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On-Column Approach in the HPLC-UV Analysis of Non-chromophoric Compounds Using Azelaic Acid as a Model

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ABSTRACT

The manuscript introduces a detection solution of compounds lacking chromophoric properties (e.g. azelaic acid) by implementing a HPLC-UV analysis with on-column derivatisation of the analyte. Azelaic acid was used to test the feasibility of the method at λ_{max} = 265 nm. Its chromatographic analysis shows linear (R = 0.999), precise (RSD < 2.0%) and accurate (97.0 - 103.5 %) behavior. Furthermore, the method was found selective for azelaic acid in a prepared cream which contains other ingredients such as triethanolamine, vaseline and stearic acid. The limit of detection (LOD) and limit of quantification (LOQ) of azelaic acid were 9 and 30 µg/ml, respectively.

Keywords: HPLC-UV, Azelaic acid, On-column derivatisation.

INTRODUCTION

The HPLC analysis of non-chromophoric compounds involving derivatisation of molecules is used to increase the selectivity of molecules or to improve their detection sensitivity. For example, amino acids and sugars are among compounds whose separation are considered challenging by reversed phase HPLC systems. Thus, derivatisation is used to improve their separation.[1-3] On the other hand, derivatisation of compounds exhibiting low UV or fluorescent sensitivity by fluorogenic or chromophoric agents is a rather common practice in HPLC analysis.[4-6] Derivatisation is either accomplished by off-line or on-line procedures.[7-9] Off-line derivatisation is carried out separately from an HPLC column, either pre-column or post-column.[10] However, off-line pre-column derivatisation is more feasible where the derivatisation reaction is carried out in a laboratory vessel. Among the drawbacks of this procedure is the product instability.[11, 12] Reproducibly of the reaction is also a major concern.[13] Moreover, the reaction is often time-consuming and requires a good experience in organic and inorganic chemistry.[10, 14]

Post-column derivatisation has been carried out by adding a special resistant pump suitable for harsh chemicals between an HPLC column and a detector. This means that the system requires extra parts to be added to the cost of the HPLC instrument. Additionally, post-column derivatisation complains of additional dilution for separated molecules.[15] Consequently, this might induce peak broadening of the separated analytes and reduce their detection sensitivity. Post-column derivatisation might not require a long time for derivatisation in comparison with off-line procedure, but the short time for the analyte transfer between the column and the detector might not be enough for the reaction to proceed completely. Moreover, the reaction solvent should be miscible with the mobile phase and also the derivatised product should be soluble with the resulted mixture.

In spite of the fact that on-column derivatisation is described as on-line modification in electrophoretic systems,[14] it is not mentioned in the literature for HPLC

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to the best of our knowledge. Basically, on-column derivatisation has some advantages over pre- and post-column derivatisation: No need for extra-configurations in the HPLC instrument and no further dilution of the samples by reaction solvents. Consequently, the broadening of the analyte peak is reduced and its limit of detection is improved. Furthermore, on-column derivatisation does not require a long time of operation and also the purification and the stability of the product is not problematic.

Azelaic acid as a model of non-chromophoric acidic analyte (Figure 1) was used to test the applicability of the on-column modification mentioned here. It is a naturally occurring saturated dicarboxylic acid used in the treatment of comedonal acne because of its antimicrobial and its anti-inflammatory effect.[16, 17] In the literature, azelaic acid is analyzed by LC-MS.[18, 19] Preparations of azelaic acid are also analyzed by volumetry.[20] HPLC pre-column derivatisation with phenacyl bromide,[21] l-leucine-4-methyl-7-coumarinylamide,[22] 2-bromoacetyl- 6-methoxynaphthalene,[23] tosylate derivatives,[24] phenacyl esters [25] and methyl methoxycoumarin were also described to analyze azelaic acid.[26] However, where the sensitivity and selectivity of azelaic acid are not a priority, azelaic acid can be determined without derivatisation under the high energy of UV, such as 210 nm.[27]

Implementation of on-column modification of azelaic acid here enabled its detection by UV at a relatively long wavelength where ionized azelaic acid desorbed oppositely charged dye from the stationary phase as a complex. The complex of the dye and azelaic acid reached the UV detector and, consequently, a positive peak proportion with the azelaic acid concentration appeared. The method was found valid according to ICH guidelines for HPLC validation. This method of analysis can be exploited for other acidic compounds lacking substantial chromophoric properties and also it might be further modified by using acidic dye to suit basic non-chromophoric compounds.

