Studies on the degradation of blue gel pen dyes by ion-pairing high performance liquid chromatography and electrospray tandem mass spectrometry

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Abstract

Ion-pairing high performance liquid chromatography (IP-HPLC) was utilized to monitor the composition changes of blue gel pen ink entries on paper stored in different light conditions and natural environment. The chromatographic conditions were optimized by comparing the separation efficiencies of the blue gel pen inks using a series of ion-pairing reagents, including ammonium carbonate, ammonium acetate, triethylamine acetate, tributylamine acetate, tetrabutylammonium bromide and dihexylammonium acetate. It has been found that tributylamine acetate was a suitable ion-pairing reagent for separation of the inks on the common C\textsubscript{18} column. The analysis results of the ink entries on paper in different aging conditions showed that the tendency of composition change in natural aging condition was similar with those in fluorescent light and UV light conditions, respectively. One main component dye of the blue gel pen ink, Acid Blue 9, and its degradation products were identified by ion-pairing high performance liquid chromatography coupled with electrospray tandem mass spectrometry. The results showed that the main degradation products originated from the Acid Blue 9. It gave a reasonable explanation for the changing rules of the relative content of the dyes in the blue gel pen ink. The results obtained can provide scientific evidences for dating of the blue gel pen ink entries on documents.

Keywords: Blue gel pen inks; Ion-pairing reagent; HPLC; ESI–MS/MS; Photodegradation; Questioned document

1. Introduction

Gel pens are becoming a prominent type of writing instruments due to their smooth writing characteristics, vivid color, low cost and environment friendly property [1]. They are used frequently throughout our lives to sign contracts, checks, loans and other documents, and thus forged documents signed by gel pens have increased rapidly in recent years. Crimes committed with documents involve billions of dollars annually and actually have a bigger impact on society than do violent crimes [2]. Therefore, the validity of questioned documents is often queried during litigation, and adequate and accurate approaches for the analysis of the gel pen inks in questioned documents are urgently needed.

Gel pen inks were water-based inks that were first created in 1984 by Sakura Color Products Corp. of Japan. These inks contain dyes or pigments as colorants, water as vehicles, resins, nonionic surfactants and other additives [2]. Acid dyes and related compounds, usually containing several sulphonic groups, have been widely used as the coloring agents in gel pen inks [1,2].

The analysis of the ink dyes would supply more useful information for ink dating than that of other additives did. Many analytical methods were developed to analyze sulphonated compounds in various matrix, including capillary zone electrophoresis [3–5], micellar electrokinetic capillary chromatography [6], spectroscopic methods [7,8], and thin layer chromatography [9]. Ion-pairing high performance liquid chromatography (IP-HPLC) can be utilized to separate ionic compounds effectively [10–15], and it would be a suitable method for analyzing the ionic dyes in gel pen inks. High performance liquid chromatography and mass spectrometry are powerful methods for identification of compounds in complex matrix [16–24] and can be
used to analyze quantitatively the components of the blue gel pen inks.

In this study, ion-pairing high performance liquid chromatography has been utilized to analyze the blue gel pen inks and their photodegradation products. The chromatographic conditions were optimized by selecting suitable ion-pairing reagent for achieving a satisfactory separation of the dyes. The blue gel pen ink entries on paper stored in different light conditions and natural environment were analyzed. Photodegradation and high performance liquid chromatography–electrospray tandem mass spectrometry (HPLC–ESI–MS/MS) have been used to identify the dyes and their degradation products. The degradation processes of the dyes were inferred and it would provide scientific foundation for dating of the documents written by blue gel pen inks.

2. Experimental

2.1. Reagents and instruments

HPLC grade acetonitrile was from Dima (USA); tetrabutylammonium bromide (99+%, TBABr), tributylamine (99%, TBA) and dihexylamine (99+%, DHA) were from Acros Organics (NJ, USA); ammonium acetate and triethylamine (TEA) were analytical grade and from Tianjin No.6 chemical reagent factory and Beijing Yili Fine Chemical; ammonium bicarbonate and from Liulidian chemical reagent factory, respectively. Other chemicals (NJ, USA); ammonium acetate and triethylamine (TEA) were analytical grade and from Tianjin No.6 chemical reagent factory and Beijing Liulidian chemical reagent factory, respectively. Other chemicals were analytical grade and used as supplied. Water for buffer preparation was Milli-Q ultra pure water.

