Journal of Chromatography A, 1218 (2011) 3502–3510

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Chromatographic studies of unusual on-column degradations of aniline compounds on XBridge Shield RP18 column in high pH aqueous mobile phase

Fang Wang*, Xiao-Keng Liu*, Susanna Lai, Jan Fang, David Semin

Department of Analytical Research and Development, Amgen Inc., Thousand Oaks, CA 91320, USA

ARTICLE INFO

Article history:
Received 15 February 2011
Received in revised form 31 March 2011
Accepted 1 April 2011
Available online 8 April 2011

Keywords:
Unusual on-column degradations
High pH aqueous mobile phase
Aniline compounds
Column temperature programs
XBridge Shield RP18 column

ABSTRACT

This paper reports unusual on-column degradations of aniline compounds on Waters XBridge Shield RP18 column when ammonium hydroxide in water and acetonitrile were used as mobile phases in liquid chromatography. The change of the level of on-column degradation of a model compound (Compound 1) with time was observed in the first fifteen injections when started at 60 °C. During a subsequent cooling program from 60 °C to 10 °C with a 10 °C interval, the levels of the degradation products of Compound 1 changed with the change of temperature and reached a maximum at 40 °C. The on-column degradation of Compound 1 was observed when started at 10 °C in the first injection, however, the magnitude of the change of the level of on-column degradation of Compound 1 with time in the first fifteen injections was much smaller than that at 60 °C. During a subsequent heating program from 10 to 60 °C with a 10 °C interval, the levels of the degradation products of Compound 1 increased with the increase in temperature but without a maximum. The change of the degradation product levels of this model compound in the heating process is not super-imposable with that in the cooling process, which demonstrates the degree of the degradation also depends on the heating or cooling process. Column history studies demonstrated that the on-column degradation of Compound 1 changed dramatically on the used columns at both starting temperatures while the dependency of heating and cooling processes on on-column degradation still existed. The unusual on-column degradation of Compound 1 on the used columns can be regenerated in a very similar fashion with an acetic acid column-wash procedure, but is not identical to that on the new column. Similar degradations of other commercially available aniline compounds were also observed with this high pH aqueous mobile phase system.

1. Introduction

High-performance liquid chromatography (HPLC) has been widely used in pharmaceutical industry in drug substance and drug product research and development. Reversed-phase liquid chromatography (RPLC) is the most commonly used analytical technique in the industry. Although most basic pharmaceutical compounds have been separated on typical silica-based columns using acidic and/or neutral pH aqueous mobile phases in RPLC [1], the recent advancement in column technology provides more opportunities to separate basic compounds at high pH aqueous mobile phases (pH > 9) as free-base forms without the concerns of the dissolution of the regular silica supporting materials [2–10]. The separation of basic compounds as free-base forms results in better peak shapes, longer retention times, higher column efficiency and potentially better compatibility with mass spectrometry (MS) detectors. This separation mode also offers some flexibility of having secondary or potentially "orthogonal" methods to confirm impurity profiles obtained at lower pH mobile phases. However, some potential limitations of using high pH aqueous mobile phase methods, such as fronting peaks, shorter column-life, and unexpected degradation products at different pHs, may occur under certain conditions [10]. In one of the specific applications, it was reported that an on-column dimerization phenomenon (structure not shown) was observed on a particular column [10]. When that particular column was washed with 0.5% acetic acid, the dimer disappeared. They concluded that the on-column dimerization was caused by the presence of residual metal iron at the head of the column [10].

During the course of the development of an impurity method for our aniline model compound, Compound 1 (Fig. 1 for structure), a high pH aqueous mobile phase (0.1% of 28% ammonium hydroxide (NH_4OH) in water, pH ~10.5) on Waters XBridge Shield RP18 column had been used to determine the impurity profile of the compound. From our experience of impurity method development for the same class of compounds, the major advantage of using high pH aqueous mobile phase to separate aniline compounds and its impurities is to minimize the effect of ionic strength of aqueous

* Corresponding authors. Tel.: +1 805 313 4117; fax: +1 805 447 8673.
E-mail addresses: fwang@amgen.com (F. Wang), xiaol@amgen.com (X.-K. Liu).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.04.004

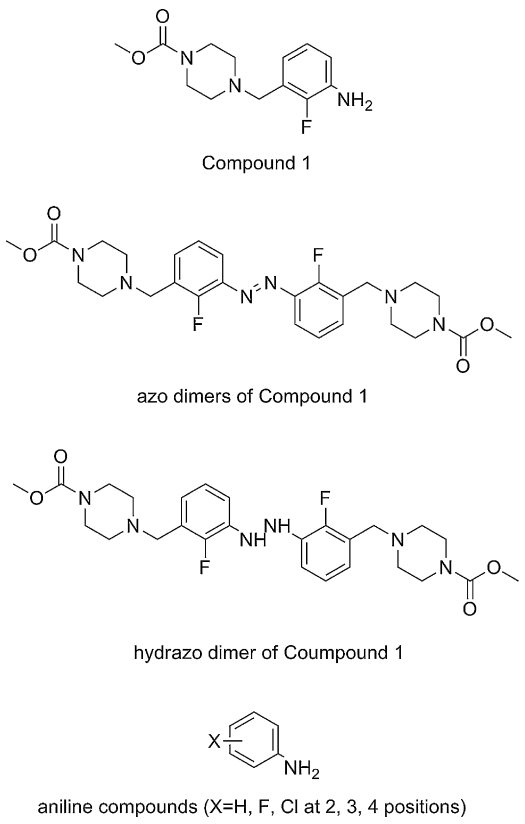

Fig. 1. Structures of Compound 1, its potential degradation products and aniline compounds.

mobile phases on the reproducibility of retention times when ion-pair reagents, such as perchloric acid or trifluoroacetic acid (TFA), are used to increase the retention times of these compounds. Since NH_4OH modified aqueous mobile phase is also compatible with MS detectors without ionization suppression from ion-pair reagent such as TFA, it also provides better sensitivity for impurity analysis than that of TFA modified aqueous mobile phase. Besides, both positive and negative ionization modes can be used with aqueous NH_4OH mobile phase on MS detectors. During the application of the method, the discrepancy of apparent purity (area %) of Compound 1 from 95 to 99% was observed among different end-users. Unusually high level of the azo impurities (Fig. 1 for structures) from 1 to 3% of each impurity was observed when using this high pH method. Since these impurities are the potentially genotoxic, it is a regulatory requirement to quantitatively analyze and track their clearance in the downstream chemical synthesis to ensure the safety of human subjects in clinical trials [11]. Therefore, the analytical method must accurately determine these impurities without generating any ambiguity from artifacts by the method itself. The first step to ensure the reliability of the method will be the establishment of a well characterized reference standard with known purity and impurity profile. In order to determine the true purity of the standard in solid state, other analytical methods, such as differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR), were used to cross-check the purity of the standard. The results from DSC and NMR indicate that the standard is relatively pure in solid state (purity ≥99%). In order to confirm the existence of these impurities and levels in the reference standard in the liquid solution, an HPLC method using neutral pH aqueous mobile phase with MS compatibility was also developed to check the purity and to confirm the formation of the degradation products (if any) in the mixture of the basic mobile phase and acetonitrile as the diluent. The results from the neutral pH aqueous mobile phase method matched the purity data from DSC and NMR. Neither azo nor hydrazo impurity was observed in the reference material.

