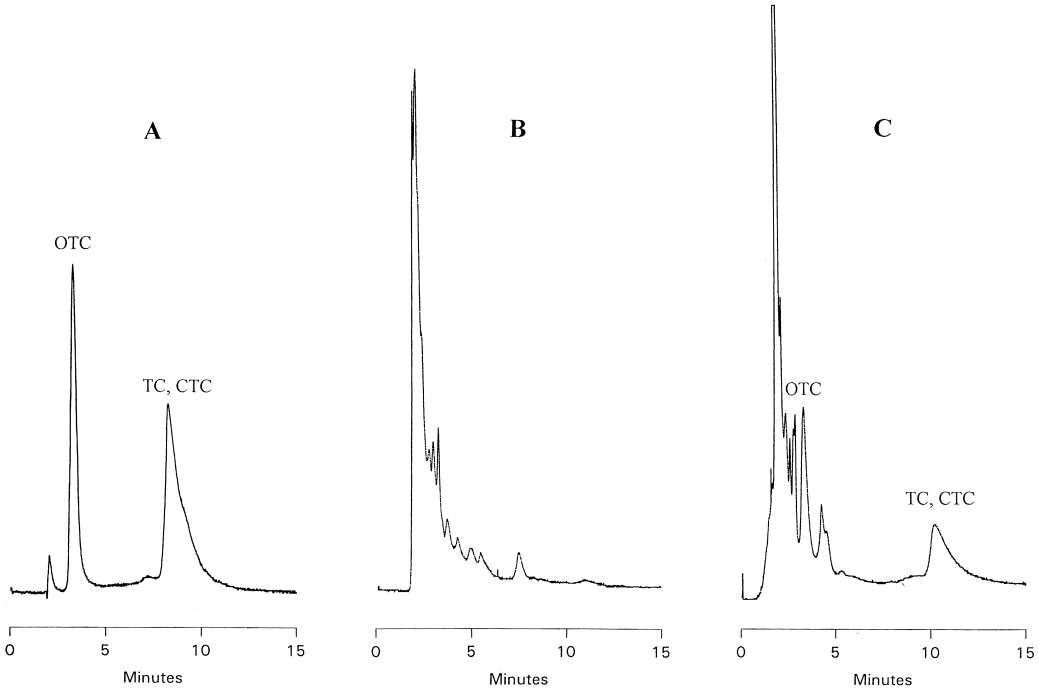

Fig. 2. UV-Chromatograms from analyses on preparative columns. (A) Standard mixture of CTC, OTC and TC, 5 ppm of each in 250 μl injected (phosphate buffer); (B) urine and EDTA buffer, 50 μl injected; (C) Spiked urine (5 ppm) and ammonium acetate buffer, 50 μl injected.

tetracyclines can be retained on the preparative column (Fig. 2A). In Fig. 2 are shown UV-chromatograms of eluates from the preparative column when a tetracycline standard solution and raw urine with and without tetracyclines are injected onto the column. The chromatogram obtained from the spiked sample (Fig. 2C) was obtained with ammonium acetate buffer as the eluent. The peaks from the urine components are identical in the two chromatograms (Fig. 2B and C) which show that ammonium acetate and the EDTA buffer give identical results. The chromatogram in Fig. 2C also shows that oxytetracycline apparently elutes together with more impurities than tetracycline and chlortetracycline. As can be seen from the chromatograms, the majority of the UV-absorbing compounds in urine elutes very quickly from the column. Consequently oxytetracycline will be transferred to the analytical column (column 2 in Fig. 1) together with a considerable amount of impurities. Tetracycline and chlortetracycline seems to be better separated from the majority of the urine compounds, and a big time-window can be used for the transfer of tetracycline and chlortetracycline to the analytical column. Since the two peaks are tailing, a big time-window is necessary to ensure efficient transfer of the two tetracyclines to the analytical column.

