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Using temperature to optimize resolution and reduce analysis times for bioanalytical diastereomer LC–MS/MS separations

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ABSTRACT

A rugged and reproducible liquid chromatographic tandem mass spectrometric bioanalytical method was developed for the quantitation of drug stereoisomers in human plasma. Column temperature was shown to be an important variable toward optimizing diastereomer selectivity, resolution and analysis cycle time. Non-linear Van't Hoff plots and changes in peak shape with temperature suggested that selectivity was governed by multiple retention mechanisms. The high temperature chromatography method was validated and used to analyze samples from human clinical trials. Utilization of high temperature chromatography offered alternative selectivity and is a viable approach for difficult separations in regulated bioanalysis.

1. Introduction

Metabolite identification and their quantitation is central to many of the activities in the discovery and development of drugs. A majority of drugs are metabolized by cytochrome P450 (CYP) enzymes to form metabolites by hydrolysis, oxidation and/or reduction. The promiscuity of the CYP enzymes on occasion results in diastereomer metabolites being formed [1–3]. Because of the potential differences in metabolism, distribution, and biological activity of the different metabolites, measurement of plasma concentrations of the individual isomers is normally required by regulatory agencies. This is particularly challenging in the high-throughput bioanalytical environment that requires fast yet robust methods. Diastereomer LC methods are often a compromise of analysis time, resolution, and peak shape. An often overlooked parameter for optimization of diastereomer separations is temperature. The conformational changes that occur with long chain alkyl stationary phases as a function of temperature can produce selectivity differences among stereoisomers [4]. Moreover, temperature is an easily adjustable parameter that can lead to increased resolution of critical isomer pairs along with an overall reduction in analysis times.

The virtues of using high temperature liquid chromatography (HTLC) were recognized in the 1960s when pioneers such as Giddings and Snyder illustrated that increasing column temperature could lead to decreased separation times and altered selectivity [5,6]. But only recently have every day chromatographers taken advantage of the potential offered by HTLC such as increased efficiency, lower system backpressures, and faster analysis times [4,7–14]. Several articles have reviewed the recent advances and applications of HTLC [15–19].

Increasing temperature alters analyte retention by changing the free energy (enthalpy and entropy) between the analyte and the stationary phase. The temperature-dependent free energy changes can be depicted using a Van't Hoff plot (ln retention factor vs. 1/temperature) [18,20]. Van't Hoff plots are often linear and the slope of the line can be used to estimate enthalpy, assuming the analyte retention is governed by only one retention mechanism [17,21]. Several studies, however, have suggested that changes in temperature result in numerous phenomena that may contribute to analyte retention including: (1) multiple retention mechanisms; (2) acid–base dissociation rate variations; (3) varying adsorption/desorption kinetics in the stationary phase; (4) conformational changes in the analyte and/or stationary phase; and (5) phase ratio changes. The end result of these contributions may be non-linear Van't Hoff plots [22–25]. This is especially true for ionizable analytes that are particularly prevalent in LC–MS/MS bioanalysis [18,26].

Retention is only one of the parameters that affect chromatographic resolution (Rs) as shown in the following equation:

$$\mathrm{Rs} = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{k' + 1}\right)$$

where N is peak efficiency, α is selectivity, and k' is the retention factor. Peak efficiency increases as temperature increases at higher flow rates (where the C-term of the Van Deemter curve dominates)

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[7]; one would therefore expect a slight increase in resolution as temperature increases if only efficiency is considered. Selectivity can either increase or decrease depending on temperature, and with several other parameters also affecting selectivity (pH, mobile phase, stationary phase, etc.), it is very difficult if not impossible to predict how selectivity will change with temperature. Decreases in mobile phase viscosity and/or polarity and increases in diffusivity with temperature could result in a decrease in k'. The decrease in k' would suggest a decrease in resolution with increasing temperature. With the effects of efficiency, selectivity, and retention factor combined, it is impossible to predict how resolution will change with increasing temperature.