EXPERIMENTAL

Chromatographic Apparatus and Conditions

HPLC Waters: A 515 HPLC pump and a Waters 2487 dual λ detector were used. A filtered and degassed mixture of 2 µg% (w/v) of 1,10 phenanthroline was dissolved in (2:8) water; a methanol solution adjusted to pH = 5 was used as an isocratic mobile phase with a flow rate of 0.5 ml/min. The HPLC column was Hypersil C18, 250*4.6 mm (Φ = 5µm). Samples were injected within a loop of 20 µl volume. The detector was set at λ_{max} = 265 nm.

Materials and Methods

Methanol HPLC grade (Fisher Scientific, UK), 1,10-phenanthroline monohydrate AR (Pharmacros, England) and deionized water were used as mobile phase components. Glycerol (GCC, UK), 98% azelaic acid (Acros, USA), vaseline (Medical Scientific and Chemicals, Jordan), triethanolamine (Riedel-Dehaen, Germany), stearic acid (Loba Chemie, India) and paraffin oil (Sigma-Aldrich, US) were used in the in-house cream. Hydrochloric acid (Carlo Erba, Italy), sodium hydroxide (Lonver, England), acetic acid (Gainland, England), ammonia (Gainland, England), 30% hydrogen peroxide solution (Sigma-Aldrich, US) were also used.

Preparation of In-house Azelaic Acid Cream

Stearic acid (13g) was triturated with triethanolamine (3 g), and then vaseline (10 g) was added and heated to 60 ^{o}C to produce mixture A. Mixture B consisted of paraffin oil (36 g), glycerol (18 g) and azelaic acid (10 g) which were added gradually to mixture A with continuous mixing. Finally, deionised water (10g) was incorporated to produce 100 g of cream.

Procedure for Preparation of Standard Solutions

Azelaic acid (200 mg) was transferred to a 100 ml volumetric flask and then dissolved in a mixture of methanol:water (8:2) to prepare a stock solution. Six samples were prepared from the stock solution to cover a linearity range of 0.03-2.0 mg/ml.

Procedure for Analysis of Dosage Form

Azelaic acid cream (1g) was transferred to a 50 ml volumetric flask and then mixed in a vortex and diluted with a methanol:water (8:2) mixture.

RESULTS AND DISCUSSION

Adequatel lipophilic dye such as 1,10-phenanthroline (log $P_{o/w}$ = 1.78, 28) was used in order to be sufficiently retained in a reversed phase column such as an ODS

column. Significant retention of the dye is essential for this technique to work properly. Also, the dye should have the capability to form salt bonds with the analyte. Since azelaic acid is acidic with pK_a values of (4.55 and 5.41 [29]) and with partial ionization at pH values near to 5, it can form a complex with partially ionized amines of 1,10-phenathroline (pK_a = 4.27 [28]) at a pH of 5 (Figure 1). The flexibility of an azelaic acid structure and its diacidic character are presumable reasons behind the tendency of an azelaic acid - 1,10-phenanthroline complex formation. As demonstrated in Figure 2, once azelaic acid is eluted from an HPLC column, an extra amount of adsorbed dye is eluted as a complex with azelaic acid and, consequently, a proportional detection with the concentration of azelaic acid is obtained. The complex has a long wavelength maximum of absorption which is far from solvent interference and also stable readings are obtained from a UV detector at long wavelength values. The complex formation between azelaic acid and 1,10-phenanthroline possesses two maxima at 225 and 265 nm. A value λ = 265 nm was chosen to avoid solvent interference and to obtain stable UV readings.