To get maximum sensitivity, the eluted compounds from the preparative column must be efficiently bound and concentrated in a narrow band at the front of the analytical column. Consequently the analytical column has to retain the tetracyclines at an eluent composition by which they are not retained on the preparative column. This can be done by using either another retaining principle or a more apolar column. Often HPLC separations of tetracyclines are performed on C-18 or analogous polymeric columns [13–24]. These are more apolar than the internal surfaces of the BioMatrix column used as preparative column in this work in which the stationary phase is based on phenyl groups. It is known that retention of tetracyclines on reversed-phase C-18 columns require eluents which either contains EDTA or has a pH below 3 [25]. EDTA is incompatible with the

mass spectrometric detection and consequently an eluent with a content of 0.1% TFA was chosen for the analytical column. The low pH necessary for retention of the tetracyclines on the analytical column indicate that it is important to keep a low pH in the solvent used for the preparative column. With either ammonia acetate or KH_2PO_4 buffers as solvent for the preparative column, analyses of standard solution showed that the tetracyclines were bound to the analytical column, and 0.020 *M* KH_2PO_4 (pH=4.7) was chosen in preference to the ammonium acetate (pH=7.0). The eluate from the preparative column is only led to the analytical column during elution of the tetracyclines and presumably this helps to maintain the pH of analytical column low enough for retention of the tetracyclines. However, in analyses of spiked urine samples, oxytetracycline was not retained on the analytical column. This problem was solved by adjusting the pH of the urine by addition of a small amount of concentrated KH_2PO_4-buffer to the urine prior to the analysis.

Two column materials with two different pore sizes were tried in analytical column. C-18 material with a pore size of 80 Å retained the tetracyclines very well with the eluent containing 0.1% TFA. Material with a pore size of 300 Å was also tested, but the resulting peaks were broad and small.

When the tetracyclines have been trapped on the analytical column, buffer residues and any weakly retained compounds are removed from the analytical column by an isocratic elution without organic modifier. The tetracyclines are retained under these conditions and are subsequently eluted in sharp peaks by a gradient from 0 to 80% acetonitrile in 5 min. Since a mass spectrometer is used as a detector, baseline separation on the analytical column is not necessary. In order to optimise the sensitivity of the detection a short and narrow analytical column is chosen, and the elution of the tetracyclines with a steep gradient ensures that they enter the ion source in a narrow band and in high concentration. The eluent profile for the analytical column is terminated by an isocratic part with a high organic content. This is included to wash out strongly retained compounds from the column before analysis of the next sample.

Since oxytetracycline elutes as a sharp peak from the preparative column it was initially attempted to use a narrow time window for the transfer of oxytetracycline to the analytical column. For oxytetracycline the use of a narrow time window could especially be a benefit as a lot of compounds from the urine sample eluted at the same time from the preparative column (compare Fig. 2A and B). The situation for tetracycline and chlortetracycline was exactly the opposite: a very broad time window had to be used as the peaks tailed a lot. Fortunately only few other components seem to elute at the same time as tetracycline and chlortetracycline. However, if a break was made between collection of oxytetracycline and tetracycline, the transfer of oxytetracycline was inefficient and irreproducible. Indeed, if the eluate from the preparative column was collected in the time interval between the peaks from oxytetracycline and tetracycline, it did appear to contain a significant amount of oxytetracycline. Consequently, the best results are obtained when, everything eluting between the 3rd and the 17th minute, is transferred to the analytical column.

UV-analysis of the eluate from the analytical column showed that significant amounts of urine components elute at the same time as the tetracyclines (data not shown).

3.2. Mass spectrometry

By using loop injection the mass spectrometer had been tuned to give the maximum signal for the three tetracyclines. The electrospray mass spectra showed no fragmentation and only singly charged molecular ions MH^+. The fragments which were produced by collisional activation of MH^+ could be assigned to $[M+H-NH_3]^+$, $[M+H-H_2O]^+$ and $[M+H-NH_3-H_2O]^+$. As loss of both NH_3 and H_2O is likely to be more specific than the loss of only one of the molecules, the transition MH^+ to $[M+H-35]^+$ was used. In optimisation of the mass spectrometric detection on pure tetracylines, an eluent consisting of 50% methanol+5% acetic acid turned out to give the most intense signal. The remaining optimum parameters are shown in the experimental section.