One of the important aspects of LC–MS/MS chromatography development is the need to balance analysis cycle time with quality of analysis. In cases where long cycle times are required, for example, a diastereomer separation, elevating column temperature can often significantly shorten the run time while maintaining resolution. In a recently published article from our group, elevated column temperatures of up to 100 °C were successfully applied for the quantitation of a serine protease inhibitor using LC–MS/MS [4]. Not only did the application of the elevated column temperature significantly reduce the cycle time of the assay, a five-fold sensitivity improvement was also gained. While most of the work examining the effect of temperature on resolution was performed using neutral compounds, basic compounds such as those frequently encountered in the pharmaceutical environment often have different trends. In this communication we will discuss our approach to optimize resolution and analysis time for the quantitation of diastereomers using a model ionizable compound. By examining the role temperature plays in the resolution of diastereomers, we demonstrate that changes in temperature can modulate both selectivity and the secondary interactions of the chromatographic separation.

2. Materials and methods

2.1. Reagents and materials

All reagents were purchased from Thermo Fisher Scientific (Fair Lawn, NJ) unless otherwise noted. Methanol, acetonitrile, isopropanol, and acetone were HPLC grade, and acetic acid, formic acid, and ammonium hydroxide were ACS reagent grade. Phosphoric acid (10%) was purchased from Ricca Chemical Company (Arlington, TX). Ultra-pure water was from a Millipore Milli-Q® water system (Millipore Corporation, Bedford, MA). Human plasma (with K_2EDTA as anticoagulant) was purchased from Bioreclamation Inc. (Hicksville, NY). SCH 900518W and $^2H_{10}$-SCH 900518W (the internal standard, IS) were synthesized by Merck Research Laboratories (Kenilworth, NJ).

Experiments were performed using a Waters Acquity™ BEH C18, 2.1 mm × 50 mm, 1.7 μm column (Waters Corporation, Milford, MA) or a Zorbax Stable Bond C18, 2.1 mm × 50 mm, 3.5 μm column (Agilent Technologies, Santa Clara, CA). Both of these columns were chosen for their symmetrical peaks and excellent chemical and thermal stability. During method development experiments with extracted human plasma samples, the Zorbax Stable Bond 3.5 μm column proved more rugged than the Waters Acquity™ BEH 1.7 μm column, and therefore validation experiments were performed using a Zorbax Stable Bond C18 column (see Section 3.3).

2.2. LC–MS/MS conditions

2.2.1. Method development experiments

SCH 900518W has six chiral centers (four of which are fixed). The two freely rotating chiral centers result in two diastereomer pairs (see Fig. 1 for structure of SCH 900518W. The asterisks denote the chiral centers responsible for the diastereomer pairs.) The combined diastereomer mixture concentration was 1 μg/mL (~250 ng/mL each) in 60:40 (v:v) water:acetonitrile. Separations were performed on a Waters Acquity™ LC system (Waters Corporation) and detection was performed using an AB Sciex API 4000 triple quadrupole mass spectrometer (AB Sciex, Concord, Ontario). Mobile phase A (MPA) was 0.1% acetic acid in water and mobile phase B (MPB) was acetonitrile. Separations were performed in isocratic mode (65:35, v:v, MPA:MPB) at a flow rate of 0.5 mL/min. Column temperature ranged from 10 to 90 °C. Injection volume was 3 μL. Once critical pair resolution with temperature was characterized on the Waters Acquity™ system, method development experiments were also performed using similar LC conditions on an Agilent 1200 RRHT HPLC system (Agilent Technologies) to take advantage of the higher temperature capabilities.

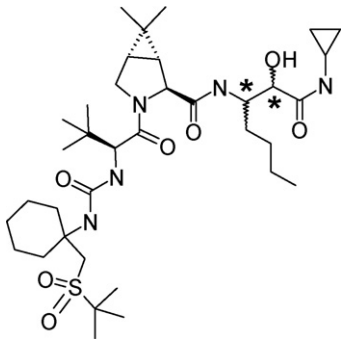

Fig. 1. Structure of SCH 900518W. The asterisks denote the chiral centers responsible for the diastereomer pairs.

SCH 900518W was infused under multiple reaction monitoring (MRM) conditions to optimize various MS/MS parameters. The mass spectrometer was tuned for unit mass resolution. Samples were ionized using a TurboIonSpray® probe in the positive-ion mode. Analyte and instrument dependent parameters such as ionization voltage, gas settings, source temperature, declustering potential, collision energy, and collision exit potential were optimized by teeing in analyte flow with mobile phase flow. The TurboIonSpray® voltage and temperature were set to 4500 V and 650 °C, respectively. The declustering potential was 85 V and the collision energy was 31 eV. The collision gas was set to maintain an indicated pressure of ~ 3.5×10^{-5} torr. The mass transitions used to detect SCH 900518W and its IS were m/z 710 → m/z 451 and m/z 720 → m/z 451, respectively. The dwell times for SCH 900518W and the IS were 400 ms and 100 ms, respectively.

