Simultaneous determination of tetracycline, oxytetracycline, and chlortetracycline using liquid chromatography tandem mass spectrometry

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Overview

Tetracyclines are a group of broad-spectrum antibiotic compounds that have a common base structure. Tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC) are widely used in veterinary medicine due to their antimicrobial activity, low toxicity, and low cost. Tetracyclines rapidly go through isomerization in aqueous solutions at pH 2-6 and form 4-epitetracyclines [1]. CTC tautomerization is also reported, and it is shown that elevated temperature selectively affects the equilibrium between the keto-enol forms [2-3]. In addition, IsoCTC, which is the metabolite of CTC, also forms at higher pH [4]. The presence of all these species potentially makes the analysis and quantitation of tetracyclines, CTC in particular challenging. Inaccurate detection and quantitation may lead to erroneous results. To address these analytical difficulties, a liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been developed to detect and distinguish different forms of TC, OTC, and CTC standard solutions. Epimer and tautomer products are isobaric compounds and cannot be distinguished by their respective mass. Therefore, optimization of the chromatographic conditions is desirable to separate and identify different forms of each drug. Quantitation is performed by comparing each product quantifier ion peak area in the samples to that of an internal standard demeclocycline, which is structurally similar. Additional results are presented to reveal the effect of temperature and pH on the dynamic of CTC isomerization and metabolite formation.

References:

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2. W. Naidong, E. Roets, R. Busson, J. Hoogmartens, J. Pharm. Biomed. Anal. 8 (1990) 881.
3. M. Cherlet, S. Croubels, P. De Backer, J. Chromatogr. A 1102 (2006) 116.
4. M. Gaugain, S. Gautier, S. Bourcier, A.M. Jacques, M. Laurentie, J.P. Abjean, D. Hurtaud-Pessel, E. Verdon, Food Additives & Contaminants: Part A, 32 (2015) 1105.

Materials and Methods

To detect TC, OTC, and CTC simultaneously, LC-MS/MS method using Shimadzu Prominence LC coupled to Sciex 4000 qTrap mass spectrometer was developed. Samples were chromatographically separated using a Waters Acquity HHS T3 column (100 x 2.1 mm, 2.6 µm). The gradient consisted of mobile phase A, water with 5% MeOH and 0.1% formic acid and mobile phase B, MeOH and 0.1% formic acid. The oven temperature was set to 40 °C and injection volume was 2 µL. The mass spectrometer operated in the multiple reaction monitoring (MRM) mode to monitor three transitions for each product. Data acquisition was performed using Analyst software v. 1.7.1.

Stock and mixed standard solution of TC, OTC, and CTC were prepared in MeOH and kept frozen at -80ºC. At the day of analysis, samples were further diluted at different solutions. To evaluate the effect of pH, solutions at different pH i.e., 4.0, 4.7, 7.1, and 7.8 were freshly prepared. LC/MS/MS analysis was performed over time on the same LC vial. Likewise, the autosampler temperature was held at 35ºC to assess the effect of temperature.

LC Gradient

Time	A	B
0.00	90	10
2.00	70	30
5.50	45	55
6.00	45	55
6.50	90	10
8.50	90	10

Results and Discussion

Linearity

The linearity of the assay was checked by injecting a series of standards from 0.1 to 8 ppm. The calibration curves used for quantitative measurements were fitted by a quadratic equation using $1/X^2$ weighting. The R^2 values for TC, OTC, and CTC were 0.998, 0.997, and 0.998, respectively.

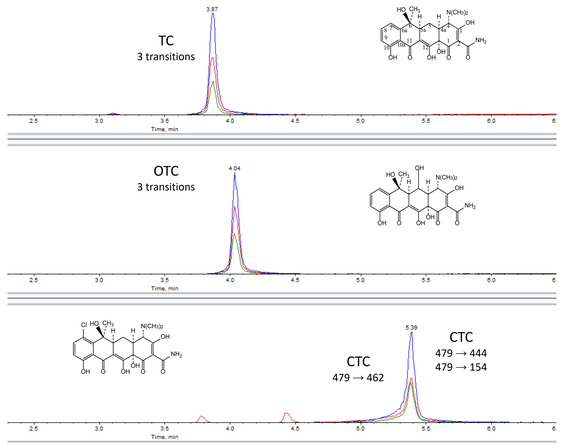

Figure 1. Ion chromatograms of freshly prepared 0.8 ppm solution of mixed standard consisting of TC, OTC, and CTC. All three transitions for TC and OTC eluted together. However, the CTC (479 →462) transition had multiple peaks.

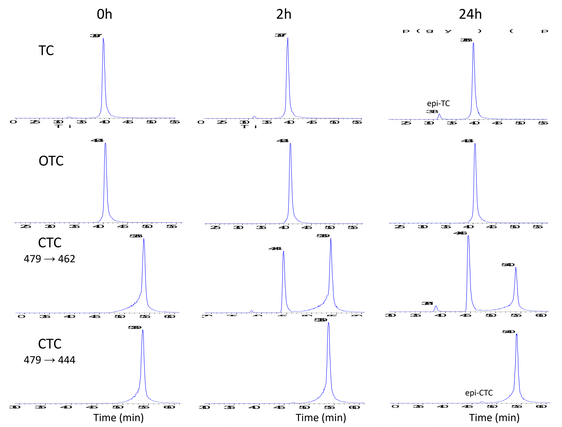

Figure 2. Tetracyclines mixed solution at pH 7.8 was followed over time. TC and 4-epi-TC were well separated eluting at 3.8, and 3.1 min, respectively. We did not observe elution of epi-OTC, whereas, CTC and 4-epi-CTC eluted at 5.39 and 4.47 min, respectively. The major CTC peak was observed as enol at time zero with virtually no keto peak. At 12 hr, equilibrium was established with equal enol and keto peaks, whereas at 24 hr, not only was keto peak dominant but the epi-iso CTC peak was also observed to grow. This indicates that the peak eluted at 4.4 min is more than likely a mix keto-CTC and IsoCTC.

