Column Robustness Case Study for a Liquid Chromatographic Method Validated in Compliance with ICH, VICH, and GMP Guidelines

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Abstract

This article presents a case study in dealing with robustness investigations and attempts by our analytical laboratory to address these issues without sacrificing valuable time in revamping the method validation prior to submission. A liquid chromatographic method is developed for the analysis of a novel triazinetrione anticoccidial product. The method effectively separates the active pharmaceutical ingredient (API), impurities, and preservatives in the API and product formulation. For much of the validation, the method holds up to the rigorous guidelines of the International Conference of Harmonization, the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products, and the Good Manufacturing Practices. However, in analyzing a base-degraded sample one of the impurity peaks yields inconsistent retention times (RTs) during a series of injections. When switching the system to another analytical column from the same supplier, this impurity peak elutes at a different retention window and the remaining peaks in the chromatographic profile remain essentially the same. This RT variation of a single peak in the chromatographic profile is observed with additional columns from the same supplier and from different manufacturing lots. This suitability problem is not encountered with the columns used in the method development stage. The method no longer meets the robustness criteria established for pharmaceutical methods. An investigation is commenced and it is discovered that with the addition of tetrabutylammonium hydroxide to the mobile phases, the impurity peak gives a consistent RT in relation to the active peak. The peak shows comparable RTs relative to that of the API peak with columns of different silica lots and bond lots. All peaks, including the aforementioned impurity peak, are well-resolved under the revised high-performance liquid chromatographic conditions. This temporary solution enables continued submission work for FDA, but the robustness of this method is still a concern. After further investigation, it is determined that inhomogeneity of the active sites on the column's stationary phase is the likely culprit. Fortunately, a new column is found to be more suitable for this method and a column qualification study is initiated.

Introduction

Running chromatographic method validation investigations is a routine practice in the pharmaceutical industry. Compendial guidelines are readily available from the International Conference on Harmonization (ICH), the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), and Good Manufacturing Practices (GMP) as interpreted by the U.S. Food and Drug Administration (FDA) and United States Pharmacoepia (USP) (1–7). Often, these method validations are quite important to the company developing a new drug application. The analytical methods employed set the foundation for data collection and ultimately the FDA approval of a new drug. In today's fast-paced submission schedules and bottom-line economics, method validations are under strict timelines imposed by management. When these timelines are upset, the whole drug submission process can be delayed. Thus, the analytical laboratory tries to operate with minimal error and maximum efficiency. When some aspect of the validation plan goes awry, it is often a time of major stress and concern.

Such was the case recently during a validation study that was initiated in our laboratory to qualify a liquid chromatographic (LC) method as a stability-indicating method for the analysis of a novel triazinetrione anticoccidial product. The method employed a bonded alkyl amide reverse-phase column with endcapping to achieve its most efficient separation. In accordance with pharmaceutical industry norms, the validation tested for specificity, precision (repeatability and intermediate precision), linearity, range, accuracy, robustness, and the quantitation limit of the triazinetrione anticoccidial product as well as the degradation products of the API. During this validation project it was discovered that a minor impurity was not illustrating acceptable method ruggedness. This ruggedness was not suspect in the method development and preliminary sample analysis investigations. The retention time (RT) for this impurity not only changed between column lots, but also did not hold constant on individual columns. Under the allotted time constraints for the project that were imposed by management, it was uncertain what we at the

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laboratory were going to do. Although the issue was of less concern in that it involved an impurity, the ruggedness of the column was in question. Fortunately, we did not have to abandon the current method and add significant delay for the regulatory submission of this drug. We acted quickly to investigate alternates, thus allowing the validation to finish with minimal interruption to the project timeline.

