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A Study of the Critical Criteria for Analyte Stability in High-Temperature Liquid Chromatography

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There are three major impediments to the use of high-temperature ultrafast liquid chromatography. First, the stationary phase must be thermally stable. Over the past decade, a series of thermally stable, highly efficient stationary phases have been developed that can withstand temperatures exceeding 200 °C. Second, the temperature mismatch between the incoming eluent and the column must be minimized (<5 °C), because such a mismatch is a very serious cause of peak broadening, especially in ultrafast separations. The thermal mismatch problem can be significantly ameliorated at high column linear velocities by using narrow-bore columns (2.1-mm i.d.). Third, analytes that are exposed to high temperatures must be thermally stable *on the time scale of the chromatographic run*. We report here a study of the ability of a number of pharmaceuticals to withstand superambient temperatures on the time scale of fast separations. We propose criteria by which a particular analyte may be rejected as a candidate for high-temperature analysis, and we demonstrate that complex molecules are amenable to quantitation, even at temperatures in excess of 100 °C in the aqueous media. We also show that as the time an analyte spends on hot column decreases, the extent of on-column reaction decreases for those analytes that do react. Although the seminal work of Antia and Horvath addresses these issues from a theoretical perspective, we hope to further alleviate fear of the use of high temperatures in liquid chromatography through the empirical approach used here.

The advantages of doing liquid chromatography at high temperature (>100 °C) are clear. The 5−10-fold decrease in eluent viscosity[1,2] that comes from using temperatures of 200 °C and the concomitant increase in analyte diffusitivity[3] work in combination to dramatically improve analysis speed.[4−6] The lower viscosity decreases the pressure drop across the column and allows higher linear velocities as the limit of pump pressure is approached. Simultaneously, at high linear velocities where interphase mass transfer broadening dominates, the increased analyte diffusion coefficient improves efficiency.[4,7−9]

Antia and Horvath[6] predicted that a 20-fold improvement in analysis time would result when a column is operated at high temperature (150−200 °C). High temperatures are not commonly used in liquid chromatography, because there are some stringent requirements and implementation problems. First, the traditional silica-based stationary phases simply are not sufficiently stable at the temperatures needed to achieve the 20-fold improvement in analysis time. In fact, reversed-phase silica columns allow only a relatively small increase (30−40 °C) in temperature over ambient.[10−14] However, a series of ultrastable, high-efficiency, metal oxide stationary phases that are capable of withstanding superambient temperatures (>150 °C)[8,9,15,16] have been produced in this laboratory; such novel materials enable substantial improvements in analysis speed.

One of the chief problems of high-temperature LC is the design of a chromatographic system that minimizes thermal mismatch broadening[17,18] and balances heat transfer in the precolumn heater with extracolumn broadening in the heater.[4,5] In a previous paper,[5] we addressed and provided recommendations for minimizing the thermal mismatch broadening in high-temperature, high-speed liquid chromatography. In this study, we will focus on the issue of chromatographic reliability and analyte stability at high temperature.

On-column reactions have been observed, even under the typically mild conditions used in conventional LC. Indeed, various eluent additives, pH control, and the use of a chilled auto sampler are all measures intended to stabilize the analyte. Hydrolysis, oxidation, isomerization, and epimerization are the major types of

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chemical reactions that affect the stability of analytes and are considered undesirable.[19−22] As early as 1960, Keller and Giddings[23] showed that first-order reactions during separation would be detrimental to elution profiles. Horvath and co-workers[24] found that on-column catalysis by residual iron present in silica stationary phases actually increased the oxidation/reduction rate for substituted methoxyhydroquinones, as compared to the catalyzed rate in free solution. They also showed that proline dipeptides isomerize on the time scale of a typical chromatographic separation (2−3 min) to produce skewed, bimodal elution profiles.[25−27] They found that temperature, column length, pH, and flow velocity could be adjusted to improve the peak profile and decrease broadening due to the reaction. A potential disadvantage of the use of superambient temperatures in liquid chromatography stems from the possible acceleration of on-column reactions for thermally labile analytes.[6,20] We note in passing that exposure of analytes to low (pH = 2) and high (pH = 11−12) pH is rather common in RPLC, yet extremes in pH can and often do accelerate reaction kinetics.[21]