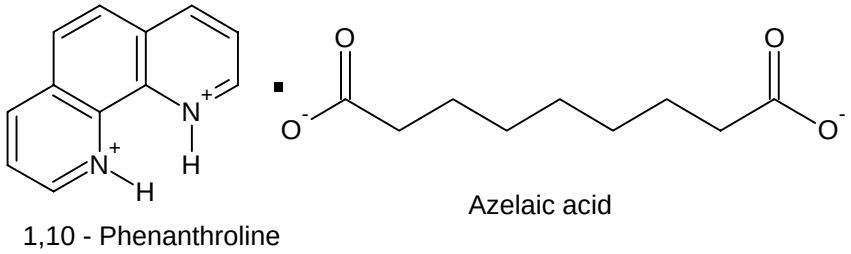

Figure1 Salt formation between 1,10-phenathroline and azelaic acid at pH=5.

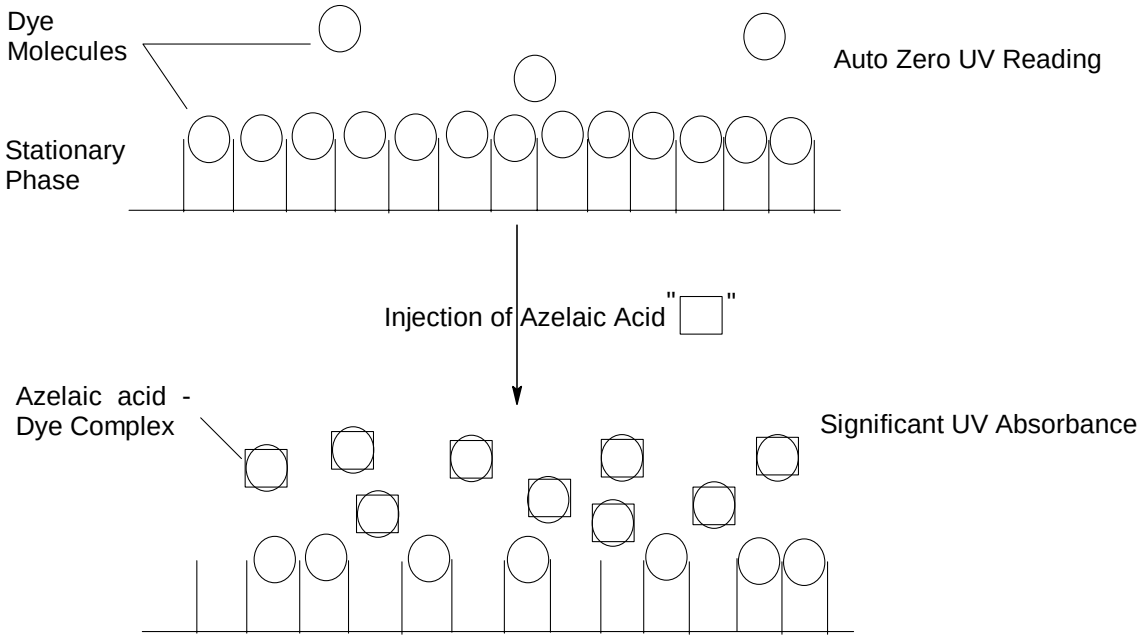

Figure 2: Sketch depicts on-column complex formation of azelaic acid with 1,10-phenanthroline (dye).

A high ratio of organic modifier was used in the mobile phase to decrease the chance of hydration of ionized groups in azelaic acid and the dye. Hydration minimizes the chance of ionic interaction between azelaic acid and the dye. A high content of methanol in the mobile phase (80%) was used for this purpose and also to facilitate the elution of the lipophilic complex of azelaic acid with 1,10-phenanthroline.