The key to any such remedy in this scenario is that the retention parameters for the major components and chromatographic profile retain their integrity under laboratory conditions. We determined that a minor addition to the mobile phase of approximately 5mM tetrabutylammonium hydroxide (TBAH) minimized the earlier ruggedness problem. TBAH is a common ion-pairing reagent. It was added solely to control the ruggedness of this problem-causing minor impurity peak. The major components of the chromatographic profile held their RTs within individual columns and those of the minor impurity within no interference for differing columns. No significant effect upon the validation parameters that were already established for the LC method was detected and no other effect upon the chromatographic profile or response was noted. With this solution in effect, the validation project was completed and the project timeline met.

We at the analytical laboratory realized that the ion-pair solution was temporary. The method worked, but the concerns about the ruggedness of the method remained. Because the method validation was now back on the project timeline, we were free to proceed to examine alternative columns and conditions. It was later found that a switch to a specific octadecyl stationary phase provided similar profile characteristics to the alkyl amide used in the validation, with the added bonus of removing the ion-pair reagent.

Experimental

A high-performance liquid chromatograph (HPLC) equipped with a UV detector was used. The mobile phase was a binary gradient consisting of ammonium (pH 5), acetate buffer, and methanol. The analytical column was a C16 alkyl amide (3 µm, 150 × 4.6 mm), and the guard column was also a C16 alkyl amide (3 µm, 20 × 4.0 mm). The column oven was set at 40°C ± 2°C, and the injector was 10 µL. All chemicals used were of HPLC grade.

Results and Discussion

The C16 alkyl amide columns provided adequate resolution for the API, formulation components as well as impurity peaks for production release, and stability studies. However, during the course of the method validation, impurity peak levels increased significantly under stressed conditions and the determination of those peaks became more important. It was discovered that one of the impurity peaks showed shifting RTs. Within sequential injections of a single sample, this minor impurity peak (annotated as Imp X) gave shorter and shorter RTs from injection to injection while the rest of the peaks kept relatively consistent retention.

The Imp X peak also showed variable RTs when different C16 alkyl amide columns were used. Thus, there were two issues associated with the Imp X peak: (*a*) the peak gave a shorter and shorter RT during a sequence of injections and (*b*) the relative retention time (RRT) varied from one column to another.

After reviewing the data collected in previous work it was discovered that the Imp X peak actually existed in the API as well as in product samples. Although present at very low levels, this minor peak raised a concern for proper peak identification and integration. This shift in RRT also raised an issue of the method ruggedness.

The migration of absolute RTs between sequences is understandable because of the minor variation in mobile phase preparation each time. A shift in RTs may also be a result of lot inconsistency of packing material or bond phase in column production. However, it was an unusual situation that one particular peak shifted towards a certain direction in a series of injections while all other peaks were kept relatively stable. A drifting peak not only causes uncertainty in component identification but may also interfere with proper identification and integration of other peaks.

It was concluded that under the original HPLC conditions the C16 alkyl amide columns may face difficulties in analyzing samples that are potentially degraded from stability studies or under stressed conditions.

Attempts of column conditioning

A nonequilibrated column may result in peak drift. Therefore, the first attempt to resolve this problem was to further condition the columns. It was found that the Imp X peak tended to slow down its RT shift on an extensively used column. By injecting an excessive amount of API onto the column, it was in hopes that the Imp X peak would stop moving. However, the peak failed to give a consistent RT with a column injected with 0.2 mg of the drug (approximately 60 times the normal level).

Mobile phase modifiers were investigated to resolve this problem. Trifluoroacetic acid (TFA) and triethylamine (TEA) were each added to the mobile phases and gradually passed through a column at a slow flow rate overnight before samples were analyzed. Although TFA failed to stabilize the Imp X peak, TEA was able to give consistent RT for the Imp X peak. However, the peak was found to elute at the front of the API peak with at least one of the columns tested, which was a significant change in the chromatographic profile.

Another possible cause for a shifting peak would be the presence of a trace amount of metal ions, which might affect the elution of Imp X. Metal chelation has previously been used to address this effect. After a column was purged with an ethylenediamine tetraacetic acid–disodium salt solution (a chelating agent for removing metal ions), a base-decomposed sample was analyzed on the system. The Imp X peak gave less than a 0.1-min shift in RT in reference to the API peak. It was encouraging, however, under this condition that the Imp X peak was later found to coelute with an aldehyde impurity.