One key issue in determining if an on-column reaction will occur is the interrelationship between the analyte residence time on the column and the rate of the reaction under column conditions. The Damköhler number (*Da*) is a measure of this interrelationship. It is defined as the ratio of the on-column residence time to the time needed for reaction and is given by

$$Da = \frac{kL(1 + k')}{u_0} \qquad (1)$$

where k is the first-order (pseudo-first-order) reaction rate (s^{-1}), L is the column length (cm), k' is the retention factor, and u_0 is the interstitial linear velocity (cm/s). When there is no reaction ($Da \ll 1$) or the reaction is very fast ($Da > 50$), single peaks are expected. In HPLC, excessive peak broadening and irregularly shaped elution profiles are observed at intermediate values of the Damköhler number ($0.1 < Da < 50$).[24,25] In this case, the analyte decomposes *on the time scale of the separation* and the intermediates and fragments will differentially migrate over the length of the column to produce a broad bimodal elution profile. All things being equal, the Damköhler number is expected to improve as the column length decreases, the linear velocity increases, the retention factor decreases, and when the rate of the reaction is small.

Horvath et al. developed a theory to predict the effect that analysis time has on the extent of the on-column reaction broadening.[6,25,28] As the column temperature is increased, the on-column reaction rate increases, which suggests that lower temperatures are preferable. However, they showed that the Damköhler number could improve as the eluent temperature is raised *if the dramatic decrease in column residence time that results from use of the higher temperature proves to be more important than the increase in the reaction rate.* The critical parameter necessary to determine if there will be a decrease in the extent of the on-column reaction with increased speed is the difference between the enthalpy of transfer (ΔH°_{retn}) and the activation energy (ΔE_a) for the on-column reaction. Under favorable initial conditions, the analyte comes out faster than it reacts, and the on-column reaction will be unimportant at elevated temperature.

An on-column reaction could potentially harm the results of a quantitative separation because the peak shape, area, and height will likely change when the Damköhler numbers are intermediate. In this study, we establish criteria by which a particular analyte should be rejected as a candidate for high-temperature analysis because of an interference of the on-column reaction with quantitation. We believe the following to be the key issues in using elevated temperature methods: 1. Does it decrease the reliability of the analytical calibration curve? 2. Does it induce a significant intercept in the calibration curve? 3. Does it significantly diminish the sensitivity? 4. Does the on-column reaction distort the peak shape? 5. Does it introduce new peaks that interfere with the resolution/quantitation of the analyte or impurities? 6. Pursuant to an impurity analysis, does a high-temperature eluent cause chemical reactions that alter the concentration or produce products that would interfere with quantitation of the impurity? If the answers to these questions for a particular analyte are no, then the on-column reaction is not important and quantitation at high-temperature ought not be problematic.

In this study, we analyzed a small set of basic drugs under high-temperature, high-speed conditions. We determined that analyses at high temperature are as reliable as analyses under ambient conditions. We applied the proposed criteria to the analytes and showed that there can be quantitation problems when an analyte decomposes on the column. We also show that as the residence time of a thermally unstable analyte is decreased, the extent of the on-column reaction is decreased, and the problem of analyte stability is diminished.

EXPERIMENTAL SECTION

Instrument. All chromatographic experiments were conducted using a Hewlett-Packard 1090 chromatographic instrument controlled by Chemstation software (Hewlett-Packard, Wilmington, DE). The instrument was equipped with a ternary pump, a helium sparger, an auto sampler, a thermostated column compartment, and a diode-array UV detector. An additional prototype heating apparatus from Systec (Systec, Inc., New Brighton, MN) was used as a second heating zone. This device consists of a mobile phase preheater assembly and an insulating jacket and allows the column to be heated to 200 °C. A heat exchanger was placed between the two columns to cool the eluent before it encountered the ODS column (see Figure 1). An in-line filter with a 0.45-μm frit was inserted before the analytical columns to prevent

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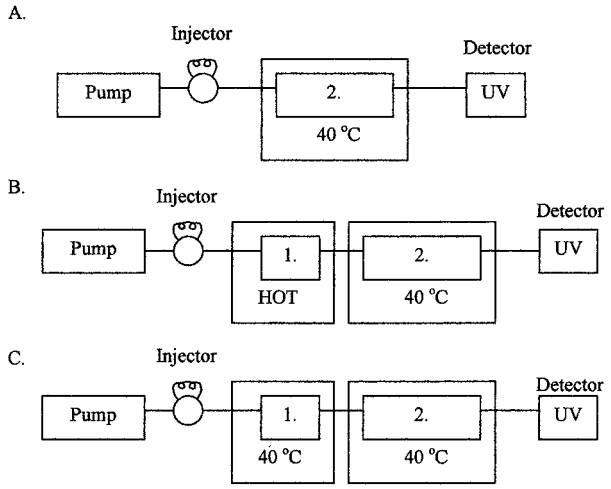

Figure 1. Block diagrams showing the column configuration sets (A, B, C) used for testing analyte stability on the timescale of a fast separation. 1. Column is 2.1 × 50 mm PBD-ZrO_2. 2. Column is 4.6 × 100 mm C18-SiO_2.

tubing or frit blockage. The flow rate was checked for accuracy using a small volumetric flask and stopwatch.

