HPLC Separation of Tetracycline Analogues: Comparison Study of Laser-Based Polarimetric Detection with UV Detection

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Abstract

A sensitive and specific high-performance liquid chromatography (HPLC) method based upon laser-based polarimetric detection is developed for the determination of six tetracycline analogues. By interfacing the laser-based polarimeter online with an HPLC system, the specific rotation of each analogue is obtained as compounds elute from the separation system. The six structurally similar tetracycline analogues exhibit significant differences in specific rotations. The experiments suggest that specific rotation can be useful in identifying closely related tetracycline analogues. Linear relationships are found to be in the range of 0.342–0.0043 mg for the tetracycline analogues. Five of the six analogues exhibit excellent linearity (R^2 value ≥ 0.99). The polarimetric results are compared with UV detection. The HPLC–laser-based polarimetric detection instrument is able to quantitate the studied tetracycline analogues with high precision, accuracy, and sensitivity, which make it useful for the development of a standard method for the determination of tetracyclines in biological specimens. The performance of the HPLC–polarimetric system for the analysis of tetracyclines in a biological matrix is evaluated. The selectivity of polarimetric detection provides a distinct advantage in the analysis of tetracycline analogues in milk. The HPLC–polarimetric system provides a rapid and sensitive technique that involves minimal sample cleanup and pretreatment for the analysis of tetracyclines in milk.

Introduction

Tetracyclines are broad-spectrum antibiotics active against a wide range of gram-positive and gram-negative bacteria, as well as a range of organisms such as mycoplasma and chlamydia. They are widely used in human and veterinary medicines, as well as feed additives. One of the analogues, doxycycline, has recently been identified as the optimal antimicrobial therapy for inhalational anthrax (if the strain is susceptible) (1,2). As a general rule, these analogues exhibit differences in their efficacy and toxicity.

The basic structure of a tetracycline consists of a hydro naphthacene backbone containing four fused rings. As shown in Figure 1, the various analogues differ primarily by substitutions of the fifth, sixth, or seventh position on the backbone.

In pharmaceutical preparations of tetracyclines, several of the analogues may be present, thus it is important to understand the chromatographic characteristics of each analogue in order to monitor for its presence in pharmaceutical preparations. For example, studies had shown that chlortetracycline is commonly found as an impurity in pharmaceutical preparations of tetracycline (3–5). Besides the presence of analogues in pharmaceutical preparation, it is known that 4- and 6-epimers can be formed from their parent compounds under abnormal condition such as heat, pH, and humidity (6). Consequently, it is also important to monitor the presence of the epimers in their parent compounds.

Many methods have been described for the determination of tetracyclines in both pharmaceutical preparations and various biological matrices. For example, in previous studies, tetracycline analogues in pharmaceutical preparation were determined by high-performance liquid chromatography (HPLC) with UV detection (6) or fluorsensor (7). In addition, methods have been developed for the determination of tetracyclines in biological matrices such as milk (8,9), animal tissue (10–12), and serum (13). The most common method for extraction of tetracyclines from biological matrices is solid-phase extraction (9,11–13). The most common analysis technique for tetracycline is HPLC with UV detection (14,15). Alternatively, with increased time and cost per analysis, fluorescence detection using derivatization with metal salts (16), or conversion to an isoderivative under alkaline conditions, has been reported (17,18). In general, fluorescence detection is more specific than UV detection and is less affected by interference from other compounds in the sample matrix. However, fluorescence detection involves postcolumn derivatization that may be time-consuming, and it adds another level of complexity to the

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analysis. In addition, as mentioned previously, the derivatives are usually unstable and must be analyzed immediately, which adds a time constraint to the analytical technique.

Optical activity is an extremely important property that is usually indicative of biological activity. The specific rotation [α] is the parameter used to assess the magnitude of the chirality of a system, where [α] is defined as:

$$[\alpha] = a/lc \qquad \text{Eq. 1}$$

where α is rotation in degrees, *l* is the path length in decimeters, and c is concentration in g/mL (19). [α] can be used to provide important and unique information about the arrangement of atoms at, or near, a chiral center. Polarimetric detection, which can provide direct information on the chirality of compounds in analytical samples, has the potential to differentiate between optically active compounds that have similar structures. Most antibiotic materials and their derivatives possess the property of chirality, that is, they are optically active. This is because of the fact that they are produced by living organisms, which use chirality as part of their system of molecular recognition. Because optical activity is such a rare property, application of optical activity detection to the determination of tetracycline analogues and their epimers should provide some unique advantages, which can be used to overcome the limitations of the techniques described previously.