Although these attempts failed, each condition did not alter the chromatographic profile of the major components. Under these modified conditions, the API and the preservatives eluted at RTs

similar to the original method assays that had no significant column conditioning.

Attempts of other types of columns

During method development, several LC columns were tested in order to optimize the chromatographic separation. The C16 alkyl amide provided a superior separation for this API and product formulation. Therefore, we then looked toward other LC columns to maintain the profile as it generated with the C16 alkyl amide column. It was hoped that another type or manufacturer of columns could replace the C16 alkyl amide column with minimal disruption to the project timeline. A column with an equivalent stationary phase to the C16 alkyl amide was obtained from another manufacturer. The major drug component showed a broad peak with significant tailing for a 5-µm particle size column. The 3-µm particle size column provided the same chromatographic profile as the original C16 alkyl amide columns; however, it failed to stabilize the Imp X peak within a defined time window. A phenylhexyl column changed the selectivity patterns of the chromatogram and was later abandoned. At this point in the investigation there was no advantage in changing to other types of columns from the original C16 alkyl amide column. The other columns required method reoptimization and would cause potential project delays.

Addition of ion-pair reagents

The original C16 alkyl amide column was advertized to be end-capped and used without the need of ion-pair reagents. Proponents for these types of columns claim that the active sites are shielded from the surface chemistry of the silica. However, at this point we suspected that the blockage of active sites on the stationary-phase original C16 alkyl amide column was incomplete, causing an interaction between the remaining active spots and Imp X. Therefore, using certain ion-pair reagents in the mobile phase was considered in an attempt to reduce or control this interaction. The addition of heptanesulfonate to the buffer was first tried. At a concentration of 15mM 1-heptanesulfonic acid in the buffer, the retention time of the IMP X peak relative to that of the API peak appeared to remain consistent.

A systematic study was initiated using the C16 alkyl amide column with and without the addition of heptanesulfonate to the mobile phases, the C16 alkyl amide column of the second manufacturer, and the phenylhexyl column without using the ion-pair reagent. Standards and samples were run with full system suitability checks using these columns on four HPLC systems. The results clearly indicated that the addition of heptanesulfonate provided a consistent RT for the Imp X peak. The chromatograms of a base-degraded sample are given in Figure 1.

Although the addition of heptanesulfonate kept the Imp X peak consistently within an RT window without interfering with other peaks of API samples, it failed to give a baseline separation for a formulation component (preservative 2) and one of its adjacent peaks. The problem was considered serious because the recovery of a formulation component is a critical parameter in product sample analysis. Therefore, we decided to investigate the use of another ion-pair reagent.

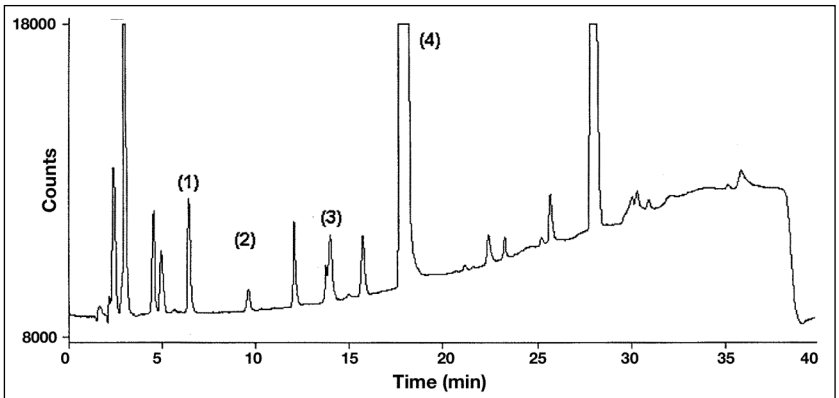

Figure 1. Representative chromatogram of a base-degraded API paste sample analyzed after the addition of 1-heptanesulfonic acid and sodium salt to the buffer: (1) preservative 1, (2) Imp X impurity, (3) preservative 2, and (4) API.