The mobile phase pH was adjusted to 5 to give a chance for both azelaic acid (pKa = 4.55 and 5.41) and 1,10-phenathroline (pKa= 4.27) to be ionized and, consequently, to form the complex.[28, 29] However, buffers were not used in the mobile phase because we found that they reduced the HPLC-UV sensitivity of azelaic acid. This might be due to the ionic strength of buffers which stabilizes ionized groups by counter ions and, consequently, this reduces the chance of ionic interaction between the dyes and analytes. Instead, the pH of the mobile phase was adjusted with a few drops of acetic acid or ammonia, whenever necessary.

Validation of the Method

Linearity

Six azelaic acid solutions were appropriately diluted with methanol:water (8:2) to obtain solutions in the concentration range 0.03-4.0 mg/ml. Each solution was injected in triplicate into the column under the described chromatographic conditions. Examples of chromatograms of standard solutions are shown in Figure 3. The least squares method was used to calculate the slope, intercept and the correlation coefficient (r) of the regression line. The mean value of the peak area is related to azelaic acid concentration according to Y = 1E+06 X + 5479.5 (r = 0.999).

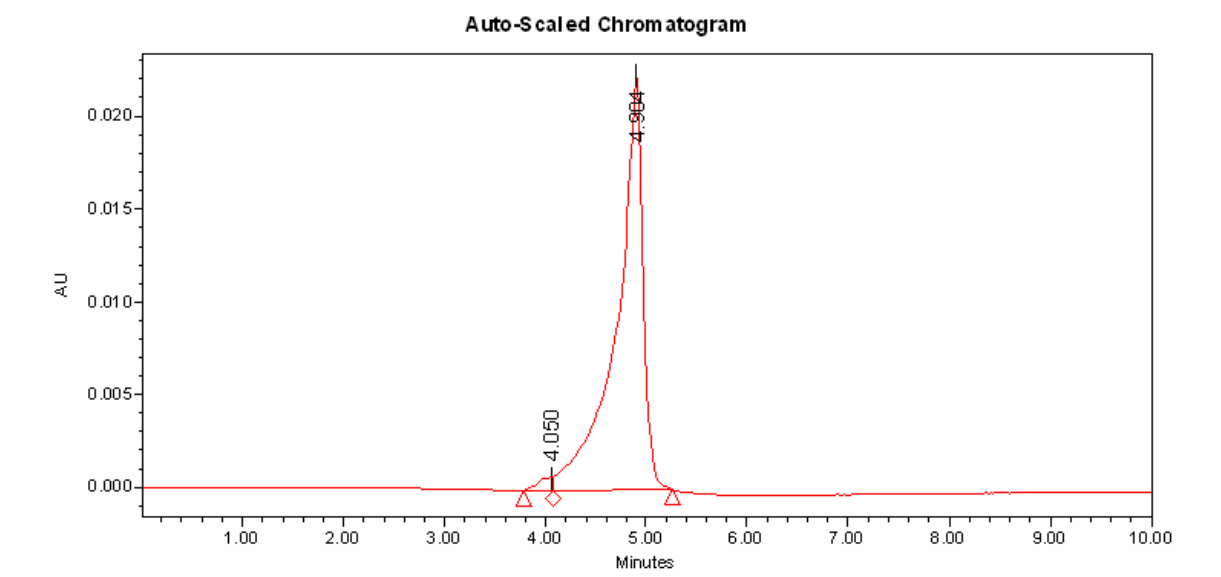

Figure 3: Example of chromatogram of standard solution of azelaic acid.

Limits of Quantification (LOQ) and Detection (LOD)

The limit of quantification (LOQ) was determined according to the least concentration below which the linearity of the calibration curve deviated from the norm. It was found that LOQ = 30 µg/ml. LOQ was also found to be ten times the signal-to-noise ratio (10 S/N). The limit of detection (LOD) was determined according to three times signal-to- noise ratio (3 S/N) procedure. It was found to be 9 µg/ml.