Reagents. All chemicals were reagent grade or better. The mobile phase contained ChromAR HPLC grade acetonitrile (ACN) from Mallinckrodt Chemical Co. (Paris, KY). HPLC water was obtained from a Barnsted Nanopure II deionizing system (Dubuque, IA) using an "organic-free" cartridge and a 0.2-μm filter. The water was subsequently boiled to remove carbon dioxide. All solvents were filtered through a 0.45-μm filter (Lida Manufacturing Corp., Kenosha, WI) before use. All analytes used in this study were purchased from Sigma-Aldrich (Sigma-Aldrich Chemical Co., Milwaukee, WI).

Analytical Columns. Polybutadiene-coated zirconia (PBD-ZrO_2) particles (3 μm) with a 3% (w/w) carbon loading were provided by ZirChrom (ZirChrom Separations, Inc., Anoka, MN) and packed into a 2.1 × 50 mm column (Isolation Technologies, Inc., Hopedale, MA) using a downward slurry method that was developed in-house. An additional 2.1 × 10 mm column packed with PBD-ZrO_2 (3 μm) was provided by ZirChrom Separations. A 4.6 × 150 mm ACE column packed with C-18 INERT particles (5 μm) was purchased from Mac-Mod Analytical, Inc. (Chadds Ford, PA). A 4.6 × 50 mm ACE column packed with C-18 INERT particles (5 μm) was used to determine system suitability.

Chromatographic Conditions. All measurements were taken at a flow rate of 1 mL/min. The eluent was 55:45 ACN/water (v/v) buffered with 20 mM ammonium dihydrogen phosphate ($NH_4H_2PO_4$) that was adjusted to pH = 7.02 with ammonium hydroxide. The eluent was sparged with helium to maintain precision and remove oxygen. The 4.6 × 150 mm column was thermostated at 40 °C. The temperature of the narrow-bore column (2.1 × 50 mm) was varied from 40 to 190 °C. The 2.1 × 10 mm column was operated at 190 °C. Detection was monitored at 254 and 230 nm. The retention factors (k') corrected for the extra-column volume were calculated from the following equation,

$$k' = \frac{t_R - t_0}{t_0 - t_{ex}} \tag{2}$$

where t_R is the retention time, t_0 is the dead time, and t_{ex} is the time that a solute spends outside the column (in the injector, connection tubing, and detector). The dead time was determined with uracil and acetone for the ODS and PBD-ZrO_2 column, respectively. The value of t_{ex} was determined by injecting uracil without any columns.

Standard Solutions. Standards of each analyte (alkylbenzenes, caffeine, anisole, methylbenzoate, and the basic drugs) were prepared in 100% acetonitrile. The concentrations ranged from 400 to 500 μg/mL. The volume injected was varied from 5 to 20 μL. It follows that the calibration range is ~2−10 μg. To construct calibration curves for the analytes, we plotted peak area vs amount injected (μg) and fit the data using linear least-squares regression. The statistics generated from the analysis of variance were used to determine if the intercept was significant. Regression line slopes and peak areas from each column configuration were compared using the Student's t-statistic that was calculated from

$$t = \frac{(\bar{x}_1 - \bar{x}_2)}{s}\sqrt{\frac{n_1 n_2}{n_1 + n_2}} \tag{3}$$

where $\bar{x}_1$ and $\bar{x}_2$ are the sample means, n_1 and n_2 are the sample size, and s is the pooled standard deviation.

Instrument Suitability. Since peak area reproducibility is a strong function of the instrumental parameters, we determined the suitability of our chromatographic system by constructing calibration curves (range: 0.25−25 μg) for caffeine standards prior to the collection of experimental data.

Peak area precision was better than 0.5% RSD, and retention time precision was typically better than 0.05% RSD. The calibration curves did not have significant intercepts (at the 90% confidence interval) and had correlation coefficients better than 0.999. Examination of the residuals showed that the deviation was random. The calibration curves also determined the upper and lower limits for which the analytical method has adequate precision and linearity, and the analyte standard solutions were prepared accordingly.