This paper reports the chromatographic studies of unusual on-column degradations of aniline compounds on XBridge Shield RP18 column with high pH aqueous mobile phase. From the temperature program studies as reported previously [12–15], we discovered that the on-column degradation process of the model compound depended on the history of the columns. To the best of the authors' knowledge, this type of unusual on-column degradations has not been reported. The magnitude of the change of the level of on-column degradation of Compound 1 with time on the used columns is much smaller than that of the new columns while the change of the level of degradation with heating or cooling processes still exists regardless the history of the columns. However, the change of the level of the degradation with time on the used columns can be regenerated by washing the column with 0.5% acetic acid for 2 h at 40 °C. During the heating/cooling programs, the van't Hoff plots of retention and selectivity factors of Compound 1 are super-imposable and independent of the thermal pathway, which indicates that the conformations of the stationary phase and Compound 1 remain the same. The column history has no impact on the retention and selectivity factors of the degradation product peaks of Compound 1 on the new, used and re-generated columns. Other commercially available aniline compounds were also studied with the same temperature programs. Similar on-column degradations were also observed in the high pH aqueous mobile phase system.

2. Materials and methods

2.1. Chemicals

Compound 1 (Fig. 1 for structure) was synthesized by Chemical Process Research and Development Department at Amgen Inc. (Thousand Oaks, CA, USA). All other aniline compounds (Fig. 1 for structures) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). HPLC grade methanol (MeOH) and NH_4OH (28% in water with purity ≥99.99%) were purchased from Sigma–Aldrich (Milwaukee, WI, USA). HPLC grade acetonitrile was purchased from J.T. Baker (Philipsburg, NJ, USA). Ammonium carbonate was purchased from Acros (Morris Plains, NJ, USA). Ammonium acetate (NH_4OAc) was purchased from MP Biomedicals (Morgan Irvine, CA, USA), respectively.

2.2. Apparatus

All HPLC experiments were performed on an Agilent G1100 HPLC system with a standard column oven and a photodiode array detector (Santa Clara, CA, USA). The system was controlled by Agilent ChemStation software or Dionex Chromeleon Chromatography Data System (Sunnyvale, CA, USA). A Thermo Finnigan LCQ Deca (San Jose, CA, USA) with an electrospray ionization (ESI) source with an Agilent HPLC system was used for LC/MS experiments. LC/MS data were collected using Thermo Finnigan Xcalibur software (San Jose, CA, USA). The positive mode of ESI source was operated under typical conditions [16]. XBridge Shield RP18, C18 and Phenyl (4.6 mm × 150 mm, 3.5 μm) columns were purchased from Waters (Milford, MA, USA). Gemini NX C18 columns were purchased from Phenomenex (Torrance, CA, USA). DSC experiments were performed on a TA Instrument's Q2000 DSC module (New Castle, DE, USA). NMR experiments were performed on a Bruker 400 MHz instrument (Billerica, MA, USA). Inductively coupled plasma (ICP)-MS experiments were performed on a Perkin-Elmer Elan DRC II instrument (Waltham, MA, USA). The normal operation conditions of the Elan DRC II instrument were used in this application [17].

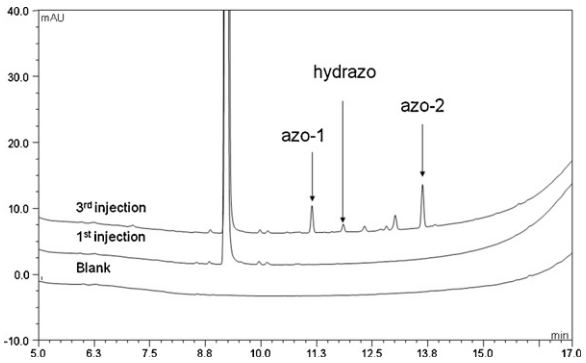

Fig. 2. Overlays of chromatograms of blank, the first and third injections of Compound 1 standard solution at 40 °C. Chromatographic conditions: the same as Section 2 except that the gradient profile: 5% acetonitrile from 0 to 2 min, 5–90% from 2 to 15 min.

2.3. Chromatographic conditions and column temperature programs

The aqueous mobile phases were prepared by the addition of the chemicals into HPLC grade water. Samples were prepared at *ca.* 0.5 mg/mL in 50% MeOH in water as diluent if not specified in the text. The flow rate was 1.0 mL/min. A 5 μL volume of each sample was injected. The HPLC conditions for the high pH method on Waters XBridge Shield RP18 columns: mobile phase: (A) 0.1% NH_4OH in H_2O, (B) acetonitrile; gradient program: 5–95% B in 15 min; column temperature: 40 °C; detection wavelength: 230 nm. The HPLC conditions for the neutral pH method on Waters XBridge Shield RP18 column: mobile phase: (A) 25 mM NH_4OAc in H_2O (pH 6.9), (B) acetonitrile; the other conditions were the same as the high pH method.

The column temperature programs were the similar as reported previously [12–15]. Heating and cooling temperature cycle program: the column temperature changed from 10 to 60 °C by a 10 °C interval during the heating/cooling processes. After each temperature change, the column was equilibrated with the mobile phases for 1 h before the injection of a compound. Unless it is otherwise indicated in the text, all the columns used in the studies were brand new columns.

3. Results and discussion

3.1. Discovery of unusual on-column degradation of Compound 1

The original RPLC method for the analysis of Compound 1 (Fig. 1 for structure), an aniline analog as an intermediate of a drug substance, was developed on an XBridge Shield RP18 column using 0.1% NH_4OH in water as the aqueous mobile phase. During the course of the development of this intermediate (hydrogenation product of its nitro starting material with palladium (Pd) on carbon as the catalyst to produce Compound 1), some discrepancies of the apparent purity of the compound were observed. Without the awareness of the history of the columns, the apparent purity of the compound could vary from 95% to 99% by area from day-to-day. The level of three major impurities, azo-1, azo-2 and hydrazo degradation products (Fig. 1 for structures), varied from 0.2 to 3% in routine operations (without multiple injections of the reference standard solution for in-process analysis per industry common practice). Fig. 2 shows the chromatograms of a blank, the first and third injections of Compound 1's standard solution on a used XBridge Shield RP18 column using high pH aqueous and acetonitrile mobile phases at 40 °C with a gradient elution. The first injection of the standard solution of Compound 1 (at retention time of 9.23 min with the asymmetric factor of 1.02) showed that the apparent purity of the compound was 99.9% with no impurity above the reporting limit of the method (0.05%). At the third injection of the same standard solution from the same vial, the apparent purity of the main peak reduced to 97.5%. There were five major impurities observed at 0.10% or above (Fig. 2). LC/MS analysis demonstrated that two major impurities, at retention times of 11.1 and 13.6 min (with the asymmetric factors of 1.00 each), were the azo impurities at the levels of 0.60 and 0.83%, respectively. The hydrazo impurity, at retention time of 11.9 min (with the asymmetric factor of 0.83), was also observed at 0.19%. Again, because of the potential genotoxic nature of these impurities, it is very critical for the analytical method to generate accurate results without any ambiguity from artifacts such as the undesired on-column degradations or decompositions in the diluent before injections.