The application of laser-based polarimetry to the detection of tetracycline analogues separated by reversed-phase (RP) chromatography will be discussed in this paper. The results are used to compare laser-based polarimetry with UV absorbance detection under the same chromatographic separation conditions. The determination of the specific rotations of each of the tetracycline (six) analogues and the use of the specific rotations for the identification of these analogues will be discussed. The application of the HPLC–polarimetric detection system for the detection of tetracycline analogues in milk will also be evaluated.

Experimental

Chemicals

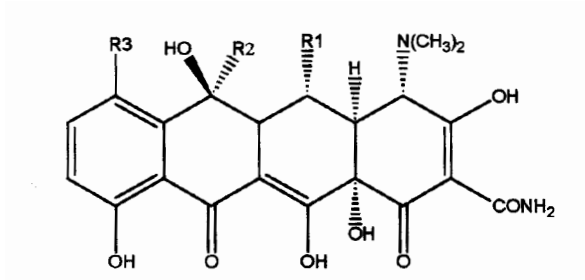
All solvents were HPLC grade, and all chemicals were analytical grade. All reagents were used as received, except for degasification using ultrasonic agitation under vacuum prior to use. Chlorotetracycline (CTC), demeclocyline (DEM), doxycycline (DC), minocycline (MC), oxytetracycline (OTC), and tetracycline (TC) as their hydrochlorides were obtained from Sigma Chemical (St. Louis, MO). Acetonitrile, methanol, oxalic acid, trifluoroacetic acid (TFA), and aqueous ammonia were obtained from Fisher Scientific (Fairlawn, NJ). Bovine milk samples were obtained from commercial sources.

The HPLC column packing material was Axxi-Chrom Octyl (25- × 4.6-mm i.d., Cole Scientific, Moorpark, CA), with a particle size of 5 mm.

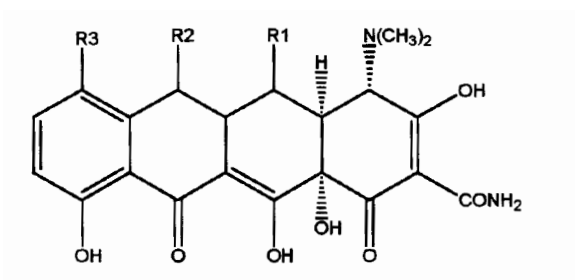
Instrumentation

The UV–vis spectrometer was from Shimadzu (model 101 PC, Kyoto, Japan). The chromatographic system was conventional, consisting of a constant-flow pump from Shimadzu coupled to a 5-mm C-8 column (25-cm × 4.6-mm i.d., maintained at ambient temperature) through a high-pressure injection valve from Rheodyne (model RH-7725i,Cotati, CA) with a 20-µL sample loop. For the UV study, the HPLC system was used together with a UV–vis detector from Shimadzu (model SPD-10Avp) operated at 355 nm. Data was transferred to a recording data processor from Shimadzu (model C-R8A).

For the specific rotation study, the HPLC system was connected to an advanced laser polarimeter from PDR-Chiral (model CH 96.001, Lake Park, FL) with a detection cell volume of 21.5 µL and a path length of 0.25 dm. Data was sampled at a 2-Hz rate and transferred to a personal computer via an Institute of Electrical and Electronic Egineers (IEEE) interface for storage and analysis. For the study of milk samples, data was sampled at 5 Hz. All data were taken with a 3-s time constant. The polarimetric system demonstrated a baseline noise



	R_1	R_2	R_3
CTC	H	CH_3	Cl
DEM	H	H	Cl
OTC	OH	CH_3	H
TC	H	CH_3	H



	R_1	R_2	R_3
DC	OH	CH_3	H
MC	H	H	$N(CH_3)_2$

Figure 1. Structures of tetracycline analogues. R_1, R_2, and R_3 correspond to substitutions of the 5th, 6th, and 7th positions on the backbone of each tetracycline analogue, respectively.

level corresponding to 25 µdeg.

Mobile phase

The mobile phase for the HPLC separation of the tetracyclines was a 1:1.5:5 mixture of methanol–acetonitrile–0.01M aqueous oxalic acid solution (the pH of the oxalic acid was adjusted to 2.0 with concentrated ammonia solution) (20). The flow rate was 0.8 mL/min.