RESULTS AND DISCUSSION

Description of the Column Configurations. Figure 1 shows the system and column configurations that were used to test the chromatographic reliability at high temperatures and analyte stability on the time scale of a fast separation (<30 s). In column set A, the analyte is passed through an ODS analytical column held at 40 °C, and a four-point (four-concentration) calibration curve for each analyte was constructed. We assume that there is no degradation of the analyte at 40 °C. In column set B, a hot, narrow-bore column is used to emulate a high-temperature, high-speed separation. The analyte encounters the hot, narrow-bore column for a certain residence time and is then cooled to about 40 °C prior to entering the ODS column. Note that the column linear velocity of the narrow-bore column (1.3 cm/s) is about 5-fold greater than the wide-bore column, and it does not have the efficiency needed to resolve potential degradation products from the parent peak. The second conventional column operated at 40 °C is used to allow us to see what is happening to the analyte on the first hot column. A four-point calibration curve was constructed for each compound to determine the reliability of the calibration curve. The calibration curves for the first two column sets were

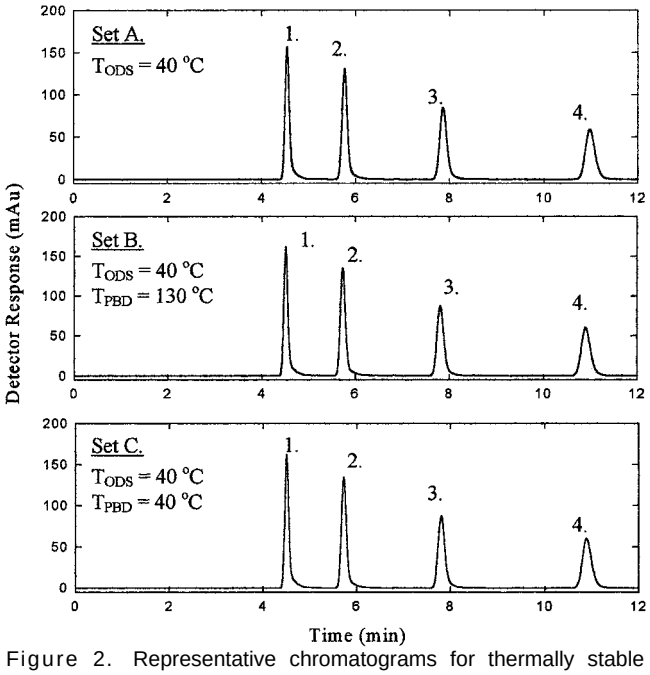

Figure 2. Representative chromatograms for thermally stable solutes on the three column sets (A, B, C). The flow rate was 1 mL/min, and the eluent was 55:45 ACN/water (v/v) buffered with 20 mM $NH_4H_2PO_4$ at pH 7.02. Column temperatures are specified with each chromatogram. The columns are the same as given in Figure 1. Detection wavelength was 254 nm. Analytes were 1, toluene; 2, ethylbenzene; 3, propylbenzene; and 4, butylbenzene.

compared to elucidate significant differences in correlations, intercepts, and sensitivities. Comparison of the chromatograms between these two sets will show the effect that the hot column had on resolution and peak shape. Column set C was used to show that the addition of the hot PBD-ZrO_2 column did not significantly change the calibration curve or peak shape characteristics. None of the analytes in this study showed a significant difference in peak shape or calibration curve characteristics solely as a result of the addition of the PBD-ZrO_2 column.

Method Controls. We employed a series of controls in order to show that the method was capable of determining the chromatographic reliability when the analytes are exposed to high temperature for a certain time period. Figure 2 shows chromatograms for thermally stable solutes (alkylbenzenes) on each column set. By inspection, the chromatograms show no new or split peaks due to analyte decomposition. Clearly, the peak shape is independent of the column set used.

The basic retention characteristic for an isothermal, isocratic set of columns connected in a series is additive.[29] Figure 2 shows that when the hot narrow-bore column was placed upstream of the analytical column, retention actually decreased (compare A and B). This can be attributed to inadequate cooling of the eluent between the columns, thereby increasing the average column temperature of the second "cool" column. Because the retention factor on the second column overwhelmingly dominates analyte retention on the narrow-bore column, a shift to shorter retention time is observed as the second column temperature increases. Retention on the narrow-bore column is minimal, because the linear velocity is high.