2.2.2. Bioanalytical validation experiments

Extracted plasma samples were analyzed using an Agilent 1200 RRHT HPLC system coupled to an AB Sciex API 4000 mass spectrometer. Chromatography was performed using an Agilent Zorbax SB-C18, 2.1 mm × 50 mm, 3.5 μm column. MPA was 0.1% formic acid in water and MPB was acetonitrile. SCH 900518W and the IS were separated from endogenous matrix components in isocratic mode (65:35, v:v, MPA:MPB) for 4.9 min. The column was then washed for 0.6 min (5:95, v:v, MPA:MPB) before re-equilibrating at initial conditions for 0.5 min. The flow rate was 1.0 mL/min and the cycle time for a single analysis was 6.0 min. A 5–10 μL sample aliquot achieved a system suitability requirement of S/N ≥ 10:1 at the lower limit of quantitation (LLOQ, ~500 pg/mL, depending on the diastereomer). MS/MS conditions were the same as those listed in the method development experiments.

2.2.3. Bioanalytical validation sample preparation

Samples were prepared by aliquotting 200 μL of plasma sample containing SCH 900518W and 200 μL of an acidified (10% phospho-

ric acid) internal standard working solution into individual 96-well format dilution tubes. Sample tubes were capped, briefly vortexed, and then centrifuged at 3000 rpm for 10 min.

SPE was performed using 96-well format SPEC™ IQe® C18-A extraction cartridges (Varian Inc., Lake Forest, CA) and a Tomtec Quadra96® automated liquid handler system (Tomtec Corp. Hamden, CT). The SPE bed was initially conditioned with 300 μL of methanol followed by 400 μL of water. Samples were then loaded, washed with 300 μL of water followed by 200 μL of water:acetonitrile (80:20, v:v). Samples were eluted into a 96-well True Taper™ collection plate (Analytical Sales and Services, Pompton Plains, NJ) with 2× 200 μL of acetonitrile and the eluent was evaporated to near dryness using nitrogen and a SPE Dry Dual evaporator (Argonaut Technologies, Inc., Foster City, CA). The dried samples were reconstituted with 150 μL of water:acetonitrile (60:40, v:v) and centrifuged at 3000 rpm for 10 min prior to being placed in the autosampler for analysis.

2.3. Data analysis

MRM data were acquired and analyzed using the Sciex Analyst (version 1.4.2) application. Data regression was performed using Watson™ Laboratory Information Management System 6.4.0.03 (Thermo LabSystems, Philadelphia, PA). Resolution was calculated using Cutter [27], and retention factors were calculated in Microsoft Excel. Peak asymmetry was calculated using the ratio of peak tail width to the peak front width at the baseline. Peak asymmetry was calculated at the baseline and not the more standard 5–10% of the peak height [28] because the peaks were manually integrated in Cutter for peak asymmetry calculations. Error is reported as ±standard deviation.

3. Results and discussion

3.1. Method development approach to determining optimum resolution and retention time

Because it is difficult to predict how temperature affects selectivity, our lab routinely conducts overnight temperature screens to determine the optimum temperature for a given separation by creating methods with separation temperatures ranging from 10 to 100 °C with temperature increasing in 5–10 °C increments. (The temperature increment size may vary depending on the resolution-temperature dependency.) Typically, a sample is injected 5–6 times at each temperature, with the methods being run consecutively overnight. Only the last three chromatograms at each temperature are used in resolution and retention time calculations to account for column temperature equilibration during the first 2–3 injections. The temperature screen is usually performed at lower flow rates than those used in bioanalysis to avoid over-pressuring the LC system at low temperatures when the mobile phase is the most viscous. (Flow rates in a typical temperature screen may be ~0.3–0.5 mL/min, whereas flow rates in a bioanalysis assay may be 0.6–1.0 mL/min.) If necessary, a larger particle size (of the same stationary phase) may be used in the temperature screen to avoid over-pressuring the LC system. Often more than one column is evaluated in case ruggedness issues are encountered during routine bioanalysis.