3.3. HPLC-mass spectrometry

Coupling of the mass spectrometer and the HPLC system was straightforward and a schematic drawing of the set-up is shown in Fig. 1. A switching valve

(V2) is placed between the analytical column and the mass spectrometer in order to direct the eluent from the analytical column to the mass spectrometer only when the tetracyclines are eluting from the analytical column. This minimises the contamination of the ion source. In order to limit the interference from peaks at neighbouring masses a resolution of 0.7u was used in both quadrupoles. The remaining KH_2PO_4 buffer from the preparative column which is incompatible with efficient ionization is removed from the analytical column in the step shown as Fig. 1C.

Three different organic eluents (eluent 2B) for the analytical column were tested. With 80% aqueous acetonitrile+0.1% TFA and with 100% methanol the signal obtained was only one sixth of that obtained with the optimal eluent described above. With 90% methanol+10% acetic acid the decrease in signal was only a factor of three but with a poorer

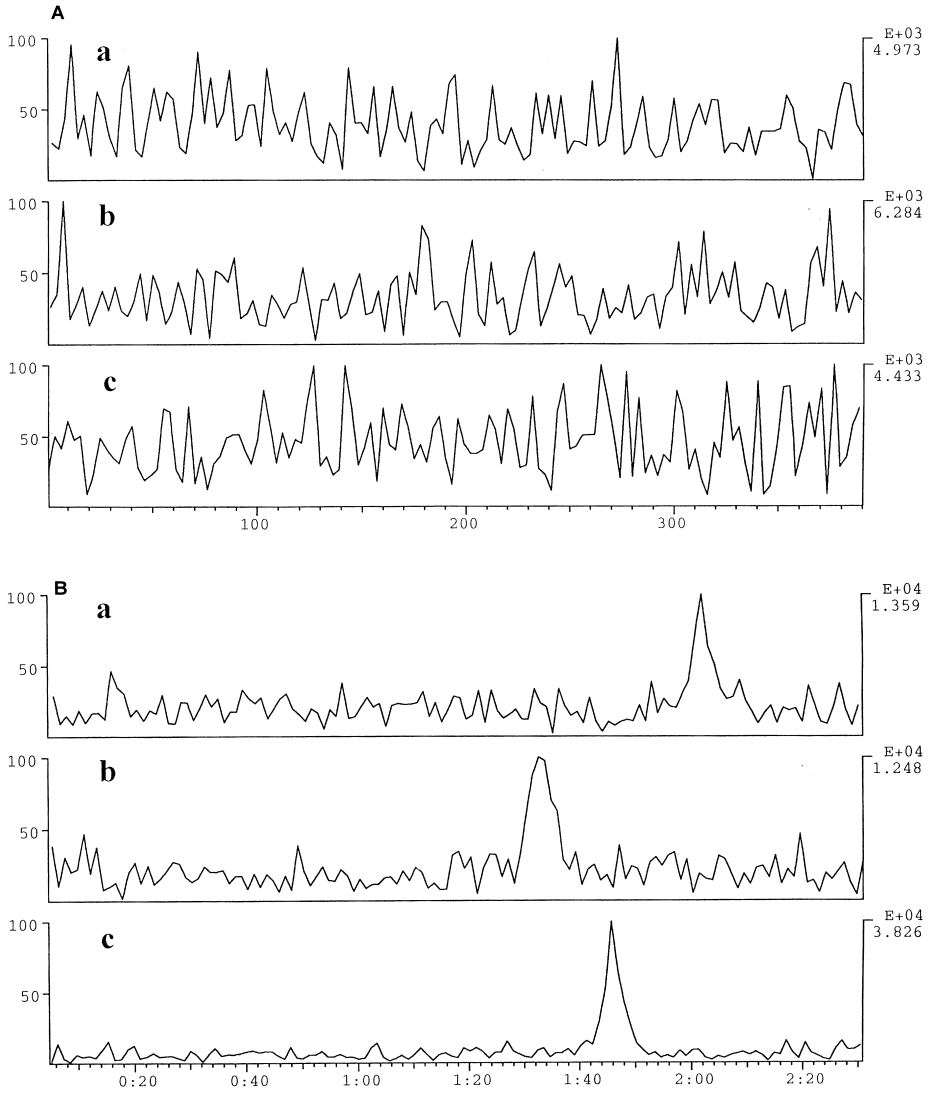

Fig. 3. Mass chromatograms from urine analyses (250 μl injected). (A) urine; (B) urine with 20 ppb of each tetracycline. The mass chromatograms show the fragmentations: (a) m/z 479→m/z 444 (chlortetracycline); (b) m/z 461→m/z 426 (oxytetracycline); (c) m/z 445→m/z 410 (tetracycline).

chromatographic separation. From the retention times and the elution profile, it was calculated that with all three non-polar eluents the tetracyclines eluted with an eluent comprised of a 50:50 mixture of the polar and non-polar eluent. Consequently, the tetracyclines are eluted with a solvent containing at least 0.05% TFA. It is well known that TFA in the eluent leads to inefficient ionization, and much of the difference between the signal from the spiked samples and the standards is likely to have been caused by TFA in the eluent [26]. However, as described above, the content of TFA is necessary in order to obtain sufficient retention of the tetracyclines on the analytical column.