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Table 1. Correlation Coefficients from Calibration Curves[a] for Thermally Stable Analytes

	correlation coeff, R^2		
solutes	ODS[b] (40 °C)	ODS[b] (40 °C) + PBD[b] (40 °C)	ODS[b] (40 °C) + PBD[b] (130 °C)
toluene	0.9999	0.9999	0.9999
ethylbenzene	0.9999	0.9999	0.9995
propylbenzene	0.9999	0.9999	0.9996
butylbenzene	0.9999	0.9999	0.9995
ethylbenzoate	0.9993	0.9999	0.9998
anisole	0.9994	0.9999	0.9999

[a] $N = 4$, range = 2−10 μg. [b] Conditions and columns described in Figure 2.

Table 1 shows the calibration curve reliability for all thermally stable analytes tested. Using column sets A and B, four-point calibration curves were obtained for the alkylbenzenes plus anisole and ethylbenzoate. The correlation coefficients show that the chromatographic reliability is also independent of the column set used. The intercepts ranged from 2 to 5% relative to the target response, which is typical for most analytical methods.[12] The analytes were injected pre- and postsequence to show that the instrument and sample did not change over the sequence time. This control was used throughout the study. Comparison of the calibration curve slopes between column sets A and B via the *t*-statistic show that the slopes *are significantly different* at the 95% confidence level, even for those analytes that are thermally stable. The origin of this difference will be discussed later.

Once it was established that highly reliable separations can be done on each column set, we moved to testing the thermal stability and analytical reliability of basic drugs by HPLC at high temperature. Figure 3 shows the basic drugs that were used.

Effect of Hot Column on Peak Shape. We grouped the basic drugs to avoid peak overlap problems, and then optimized the eluent composition to balance resolution and retention time. Qualitative evidence for an on-column reaction can be obtained by observing the peak shape.[24,28,30] Figure 4 shows the separation of basic drugs 1−8 on column sets A and B. The figure shows that for basic drugs 1−4, both peak shape and resolution are maintained on each column set with no new peaks being formed. Since the analytes are strong bases, the peaks are strongly tailed as a result of the large extra-column volume and the interactions with the negatively charged residual silanols on the ODS column at pH 7. The separation of basic drugs 5−8 on both column sets is shown in the right panel. Peak shapes for analytes 6−8 appear to be unaffected by the presence of the hot column. The skewed elution profile of peak 5 is evidence of a reaction involving norpseudoephedrine. It is not our purpose to elucidate the reaction specifics, but it is important to understand that the reaction could involve a combination of other analytes, the eluent, and the stationary phase. Another small peak also emerged between peaks 6 and 7. The origin of this peak is not obvious and is indicative of further analyte decomposition. Clearly, norpseudoephedrine would violate criteria 4 and 5, and the column temperature should not exceed the temperature at which norpseudoephedrine will react.

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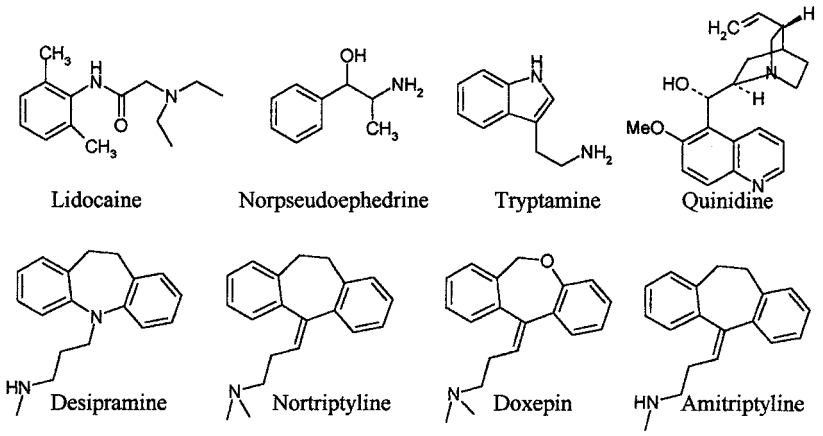

Figure 3. Structures of the basic drugs.