After the initial temperature screen, separation conditions are tested using extracted samples to confirm that the resultant temperature remained optimized for resolution and/or retention time. More specifically, temperatures around the optimum conditions (balancing acceptable resolution with fast cycle time) are re-tested with the final flow rate, mobile phase, column, and sample extraction procedure that will be used in bioanalysis.

The following sections will illustrate how the temperature screen was used to develop and validate a robust and rugged bioanalytical method for SCH 900518W.

3.2. The effect of temperature on the SCH 900518W diastereomer separation

SCH 900518W is a mixture of two diastereomer pairs resulting in four stereoisomers. Because the four isomers of SCH 900518W are diastereomers and not enantiomers, they have different physical properties and a chiral column should not be required to resolve them; i.e., resolution should be achievable on a standard C18 column. Initial method development focused on finding suitable column stationary phase and mobile phase conditions to partially resolve all four stereoisomers, paying particular attention to the critical pair (peaks 2 and 3). Preliminary experiments suggested that both the Waters Acquity™ BEH C18 1.7 μm and Zorbax SB C18 3.5 μm phases were able to partially resolve the critical pair, and so temperature screens were conducted on both columns. The column temperature was varied in 5 °C increments (for separation temperatures ranging from 10 to 30 °C) or 10 °C increments (for separation temperatures ranging from 30 to 90 °C) to examine the effect of temperature on resolution.

Chromatograms of the stereoisomer separation on the Waters Acquity™ column at varying temperatures are illustrated in Fig. 2A. At 10 °C, the critical pair had a resolution of ~1.0 with the more abundant isomer eluting earlier than the less abundant isomer (peaks 2 and 3, respectively). As the temperature increased, peaks 2 and 3 began to co-elute until peak 3 eluted at an earlier retention time than peak 2. At 90 °C, the critical pair had a resolution of ~1.6. Clearly, increasing the temperature increased resolution, but there was also a net reduction in analysis time, peak shape was improved, and the LC system backpressure was reduced. The Zorbax SB C18 3.5 μm column showed similar trends but critical pair resolution was lower (Rs = 1.2) compared to separations on the Waters Acquity™ 1.7 μm column (Rs = 1.6) at 90 °C, likely due to the particle size difference.

3.2.1. The effect of temperature on resolution

A primary goal was to find a column temperature that resulted in baseline resolution of the critical pair with a reduced run time. Fig. 2B displays the resolution of the critical pair from 10 to 90 °C using the Waters Acquity™ 1.7 μm column. The critical pair was partially resolved at 10 °C (Rs ~ 1.0). Increasing the temperature deteriorated the resolution, resulting in co-elution of the critical pair for temperatures between 30 and 50 °C. As the temperature further increased, the critical pair elution order switched and resolution improved until baseline resolution was achieved at 80 °C (Rs ~ 1.5). This switch in retention could suggest that different (or additional) retention mechanisms were introduced at elevated temperatures, and that the differences in retention were most divergent at higher temperatures (90 °C in this case). It is likely that the increase in temperature slightly changed the conformation of the critical pair, resulting in one of the diastereomers being preferentially retained over the other thereby increasing resolution.

3.2.2. The effect of temperature on analysis time

As mentioned above, the goal of the separation was to reproducibly and robustly resolve the diastereomers with the shortest possible cycle time. The retention time of the last eluting peak, peak 4, is plotted vs. temperature in Fig. 2B. Most studies have shown that increasing temperature resulted in decreased retention times because mass transfer was increased. The retention time of