The change of the impurity levels with the change of the number of injections was confirmed by another sequence with 30 injections of the standard (prepared and used in the previous day) and sample solutions in the next day on the same column (data not shown). In that sequence, the first and last 6 injections were the same standard solution from two different vials to bracket 18 injections of different sample solutions. The first injection of standard solution showed the apparent purity of the standard was 99.8% without any reportable impurities, which indicates that the standard solution was stable over 24 h. At the 30th injection of the same standard solution from a different vial (un-used), the apparent purity reduced to 92.4% with the azo impurities at 1.7 and 2.3% (chromatograms not shown), respectively. Obviously, these impurities, or more accurately, the degradation products, were generated from the on-column degradations. The level of the degradation impurities changed with the change of time.

Since the unusual on-column degradation has not been reported (to the best of the authors' knowledge), we first tried to confirm the true purity and stability of the solid reference standard using other orthogonal methods such as DSC [18] and NMR. From DSC and NMR measurements, the data show that the apparent purity of the solid standard is more than 99%. Secondly, in order to confirm the source of the degradation by on-column reactions in high pH aqueous and acetonitrile mobile phases, but not by decomposition of the compound in the mixture of the mobile phases, a standard solution of Compound 1 was prepared in the mixture of 50% of 0.1% NH_4OH aqueous solution with 50% acetonitrile and injected into HPLC using 25 mM NH_4OAc aqueous mobile phase (pH 6.9, see Section 2 for details). The apparent purity of this standard solution was larger than 99.8%. On-column degradation was not observed by LC/MS over 10 injections of the standard solution (data not shown). Therefore, we were quite convinced that the azo impurities came from the on-column degradations rather than that from the solution degradations. Since the other degradation products' structures have not yet been elucidated and the potential co-elution issue of the hydrazo impurity with another degradation product, we will only focus on the chromatographic studies on the azo degradation products of Compound 1 throughout the text. Unless it is specified in the text, all the mobile phases used in the paper were 0.1% NH_4OH in water as mobile phase A and acetonitrile as mobile phase B.

3.2. Effect of temperature programs on on-column degradation of Compound 1 on XBridge Shield RP18 column

From our previous thermodynamic studies of the coated cellulose and amylase chiral stationary phases (CSPs), we discovered the thermally induced hysteresis of these CSPs when different heating

(from 10 to 50 °C by a 5 °C interval) or cooling (from 50 to 10 °C by a 5 °C interval) programs applied on the phases [12–15]. While thermal hysteresis of synthetic polymers caused by the conformation changes during the temperature programs is quite common [19], it is very rare for reversed-phase columns unless they were deliberately modified before the application of temperature programs [20–22]. In order to study the thermodynamic effect on the on-column degradation, similar heating (from 10 to 60 °C by a 10 °C interval) or cooling (from 60 to 10 °C by a 10 °C interval) programs have been applied to XBridge Shield RP18 columns with the high pH aqueous mobile phase. Fig. 3 shows the overlays of chromatograms of Compound 1 on two brand new columns at different starting temperatures. Fig. 4 summarizes the effect of column temperature programs on the unusual on-column degradations. Starting at 60 °C before the cooling program, the apparent purity of Compound 1 was 99.6% from the first injection and decreased to 96.6% in the fifteenth injection (Figs. 3 and 4A). Compared with the apparent purity changes from the first to the third injections of the same standard solution at 40 °C on the used column (without knowledge of the column history), the magnitude of reduction of apparent purity at 60 °C is smaller (2.4% in the third injection at 40 °C on the used column vs. 2.0% in the fifteenth injection at 60 °C on a brand new column). The levels of the azo degradation products were 0.00 and 0.04% in the first injection at 60 °C (Fig. 4B), which demonstrates that the degradation was nearly zero at the beginning of the injections. With the increase in the number of injections, the levels of the azo degradation products increase drastically at 60 °C (Fig. 4B). The effect of flow rates on on-column degradation was also studies at 60 °C from 0.5 to 2 mL/min by a 0.5 mL/min interval on a used column. The levels of the azo degradation products changed from 0.83 and 1.71% at 0.5 mL/min to 0.57 and 0.97% at 2.0 mL/min, respectively (data not shown). Obviously, the slower the flow rate, the longer the retention of the compound on the entire column to generate more degradation products. We also evaluated the effect of gradient time on the degree of on-column degradation on a used column. The levels of azo degradation products increased from 0.16 and 0.25% to 0.29 and 0.61% when gradient time changed from 5 to 120 min (data not shown). Again, the increase in gradient time increases the exposure time of the sample plug on the entire column. After cooling the column from 60 to 10 °C (1 injection/temperature step (instead of fifteen injections at 10 or 60 °C) after the column reached equilibrium) (Fig. 3B), the overall apparent purity of Compound 1 in the standard solution decreases with the decrease of the temperature. It reaches a valley at 30 °C with a value of 95.5% (Fig. 4C). When the column temperature changes from 60 to 10 °C, the levels of the degradation products increase with the decrease of column temperature first. Both peaks reach the maximum values at 40 °C. Then, they decrease with the decrease in column temperature (Fig. 4D).

Starting at 10 °C before the heating program, the apparent purity of Compound 1 in the standard solution was 99.0% from the first injection on a brand new column (but not 99.6% or above as the first injection at 60 °C, which is an indication of the decomposition) and slightly decreased to 98.7% in the fifteenth injection (Figs. 3C and 4A). It was surprised to observe the on-column degradation even in the first injection on this brand new column at the lower temperature. Compared with the apparent purity changes from the first to the fifteenth injection of the same standard solution at 60 °C, the magnitude of reduction of the apparent purity of Compound 1 is much smaller (Fig. 4A). The levels of the azo degradation products changed from 0.13 and 0.32% in the first injection to 0.19 and 0.40% in the fifteenth injection (Figs. 3C and 4B), respectively. Both indicate that the magnitude of the on-column degradation at 10 °C is much smaller than that at 60 °C. After heating the column from 10 to 60 °C in the same fashion as the cooling program, the overall apparent purity reduced from 98.6 to 96.5% with the increase of column temperature (Fig. 4C). The levels of two azo degradation products increased from 0.19 and 0.40% to 0.64 and 1.24% with the increase of the temperature (Figs. 3D and 4D). The non-superimposable nature of all the curves in Fig. 4 indicates that the on-column degradation depends on not only the time of exposure to the mobile phases, but also heating and cooling processes.