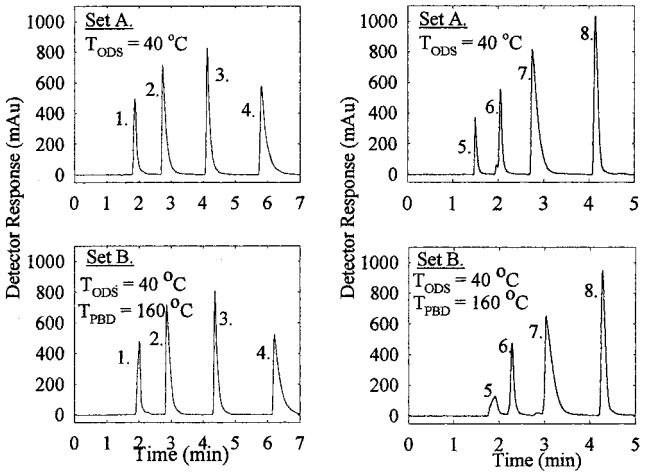

Figure 4. Representative chromatograms for basic drugs (1−8) and corresponding column sets (A and B). Conditions were the same as given in Figure 2. Analytes were 1, tryptamine; 2, desipramine; 3, doxepin; 4, amitryptyline; 5, norpseudoephedrine; 6, lidocaine; 7, quinidine; and 8. nortryptyline.

Figure 5 further illustrates our conclusion that norpseudoephedrine is reacting on-column. A chromatographic peak that is free from degradation or impurities should be spectrally pure. Figure 5 shows that with the exception of norpseudoephedrine, the ratio of the peak areas at two wavelengths (254 and 230 nm) is largely independent of temperature. As the reaction progresses, the spectrum shifts and alters the area ratio as is evident by the finite slope (see Figure 5B, •).

Effect of a Hot Column on Analytical Reliability. Calibration curves were obtained for the drug standards on column sets A and B. Table 2 shows the correlation coefficients for each drug tested. Again, the reliability is independent of the column set and analyte. The table shows that the correlation coefficient for norpseudoephedrine is acceptable, even though it apparently decomposes on the column. This is to be expected, because as Figure 5 shows, the degradant is not separated from the parent peak and a good coefficient merely indicates that the peak area is reproducible, even though the peak shifted and the profile is skewed. Table 3 shows that the reliability is also independent of the temperature of the hot narrow-bore column. Even when the first column is heated to 190 °C, the correlation coefficient essentially does not change with column temperature. This demonstrates that it is possible to do highly reliable separations at a very high temperature; however, the correlation coefficient should not be used as a test to indicate method equivalence as a function of temperature.

Table 2. Correlation Coefficients from Calibration Curves[a] for Basic Drugs

	correlation coeff, R^2	
solutes	ODS[b] (40 °C)	ODS[b] (40 °C) + OBD[b] (130 °C)
nortryptyline	0.9997	0.9999
desiprimine	0.9996	0.9999
doxepin	0.9999	0.9999
amitryptyline	0.9999	0.9999
tryptyline	0.9998	0.9997
lidocaine	0.9996	0.9994
quinidine	0.9999	0.9998
norpseudoephedrine	0.9999	0.9999

[a] $N = 4$, range = 2−10 μg. [b] Conditions and columns described in Figure 2.

Table 3. Correlation Coefficients from Calibration Curves[a] for Basic Drugs

	correlation coeff, R^2			
solutes	100 °C[b,c]	130 °C[b,c]	160 °C[b,c]	190 °C[b,c]
nortryptyline	0.9997	0.9997	0.9997	0.9997
desiprimine	0.9996	0.9996	0.9996	0.9996
doxepin	0.9999	0.9999	0.9999	0.9999
amitryptyline	0.9999	0.9999	0.9999	0.9999
tryptyline	0.9998	0.9998	0.9998	0.9998
lidocaine	0.9996	0.9996	0.9996	0.9996
quinidine	0.9999	0.9999	0.9999	0.9999
norpseudoephedrine	0.9999	0.9999	0.9999	0.9999

[a] $N = 4$, range = 2−10 μg. [b] Conditions and columns described in Figure 2. [c] Temperature of PBD column. ODS column temperature was kept at 40 °C.