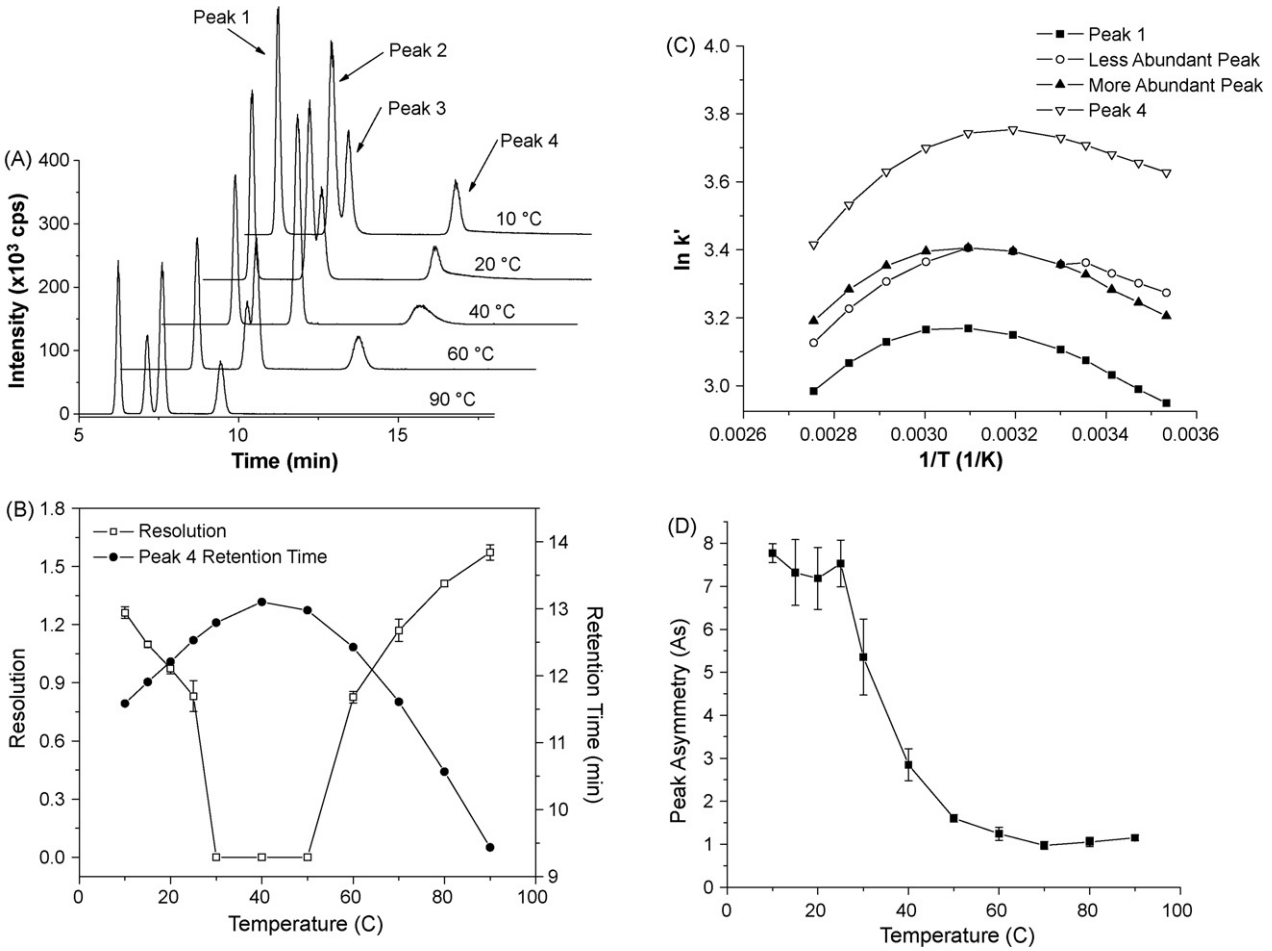

Fig. 2. The effects of temperature on resolution and analysis time. (A) Chromatograms of the four-analyte diastereomer mixture of SCH 900518W at 10, 20, 40, 60, and 90 °C. The critical pair is peak 2 and peak 3. The *x*- and *y*-axes are offset for clarity. (B) The resolution of the critical pair (left *y*-axis) and retention time of peak 4 (right *y*-axis) were measured as a function of temperature. (C) Retention factors of the four diastereomers were measured as a function of temperature and represented in a Van't Hoff plot. The less and more abundant peaks are illustrated as Peaks 3 and 2, respectively, in (A). (D) Peak asymmetry of peak 4 is calculated as a function of temperature. Results are representative of at least 3 injections, and all data points have error bars expressed as ±one standard deviation unit. Experimental details are found in the method development portion of Section 2.

peak 4 unexpectedly increased from 10 to 40 °C, and then followed the expected trend by decreasing from 40 to 90 °C. The concave shape of the retention time vs. temperature curve suggests that the dominant retention mechanism changed as temperature increased. Not only was the critical pair better resolved at 90 °C compared to 10 °C, but the analysis time also decreased from 13 min (at 10 °C) to 10 min (at 90 °C).