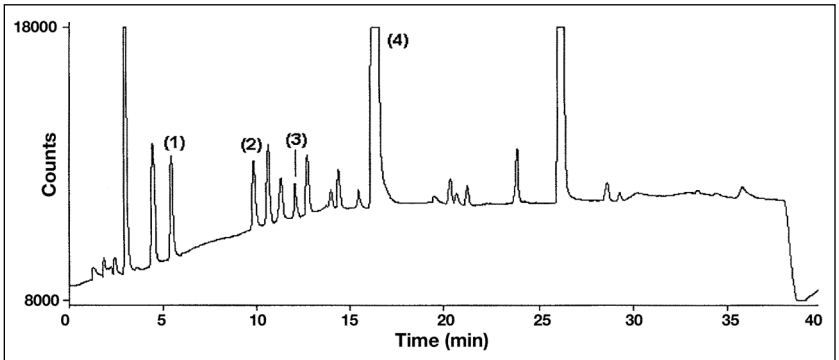

Figure 2. Representative chromatogram of a base-degraded API paste sample analyzed with the addition of TBAH to the buffer: (1) preservative 1, (2) Imp X impurity, (3) preservative 2, and (4) API.

TBAH was chosen to replace heptanesulfonate. The addition of 7.5mM TBAH in the buffer showed a baseline separation of all detectable peaks, including preservative 2 and its adjacent peaks (as illustrated in Figure 2). The migrating Imp X peak eluted consistently in an acceptable time window without interfering with any major component or impurity peaks. More importantly, the peak response factors of the API and formulation components remained essentially the same as those from the initial validation (Table I).

The comparable K values (mass of the standard multiplied by the purity and then divided by the area of the standard) in Table I indicate that the addition of TBAH has no impact on the quantitation of the API or formulation components. Because impurities are calculated using their response factors relative to API itself, there should be no effect upon the quantitation of the known impurities as well.

It should be pointed out that the chromatographic profile of the major components was not significantly changed as compared with those obtained in the absence of ion-pair reagents or column modifiers (Table II).

In order to verify this improved robustness, mobile phases with TBAH added were used on three C16 alkyl amide columns with different silica and bond phase lots. As seen in Table III, all

three columns provided stable RTs for the Imp X peak while maintaining suitable RT windows and the integrity of the other peaks.

In summary, RTs of the Imp X peak under various method development modifications are given in Table IV.

Effect upon the validation of the C16 alkyl amide column

Now that the robustness of the method was under control, a study was undertaken to determine the effect on the validation. A change was made to the mobile phase, but this change only affected the impurity peak in question. No significant effect upon the other components in the chromatographic profile was seen.

Table I. Response Factors of Major Peaks of Interest

	K value*		
	Drug	Preservative 1	Preservative 2
Initial validation	1.1×10^{-8}	3.8×10^{-7}	2.9×10^{-6}
With 7.5mM TBAH	1.1×10^{-8}	3.5×10^{-7}	2.8×10^{-6}

* K value = (mass of standard × purity) / (area of standard).

Table II. RRTs of Major Peaks of Interest

	RT (min)	RRT*	
	API	Preservative 1	Preservative 2
Initial validation	18.70	2.18	1.30
With 7.5mM TBAH	16.30	2.21	1.36

* RRT = (RT of antioxidant) / (RT of API)

Table III. RT Change of Imp X Peak

	RT (min)		
Column	Imp X	Drug	RRT
1	9.86	16.29	0.61
2	8.41	16.76	0.50
3	8.57	17.59	0.49

Table IV. RT and Peak Tailing Comparison on the Chosen C18 and C16 Alkyl Amide Columns

