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Separation Differences Among Phenyl Hydride, UDC Cholesterol and Bidentate C8 Stationary Phases for Stability Indicating Methods of Tetracyclines:

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Abstract

Formulation extracts of tetracycline hydrochloride (HCl), minocycline hydrochloride (HCl), and doxycycline hyclate were degraded by strong acidic conditions and heating. Subsequently, components of the extracts were separated by Bidentate C8, Phenyl Hydride and Cholesterol (UDC) HPLC columns operating in the reverse phase mode. The Phenyl Hydride column was able to baseline separate minocycline from the observed degradant, while partial or total co-elution was observed with the other two columns using otherwise identical method conditions. For both the degraded tetracycline HCl and doxycycline hyclate extracts, the UDC column gave the best resolution for the critical pair. The findings suggest that the postulated secondary retention mechanisms of π - π interactions from the Phenyl Hydride and shape selectivity from the UDC can provide superior resolution for structurally similar analytes compared to hydrophobic interactions alone.

Keywords

tetracycline; minocycline; doxycycline; silica hydride

Introduction

Tetracyclines are broad-spectrum antibiotics used for a variety of applications in both human and veterinary medicine. Under acidic conditions, tetracycline is known to degrade primarily by two pathways: epimerization and dehydration. Epimerization at the 4-carbon position is a reversible first-order process that occurs most readily in the pH range of 3–5. The resulting epimer, 4-epitetracycline, is reported to be inactive and non-toxic *in vivo*.^[1] Dehydration and subsequent aromatization is most favorable at pH < 2 and leads to formation of anhydrotetracycline. The anhydro form can also reversibly epimerize at the C4 position as well. Anhydrotetracycline and 4-epianhydrotetracycline are reported to be toxic *in vivo*^[2] and have been implicated in the development of Fanconi's Syndrome from use of

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degraded tetracycline medication.^[3] For this reason, it is crucial to have a reliable analytical method for discriminating amongst tetracycline and its major degradation products.

Semisynthetic derivatives of naturally occurring tetracyclines are also available. Two such examples are minocycline and doxycycline. They can be used to treat a variety of bacterial infections such as acne and Lyme disease. They are often preferred over naturally occurring tetracyclines for these types of applications due to easier dose schedules and fewer side effects. Also, minocycline is more lipophilic than tetracycline and therefore can more readily cross the blood–brain barrier. For this reason, research has been conducted to study its efficacy in treatment of neurodegenerative conditions such as stroke^[4, 5] and spinal cord injury.^[6] Like tetracycline, minocycline can also reversibly epimerize at the C4 position in acidic conditions. Doxycycline has three main reported degradation products under heating conditions: 4-epidoxycycline, 6-epidoxycycline, and methacycline.^[7, 8] The structures of tetracycline, minocycline, doxycycline, and their degradants are shown in Figure 1.

The United States Pharmacopeia (USP) assay methods for many tetracyclines often lack robustness. With doxycycline hyclate for example, an unusual water/tert-butanol based mobile phase is used in combination with an ion pair agent and other additives.^[9] A bare silica column is used for the separation and the ion pair agent is believed to be electrostatically adsorbed onto the silanolic surface. Retention is thought to be due to interaction with the hydrophobic moieties of the ion-pair agent adsorbed onto the silica surface. The use of ion pair agents is generally less robust than use of columns with covalently bonded organic moieties due to slow and variable loading of the ion pair agent.^[10] However, even methods that use ordinary covalent bonded phases may need to use ion pair agents in order to reduce silanolic peak tailing of basic analytes such as tetracyclines.

Silica hydride is a stationary phase material which exhibits a number of unique and desirable chromatographic properties which may be useful for stability indicating methods of tetracyclines. One such property is that because over 95% of the surface silanols are replaced in its synthesis,^[11] the resulting relatively hydrophobic silica hydride surface will not promote silanolic adsorption of basic analytes like a typical silica-based stationary phase will. Because tetracyclines and their main degradants contain one or more dimethylamine groups, this is an important feature for reducing peak tailing for their stability indicating HPLC methods. Another advantage of silica hydride is that unique stationary phase moieties can be obtained since the moieties are not limited by the conventional organosilane bonding chemistry used for type B silica materials. For example, the Undecanoyl Cholesterol (UDC) column is unique to silica hydride-based materials and is well-suited to the separation of isomers. The UDC moiety is a liquid crystal and can therefore potentially differentiate between structurally similar analytes, such as the degradants of tetracyclines, on the basis of shape selectivity.^[12]