The change in retention factor with temperature for all four peaks is depicted in a Van't Hoff plot of $\ln k'$ vs. 1/temperature (Fig. 2C). The Van't Hoff plots of most analytes exhibit a positive, linear slope because retention time is expected to decrease as temperature increases [21]. The positive slope indicates that the interaction is exothermic. All four diastereomers exhibited a concave shape from the initial increase in retention time at low temperatures (right side of graph) before a decline in retention time as temperature further increased (left side of graph). The concave slope suggests that the thermodynamics of retention (in terms of enthalpy and entropy) were changing as temperature increased [29]. Each of the four diastereomers reached its maximum retention at a different temperature. The differences in retention change with temperature (slope of the curve) resulted in resolution differences with temperature. These resolution differences could have been caused by differences in secondary ionic interactions and/or conformational changes among the diastereomers or stationary phase.

3.2.3. The effect of temperature on peak shape

Basic compounds that interact with silica particles in the stationary phase are notorious for peak tailing, and increasing temperature may lead to improved peak shape and symmetry. The improved peak symmetry at high temperature is usually attributed to increased adsorption/desorption kinetics (mass transfer) [30–32] or to mobile phase pH and/or analyte pK_a changes that alter the ionization state [33]. Peak 4 exhibited some noteworthy changes in peak shape as temperature was varied (see chromatograms in Fig. 2A). Fig. 2D illustrates the peak asymmetry of peak 4 at varying temperatures (see Section 2.3 for peak asymmetry calculation method). At 10 °C, peak 4 did not return to the baseline causing severe peak tailing. The peak had an asymmetry factor of 7.0–8.0 from 10 to 25 °C, but steadily became more symmetric as temperatures increased to 60 °C. Above 60 °C, peak 4 was symmetric with asymmetry factors ranging between 1.0 and 1.5. The mobile phase pH and/or analyte pK_a changes are not likely responsible for the improved peak symmetry because the same peak shape improvement occurred when both formic acid and acetic acid were used as mobile phase modifiers. Instead, the improved peak shape is likely due to increased kinetics and faster desorption from the stationary phase at higher temperatures. The use of high temperatures not only improved resolution and reduced analysis times, but also improved peak shape for this analyte.

Table 1
Accuracy and precision data for peaks 1 and 2 in extracted SCH 900518W quality control samples in human plasma. In three separate analytical runs, data was fit using a quadratic regression ($y = Ax^2 + Bx + C$) with a weighting of 1/concentration^2 (QC samples at $n = 6$ in each run). y is the peak area ratio, x is the concentration of Compound A, and A, B, and C are the calibration curve parameters. LLOQ: lower limit of quantitation; QCH: quality control of high concentration; QCL: quality control of low concentration; QCM: quality control of medium concentration; QCML: quality control of medium-low concentration. %CV: percent coefficient of variation (precision).

	LLOQ	QCL	QCML	QCM	QCH
Peak 1					
Theoretical concentration (ng/mL)	0.453	1.36	13.6	36.3	363
Calculated concentration (ng/mL)	0.433	1.36	13.5	36.3	348
Standard deviation	0.0265	0.0545	0.669	1.6	14.1
%CV	6.12	4.00	4.96	4.36	4.05
%Theoretical	95.7	100.3	99.1	100.0	95.9
n	18	18	18	18	18
Peak 2					
Theoretical concentration (ng/mL)	0.393	1.18	11.8	31.4	314
Calculated concentration (ng/mL)	0.391	1.19	11.6	31.1	307
Standard deviation	0.0256	0.0740	0.551	1.08	20.2
%CV	6.54	6.23	4.73	3.46	6.59
%Theoretical	99.5	100.6	98.6	99.1	97.8
n	18	18	18	18	18

It is interesting to note that of the four diastereomers, only peak 4 exhibited peak tailing at separation temperatures <60 °C. This would again suggest that the four diastereomers have slightly different conformations that uniquely interact with the stationary phase.