The Effect of the Hot Column on Sensitivity. When the analyte peak area is altered, the slope of the calibration curve changes. When an appropriate internal or external standard is used, an area change will usually cancel out when the area ratio is calculated. Theoretically, there should be no change in analyte peak area when column set A is changed to column set B. The equation for peak area for a concentration-dependent detector is

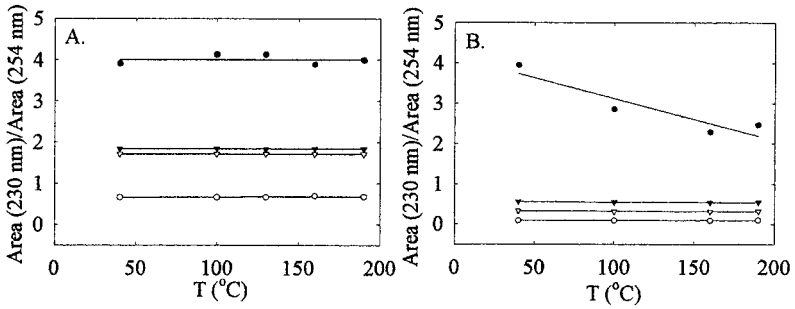

Figure 5. Plots of area ratios at two wavelengths (230 and 254 nm) vs. the temperature of the PBD-ZrO_2 column for the two sets of basic drugs. A, basic drug set 1−4: ●, tryptamine; ▼, doxepin, ▽, amitriptyline; ○, desipramine. B, basic drug set 5−8; ●, norpseudoephedrine; ▼, quinidine; ▽, nortriptyline (offset by 0.2); and ○, lidocaine.

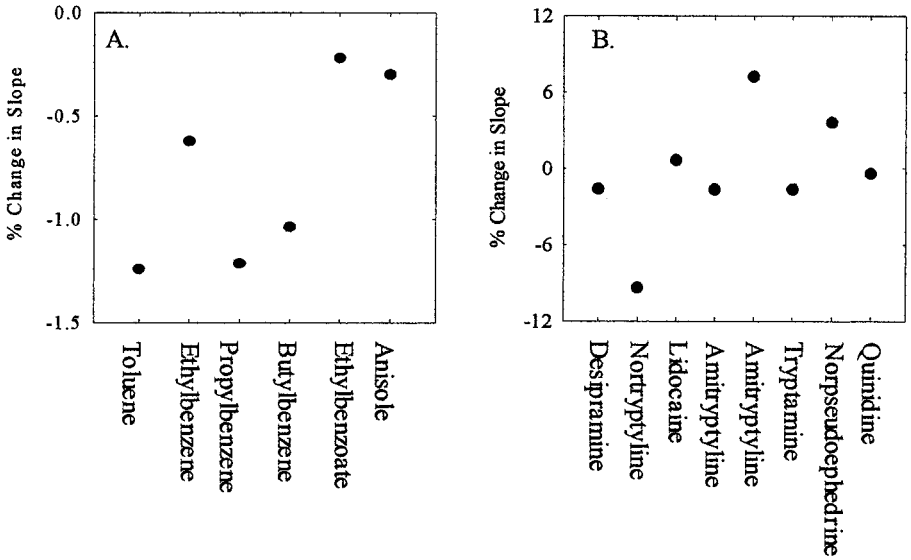

Figure 6. Plot showing the percent change in calibration curve slope for each solute in the case when there is a hot column present (set B) at 130 °C relative to when there is no hot column (set A): A, thermally stable solutes; B, basic drugs (1−8).

$$\text{Area}_{\phi} = \frac{\epsilon b W_a}{F} \tag{4}$$

where the subscript ϕ refers to a specific eluent, ϵ is the molar absorptivity for the analyte, b is the flow cell path length, W represents the total mass of the analyte, and F is the flow rate. Clearly, the peak area ought to be independent of the column set used.

Peak areas changed significantly as a function of column set, despite the above theoretical considerations. Figure 6 shows the percentage change in calibration curve slope when we change from column set A to B. All of the slopes change significantly, as determined by a *t*-test at the 95% confidence level. The trend observed for the thermally stable solutes (Figure 6A) was a significant decrease in sensitivity when the hot column was added. Conversely, there was no trend observed for the basic drugs. In fact, three of the eight had a positive change and five of the eight had a negative change; the effect was almost randomly distributed for the eight test drugs.

There are many factors that will alter peak area as a function of eluent temperature. As previously mentioned, a control sample of the thermally stable and basic drugs showed no significant area change over the sequence time. Other possibilities include the following: 1, the compound may have decomposed; 2, a change in peak shape may have changed how the peak is integrated; 3, the flow rate could have changed; 4, solvent decomposition on the hot column could affect the solvent background spectrum; and 5, a change in the average column temperature of the second column could produce a thermochromic shift in the spectrum.

Since peak area is proportional to the mass of analyte injected, the parent peak area should decrease as a reaction proceeds and alter the calibration curve slope. Certainly, the decomposition of norpseudoephedrine contributes to the change in slope. However, this is not the best explanation for the other analytes, because in a future paper, we show that they are thermally and chemically stable, and yet still exhibit the slope changes shown in Figure 6.