	C18			C16 alkyl amide		
Identity	RT (min)	RRT	USP tailing	RT (min)	RRT	USP tailing
Preservative 1	3.83	0.24	1.13	4.82	0.32	1.11
Imp X	5.58	0.35	1.09	7.69	0.51	1.86
Preservative 2	8.89	0.56	1.11	10.56	0.70	1.06
Alcohol impurity	9.52	0.60	0.98	9.11	0.60	1.13
Aldehyde impurity	10.00	0.63	0.95	9.78	0.65	1.68
Sulfoxide impurity	10.81	0.68	0.98	9.78	0.65	1.68
Desmethyl impurity	13.52	0.85	1.12	13.07	0.86	1.10
API	16.00	1.00	1.05	15.15	1.00	0.96
Ethyl impurity	21.43	1.34	1.00	19.92	1.32	1.31

The key point in a validation is to have established control of a method, which we believed we had. The validation protocol was amended to reflect this.

Relative response factors

The response factors for the known impurities relative to API were determined using the original C16 alkyl amide mobile phase. The investigation report confirmed that the addition of TBAH to the acetate buffer had no significant effect upon the previous response factors established. Therefore, the relative response factors did not need to be reestablished.

This equivalence was also to be verified through the intermediate precision investigation for the API.

Specificity

Check for interference. No change in the chromatographic profile was observed. Thus, a check for interferences did not need to be reestablished.

Accelerated degradation and peak purity. The investigation report compared the profiles for oxidative, base, and thermal degradation for both the API and the product components. The addition of TBAH to the acetate buffer had no significant effect upon the profile of the API or product components. Because the original data illustrated that the base, oxidative, and thermal degradation samples of the paste represent a worst-case scenario for both the API and product, the investigation was not duplicated.

Resolution and tailing factor. Because the addition of the ion-pair reagent tightened peak shape and provided increased resolu-

Table V. Resolution Comparison of the Chromatographic Profile of the C18 Column at Optimized Conditions to Those of the C18 and C16 Alkyl Amide Columns Using the Original Method Conditions

Compound pair	USP resolution (optimized condition)	USP resolution (original condition)
C18		
Preservative 1–Imp X	13.74	8.27
Imp X–preservative 2	15.27	13.60
Preservative 2–alcohol impurity	4.22	2.40
Alcohol impurity–aldehyde impurity	1.62	1.51
Aldehyde impurity–sulfoxide impurity	1.34	0.95
Sulfoxide impurity–desmethyl impurity	10.29	9.40
Desmethyl impurity–API	8.90	8.40
API–ethyl impurity	18.44	17.80
C16 alkyl amide		
Preservative 1–Imp X		7.67
Imp X–preservative 2		3.63
Preservative 2–alcohol impurity		2.11
Aldehyde impurity–sulfoxide impurity		coelute
Sulfoxide impurity–preservative 2		2.40
Preservative 2–desmethyl impurity		11.21
Desmethyl impurity–API		9.15
API–ethyl impurity		19.50

tion, we established resolution and tailing factor specifications for the new mobile phase.

Linearity and range. The system linearity and range for the API and formulation components were established using the original mobile phase. Instead of repeating this study, a system suitability check at 80% and 120% and the relative response investigation were used to confirm that the addition of TBAH to the acetate buffer had not altered the response of the chromatographic profile. Therefore, linearity was not reestablished.

Accuracy, precision, and robustness. The accuracy, precision, and robustness of the method with respect to the quantitation of the API and the formulation components were determined using a mobile phase containing the TBAH ion-pair reagent. The accuracy of the method for the quantitation of the degradants detected was inferred from the criteria for precision, linearity, and specificity with respect to the major drug impurities.

Feasibility of changing LC columns in the analysis of the API

The C16 alkyl amide column provided adequate resolution for the API, formulation components, and impurity peaks for release and stability assays. However, it was found that TBAH had to be added to the mobile phases solely to minimize a demonstrated shifting of RTs for a single impurity peak (Imp X) from injection to injection and with varying column lots.