3.3. From method development to high-throughput bioanalysis

Initial method development experiments performed on the Waters Acquity™ LC system determined that 90 °C resulted in the best resolution and shortest analysis times for the SCH 900518W diastereomers (see Section 3.1). The high temperature chromatography conditions developed for SCH 900518W were adjusted for high-throughput analysis of human plasma samples. More specifically, the analytical column was switched from a Waters Acquity™ BEH C18, 2.1 mm × 50 mm, 1.7 μm to an Agilent Zorbax SB-C18, 2.1 mm × 50 mm, 3.5 μm to reduce the possibility of column plugging and subsequent system overpressure and shutdown, being a primary concern with human plasma analysis from clinical studies. Because the temperature screen had already been conducted on two columns and results demonstrated that the Zorbax SB-C18 column had the same resolution vs. temperature profile as the Acquity™ BEH C18 column (see Section 3.2), switching the column at this point was relatively straightforward. The Zorbax SB-C18 column was also chosen because previous work in our lab suggested that the performance of this column (measured by peak shape and resolution) remained consistent for >300 injections at 100 °C under acidic conditions [4]. The Zorbax SB-C18 columns yielded longer retention times when compared to the Waters BEH C18 columns and therefore, the flow rate was increased to 1.0 mL/min to compensate. An Agilent RRHT system was used instead of a Waters Acquity™ system to take advantage of the increased column oven temperature capabilities of the former (the Agilent RRHT column oven can maintain 100 °C vs. the Waters Acquity™ column oven which has a maximum temperature of 90 °C). Operating at a column temperature of 100 °C not only increased resolution, but also further reduced retention times. Moreover, the mass spectrometer run time was reduced to 5 min because quantitation of peaks 3 and 4 were not required for pharmacokinetic profiling (they were formed at <10% of parent drug systemic exposure at steady state). A strong organic wash step was added after the diastereomer separation to rapidly elute the remaining diastereomers and any endogenous materials from the column. The end result of these changes is that the cycle time was reduced from 10 min in method development to 6 min for routine application.

After the final bioanalytical flow rate, column, and mobile phase parameters were established, a temperature screen from 80 to 100 °C was performed for SCH 900518W to optimize and fine-tune the column temperature. A separation temperature of 100 °C resulted in the best resolution with the shortest analysis time, therefore bioanalytical experiments were performed at 100 °C.

3.4. Application of high temperature chromatography in bioanalysis

A bioanalytical method with a calibration range of ~0.500–500 ng/mL SCH 900518W (depending on the diastereomer) was successfully validated in accordance with the FDA guidance for Bioanalytical Method Validation [34–36]. Specifically, three analytical runs were processed and analyzed to assess sensitivity, reproducibility, accuracy and precision. Each analytical run contained ten calibration standards defining the analytical range ($n = 2$ at each level), two control blanks (blanks with no IS), two zero standards (blanks with IS), and quality control samples ($n = 6$ at each level). The inter-run QC precision for peaks 1 and 2 (quantitation of peaks 3 and 4 in human clinical samples was not required) are shown in Table 1. For both peaks 1 and 2, the accuracy and precision were within the acceptance criteria of ±15% (±20% at the LLOQ) and ≤15% (≤20% at the LLOQ), respectively.

Two indicators of a rugged assay are reproducible retention times and consistent chromatographic resolution throughout sample analysis. In our lab, we strive for resolutions between 1.3 and 1.7. Fig. 3A illustrates the resolution between the critical pair (peaks 2 and 3) for every tenth injection over the course of 183 injections ($n = 19$). Of the 19 chromatograms evaluated for resolution, 18/19 fell within the range Rs = 1.3–1.7, with the exception of one chromatogram with Rs > 1.7. The 19 chromatograms demonstrated consistent resolution with a RSD of 6%. Chromatographic peak shape and resolution between the critical pair (peaks 2 and 3) for the 3rd and 176th injections are illustrated in Fig. 3B. The consistent peak shape and resolution between the two injections demonstrate that the column performance remains steady during the 21 h analytical run. Fig. 3C illustrates the retention times of peaks 1, 2, and 3 over the course of 183 injections. Over 21 h of continuous analysis, peaks 1, 2, and 3 maintained their retention times with %RSDs of 0.5, 0.6, and 0.5, respectively. The consistent retention times and resolution demonstrates that the Zorbax SB-C18 column is capable of withstanding high temperatures (100 °C) for extended periods of time without loss of performance.