The goal of this work was to study and compare the separation characteristics of three silica hydride-based columns for use in stability indicating methods of tetracyclines. The first was a Bidentate C8 column, in which only hydrophobic interactions with the alkyl chain would be expected to play a role in selectivity among the analytes. The second was a Phenyl

Hydride column, which can potentially provide additional selectivity between compounds through postulated π – π interactions between the phenyl group and an appropriate moiety from the analyte. The third was the UDC column, where shape differences between analytes could lead to secondary selectivity. Due to both the peak shape and selectivity difficulties associated with stability indicating tetracycline methods, it was of interest to investigate the use of silica hydride-based materials for the analyses. The silica hydride surface would be expected to minimize peak tailing issues while a comparison of the selectivities obtained from data using the three stationary phases would elucidate the roles of the secondary retention mechanisms.

Method and Materials

Reagents and Chemicals

Tetracycline hydrochloride (HCl), minocycline HCl, doxycycline HCl, and methacycline HCl were purchased from Sigma–Aldrich (St. Louis, MO, USA). 4-Epitetracycline HCl, 4-epianhydrotetracycline HCl, and anhydrotetracycline HCl were from Janssen Chimica (Beerse, Belgium). Trifluoroacetic acid of analytical grade and sodium hydroxide pellets were from J. T. Baker (Phillipsburg, NJ, USA). Concentrated hydrochloric acid was from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water (DI H₂O) was prepared on a Milli-Q™ purification system from Millipore (Bedford, MA, USA). Acetonitrile (HPLC grade) was obtained from EMD (Gibbstown, NJ, USA). Tetracycline HCl capsules (500 mg strength), minocycline HCl capsules (100 mg strength), and doxycycline hyclate tablets (100 mg strength) of USP grade were purchased from commercial sources.

Instrumentation

For HPLC analyses, a Hewlett–Packard (Palo Alto, CA, USA) 1100 HPLC system consisting of an autosampler, degasser, binary pump, and variable wavelength UV detector was used. The system was interfaced with Agilent Chemstation™ (Santa Clara, CA, USA) software. The analytical columns were packed with Bidentate C8™, Phenyl Hydride™ and UDC-Cholesterol™ stationary phases (MicroSolv Tech. Corp. Eatontown, NJ, USA). All three columns had dimensions of 4.6 mm (I.D.) × 75 mm, particle diameters of 4 μ m, and pore sizes of 100Å. Mobile phase A was DI H₂O + 0.1% trifluoroacetic acid and mobile phase B was acetonitrile + 0.1% trifluoroacetic acid. For all methods, the injection volume was 20 μ L, the column temperature was 25 °C, and the flow rate was 1.5 mL/min. The blank run (a sample mixture of 50% solvent A/ 50% solvent B) was subtracted from all the chromatographic data.

The tetracycline HCl capsule extracts were analyzed using a wavelength program of 360 nm from 0–9 min and 430 nm from 9–13 min. The gradient elution program used was Gradient 1 shown in Table 1. A three minute equilibration time was allowed between runs, leading to a total run time of 16 min. A given analyte was identified by comparing its retention time in a formulation extract with that of the appropriate standard.

The minocycline HCl capsule extracts were analyzed using a wavelength of 280 nm. The gradient elution program used was Gradient 2 shown in Table 1. A three minute equilibration time was allowed between runs, leading to a total run time of 16 min. The

minocycline peak was identified by comparing the retention time in the degraded formulation extract with that of the standard.

The doxycycline hyclate tablet extracts were analyzed using a wavelength of 350 nm. The gradient elution program used was Gradient 3 shown in Table 1. A three minute equilibration time was allowed between runs, leading to a total run time of 16 min. The doxycycline and methacycline peaks were identified by comparing the retention times in a formulation extract with those of the standards.

All three gradient methods were run with each of the three columns for a total of nine separation methods. All chromatographic measured and calculated values were obtained from Agilent Chemstation™ data analysis.