The manufacturer confirmed that the C16 alkyl amide had this "moving peak problem". Again, it was postulated that there are two possible causes for the "moving peak problem". If the silica surface of the packing material is not completely covered by the bonded stationary phases, the residual silanol groups might interact with the analyte. Secondly, the specific amide functional group on the C16 alkyl amide bonded phase can interact with the impurity. Although adding ion-pairing reagents to some extent solved this problem, it was not the ultimate solution. The goal was to find a more viable solution to this ruggedness problem, such as finding an alternative HPLC column.

In regards to alternate columns, the aim of this post-development column investigation was to retain the chromatographic integrity of the C16 alkyl amide profile on a more robust column.

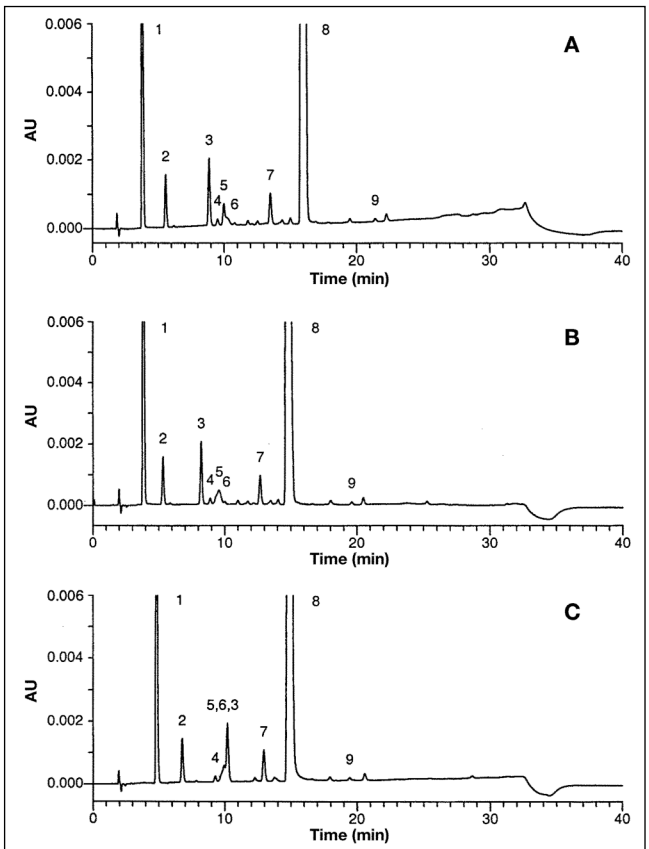

Figure 3. Comparison of the elution profiles for the API on (A) alternative C18, (B) C8, and (C) RP-amide C16 columns: (1) preservative 1, (2) Imp X impurity, (3) alcohol impurity, (4) aldehyde impurity, (5) sulfoxide impurity, (6) preservative 2, (7) desmethyl impurity, (8) API, and (9) ethyl impurity.

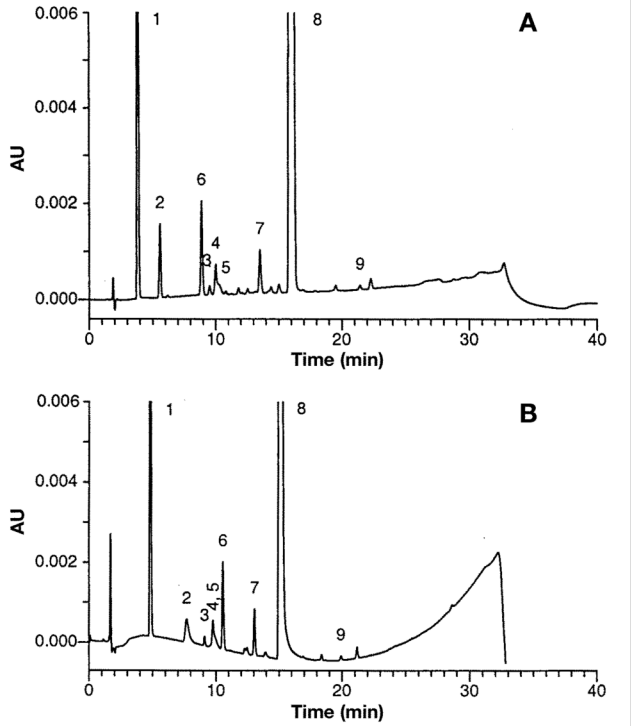

Figure 4. Elution profile comparison for the API on (A) the replacement C18 and (B) original C16 alkyl amide columns: (1) preservative 1, (2) Imp X impurity, (3) alcohol impurity, (4) aldehyde impurity, (5) sulfoxide impurity, (6) preservative 2, (7) desmethyl impurity, (8) API, and (9) ethyl impurity.