Liquid handling products (20–200 µL and 100–1000 µL) were Finnpipette Stepper from Thermo Electron (USA). Basis pH Meter PB-21 was from Sartorius AG (Germany). Ultraviolet analysis apparatus was from Haimen QL-Lab Co. Ltd. (China).

2.2. High performance liquid chromatography

The chromatographic apparatus consisted of a Waters 510 pump and a Waters 490E Programmable Multiwavelength UV Detector (Waters, USA). The separation was performed on a Luna C18 column (250 mm × 4.60 mm, 5 μm, Phenomenex, USA). The mobile phase was tributylamine acetate (eluent A, pH 7.0) and acetonitrile (eluent B), and the flow rate was 1.0 mL min⁻¹. The injection volume was 20 μL.

2.3. HPLC–MS/MS

The Waters Quattro Premier XE Mass Spectrometer with the ACQUITY ultra performance liquid chromatography (UPLC) system was used for HPLC–MS/MS analysis (Waters, USA) and the wavelength of a tunable UV (TUV) detector was set to 580 nm. Software for data processing was Masslynx and the column for separation was Bridged Ethane Hybrid Column (50 mm × 2.10 mm, 1.7 μm, Waters, USA). The chromatographic conditions were 10 mmol L⁻¹ ammonium carbonate (eluent A, pH 9.5, adjusted by ammonia) and acetonitrile (eluent B), linear gradient from 90% A to 10% A in 10 min, hold 3 min and then 2 min for re-equilibration. The column temperature was kept at 55 °C and the flow rate was 0.30 mL min⁻¹. Full-scan spectra were acquired from m/z 250–1200 using electrospray ionization (ESI) in negative and positive ionization mode. Desolvation gas was nitrogen and heated to 350 °C at a flow rate of 602 L/h. The cone voltage was 30 V. The fragmentation voltage was set to 30–60 V according to the properties of the components when performing MS/MS analysis.

2.4. Preparation of samples

Forty-seven blue gel pens were collected, which represented gel pen available from markets at home and abroad. Lines were drawn on ordinary A4 print paper for preparing the ink samples. For light aging study, ink samples were exposed to ultraviolet light in the wavelength of 254 nm and a fluorescent tube from a short distance (about 10 cm), respectively. Ink samples were stored in natural conditions (room temperature, avoiding irradiated directly by sunlight) for preparing naturally aging samples.

For each sample, 5 cm ink line was cut out and extracted by 0.5 mL 10 mmol L⁻¹ tributylamine acetate buffer/acetonitrile (v/v = 1:1) for about 10 h at room temperature, and the extract was filtered through a 0.45 μm Millipore filter prior to the HPLC analysis.

3. Results and discussion

3.1. Optimization of chromatographic conditions

Ionic organic compounds have weak retention on the ordinary reversed stationary phases, such as C₁₈, C₈, when performing the separation in high performance liquid chromatography due to their high polarities, and so ion-pairing reversed phase liquid chromatography has been becoming one of the most important approaches to analyze them [10–12]. For separation of the dyes in blue gel pen inks, cationic ion-pairing reagents were selected as the additives of the mobile phase because the dyes were usually anions. The main UV absorption bands of the dyes are at the wavelength ranged from 400 to 700 nm, and most of the dyes have maximum UV absorption near 580 nm, so the wavelength of the UV detector was set to 580 nm. The UV absorptions of the fluorescence whitening reagents in paper are usually below 500 nm and they have no interference for the detection of the gel pen dyes at 580 nm.

3.1.1. Selection of the ion pairing reagent

There are a number of factors that affect the separation of ionic organic compounds in ion-pairing reversed phase liquid chromatography [25–30], including type of ion-pairing reagents, concentration of the ion-pair reagents and pH value of the mobile phase. The choice of the cationic ion-pairing reagents is an important step for optimizing the chromatographic conditions of the gel pen dyes and it is related to the property of the analyte [30,31]. For the polysulphonated anionic dyes, an ion pairing reagent with high ability to form ion pair with the dyes is needed. A series of volatile and non-volatile ion-pair reagents, alkyl ammonium salt with different alkyl chain, including ammonium...
carbonate, ammonium acetate, TEA, TBA, TBABr, and DHA were tested as mobile phases, respectively for selecting a suitable ion pairing reagent.