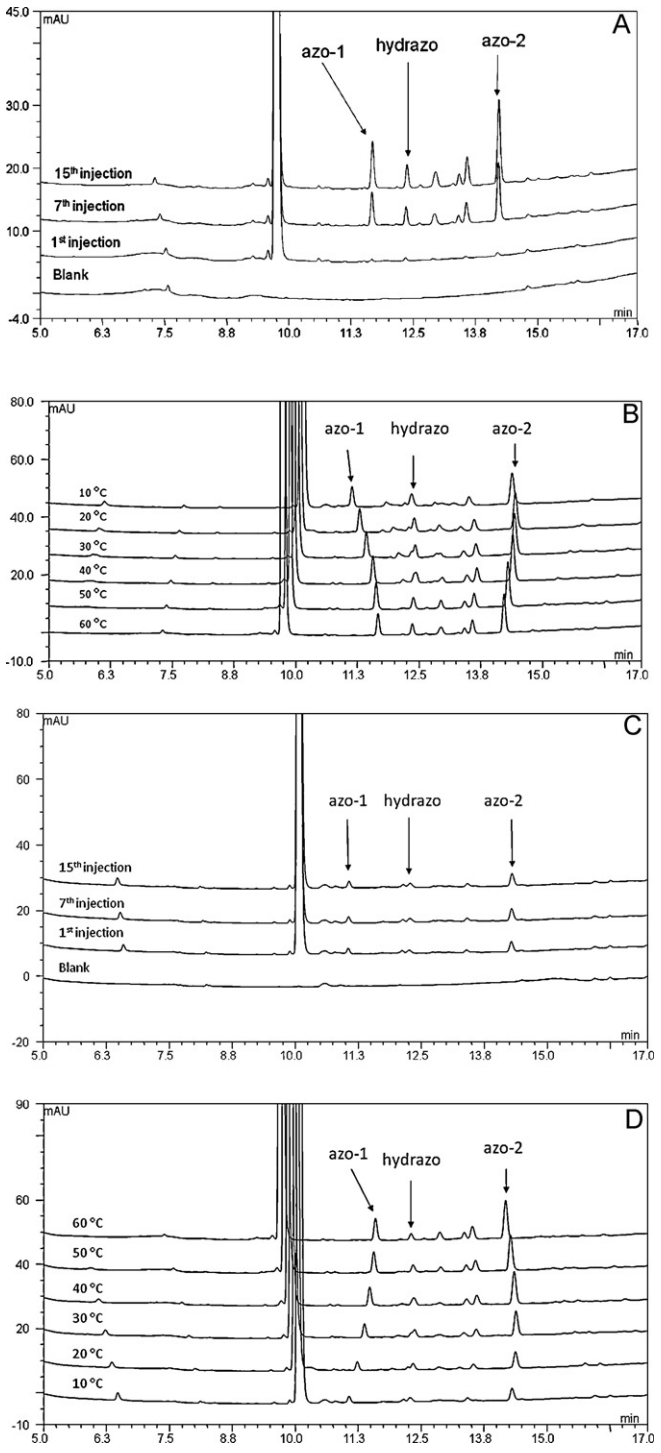

Fig. 3. Effect of starting temperatures and subsequent cooling/heating programs on the on-column degradation of Compound 1. The conditions: the same as Section 2 except: (A) effect of injection numbers on on-column degradation at 60 °C; (B) effect of cooling program on on-column degradation; (C) effect of injection numbers on on-column degradation at 10 °C; (D) effect of heating on on-column degradation.

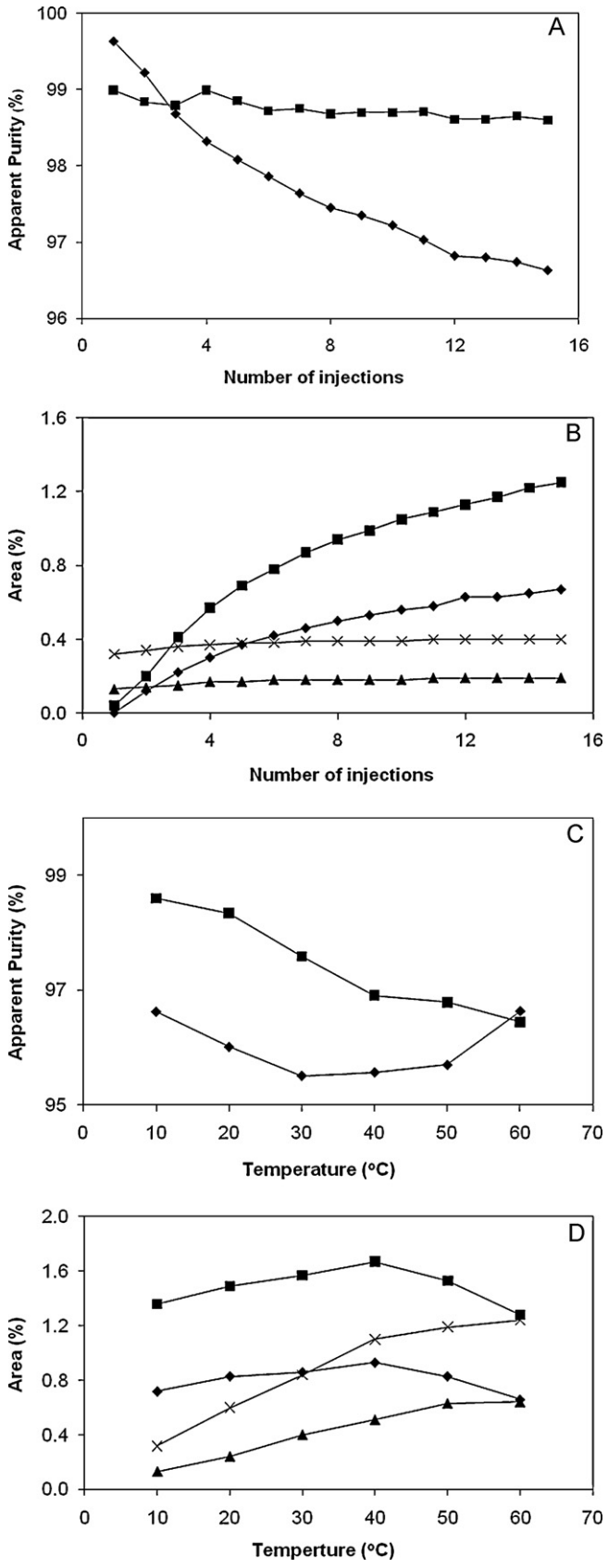

Fig. 4. Summaries of starting temperatures and cooling/heating programs on on-column degradation of Compound 1. The conditions: the same as Fig. 3. (A) Comparison of starting temperature on apparent purity of Compound 1, column temperature: 60 °C (filled diamond), 10 °C (filled square); (B) comparison of starting temperature on the formation of azo degradation products, column temperature: azo-1 at 60 °C (filled diamond), azo-2 at 60 °C (filled square), azo-1 at 10 °C (filled triangle), azo-2 at 10 °C (cross); (C) effect of temperature programs on apparent purity of Compound 1, temperature programs: cooling from 60 to 10 °C (filled diamond), heating from 10 to 60 °C (filled square); (D) comparison of temperature programs on the formation of azo degradation products, temperature programs: azo-1 cooling from 60 to 10 °C (filled diamond), azo-2 cooling from 60 to 10 °C (filled square), azo-1 heating from 10 to 60 °C (filled triangle), azo-2 heating from 10 to 60 °C (cross).