Table VI. Comparison of RT, RRT, and Resolution of the Chosen C18 Column Under Different Buffer pHs

	C18					
	RT (min)		RRT		USP resolution	
Identity	pH 4.5	pH 5.4	pH 4.5	pH 5.4	pH 4.5	pH 5.4
Preservative 1	4.80	4.78	0.25	0.25	–	–
Imp X	7.78	7.38	0.41	0.39	13.74	11.89
Preservative 2	11.54	11.52	0.60	0.61	15.27	16.69
Alcohol impurity	12.65	12.47	0.66	0.66	4.22	3.44
Aldehyde impurity	13.44	13.24	0.70	0.70	1.62	1.52
Sulfoxide impurity	14.10	13.92	0.74	0.73	1.34	1.17
Desmethyl impurity	16.78	16.44	0.87	0.86	10.29	8.00
API	19.18	19.00	1.00	1.00	8.90	9.33
Ethyl impurity	24.13	24.16	1.26	1.27	18.44	17.70

Several stationary-phase types were considered: a C18, C8, RP-amide C16, and a cyano phase. When comparing the chromatograms and the RRTs listed in Tables IV and V, the overall profiles seen in Figure 3 are similar with one minor variation. The preservative 2 peak eluted in front of the alcohol impurity, aldehyde impurity, and drug impurity peaks on the C18 column. However, on the C16 alkyl amide column the alcohol impurity, aldehyde impurity, and drug impurity peaks eluted in front of the preservative 2 peak. Clearly, the C18 column distinguished itself from the C16 alkyl amide column on several aspects: (*a*) without using TBAH as an ion-pair reagent in the mobile phase, the troublesome "moving peak" impurity eluted with a consistent RT and excellent peak shape; (*b*) peak shapes improved from the C16 alkyl amide column; and (*c*) resolution was superior for the C18 column. One of the drug impurity peaks separated from the aldehyde impurity peak, although it was not baseline separated (Figure 4).

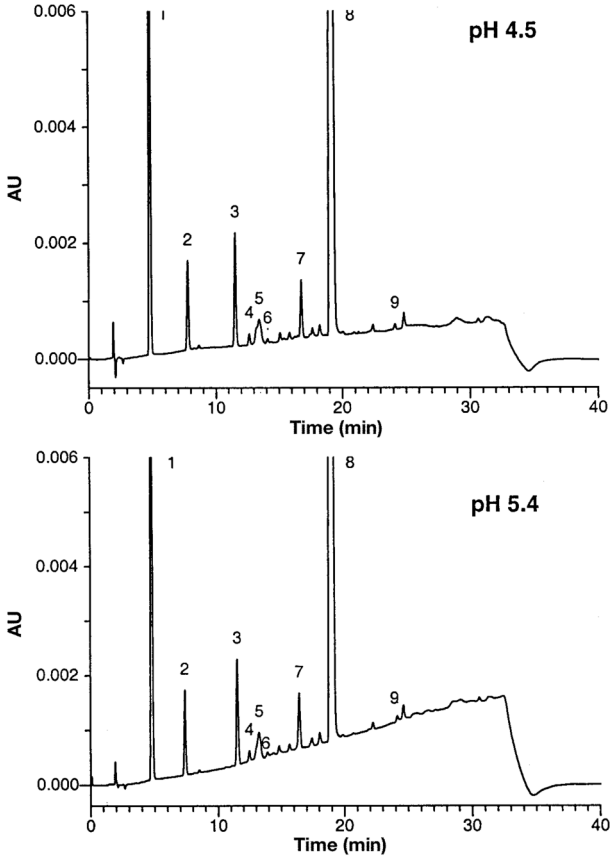

Figure 5. The effect of buffer pH on the elution profile under optimized conditions using the replacement C18 column: (1) preservative 1, (2) Imp X impurity, (3) alcohol impurity, (4) aldehyde impurity, (5) sulfoxide impurity, (6) preservative 2, (7) desmethyl impurity, (8) API, and (9) ethyl impurity.