Sample Preparation

For individual standards of the analytes (tetracycline HCl, 4-epitetracycline HCl, 4-epianhydrotetracycline HCl, anhydrotetracycline HCl, minocycline HCl, doxycycline HCl, and methacycline HCl), 1000 mg/L stock solutions were prepared by adding 1.0 mg of the appropriate analyte to an autosampler vial and diluting with 1.0 mL of 50% 10 mM ammonium acetate/ 50% acetonitrile/ 0.1% 1 N NaOH. In the case of each stock solution, a 10 µL aliquot was taken and transferred to a new autosampler vial and diluted to 10 mg/L using this diluent. These working standards were then used for HPLC analysis (see 2.2.1).

The procedure for preparing the non-degraded formulation extracts was as follows: In the case of both the 500 mg strength tetracycline HCl and 100 mg strength minocycline HCl formulations, a capsule was opened and all of the contents were added to a 50 mL volumetric flask. For the doxycycline hyclate formulation, a tablet was crushed with a mortar and pestle and then added to a 50 mL volumetric flask. Then in each case, an aliquot of 25 mL diluent (50% 10 mM ammonium acetate/ 50% acetonitrile/ 0.1% 1 N NaOH) was added. After sonicating the flask for 10 min in a Symphony™ ultrasonic bath (VWR, West Chester, PA, USA), it was diluted to mark with this diluent. Then a portion of the solution was filtered through a 0.45 µm nylon HPLC syringe filter (MicroSolv Technology Corp. Eatontown, NJ, USA). The solutions were diluted to final concentrations of 10 mg/L each. These solutions were used for HPLC analysis with each of the three columns.

The degraded formulation extracts were prepared as follows: In the case of both the 500 mg strength tetracycline HCl and 100 mg strength minocycline HCl formulations, a capsule was opened and all of the contents were added to a 50 mL volumetric flask. For the doxycycline hyclate formulation, a tablet was crushed with a mortar and pestle and then added to a 50 mL volumetric flask. Then in each case, an aliquot of 25 mL diluent (50% 1 N HCl/ 50% solvent B) was added. After sonicating the flask for 10 min in a Symphony™ ultrasonic bath, it was diluted to mark with this diluent. Then a portion of the solution was filtered through a 0.45 µm nylon HPLC syringe filter. It was heated in a Chrom™ mini dry bath at 80 °C for 30 min (MicroSolv Technology Corp. Eatontown, NJ, USA). Then the solutions were diluted to final concentrations of 10 mg/L each. These solutions were used for HPLC analysis with each of the three columns.

Results and Discussion

The pH of the mobile phase is frequently an important parameter in HPLC method development since it dictates the ionization state of the analytes and/or the stationary phase. Tetracycline HCl has three reported pK_as of 3.32, 7.78, and 9.58.^[13] According to Stephens et al., the three pK_as can be assigned to the tricarbonyl system, the protonated dimethylamine cation, and the β -diketone system respectively.^[14] A 0.1% trifluoroacetic acid additive was chosen for the mobile phase for two reasons. First, the enolic and phenolic moieties will be fully protonated (*i.e.* neutral) at this pH, thereby increasing analyte hydrophobicity and favoring greater selectivity between the active pharmaceutical ingredient (API) and its degradants. Although the dimethylamine group will be charged, it is not feasible to operate at a pH where this moiety would be neutral because of the high dissolution rate of silica in this pH range.^[15] Another reason for choosing the 0.1% trifluoroacetic acid was that any residual silanols on the column will be fully protonated, thus minimizing silanolic interactions. Indeed, superior peak shapes were observed with methods using acidic mobile phase additives when compared to those using 10 mM ammonium acetate or formate (data not shown).

For the tetracycline samples, the wavelengths selected for the analysis are local absorbance maxima of the analytes.^[1] These wavelengths were used because they are both selective for the analytes and produce minimal background interference from the gradient. For higher sensitivity, tetracycline's global absorbance maximum of 267 nm could be selected for the entire analysis, as this wavelength is also close to the global maximum of the anhydro form.