Fig. 1 showed the chromatograms using various ion-pairing reagents as the mobile phase when performing the separation of a representative blue gel pen ink (G7). There are some reports [24,31,32] that mobile phases containing ammonium acetate or TEA can be used for the IP-HPLC analysis of ionic compounds with mono-sulphonic acid, but the gel pen dyes, usually containing di- or multi-sulfonic groups, have weak retention and cannot be efficiently separated in the similar conditions (see Fig. 1a and b). From the figures, it can be seen that the dyes were nearly not reserved in the column and eluted with the solvent. Fig. 1c showed the IP-HPLC chromatogram using ammonium carbonate as the ion-pairing reagent (for preparing the ammonium carbonate solution, the pH of the ammonium bicarbonate solution should be adjusted above 9.0 with ammonia). It can be seen from Fig. 1c that one of the two main dye components can be reserved, but there was still a portion not to be retained in the common C18 column.

Tetrabutylammonium bromide has been used as ion-pairing reagent in the HPLC analysis of the acid dyes and has high ability to form ion pair with anionic analyte [24,33]. Most of the blue gel pen inks can be separated effectively, but the separation efficiencies of some dyes were poor when it was used as ion pairing reagent. The two main components of the blue gel pen ink (G7) were not achieved baseline separation (see Fig. 1f) and the separation cannot be improved when optimizing the HPLC conditions.

When TBA acetate and DHA acetate was used as the ion-pairing reagent, almost all of the blue gel pen inks collected can be separated, but the separation efficiency was higher and retention time was shorter when TBA acetate was used as ion-pairing reagent (see Fig. 1d and e). From Fig. 1d, it can be seen that the two main components in blue gel pen ink (G7) were separated well, so TBA acetate was chosen as the ion pairing reagent to investigate the aging rule of the dyes by HPLC.

3.1.2. Effects of concentration of ion-pairing reagent on HPLC separation

In order to evaluate the influence of the TBA concentration on the separation of the blue gel pen ink, a series of concentrations from 2 to 80 mmol L$^{-1}$ (2, 5, 10, 20, 40, 80, individually) as ion-pairing reagent were tested. The results showed that the dyes could be efficiently retained and separated when the concentration of TBA was over 5.0 mmol L$^{-1}$ and the retention time of the dyes significantly increased with the augment of the TBA concentration, however, the peak shape and the efficiency of separation have only minor improvement.

When the concentration of TBA was below 5.0 mmol L$^{-1}$, weak retention of some dyes was observed due to the insufficient counter-ion in the mobile phase and the relative peak area of some dyes were not consistent with those obtained in high concentration of ion pairing reagent. Different concentrations of other ion-pairing reagents were also examined. The results were similar to those of TBA when their concentrations were higher than 20 mmol L$^{-1}$, and the efficiency of separation and peak shape were not obviously improved with the variations of concentration. For the suitability of separation of most blue gel pen dyes, 10 mmol L$^{-1}$ of TBA was selected as the buffer solution to perform the HPLC analysis.

3.1.3. Effects of pH on the HPLC separation

The buffer solutions of TBA and DHA would become turbid when the pH value was adjusted above 8.0, so the separation of the blue gel pen dyes were performed on the IP-HPLC using the
buffer solutions of the ion-pairing reagents at pH 5.0, 7.0, 8.0 as mobile phases to optimized the chromatographic conditions. It has been found that the retention time of the dyes increased with the decrease of the buffer’s pH, and the pH of the ion-pairing reagent had little effect on the peak shapes and separation efficiencies of the dyes. The blue gel pen dyes belonged to the acid dyes and their $pK_a$ are usually lower than 2.0 (for strong acid dyes) or in the range from 2.0 to 6.0 (for weak acid dyes) and they would be completely dissociated above pH 6.0. At neutral pH 7.0, satisfactory separation results, symmetrical peak shape and appropriate retention time of the dyes can be achieved. Another reason for choosing pH 7.0 is its wide suitability that the pH range of most C18 column is between 2.0 and 7.5.