Preparation of tetracycline solutions

Extinction coefficient determination of the tetracyclines

The tetracycline stock solutions were prepared from reference standards by accurately weighing 20 mg of substance and dissolving in methanol in a 10-mL volumetric flask. The working standards were prepared from the stock solutions by dilution with the appropriate volume of methanol to make a final concentration in the range of 9.8–50 µg/mL. A UV–vis instrument was used to determine the absorbance at the λ_{max} of each compound evaluated. The extinction coefficients for each of the tetracycline analogues were determined from the slope of the Beer's Law plot.

HPLC separation with UV detection

Standard solutions for each tetracycline analogue were prepared and injected individually.

The tetracycline stock solutions were prepared from reference standards by accurately weighing 100 mg of substance and dissolving in methanol in a 10-mL volumetric flask. When stored at 4°C, these stock solutions were stable for approximately two days. The working standards were prepared from the stock solutions by dilution with the appropriate volume of methanol to make final concentrations in the range of 0.0092–1.1 mg/mL.

For comparison purposes, calibration curves were prepared by injecting the tetracycline analogues as mixtures at different concentrations. Stock solutions that contained the tetracycline analogues as a mixture were prepared by taking 1 mL of the standard solution of each analogue with a concentration in the range of 1.0–1.5 mg/mL and diluting in a 10-mL volumetric flask with methanol. Working standards were prepared from the stock solution by dilution with the appropriate volume of methanol to provide final concentrations in the range of 0.0076–1.1 mg/mL. The chosen analytical range is within the range of typical serum concentrations of tetracycline analogues for the standard dosage of 200 mg (13).

Separation of the tetracycline analogues was demonstrated with mixtures that contained the six analogues, with concentrations in the range of 0.85–1.70 mg/mL.

HPLC separation with polarimetric detection

Standard solutions for each tetracycline analogue were prepared and injected individually. The tetracycline stock solutions were prepared from reference standards by accurately weighing 200 mg of substance and dissolving in methanol in a 10-mL volumetric flask. The working standards were prepared from the stock solutions by dilution with the appropriate volume of methanol to make the final concentrations in the range of 0.215–17.1 mg/mL.

Separation of the tetracycline analogues was optimized using solution mixtures that contained the 6 analogues with concentrations in the range of 0.85–1.70 mg/mL. Calculation of the specific rotation was accomplished using the peak height method reported previously (21).

To test the ability of the HPLC–polarimetric system for the analysis of tetracycline analogues in a biological matrix, preliminary studies were conducted by spiking 10 mL of commercial milk samples with 4 tetracycline analogues (OTC, TC, DEM, and CTC) at a concentration of 0.2 mg/mL. The milk samples were then acidified with 1 mL of TFA and centrifuged for 15 min, at which time the supernatant liquid was drawn through a 0.2-µm syringe filter and injected directly into the HPLC system.

Results and Discussion

Extinction coefficient determination for the tetracyclines

The extinction coefficient and λ_{max} for each tetracycline analogue are reported. The results were compared with literature values and are listed in Table I. As shown in Table I, the extinction coefficients for the six tetracycline analogues studied (except for MC) are similar. The relatively close extinction coefficient values for the tetracycline analogues suggest that the difference in UV absorption among the analogues is minimal and expected, given the similarity of their structures. Consequently, UV detection of the tetracycline analogues may not be very selective, and differentiation must rely on the separation system. In order to increase the detection selectivity, alternative modes of detection must be explored. Polarimetric detection for the tetracycline analogues will be discussed and compared with UV detection in the following section.

HPLC separation with UV detection

Because of their polar nature, tetracycline analogues are

Table I. Extinction Coefficients and λ_{max} of Tetracycline Analogues

Tetracycline analogue	Reported values for molar extinction coefficient ($cm^{-1}M^{-1}$)	Literature values for molar extinction coefficient ($cm^{-1}M^{-1}$)	Reported λ_{max} (nm)	Literature values for λ_{max} (nm)	Linear regression (R^2 value)
CTC	12,725	10,400*	374	370*	0.9968
DEM	12,893	unavailable	368	unavailable	0.9990
DC	13,326	13,183†	353	351†	0.9943
MC	10,345	14,454†	343	352†	0.9987
OTC	11,583	12,890*	358	354*	0.9991
TC	13,095	13,320†	369	355†	0.9989

* Values from Sigma-Aldrich.
† Values from the Merck Index. Note: reported values were compared with values reported in the Merck Index if they were available.

usually determined by ion exchange chromatography (22–25) or RP chromatography (26–29). Previous work has shown that RP chromatography gives much better performance in terms of plate number, plate height, and resolution when compared with ion-exchange chromatography (25,30,31). In this paper, the separation was performed using a commercially available octyl column. In order to minimize the formation of isomeric analogues, which occurs rapidly in alkaline medium (32), the pH of the mobile phase was chosen as 2.0. A typical HPLC separation of six tetracycline analogues with UV detection is illustrated in Figure 2.