Chromatographic data of the degraded and non-degraded minocycline HCl formulation extracts using the Phenyl Hydride column is shown in Figure 2. With acid and heating conditions of the formulation extract, significant epimerization could be observed from the separation of the two isomers in the chromatogram overlays shown in Figure 2A. Although a standard of 4-epiminocycline could not be obtained for unequivocal identification of the degradant, epimerization at the C4 position of minocycline under acidic conditions has been described elsewhere.^[16] Since this epimerization occurs readily in acidic conditions, an ammonium acetate based diluent was selected for the non-degraded extract. However, degradation was still observed using only 10 mM ammonium acetate in the mobile phase (data not shown). With the addition of a small amount of dilute sodium hydroxide to the diluent, no epimer peak was observed in the chromatogram for the non-degraded extract (Figure 2B).

Data obtained for the minocycline samples using the Bidentate C8 column is shown in Figure 3. For the degraded extract in Figure 3A, a shoulder can be seen on the minocycline peak, indicating partial co-elution of the API with the degradant. This shoulder is not observed in the non-degraded extract in Figure 3B, showing that this is a selectivity issue and not a peak shape issue. Likewise, the data obtained using the UDC column in Figure 4 showed no indication that the API and degradant were separated. In both the degraded (Figure 4A) and non-degraded sample (Figure 4B), only one peak was observed. Data from both columns illustrate how only the Phenyl Hydride column was capable of obtaining a separation of the API from the epimer degradant under these conditions. This suggests that

π – π interactions from the Phenyl Hydride moiety may have been primarily responsible for differentiating between the epimers.

Measured and calculated chromatographic parameters for the degraded minocycline extract using each column are shown in Table 2. The tailing factors of the compounds show good peak symmetry for all three columns and are within the acceptable range of 0.9–2.0 specified by the United States Pharmacopeia–National Formulary (USP–NF) for assay of minocycline HCl capsules.^[17] The symmetrical peak shapes for these compounds suggest that the silica hydride surface is not highly susceptible to silanolic interactions with basic solutes such as minocycline and its degradant. The repeatability of the method using the columns is demonstrated by the low retention time %RSDs for the two analytes over five consecutive injections.

Tetracycline HCl capsules were analyzed in a similar manner. In addition to the 4-epimer of the API, the anhydro degradant and its 4-epimer must also be separated. Figure 5 shows the chromatograms obtained from both the degraded and non-degraded tetracycline HCl capsule extracts using the Phenyl Hydride column. As can be seen in Figure 5A, the API is separated from all three degradants. The elution order differs from the data obtained from the minocycline degradation in that the *epi* form elutes first for both tetracycline and its anhydro form. Under non-degrading conditions (Figure 5B), the dehydration product and its epimer were not observed but the 4-epimer of tetracycline was still present in a small amount. A number of other mobile phases and diluents were investigated in method development, but none of those studied could entirely prevent the reversible formation of 4-epitetracycline (data not shown). However, the most important aspect of the analysis for non-degraded tetracycline is that the method conditions do not cause the formation of 4-epianhydrotetracycline, which the USP monograph specifies must be present in no more than 3.0% concentration.^[18] Consequently, if a 4-epianhydrotetracycline peak is observed for a tetracycline HCl capsule extract under these non-degrading conditions, it can be shown that the formulation degraded prior to sample preparation and analysis.

In Figure 6, data from the tetracycline extracts using the Bidentate C8 column is shown. The degraded extract in Figure 6A illustrates improved epimer separation than for minocycline, where co-elution was observed. In this case, hydrophobic interactions from the Bidentate C8 column provided adequate selectivity between epimers. The separations using both the Bidentate C8 and the UDC (Figure 7) were superior to those of the Phenyl Hydride column, suggesting that the secondary interactions of the Phenyl Hydride did not appear to play as significant a role as they did with minocycline epimers.

Table 3 shows the average measured and calculated chromatographic values for the degraded tetracycline HCl extract using the columns. Unlike the case of minocycline, the API was adequately resolved from its degradants using all three columns. In particular, the resolution between tetracycline and 4-epianhydrotetracycline exceeds the USP requirement of $R_s \geq 1.2$ in all three cases.^[18] The data also show that use of the UDC column resulted in the highest selectivity among the compounds. This suggests that shape selectivity may have played a beneficial role in the separation. The columns all exhibit excellent stability from the run-to-run precision of analyte retention times; the calculated %RSD data of five

consecutive runs demonstrates that the methods exceed the USP criterion of %RSD 2.0.^[18]