The optimized conditions for separation of the blue gel pen dyes by ion-pairing high performance liquid chromatography were as follow: 10 mmol L$^{-1}$ TBA as ion-pairing reagent, pH 7.0.

Forty-seven blue gel pens, of various brands and models, were collected and separated into two classes by solubility test in ethanol [34]: 20 pigment-based and 27 dye-based gel inks. The pigment-based gel pen inks cannot be extracted from the paper for further HPLC analysis. The 27 dye-based gel pen inks were separated by IP-HPLC based on the chromatographic conditions optimized above, and all the components of the dye-based gel pen inks were efficiently separated. Most of the inks contained two or three main components of dyes and some inks have one main peak and several minor peaks when the wavelength of UV detector was set to 580 nm.

### 3.2. Aging of the blue gel pen dyes

Up to now, there are only reports concerning the dating and degradation of ballpoint pen ink entries on documents [35–42]. Ink dyes of ballpoint pen would undergo degradation during storage in natural or artificial light aging conditions [35–38]. The study of ink entries on document stored in different light conditions would be helpful to explore the effects of natural aging and light aging on the decomposition of ink dyes and offered theoretical foundation for determination and dating of the inks on suspicious documents [39–42].

Blue gel pen G7 was randomly selected as a representative to investigate the decomposition mechanism of the dyes under various aging conditions. The inks were extracted from the paper before and after aging in fluorescent light, ultraviolet light and natural conditions, respectively. The extracts were separated by ion-pairing liquid chromatography with the condition optimized in Section 3.1 to monitor quantitatively the variations of the components.

**3.2.1. Aging of the blue gel pen dye in light condition**

Fig. 2A showed the representative chromatograms of the G7 ink samples after exposure to fluorescent light. From the chromatograms, significant changes in composition of the ink were noted on exposure to light. The G7 ink contained two main components of dye (Fig. 2A(a)), numbered as peak 1 (retention time (RT) 6.2 min) and peak 2 (RT 8.3 min). After the ink entries on paper exposed to fluorescent light, two new peaks appeared in the chromatogram (numbered as peak 3 (RT 3.9 min) and peak 4 (RT 4.9 min)), which signified that new components have been formed. The two new peaks became more prominent with increasing the aging time, meanwhile some weak peaks appeared nearby the peaks 3 and 4 when the aging time was 36 h (Fig. 2A(b)). After the aging time was above 100 h, the weak peaks became more obvious apart from peaks 3 and 4 (Fig. 2A(c)). It can also be noted that the relative...
intensity of peak 1 decreased significantly with increasing the aging time.

Fig. 2B showed the chromatograms of G7 ink entries on paper after exposure to UV light. From the chromatograms, it can be seen that the photodegradation products of the dyes induced by UV light have the same retention time with those by fluorescent light. The relative intensities of the new photodegradation products increased with increasing the aging time (Fig. 2B(b) and B(c)), but their relative intensities were weaker than those induced by fluorescent light in the same aging time.

In order to evaluate the relative changes in dye compositions, the relative peak area of each component including the main photodegradation products to the total peak area (peak 1–4) versus the aging time was plotted (see Fig. 3). General features of these curves could be easily observed from the figure.

When the ink entries on paper were exposed to fluorescent light, the relative peak area of peak 1 decreased significantly with increasing of the aging time, however, that of peak 2 only showed minor reduction (see Fig. 3A). For the photodegradation products, the relative peak area of peak 3 augmented obviously as the aging time prolonged; however, the curve of peak 4, which did not pass through the origin of coordinates because this component existed in original ink (see Fig. 2A(a)), rose slowly with the aging time. When the aging time was more than 110 h, the relative peak area of each component was nearly constant and changed slightly with further light exposure. The results demonstrated that the photodegradation products probably came from the peak 1 and it would be validated further by HPLC–MS/MS, and the minor reduction of peak 2 may be due to the variation of total peak area.