3.3. Effect of column history on the on-column degradation of Compound 1

During the studies of thermal hysteresis on enantioseparation of DHP compounds on the coated CSPs, the authors discovered that the column history had profound impact on the chromatographic behaviors of the probe compounds in normal phase conditions [12–15]. Literature survey also shows that the adsorption/desorption hysteresis can occur under certain isothermal conditions [23,24]. Since we have observed the thermal hysteresis of the on-column degradation of Compound 1 on XBridge Shield RP18 column (Figs. 2–4), it became very clear that the surface conditions have profound impact on the on-column degradation in RPLC as well. From the beginning, based on our previous experience, we only utilized all brand new columns to study the unusual on-column degradation of Compound 1. Fig. 5 shows the effect of column history on the on-column degradation at 60 °C and in the subsequent cooling program. A used column with history of two previous cooling sequences was used for this study. Starting at 60 °C as in Fig. 4B (for the comparison purpose, the first and the third injection sequences are plotted in Fig. 5), the levels of the azo degradation products started at 0.86 and 1.46% in the first injection on the used column, respectively. The levels of the degradation products on the used column did not change with the increase in the numbers of the injections at 60 °C (Fig. 5A). The magnitude of the degradation on the used column is bigger than that of the fifteenth injection of the brand new column at the same temperature in the first sequence (Fig. 5A). Fig. 5B shows a similar trend (but not super-imposable) during the cooling program for both new and used columns. This indicates that once the column experienced the mobile phases (even in the first injection sequence after 15 injections at the starting temperature on a new column), the surface conditions of the column were no longer considered as fresh any more. Therefore, the brand new column after 15 injections of the standard solution in the first sequence showed the same trend of the effect of temperature programs as the used column. Fig. 5C shows the impact of column history on the on-column degradation of two azo peaks at 10 °C on the used column. The level of on-column degradation of Compound 1 slightly decreases with the increase in the injection times, which is opposite to the trend of the new column (Fig. 5C). The magnitude of the degradation on the used column is much larger than that on a brand new column at 10 °C, which is an indication of the accumulative effect on the on-column degradation. Again, the levels of the degradation products in the first and fourth heating sequences show similar trend (but not super-imposable) on both new and used columns (Fig. 5D). Obviously, for the used columns, the change of the level of the on-column degradation with time does not have the same trends as the new columns at both starting temperatures. The temperature programs still have impact on the degradation levels for the used columns.

In order to study the effect of acetic acid wash on the on-column degradation as previously reported [10], the used columns were isocratically washed with a mixture of 90% of 0.5% acetic acid in water and 10% of acetonitrile for 2 h at 40 °C before re-tested with 15 injections at 60 °C. Fig. 6 shows the effect of column wash on the on-column degradation of Compound 1. The initial 15 injections of

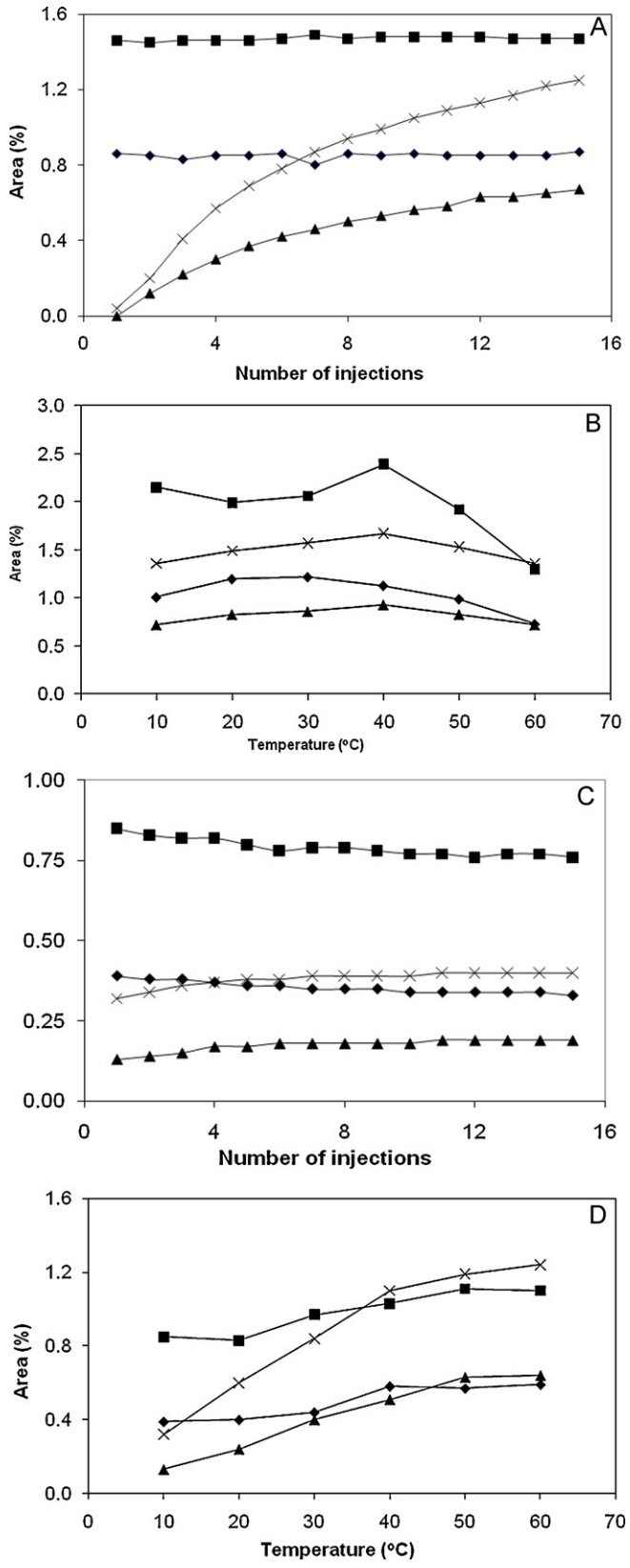

Fig. 5. Effect of column history on on-column degradation of Compound 1. The conditions are the same as Section 2 except column temperature. (A) Starting at 60 °C, column history and compound names: azo-1 on the used column (filled diamond), azo-2 on the used column (filled square), azo-1 on the new column (filled triangle), azo-2 on the new column (cross); (B) cooling program: azo-1 on the used column (filled diamond), azo-2 on the used column (filled square), azo-1 on the new column (filled triangle),