Finally, doxycycline hyclate tablet extracts were analyzed with the three columns as well. Three degradants were observed using each column. Methacycline could be identified from a standard, but the other two degradants could not be unequivocally identified. Based on the literature descriptions of doxycycline degradation, it is likely that they are the C4 and C6 epimers of doxycycline.^[7, 8] In Figure 8, separation was obtained between the analytes using the Bidentate C8 column, but resolution between the critical pair (methacycline and doxycycline) could be improved. Using the Phenyl Hydride column (Figure 9), resolution between the critical pair was slightly higher than with the Bidentate C8 column. This may be expected since methacycline and doxycycline differ only by a π bond, and additional π - π interactions could be responsible for the superior selectivity. Use of the UDC column produced the best separation between this peak pair of all three columns (Figure 10A), suggesting that the secondary separation attributes of the column are not limited to isomers.

Calculated data using all three columns for doxycycline hyclate tablet extracts is presented in Table 4. The USP assay method for doxycycline requires a resolution of not less than 3.0 between doxycycline and 4-epidoxycycline, a tailing factor of not more than 2.0 for the API peak, and a %RSD of not more than 2.0%.^[9] The data in Table 4 meet these criteria for all three columns. Although it is not known whether 4-epidoxycycline is degradant 1 or 2, sufficient resolution with respect to doxycycline is obtained in either case.

Conclusion

The data show how the use of silica hydride-based columns can be beneficial for HPLC stability testing of tetracyclines and their degradation products. With some degradants, selectivity believed to arise from π - π interactions were the most important for separation while differences in shape provided by UDC Cholesterol column, may have been responsible for the superior separation of others. The data illustrate how secondary interactions from these column types may be used to obtain better resolution than with hydrophobic selectivity alone. Less robust techniques such as ion-pairing agents were not necessary using the silica hydride-based materials since adequate peak shapes could be obtained with only the use of TFA as a mobile phase additive.

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Figure 1.

Structures of (A) tetracycline, (B) 4-epitetracycline, (C) anhydrotetracycline, (D) 4-epianhydrotetracycline, (E) doxycycline, (F) 4-epidoxycycline, (G) methacycline, (H) 6-epidoxycycline, (I) minocycline, and (J) 4-epiminocycline.

Figure 2.

Chromatograms obtained from minocycline HCl capsule extracts using the Phenyl Hydride column. (A) Five-run overlay of the degraded extract. (B) Single chromatogram of non-degraded extract. Peak identities are as follows: 1) minocycline and 2) unknown degradant. Sample preparations for the non-degraded and degraded extracts are described in the Sample Preparation section. Method conditions are described in the Instrumentation section.

Figure 3.

Chromatograms obtained from minocycline HCl capsule extracts using the Bidentate C8 column. (A) Five-run overlay of the degraded extract. (B) Single chromatogram of non-degraded extract. Peak identities are as follows: 1) minocycline and 2) unknown degradant. Sample preparation, method conditions and peak identities and are the same as Fig. 2.

Figure 4.

Chromatograms obtained from minocycline HCl capsule extracts using the UDC column. (A) Five-run overlay of the degraded extract. (B) Single chromatogram of non-degraded extract. Peak identities are as follows: 1) minocycline and 2) unknown degradant. Sample preparation, method conditions and peak identities are the same as Fig. 2.

Figure 5.

Chromatograms obtained from tetracycline HCl capsule extracts using the Phenyl Hydride column. (A) Five-run overlay of the degraded extract. (B) Single chromatogram of non-degraded extract. Peak identities are as follows: 1) 4-epitetracycline, 2) tetracycline, 3) 4-epianhydrotetracycline, and 4) anhydrotetracycline. Sample preparations for the non-degraded and degraded extracts are described in the Sample Preparation section. Method conditions are described in the Instrumentation section.

Figure 6. Chromatograms obtained from tetracycline HCl capsule extracts using the Bidentate C8 column. (A) Five-run overlay of the degraded extract. (B) Single chromatogram of non-degraded extract. Peak identities and sample preparation are the same as Fig. 5.

Figure 7. Chromatograms obtained from tetracycline HCl capsule extracts using the UDC column. (A) Five-run overlay of the degraded extract. (B) Single chromatogram of non-degraded extract. Peak identities and sample preparation are the same as Fig. 5.

Figure 8.