Fig. 3B showed the variation tendencies of the ink compositions induced by ultra violet light. The relative peak areas of both peaks 1 and 2 decreased nearly in same rate as the aging time increased, but the reduction extent of peak 1 was smaller than that caused by fluorescent light. The curves of peaks 3 and 4 ascended with the aging time in almost same extent.

3.2.2. Aging of the blue gel pen dye in natural condition

Ink dyes stored in natural condition would take place degradation or decomposition [2,43]. The changes of their components can be used to the dating of the documents [38,40,43]. The blue gel pen (G7) ink entries on paper stored in natural environment for about 1–9 months (at about 1 month interval) were extracted with the HPLC mobile phase and analyzed by IP-HPLC.

The chromatograms of the inks stored for different durations of time were shown in Fig. 4. It can be seen from the chromatograms that the natural dating process was slow. The changes of the two main peaks were not significant in comparison with those exposed to light, and no new component peaks

![Fig. 3. Plots of the relative intensity of each peak to the sum of peak 1–4 vs. the aging time. The ink samples were exposed to fluorescent light (A) and ultraviolet light (B).](image)

![Fig. 4. Chromatograms of the ink samples stored in natural environment for (a) 0 day, (b) 158 days, and (c) 284 days. HPLC conditions: eluent A (10 mmol L\(^{-1}\) TBA buffer, pH 7.0):eluent B (acetonitrile) = 60:40 (v/v), the flow rate of mobile phase was 1.0 mL/min, \(\lambda = 580\) nm.](image)
were observed from the chromatograms at the detection wavelength of 580 nm.

For detailed understanding the natural aging rule, the relative peak area of each component to total peak area was drawn versus the aging time (see Fig. 5). The variation tendencies of the two main components were obviously observed from Fig. 5. The relative intensity of peak 1 (RT 6.2 min) decreased slowly with increasing the aging time, and that of peak 2 (RT 8.3 min) rose simultaneously. This phenomenon illustrated that the component (peak 1) decomposed in natural aging condition, and the rate of its decomposition can be related to the dating of the questioned documents in certain conditions.

Unlike the results obtained by Andrasko when conducting the photodegradation of ballpoint pen ink dye, Crystal Violet [38], the differences of the ink entries on paper between natural aging condition and light aging conditions were obvious. The photodegradation products (peaks 3 and 4) emerged after exposure to light for 12 h (see Fig. 3), but the peaks of new components cannot be seen in the chromatograms for the blue gel pen inks after stored in natural aging condition for 9 months. In this case, the relative area of peak 1 to peak 2 in light aging condition was smaller than in natural aging condition. This can offer an easy way to distinguish the inks stored in natural and artificial environments.

3.3. HPLC–MS/MS analysis

Photodegradation and HPLC–MS/MS is a powerful combination of methods capable of characterizing the structure of dyes [24,42]. The degradation products can be analyzed with HPLC–MS/MS, and the results can be used for dye identification. In this study, ultra performance liquid chromatography–electrospray mass spectrometry (UPLC/MS/MS) was used to identify the ink dyes and their degradation products. Ammonium carbonate was selected as the ion-pairing reagent because TBA would introduce interferences for the ionization source. With the 1.7 μm ACQUITY UPLC Bridged Ethane Hybrid (BEH) column, the gel pen ink dyes can be successfully separated using ammonium carbonate as ion-pairing reagent, although the common C18 column cannot be effectively retained the dyes in the same condition.

Fig. 6 showed the chromatograms of blue gel pen ink (G7) before and after exposure to fluorescent light separated by UPLC using ammonium carbonate as the ion-pairing reagent. The chromatograms were similar to those obtained using TBA as an ion-pairing reagent. Sufficient chromatographic separation and satisfied mass spectrometric detection were simultaneously achieved within 4 min. Electrospray ionization is a soft ionization technique that usually yields information only about the molecular mass with little fragmentation for each compound [44]. The molecular mass of the dyes and their degradation products in the chromatograms can be determined by the mass spectra in negative and positive ionization modes simultaneously. The results of the ink sample exposed to UV light were same as those to fluorescent light.