The C16 alkyl amide column had an amide bond in the alkyl chain versus a pure alkyl chain on a C18. Therefore, the C16 alkyl amide column was less hydrophobic than most C18 columns. Despite the differences, the columns were both reversed-phase HPLC columns in nature. This is why their elution profiles were nearly equivalent, but with some selectivity difference.

Optimization of profile on a C18 column

It has been demonstrated that the chosen C18 column can replace the C16 alkyl amide column for the chromatographic profiling of the API. The resolution was further improved by adjusting the gradient to an optimized condition illustrated. The methanol concentration in solvent A was reduced while other parameters remained constant. The buffer pH in the initial method was 5.4. In this investigation, the pH of the ammonium acetate buffer used was 4.5. It was confirmed that this buffer pH range did not significantly affect the elution profile. A comparative study was carried out using the optimized gradient conditions and mobile phases prepared using ammonium acetate buffers at pH 4.5 and 5.4, respectively. The results in Table VI and Figure 5 demonstrate that the two profiles are almost identical and confirmed that there was no pH effect on the elution profile when lowering the buffer pH from 5.4 to 4.5.

Experiments were run to demonstrate that the elution profiles are very consistent from run to run and day to day on the same C18 column. Column lot-to-lot reproducibility of C18 was investigated by randomly selecting five columns from three different silica lots and five different bonding lots along with three different lots in a second laboratory. The RTs of each component are summarized in Table VII. The coefficient of variation for the RTs was 0.925 for the "moving peak" impurity, thus confirming the ruggedness of the chosen column.

Table VII. C18 Column Lot-to-Lot Reproducibility of the Method Under Optimized Conditions

	Column 1	Column 2	Column 3	Column 4	Column 5	RT (min)	Average deviation of RT	Standard %CV*
Preservative 1	4.64	4.64	4.72	4.66	4.76	4.68	0.046	0.98
Imp X impurity	7.52	7.67	7.56	7.47	7.62	7.57	0.070	0.92
Preservative 2	11.22	11.25	11.42	11.29	11.46	11.33	0.095	0.84
Alcohol impurity	12.32	12.37	12.50	12.37	12.54	12.42	0.084	0.68
Aldehyde impurity	13.11	13.16	13.29	13.18	13.32	13.21	0.080	0.61
Sulfoxide impurity	13.74	13.79	13.96	13.83	14.00	13.87	0.100	0.72
Desmethyl impurity	16.43	16.49	16.64	16.49	16.67	16.54	0.092	0.56
API	18.83	18.89	19.05	18.90	19.07	18.95	0.094	0.50
Ethyl impurity	23.81	23.84	24.02	23.88	24.04	23.92	0.096	0.40

* %CV, percent coefficient of variation.

Conclusion

It was demonstrated that the HPLC system is more rugged when the ion-pair reagent TBAH is added to mobile phases when analyzing the triazinetrione anticoccidial API, product, and their degraded samples. The mobile phase modification showed no impact on the quantitation of the API or formulation components.

This investigation confirmed that an acceptable alternative to the C16 alkyl amide column could be found and implemented. Better resolution was obtained on a specific C18 column after optimization without a corresponding increase in run time. Most importantly, the column ruggedness issue resulting from the C16 alkyl amide was eliminated and the method no longer required an ion-pairing reagent in the mobile phase. By switching the method to a specific C18 column, excellent ruggedness was once again achieved for the analysis of the anticoccidial API.

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