Mass chromatograms obtained from analyses of a blind urine sample and a urine sample fortified to a concentration of 20 ppb of each tetracycline are shown in Fig. 3. In the analyses of the blind sample no signal is observed. The calibration curves for the three tetracyclines shown in Fig. 4 are linear in the concentration range 10–1000 ppb.

In line with other chromatographic methods for analysis of tetracyclines, no internal standard has been incorporated in this analysis. Isotopically labelled standards of tetracyclines are not commonly available and difficult to synthesize. Any internal standard would have to be an analogue of the tetracyclines. However, the high degree of automation and the absence of evaporation steps and manual transfers in the described method will ensure a high degree of reproducibility.

4. Conclusion

An automatised HPLC column-switching method based on tandem mass spectrometric detection have been developed for the determination of tetracycline, oxytetracycline and chlortetracycline in urine. Based on 225 μl sample the detection limits for all three tetracyclines is about 10 ppb, and the calibration curves are linear from 10 to 1000 ppb. In a lower limit a total amount of approximate 5 pmol of the tetracycline is analysed.

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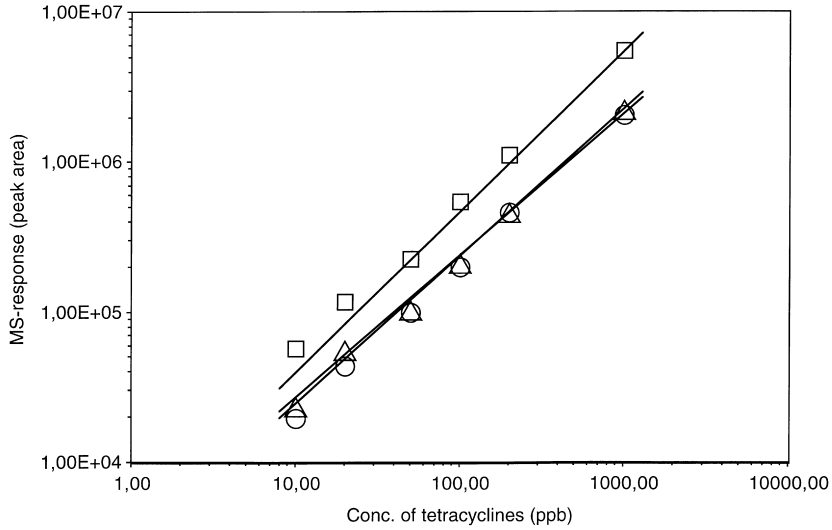

Fig. 4. Calibration curves for the three tetracyclines in double logarithmic plot. Sample concentrations: 10, 20, 50, 100, 200, 1000 ppb. Chlortetracycline (○), r^2=0.9994; oxytetracycline (△), r^2=0.9998; tetracyclines (□), r^2=0.9999.

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