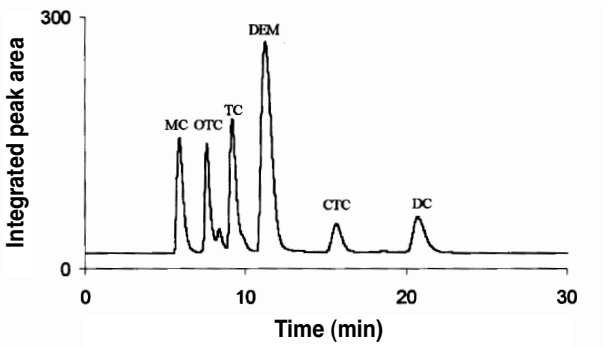


Figure 2. HPLC separation of tetracyclines with UV detection. Column, Axxi-Chrom Octyl (25- × 4.6-mm i.d.); mobile phase, methanol–acetonitrile–0.01M oxalic acid solution with a pH of 2.0 (1:1.5:5); flow rate, 0.8 mL/min; and detection, UV–vis absorbance at 355 nm. Note: the shoulder at the OTC peak is an undetermined impurity that does not affect the linearity of the calibration curve.

Table II. Retention Times (Min) for HPLC–UV Analysis of Tetracyclines*

Tetracycline analogues	Retention time (min)	Relative retention time (based on MC)
MC	5.5	1.0
OTC	7.9	1.4
TC	9.7	1.8
DEM	12.6	2.3
CTC	17.7	3.2
DC	25.3	4.6

* See Figure 2 for chromatographic conditions.

Table III. Linear Regression for Tetracycline Analogues with UV Detection

Tetracycline analogues	R^2 value for analogues injected as a mixture	R^2 value for analogues injected separately
CTC	0.9875	0.9998
DEM	0.9885	0.9982
DC	0.9933	0.9911
MC	0.9848	0.9976
OTC	0.9932	0.9995
TC	0.9936	0.9995

As shown in the chromatogram, all six of the studied tetracycline analogues eluted with favorable retention times, while maintaining adequate resolution between each analogue. Therefore, the technique used here would be useful for the

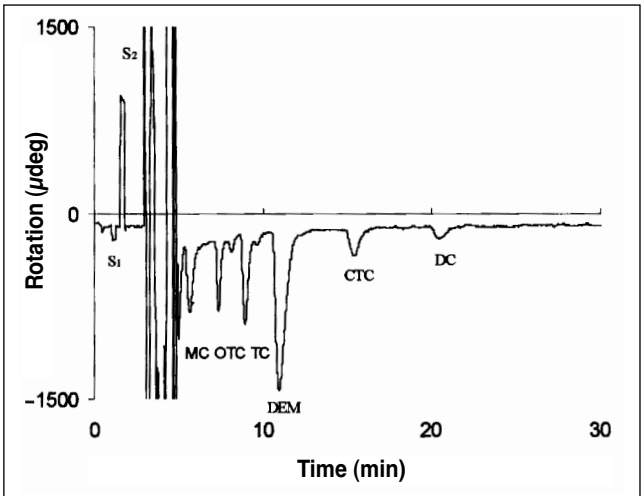


Figure 3. Separation of tetracycline analogues with laser-based polarimetric detection. S1 and S2: standard signal produced by a DC Faraday coil corresponding to a rotation of –100 μdeg and +1000 μdeg, respectively. The disturbances at approximately 3–5 min are refractive index disturbances. The flow rate is 0.7 mL/min. All other chromatographic conditions are the same as described in Figure 2.