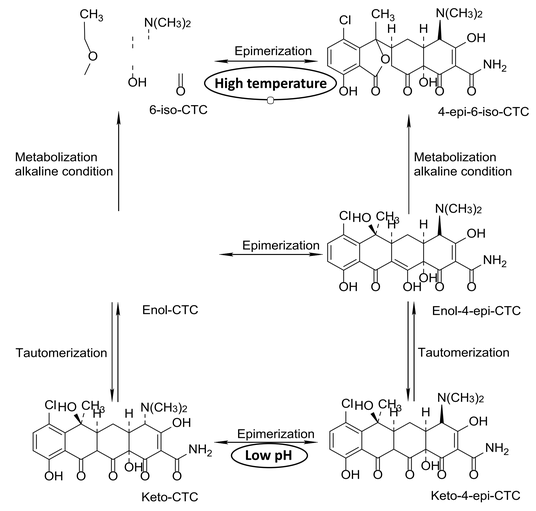

Figure 3. CTC, epimerization gives rise to the 4-epi-CTC. But in addition to epimerization, CTC undergoes keto-enol tautomerism. Keto and enol forms are present in equilibrium in the solution and the ratio depends on the pH and temperature of the solution, among other factors.

Effect of pH

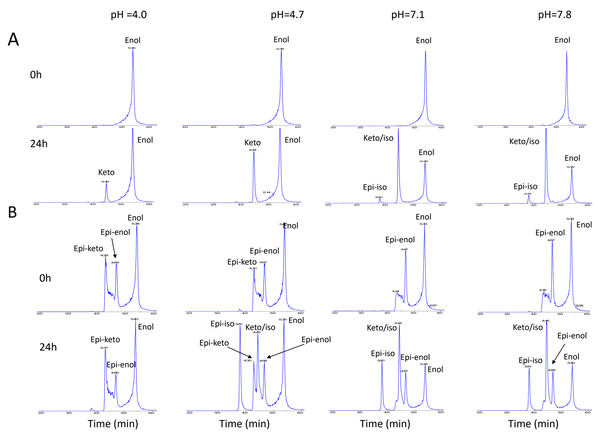

Figure 4. To evaluate the dynamic of the CTC keto-enol formation, we performed a series of experiments of A) pure CTC standard and B) CTC standard and 4-epi-CTC. The concentration level was 2.64 ppm. At time zero, the CTC solution was in enol form essentially in all pH tested. Keeping the solution for 24hrs in the autosampler shifted the equilibrium towards keto form even in low pH solutions. In the mixed solutions however, iso-CTC peak was the dominant peak in higher pH as it was evident by presence of epi-isoCTC peak at 3.8 min.

Effect of Temperature

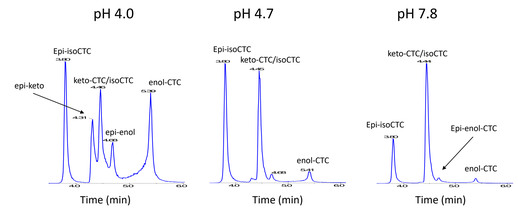

Figure 5. Extracted ion chromatograms of CTC std and 4-epi-CTC 479→462 transition after keeping the samples at 35ºC for 24 hrs. At the elevated temperature, the intensity of enol form diminished with time, while the chromatograms became dominated by isoCTC and epi-isoCTC peaks eluting at 4.4 min and 3.8 min, respectively. Although we cannot distinguish keto-CTC and iso-CTC both eluting at 4.4 min, the absence of the epi-keto-CTC peak at 4.3 min is further evidence that the peak at 4.4 min is iso-CTC.

Conclusion

- We developed an LC-MS/MS method which can simultaneously detect TC, OTC, and CTC in a standard mixed solution.
- For TC and CTC, their corresponding epimers were easily separated, however, we did not detect epi-OTC.
- We showed a freshly prepared solution of CTC is in enol form but transforms to keto-form with time. The ratio of keto/enol forms of CTC is affected by the solution pH and temperature.
- pH and temperature play important roles in the keto-enol tautomerization of CTC and 4-epiCTC, with lower pH values favoring the keto form and higher temperature favoring the formation of iso-CTC.
- CTC analysis is complicated as it forms different isoforms including epimers, keto-enol tautomers, and metabolite which potentially make the accurate quantitation of CTC more difficult. To accurately quantify CTC, these additional chemical species should be considered.

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How does this work support FDA's mission?

This study provides new tool for drug detection that may not only be more rapid and sensitive, but enables to detect multiple drugs simultaneously to ensure they are safe and effective.