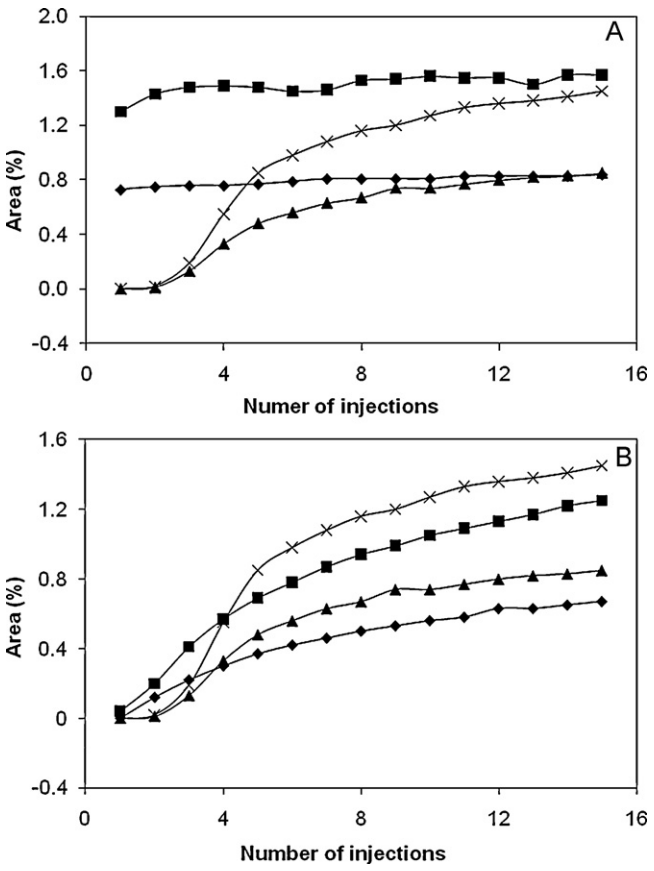

Fig. 6. Effect of column-wash on on-column degradation of Compound 1. The conditions: the same as Fig. 5 except the wash procedure. (A) Comparison of the used column before and after column wash at 60 °C, azo-1 before the acid wash (filled diamond), azo-2 before the acid wash (filled square), azo-1 after the acid wash (filled triangle), azo-2 after the acid wash (cross); (B) comparison of the time-dependency of the washed and new column at 60 °C, azo-1 on the new column (filled diamond), azo-2 on the new column (filled square), azo-1 on the used column post the acid wash (filled triangle), azo-2 on the used column post the acid wash (cross).

the standard solution on the used column before the column wash showed a small degree of the change of the level of azo-2 degradation product with time while not for azo-1 degradation product (Fig. 6A). After the acetic acid wash, the change of the level of on-column degradation with time was regenerated in the same fashion as a brand new column, but the levels of degradation on the new and re-generated column are not super-imposable (Fig. 6B). This indicates that the reactive sites on the surface of the stationary phase cannot be reproducibly re-generated as identical to those on brand new columns. The impact of temperature programs on the on-column degradation in cooling program before and after the acid wash shows very similar trend, but not identical (data not shown). The impact of temperature programs on the on-column degradation in cooling program on the re-generated column is also not identical to the brand new column (data now shown). The used columns were also washed and re-tested with 15 injections of the standard solution at 10 °C and with a subsequent heating program as well. The unusual on-column degradations can be reproduced, but not superimposable to those of the new columns (data not

azo-2 on the new column (cross); (C) starting at 10 °C, column history and compound names: azo-1 on the used column (filled diamond), azo-2 on the used column (filled square), azo-1 on the new column (filled triangle), azo-2 on the new column (cross); (D) heating program: azo-1 on the used column (filled diamond), azo-2 on the used column (filled square), azo-1 on the new column (filled triangle), azo-2 on the new column (cross).

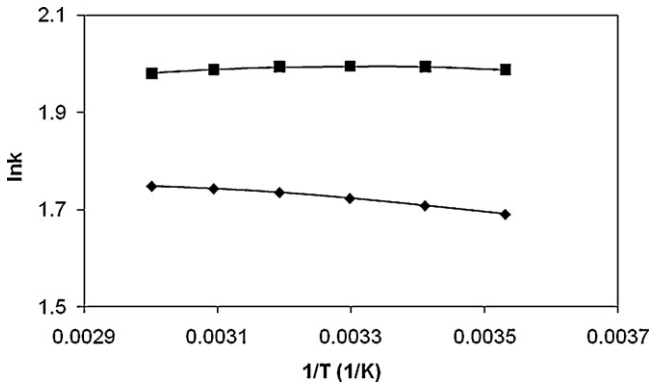

Fig. 7. van't Hoff plots of the azo degradation products of Compound 1 in cooling program. The conditions: the same as Fig. 3B. van't Hoff plots of retention factors, compound names: azo-1 (filled diamond), azo-2 (filled square).

shown). All these data indicate that the reactive sites on the surface of the columns are not reproducible at all, which in turn has a profound impact on the levels of the degradation products.

3.4. Effect of temperature programs on chromatographic behaviors of Compound 1

Fig. 7 shows van't Hoff plots of the azo degradation products during the cooling program. From Figs. 3B and D and 7, the logarithm of the retention factors of azo-1 peak of Compound 1 decreases with the decrease of temperature in a linear fashion (R^2 = 0.97). The logarithm of the retention factors of azo-2 peak increases with the decrease in temperature first and reaches a maximum at 40 °C. The van't Hoff plot of retention factors of azo-2 peak is non-linear (R^2 = 0.20) (Fig. 7). The van't Hoff plot of the selectivity factors of these two degradation products is linear (R^2 = 1.00, data not shown). The selectivity factors increase with the decrease in column temperature (data not shown). The heating van't Hoff plots of retention and selectivity factors of Compound 1 and degradation products are super-imposable with those of the cooling van't Hoff plots (difference within ±2.0%, data not shown), which indicates that the retention behaviors of Compound 1 and the azo degradation products were under thermodynamic equilibriums as the most reversed phase separations. As expected, the used columns show the same retention behaviors in the cooling and heating programs before and after the acid wash as brand new columns (difference within ±2.0%, data not shown). All the data indicate that neither the temperature programs nor the history of the columns has any impact on the retention behaviors of the degradation products.

3.5. Effect of temperature programs on on-column degradations of other aniline compounds on XBridge Shield RP18 column

Table 1 shows the preliminary screen of the commercially available compounds (purities ≥99.6% by GC per vendor's certificates of analysis) with high pH aqueous and acetonitrile mobile phases on a used column applying column-wash program before the injection of each compound. The position of the substitution (fluoro- and chloro-) groups has a major impact on the magnitude of the degradation. The degree of the degradation is: 4Cl- and 4F-anilines > aniline > 3Cl-aniline > 2Cl- and 2F-anilines. We systematically tested 4Cl- and 4F-anilines using the same temperature programs on new columns in order to demonstrate that this type of on-column degradations occurs not only on our model compound, but also on the same class of compounds as well. Fig. 8 shows the change of the level of on-column degradations of 4Cl- and 4F-anilines with time at two different starting temperatures on new columns. At the first injections of both compounds, the degradations were at minimum level for both compounds at 60 °C while those were significant at 10 °C. With the increase of the injection numbers, the degradation products increase at both temperatures (Fig. 8A–D). The plots of the apparent purities of the main peak and the largest degradation products vs. injection numbers are similar to Fig. 4A and B at both starting temperatures. However, the magnitude of the change of the level of degradation of 4Cl-aniline with time is much higher than that of 4F-aniline at both temperatures (Fig. 8A–D). Interestingly, for 4Cl-aniline at the 15th injection at 60 °C, the largest degradation peak (at retention time 15.9 min) shows severe fronting (with the asymmetric factor of 0.78) while not obvious (with the asymmetric fact of 0.95) in the 7th injection (Fig. 8B). Similar temperature program effect on both compounds was also observed during the heating/cooling processes (data not shown).