Table IV. Linear Regression Values for Tetracycline Analogues with Polarimetric Detection

Tectracycline analogues	R^2 value
CTC	0.9999
DEM	0.9999
DC	0.9999
OTC	1.000
TC	0.9999

Table V. Specific Rotations of Tetracycline Analogues

Tetracycline analogues	Reported specific rotation (deg/dm gcm^{-3}) (λ = 670 nm)	Literature values for specific rotation (deg/dm gcm^{-3}) (λ = 589 nm)
CTC	–244.5	–240.0*
DEM	–259.6	–258†
DC	–114.3	–110*
MC	–133.83‡	–166†,‡
OTC	–193.9	–196.6†
TC	–275.8	–257*

* Values from Sigma-Aldrich (2002 catalog).
† Values from the Merck Index. Note: reported specific rotations were compared with values obtained from the supplier, Sigma-Aldrich, if available.
‡ MC shows a relatively large difference between the reported specific rotation and the corresponding literature value because it has the lowest retention time and its polarimetric response is very close to the refractive index disturbance.

identification of tetracycline analogues. The retention times of the tetracycline analogues as listed in Table II were relatively short, with a maximum run time of 25 min being required in the case of doxycycline.

Calibration curves were prepared for each analogue by injecting the six analogues as a mixture. Three injections were made for each standard. Data was reported as the average of three injections. At a given concentration, CTC and DC showed smaller UV absorption signals than the MC, OTC, TC, and DEM at the chosen wavelength (355 nm). Consequently, a higher range was chosen for the study for CTC and DC. Linear relationships were found in the range of 20.3–1.1 mg for CTC and DC and in the range of 6.4–0.15 mg for MC, OTC, TC, and DEM. The relative standard deviation (RSD) values for the integrated peak areas were on the order of 3.1%.

A comparison study was conducted by injecting each of the tetracycline analogues separately. The linear regressions for both studies are listed in Table III.

As shown in Table III, the value of linear regression for each analogue does not show a significant difference between the two studies (either injected separately or as a mixture). In addition, the linear regression values for all six analogues studied are on the order of $R^2 > 0.99$, indicating that there is excellent linearity. The results showed that the separation method used here for the six tetracycline analogues is accurate and showed excellent linearity for all six analogues.

HPLC separation with polarimetric detection

A typical separation of tetracycline analogues with polarimetric detection is illustrated in Figure 3. As shown in Figure 3, all six analogues are well separated, with significant differences in the optical rotation. From the chromatogram, it can be concluded that the technique used here would be useful for the identification of tetracycline analogues in conjunction with the known HPLC retention times for the analogues under these separation conditions.

As shown in Figure 3, MC has the shortest retention time, and its polarimetric response is very close to the refractive index disturbance. Consequently, no calibration curve was prepared for MC. A linear relationship was observed between the polarimetric response and injection concentration for the five analogues with resolved peaks. Linear regression values (R^2) ranged from 0.9999 (other analogues) to 1.000 (OTC). The linearity was compatible to that obtained with UV–vis detection.

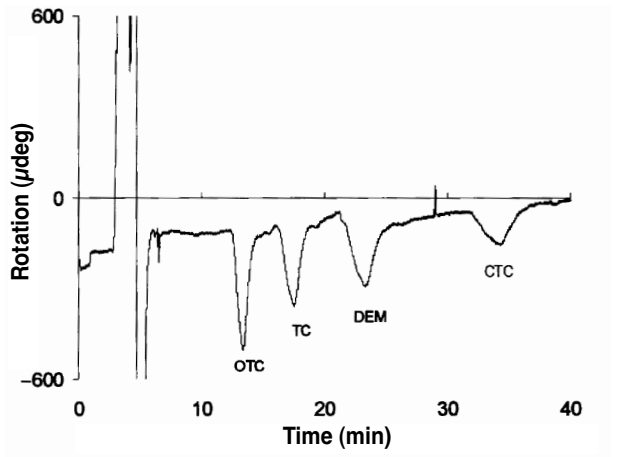


Figure 4. Laser-based polarimetric detection of tetracycline analogues in a milk sample. The disturbances at approximately 3–6 min are refractive index disturbances. The flow rate is 0.5 mL/min. All other chromatographic conditions are the same as described in Figure 2.

The linear regression for the other tetracycline analogues is listed in Table IV. The RSD values for integrated peak areas were on the order of 1.7%.

As demonstrated, linear relationships were found in the range of 0.342–0.0043 mg for the tetracycline analogues. In addition, the linear regression values for the five analogues under study have values of $R^2 > 0.99$, indicating that there is excellent linearity.

The RSD value for the integrated peak areas for polarimetric detection was 1.7%, compared with the value of 3.1% for UV detection. It can be concluded that UV detection has a lower precision than polarimetric detection under the experimental conditions of these studies. This is surprising because polarimeters suffer from increased baseline drift caused by temperature variation at the most sensitive scale. However, that did not affect the RSD for tetracycline detection. The higher precision found in the polarimeter is because of the fact that the polarimeter only responds to optically active material and, thus, provides a degree of selectivity. On the other hand, the UV detector responds to any material with a chromophore that is active at a given wavelength. Consequently, any impurity in the samples could show a response in the UV detector and thus provide a less precise quantitation. Because of the mentioned reasons, the polarimeter is more responsive to optically active compounds than using detection by UV absorption. The larger response per mass injected in the polarimeter then leads to a better precision for the analysis of tetracycline analogues.