Chromatograms obtained from doxycycline hyclate tablet extracts using the Bidentate C8 column. (A) Five-run overlay of the degraded extract. (B) Single chromatogram of non-degraded extract. Peak identities are as follows: 1) unknown degradant, 2) unknown degradant, 3) methacycline, and 4) doxycycline. Sample preparations for the non-degraded and degraded extracts are described in the Sample Preparation section. Method conditions are described in the Instrumentation section.

Figure 9.

Chromatograms obtained from doxycycline hyclate tablet extracts using the Phenyl Hydride column. (A) Five-run overlay of the degraded extract. (B) Single chromatogram of non-degraded extract. Peak identities and method conditions are the same as in Fig. 8.

Figure 10.

Chromatograms obtained from doxycycline hyclate tablet extracts using the UDC column. (A) Five-run overlay of the degraded extract. (B) Single chromatogram of non-degraded extract. Peak identities and method conditions are the same as in Fig. 8.

Table 1

Gradient elution programs used in the separations

Gradient 1		Gradient 2		Gradient 3	
time (min.)	%B	time (min.)	%B	time (min.)	%B
0	5	0	5	0	5
12	35	12	10	12	30
13	5	13	5	13	5

Table 2
Average measured and calculated chromatographic values for degraded minocycline capsule extract data*

	minocycline	degradant
Phenyl Hydride		
t_R (min.)	10.69	11.88
%RSD	0.18	0.14
T_f	1.16	0.98
α	1.11	
R_s	1.51	
UDC		
t_R (min.)	10.35	10.35
%RSD	0.23	0.23
T_f	1.01	
α	0	
R_s	0	
Bidentate C8		
t_R (min.)	9.38	9.38
%RSD	0.24	0.24
T_f^{**}	1.10	
α	0	
R_s	0	

* $n = 5$, t_R = retention time, %RSD = percent relative standard deviation of retention time, T_f = tailing factor, α = selectivity to next peak, R_s = resolution to next peak

** Non-degraded sample was used for calculation due to partial peak co-elution in degraded sample data.

Table 3
Average measured and calculated chromatographic values for degraded tetracycline capsule extract data*

	4-eTC	TC	4-eATC	ATC
Phenyl Hydride				
t_R (min.)	6.38	6.97	10.69	11.30
%RSD	0.27	0.22	0.14	0.11
T_f	0.93	0.98	1.16	1.24
α	1.09	1.55	1.06	
R_s	1.99	14.7	2.73	
UDC				
t_R (min.)	6.39	7.28	11.75	12.60
%RSD	0.41	0.38	0.23	0.23
T_f	0.93	1.01	1.14	1.19
α	1.14	1.63	1.07	
R_s	2.83	16.4	3.51	
Bidentate C8				
t_R (min.)	6.04	6.82	10.62	11.21
%RSD	0.11	0.09	0.02	0.17
T_f	0.80	0.85	1.10	1.15
α	1.13	1.58	1.06	
R_s	2.76	18.3	3.62	

* $n = 5$, t_R = retention time, %RSD = percent relative standard deviation of retention time, T_f = tailing factor, α = selectivity to next peak, R_s = resolution to next peak, 4-eTC = 4-epitetracycline, TC = tetracycline, 4-eATC = 4-epianhydrotetracycline, ATC = anhydrotetracycline.

Table 4
Average measured and calculated chromatographic values for degraded doxycycline hyclate capsule extract data*

	degradant 1	degradant 2	methacycline	doxycycline
Phenyl Hydride				
t_R (min.)	6.94	7.63	10.21	10.75
%RSD	0.11	0.11	0.06	0.05
T_f	1.20	1.02	1.10	1.19
α	1.11	1.37	1.06	
R_s	2.10	7.14	1.57	
UDC				
t_R (min.)	6.53	7.58	10.72	11.58
%RSD	0.15	0.13	0.09	0.08
T_f	1.04	1.04	0.93	1.02
α	1.18	1.45	1.08	
R_s	2.46	9.65	2.87	
Bidentate C8				
t_R (min.)	6.33	7.26	10.57	10.88
%RSD	0.04	0.04	0.04	0.05
T_f	0.85	1.20	1.24	0.96
α	1.16	1.50	1.03	
R_s	3.11	12.31	1.29	

* $n = 5$, t_R = retention time, %RSD = percent relative standard deviation of retention time, T_f = tailing factor, α = selectivity to next peak, R_s = resolution to next peak