Obviously, the conversion of aniline compounds to azo or hydrazo pseudo-dimers involves the oxidation reactions. Literature search shows that the symmetric azo and hydrazo compounds can be formed by the oxidizing reagents such transition metal compounds, for example [25,26]. Aerobic oxidation of the aniline starting materials can also produce the symmetric azo compounds on gold nano-particles under certain oxygen pressure (a couple of bars) [27]. We compared the impact of oxygen level in the mobile phases on the degree of degradation of 4F-aniline on a used column without degasser (by-passing), with HPLC degasser and with degasser plus helium sparging both mobile phases. There was no significant change in the degree of degradations at both starting temperatures (data not shown). It is also unlikely that the residual metal iron at the head of the columns plays a role since the iron level in the packing materials is ≤2 ppm per vendor's certificate of analysis. Since the synthetic process of manufacturing these commercially available aniline compounds was not known from the vendor, ICP-MS testing was conducted to monitor the potential source of oxidation reagents from the transition metals in the analytes for the purpose of understanding of degradation mechanism. The level of the transition metals in all these aniline compounds (including Compound 1) was negligible (≤1 ppm, data not shown). Therefore, it is also unlikely that the metal impurities in the analytes have major impact on the potential formation of oxidation reagents in the sample plugs to cause the on-column degradations. The on-column degradations of the aniline compounds are more likely caused by the oxidation reagent(s) formed by the complexation between some elements on the surfaces of the XBridge stationary phases and ammonia at high pH aqueous mobile phase. That is the reason that on-column degradation was never observed in the neutral aqueous mobile phase with ammonium acetate buffer. The application of the high pH ammonia aqueous and methanol

Table 1
Effect of starting column temperatures on on-column degradation of commercially available aniline compounds at 15th injection on a used column.[a]

	Starting at 60 °C		Starting at 10 °C	
	% main peak	% largest degradation peak	% main peak	% largest degradation peak
4F-anailine	96.7	2.69	95.3	2.61
3F-anailine	99.2	0.25	99.2	0.21
2F-anailine	99.1	0.25	99.5	0.19
Aniline	97.9	1.24	97.4	0.87
4Cl-anailine	96.4	2.04	94.8	1.76
3Cl-anailine	98.8	0.41	98.8	0.27
2Cl-anailine	99.4	0.13	99.3	0.22

[a] With 2 h column-wash before the injection of each compound. Other conditions: see Section 2.

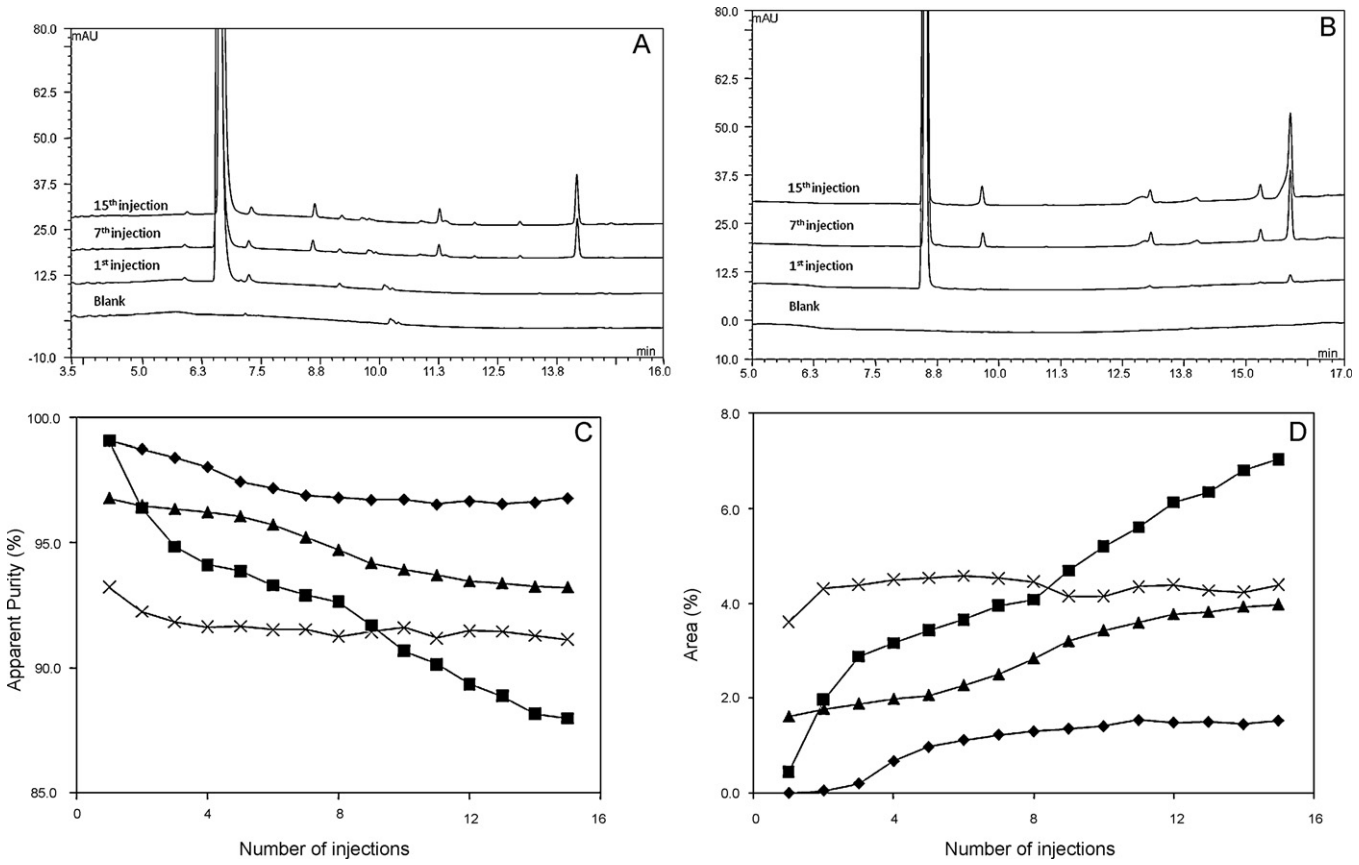

Fig. 8. Effect of starting temperatures on the on-column degradation of 4F- and 4Cl-anilines The conditions: the same as Section 2 except: (A) effect of injection numbers on on-column degradation of 4F-aniline at 60 °C; (B) effect of injection numbers on on-column degradation of 4Cl-aniline at 60 °C; (C) effect of injection numbers on on-column degradation of the apparent purity of the main peaks of 4F- and 4Cl-anilines at 60 and 10 °C 4F-aniline at 60 °C (filled diamond), 4Cl-aniline at 60 °C (filled square), 4F-aniline (filled triangle) at 10 °C, 4Cl-aniline at 10 °C (cross); (D) effect of injection numbers on on-column degradation of the single largest degradation peaks of 4F- and 4Cl-anilines at 60 and 10 °C: 4F-aniline at 60 °C (filled diamond), 4Cl-aniline at 60 °C (filled square), 4F-aniline (filled triangle) at 10 °C, 4Cl-aniline at 10 °C (cross).