Besides providing a significant advantage in terms of mass detectability, polarimetric detection has the unique capability to provide specific rotation information on eluting compounds, which is not available by any other means. Because specific rotation is sensitive to the arrangement of atoms at, or near, the chiral center, subtle structural changes within a complex molecule can be recognized by specific rotation measurements.

Specific rotations for all six analogues were obtained by the peak height method reported previously (13). The specific rotation for each analogue is reported as an average of four measurements at four different concentrations. The results are compared with literature values and are listed in Table V.

As shown in Table V, the reported specific rotations for the six analogues are comparable with the corresponding literature values. In addition, it can be seen from Table V that there is a significant difference in the specific rotation for each analogue. This suggests that polarimetric detection provides distinct qualitative, as well as quantitative, advantages over UV detection for tetracycline analogues. The measurement of specific rotation is sensitive to the native optical activity of the molecule under study and, thus, is sensitive to the subtle structural variations among the tetracycline analogues. The minor structural variations that differentiate these analogues are

attributed to only one chiral center (in the cases of CTC, DMC, OTC, and TC) or two chiral centers (in the cases of DC and MC) out of several chiral centers present in these compounds. The large differences in specific rotations exhibited by these six structurally similar tetracycline analogues suggest that specific rotation is useful for the identification of closely related tetracycline analogues.

Laser-based polarimetric detection provides a sensitive detection method for tetracycline analogues. More significantly, by interfacing the laser-based polarimeter online with an HPLC system, specific rotations for each analogue can be obtained as they are eluted from the separation system. As a result, laser-based polarimetric detection in combination with real-time specific rotation measurements can provide a selective method to detect and identify these closely related analogues.

The analysis of tetracyclines analogues in complicated matrices could also benefit from the selectivity offered by the polarimetric system. As the selectivity of the detection system is increased, the constraints placed upon the separation system are reduced. To test the ability of the HPLC–polarimetric system for detection of tetracycline analogues in a biological matrix, commercial milk samples were spiked with tetracycline analogues and extracted according to the procedures described in the Experimental section of this report. A representative chromatogram is given in Figure 4. As shown in Figure 4, the elution window from 10 to 40 min is devoid of any extraneous peaks, even though the complicated nature of milk suggests that more materials should be present. Because the polarimetric system only responds to chiral compounds, the inherent selectivity of the procedure is enhanced. The percentage recovery from this simple procedure was in excess of 85% for the tetracycline analogues. The procedure provides a simple and rapid technique for the detection of tetracyclines in milk that does not require extensive sample cleanup and pretreatment prior to analysis. More detailed studies for the detection of tetracycline analogues in biological samples are in progress and will be published in the near future.

Conclusion

The results of the present study demonstrate that a laser-based polarimeter can be used as a sensitive detector for the analysis of tetracycline analogues, with a detection limit at the microgram level. An important aspect of the present technique is that the specific rotation for each analogue is strongly dependent on molecular structure and, thus, provides a selectivity advantage for polarimetric detection over UV detection. This qualitative information can be obtained online and in real-time as the compound elutes from the separation system.

The combination of HPLC separation with laser-based polarimetric detection has been shown to provide distinct advantages for the study of antibiotic systems. The HPLC–polarimetric method can be used for the separation and accurate quantitative determination of microgram levels of tetracycline analogues. Because polarimetric detection is selective for optically active analytes, interferences from the sample matrix are minimized. Preliminary studies for the detection of tetracycline analogues in milk show that the HPLC–polarimetric system provides a rapid and sensitive technique with minimal sample cleanup and pretreatment for the detection of tetracyclines in a complex biological matrix.

In summary, the combination of HPLC separation with laser-based polarimetric detection has been shown to provide distinct advantages for the determination of tetracycline, particularly in complicated biological matrices. More importantly, the laser-based polarimetric system has the capability to provide accurate and precise specific rotation information on eluting materials. The reported method has considerable potential as a basis for the development of a standard method for the determination of tetracyclines in biological specimens. In addition, the reported HPLC–polarimetric detection can be used for impurity analysis in antibiotics by coupling with UV detection.

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