The simplest explanation for the slope change would be to attribute it to a change in flow rate. This would suggest that the percentage change in the slope should be identical for all analytes and that they should change in the same direction (+ or −). However, we see a random shift in the proportion for the thermally stable analytes and both the proportion and direction for the basic drugs. Moreover, independent testing showed the flow rate to be the same under all column conditions throughout this work. This explanation does not fit the data.

The origin of the significant change in the slope of the calibration line upon change in column set could stem from the temperature dependence of the peak area through the molar

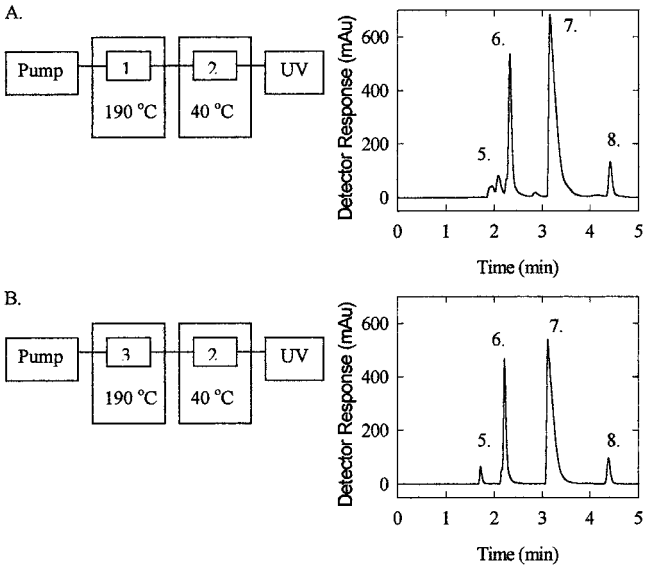

Figure 7. Chromatograms showing the effect of analyte residence time on the extent of the on-column reaction. Column 2 and other conditions are the same as given in Figure 2. The solutes are 5, norpseudoephedrine; 6, lidocaine; 7, quinidine; 8, nortriptyline. A: column 1 is a 2.1 × 50 mm PBD-ZrO_2 column. B: column 3 is a 2.1 × 10 mm PBD-ZrO_2 column.

absorptivity coefficient, ϵ. When the hot column was added, the second column's average temperature could increase if the eluent was not completely cooled. As the average column temperature increases, the temperature of the eluent going into the detector increases, thereby producing a thermochromic shift. This explanation would be consistent with the random changes observed in Figure 6. However, a thermocouple placed just prior to the detector did not register any significant change in eluent temperature upon the addition of the hot column. We are convinced that although the fluid going into the analytical column is warmer, the fluid going into the detector is very well equilibrated, and thus, the slope change is not due to thermochromism.

This series of tests shows that there is no simple explanation for the peak area changes reported here, and they should be treated solely from a phenomenological perspective when the method temperature is changed. A change in the method temperature may significantly change the calibration curve slope and may be an important parameter when determining method robustness. The fact remains that area changes caused by eluent exposure to higher temperatures make no difference in the reliability of the calibration curve.

Effect of Residence Time on Reaction Extent. Over a decade ago, Antia and Horvath[6] predicted that if the analyte went through the column faster than it reacted, then there would be no on-column reaction. To test this, we decreased the residence time of the test drugs using a 2.1 × 10 mm column in place of the 2.1 × 50 mm column. This decreases the Damköhler number 5-fold. Both columns were packed with nominally the same material to maintain retention characteristics. Figure 7 shows that a 5-fold decrease in the analyte residence time has a beneficial effect on the extent of reaction. The chromatograms show that there are no new peaks and the peak shape is no longer skewed when the shorter column is used. We contrast this result with the seemingly generally held "thermophobic" notion that high temperature is universally undesirable for thermally labile analytes.

CONCLUSIONS

We show that the chromatographic reliability at high temperature is entirely comparable to the expected reliability at ambient temperature.

The criteria articulated in the Introduction Section can be used to exclude thermally unstable analytes from measurement at high temperature.

For concentration-dependent detectors, the calibration curve slopes and intercepts will likely change as the method temperature changes, even if there is no reaction.

A decrease in the column residence time of a thermally labile analyte may decrease the extent of the on-column reaction. In the case of norpseudoephedrine, decreasing the residence time 5-fold improved peak shape.

Complex molecules can be analyzed by high-temperature LC.

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