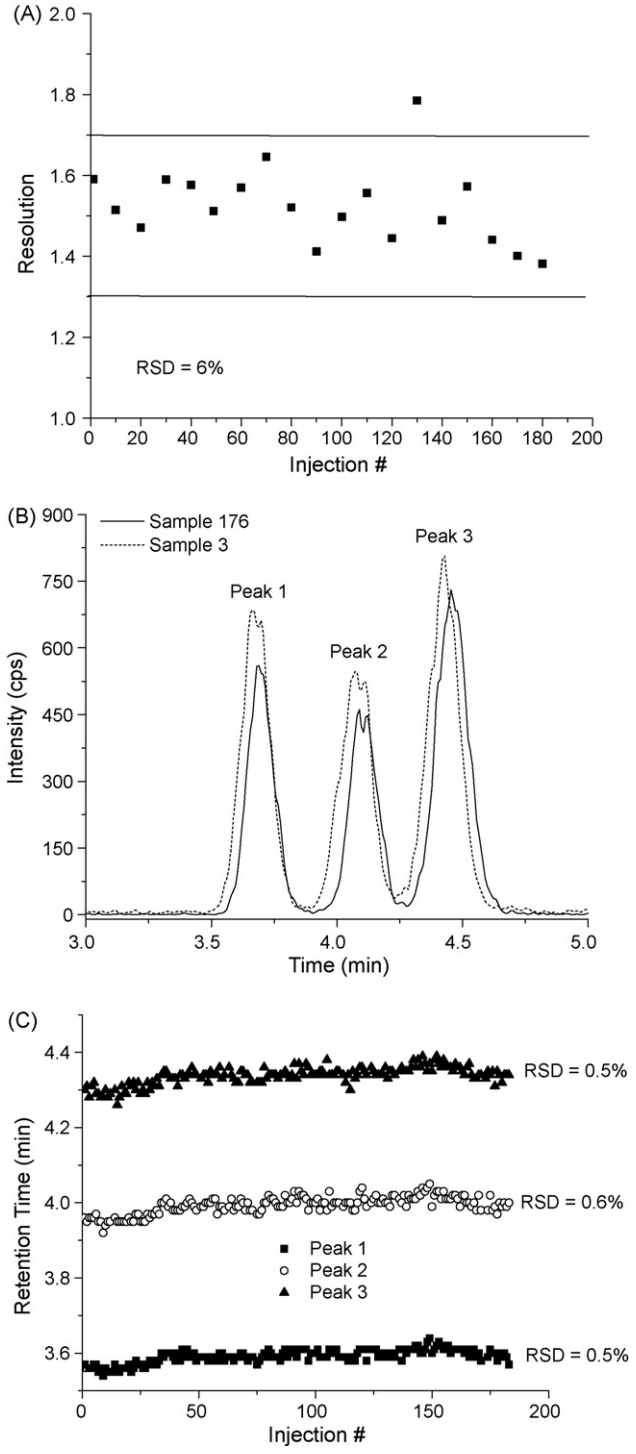

Fig. 3. Bioanalytical method performance at 100 °C. (A) Critical pair resolution was measured for every tenth injection over the course of 183 injections (21 h). The solid lines represent acceptable resolution for this assay. (B) Chromatograms from the 3rd and 176th injections are overlaid. Retention time and critical pair resolution (peaks 2 and 3) remain constant throughout the run. (C) The retention times of peaks 1, 2, and 3 are plotted over the course the run (183 injection, 21 h). Experimental details are found in the bioanalytical validation portion of Section 2.

4. Conclusions

Temperature is a powerful tool to reduce analysis time and optimize chromatographic resolution for bioanalytical diastereomer separations. In this communication, the effects of temperature on the resolution of one diastereomer quartet (four diastereomers) was examined and the maximum resolution was achieved at 100 °C. Improvements in peak shape and analysis time were also realized at higher temperatures. Chromatographic performance was maintained over 21 h under these conditions with resolution and retention time remaining consistent. Because temperature can greatly alter resolution, and the effects of temperature on resolution are impossible to predict, temperature should be explored to improve the resolution of closely eluting analytes in bioanalysis.

Conflict of interest

The authors have declared no conflict of interest.

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