For identification of the dyes and their degradation products, HPLC–MS/MS was performed on the molecular ions, and Figs. 7–9 showed their mass spectra and the origins of the daughter ions. The component, of which the molecular ion was 749 in positive ionization mode, was inferred to be the Acid Blue 9 from their molecular ion and characteristic fragments through consulting the related data (see Fig. 7). It has been reported that Acid Blue 9 was a common dye used in blue ink [43]. The parent ion at m/z 749 obtained in positive ionization mode was stable because of its big conjugated system, and it still existed with the highest relative abundance among the fragment ions at +40 eV collision voltage. The scheme of Fig. 7 gave the interpretation...
of the MS/MS spectra. The loss of the fragment B (m/z 171) from the parent ion (m/z 749) formed the fragment C (m/z 579), and the fraction D (m/z 498) came from fragment C after missing –HSO3 group; the fragment ion F (m/z 458) also can be obtained from the parent ion (A), and it broke into G (m/z 306) subsequently. Further confirmation of the dye will be discussed from its degradation products.

Fig. 8 illustrated the MS/MS spectra of the photodegradation product of which the molecular ion was 719 in negative ionization mode. This component may come from Acid Blue 9 by elimination of an ethyl group and the scheme of interpretation of the MS/MS spectra was shown in Fig. 8. The elimination of SO3 from the negative parent ion gave the fragment E (m/z 639), and from which the further loss of ethyl and phenyl groups resulted in fragment F (m/z 533). The fragment G (m/z 453) was from fragment F after expulsion of SO3. In addition, the fragment B (m/z 170) and C (m/z 260) were the anions after the parent ion (m/z 719) decomposed by breaking the C–N bond and the C–C bond in the center of the molecule [44].

Fig. 7. MS/MS spectra of the daughter ions of m/z 749 in positive ionization mode at +40 eV and the scheme of their major origins.

Fig. 8. MS/MS spectra of daughter ions of m/z 719 in negative ionization mode at −40 eV and the scheme of their major origins.
Another photodegradation product, of which the molecular ion was 551 in positive ionization mode, was inferred from Acid Blue 9. The fragments in its MS/MS spectra were similar to those of Acid Blue 9. Fig. 9 gave its MS/MS spectra and the origin of the fragments. The cleavage of C–N bond in parent ion A (m/z 551) can obtain fragment B (m/z 171) and the further loss of SO₂ from B gave fragment D (m/z 107). The fragment C (m/z 260) came from the rearrangement of the parent ion after losing B, ethyl group and the cleavage of the C–C bond in the right part of A [44]. The origins of fragment E and F were from the parent ion A like the cleavage of the bond and rearrangement of Acid Blue 9.

Another main component in the blue gel pen ink, of which molecular mass was 1117, was relative stable. There were nearly not any daughter ions with a very high collision-induced-dissociation (CID) energy as much as +60 eV except the doubly charged ion at m/z 559 in positive ionization mode. The component could not be identified by the information obtained, and further study was needed.

From the identification of the dye and degradation products, it can be inferred that Acid Blue 9 was a main component of dye used in blue gel pen inks and the main photodegradation products observed in the chromatogram originated from the decomposition of Acid Blue 9. It was consistent with the results that the relative area of Acid Blue 9 was decreased significantly with aging time when exposure to light.

4. Conclusions

Forty-seven blue gel pen inks were collected, and 27 dye-based gel pen inks were analyzed with IP-HPLC. Mobile phases containing ion-pairing agents with different alkyl chain length were tested for separation of the blue gel pen inks to optimize the chromatographic condition. It has been found that 10 mmol L⁻¹ TBA acetate (pH 7.0) was suitable ion pairing agent for the purpose and ink samples stored in different conditions were analyzed by IP-HPLC. Significant changes of ink composition were observed, of which the natural aged inks had the similar but weaker degradation trend than the light aged inks. HPLC–MS/MS with ammonium carbonate as ion-pairing reagent was used to obtain the information of the light aged inks and their photodegradation products. One dye and its main degraded products were identified, and an elementary photodegradation mechanism was investigated. The studies can supply a feasible method for determination and dating of the blue gel pen inks on questioned documents.

References