mobile phases on XBridge Shield RP 18 columns did not generate any degradation product of Compound 1 on new columns with the heating and cooling cycles (data not shown). No degradation product of 4Cl-aniline was observed on Xbrideg Shield RP 18 column when 20 mM ammonium carbonate (adjusted to pH 10.2) aqueous buffer and acetonitrile were used as the mobile phases (data not shown). In order to confirm that the XBridge packing materials play the key role in the degradations of the aniline compounds, we also performed the same heating/cooling temperature programs on a Gemini NX C18 column using 4Cl-aniline as a model compound. Starting at 10 °C, only one degradation peak grew from 0.00% in the first injection to 0.32% in the 15th injection (chromatograms not shown). After the temperature changes, no further increase in the numbers of degradation products and in the level of the degradation peak was observed in three heating/cooling sequences (data not shown). We also conducted the same systematic studies on the other XBridge stationary phases, such as XBridge C18 and Phenyl columns. Similar degradation trends were observed on these columns (data not shown). Per one of the reviewers' comments, we also conducted another control experiment with both heating first and cooling first temperature cycles on two new columns with an inert solute – toluene. After completed the first temperature cycle on each column using toluene as the testing compound, a second temperature cycle was repeated on each used column (without column wash) using 4Cl-aniline as the analyte. In the first temperature cycles, the levels of the apparent purity and impurities of toluene did not change with the temperature cycles and starting temperature. No additional impurity of toluene was observed in the first temperature cycles. Essentially no change was observed for the retention times of the main peak and impurities, the asymmetric factors and column efficiencies of the toluene peaks in the first and the last injections of each temperature cycle. During the second temperature cycles on each used column, the same column history effect as described in Section 3.3 was observed when 4Cl-aniline was injected (i.e. as Fig. 5, data not shown). In conclusion, the stationary phase does not change as the result of: (1) exposure to the high pH mobile phase over time and/or (2) temperature programs as evidenced by the chromatographic behaviors of toluene analyte before and after the temperature cycles. The reaction of the aniline analytes with the columns in the ammonia modified aqueous and acetonitrile mobile phases produced the artifacts of the on-column degradation products.

4. Conclusions

The unusual on-column degradations of aniline compounds were observed with ammonia at a high pH aqueous mobile phase and acetonitrile were applied on XBridge Shield RP18 column. The positions of halogen groups on anilines have major impact on the degree of degradations. The change of the level of the on-column degradation of Compound 1 was more profound at both 10 and 60 °C when the column surface was either brand new or re-generated. The on-column degradations belong to surface reactions. Temperature programs, the composition of the aqueous mobile phases and column history have profound impact on the degree of on-column degradation. The retention behaviors of the degradation products of Compound 1 are independent of the temperature programs, history of the columns and column wash.

The paper also provides some precautions of the artifacts created by the potential genotoxic degradation products from on-column reactions, such as azo and hydrazo impurities, using the high pH aqueous and acetonitrile mobile phases in an analytical method.

Acknowledgements

The authors thank Dr. Alan Allgeier and Sheng Cui of Chemical Process Research and Development Department for Compound 1 samples and discussions on reaction mechanism. The authors also thank Dr. Tsang-Lin Hwang for the NMR purity confirmation data and Mr. Christopher Scardino for ICP-MS measurement of aniline samples.

References

[1] L.R. Synder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, 2nd ed., Wiley, New York, 1997 (Chapter 9).
[2] J.J. Kirkland, M.A. van Straten, H.A. Classens, Anal. Chem. 70 (1998) 4344.
[3] J.J. Kirkland, M.A. van Straten, H.A. Classens, J. Chromatogr. A 797 (1998) 111.
[4] K.D. Wyndham, J.E. O'Gara, T.H. Walter, N.L. Lawrence, B.A. Alden, G.S. Lzzo, C.J. Hudalla, P.C. Iraneta, Anal. Chem. 75 (2003) 6781.
[5] N.H. Davies, M.R. Euerby, D.V. McCalley, J. Chromatogr. A 1178 (2008) 71.
[6] C. Stella, S. Rudaz, M. Mottaz, P. Carrupt, J. Veuthey, J. Sep. Sci. 27 (2004) 284.
[7] C.E. Jones, C.J. Darcy, T. Woodberry, N.M. Anstey, Y.R. McNeil, J. Chromatogr. B 878 (2010) 8.
[8] A. Espada, A. Marin, C. Anta, J. Chromatogr. A 1030 (2004) 43.
[9] W. Kiridena, C.F. Poole, S.N. Atapattu, J. Qian, W.W. Koziol, Chromatographia 66 (2007) 453.
[10] M.W. Dong, G.D. Miller, R.K. Paul, J. Chromatogr. A 987 (2003) 283.
[11] L. Muller, R.J. Mauthe, C.M. Riley, M.M. Andino, D. de Antonis, C. Beels, J. DeGeorge Jr., A.G.M. De Knaep, D. Ellison, J.A. Fagerland, R. Frank, B. Fritschel, S. Galloway, E. Harpur, C.D.N. Humfrey, A.S. Jacks, N. Jagota, J. Mackinnon, G. Mohan, D.K. Ness, M.R. O'Donovan, M.D. Smith, G. Vudathala, L. Yotti, Regul. Toxicol. Pharmacol. 44 (2006) 198.
[12] F. Wang, T. O'Brien, T. Dowling, G. Bicker, J. Wyvratt, J. Chromatogr. A 958 (2002) 69.
[13] F. Wang, R.M. Wenslow Jr., T.M. Dowling, K.T. Mueller, I. Santos, J.M. Wyvratt, Anal. Chem. 75 (2003) 5877.
[14] F. Wang, T. Dowling, D. Ellison, J. Wyvratt, J. Chromatogr. A 1034 (2004) 117.
[15] F. Wang, D. Yeung, J. Han, D. Semin, J. McElvain, J. Cheetham, J. Sep. Sci. 31 (2008) 604.
[16] P.J. Magalhaes, L.F. Guido, J.M. Cruz, A.A. Barros, J. Chromatogr. A 1150 (2007) 295.
[17] S.D. Tanner, V.I. Baranow, J. Am. Soc. Mass Spectrom. 10 (1999) 1083.
[18] B. Wunderlich, Thermal Analysis of Polymeric Materials, Springer, New York, 2005 (Chapter 4).
[19] B. Wunderlich, Thermal Analysis of Polymeric Materials, Springer, New York, 2005 (Chapter 6).
[20] R.K. Gilpin, J.A. Squires, J. Chromatogr. Sci. 19 (1981) 195.
[21] R.K. Gilpin, S.S. Yang, J. Chromatogr. 394 (1987) 295.
[22] J.W. Carr, J.M. Harris, J. Chromatogr. 481 (1989) 135.
[23] S. Lin, R. Blanco, B.L. Karger, J. Chromatogr. 557 (1991) 369.
[24] F. Britti, G. Guiochon, J. Chromatogr. A 1010 (2003) 153.
[25] E. Baer, A.L. Tosoni, J. Am. Chem. Soc. 78 (1956) 2857.
[26] H. Firouzabadi, Z. Mostafavipoor, Bull. Chem. Soc. Jpn. 56 (1983) 914.
[27] A. Grirrane, A. Corma, H. Garcia, Nat. Protoc. 5 (2010) 429.