Selectivity

Selectivity of the method was assessed by comparing chromatograms of placebo blank solutions in

methanol:water (8:2) with standard sample chromatograms. A synthetic placebo mixture of the cream was prepared as described in the experimental part without azelaic acid. The placebo blank solution showed no response at the retention time of azelaic acid (Figure 4).

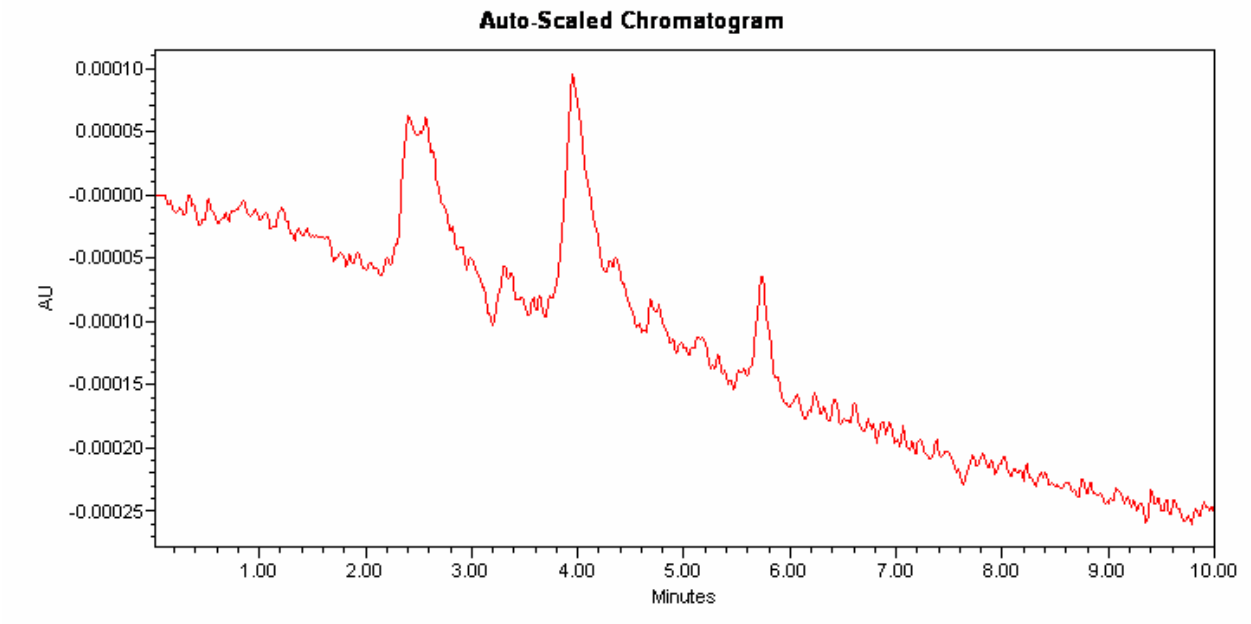

Figure 4 Chromatogram of placebo blank solution of azelaic acid cream.

The specificity of the developed method was assessed by performing a forced degradation study. A stress decomposition study was performed at the concentration of the test solution of dosage form described in experimental part. Three solutions were prepared and heated at 80 ^{o}C for 15 minutes. One of the solutions contained a few drops of a concentrated HCl solution. A few drops of H_2O_2 (30% w/v) and NaOH (40% w/v) were added to each of the other solutions. The purity of the azelaic acid peaks was tested by a purity test of photodiode array detector. The purity indices of the results were above the purity threshold limit (> 0.996). This confirms the purity of the chromatographic peak from its degradation products.

Precision and Accuracy

Six solutions of azelaic acid standard material with a concentration of 2 mg/ml were prepared to evaluate the method precision. Moreover, six other solutions of azelaic cream were tested in the same manner (Figure 5). Triplicate injections were carried out for each solution. It was found that the relative standard deviations (RSD) of inter-day and intra-day were less than 2.0%.

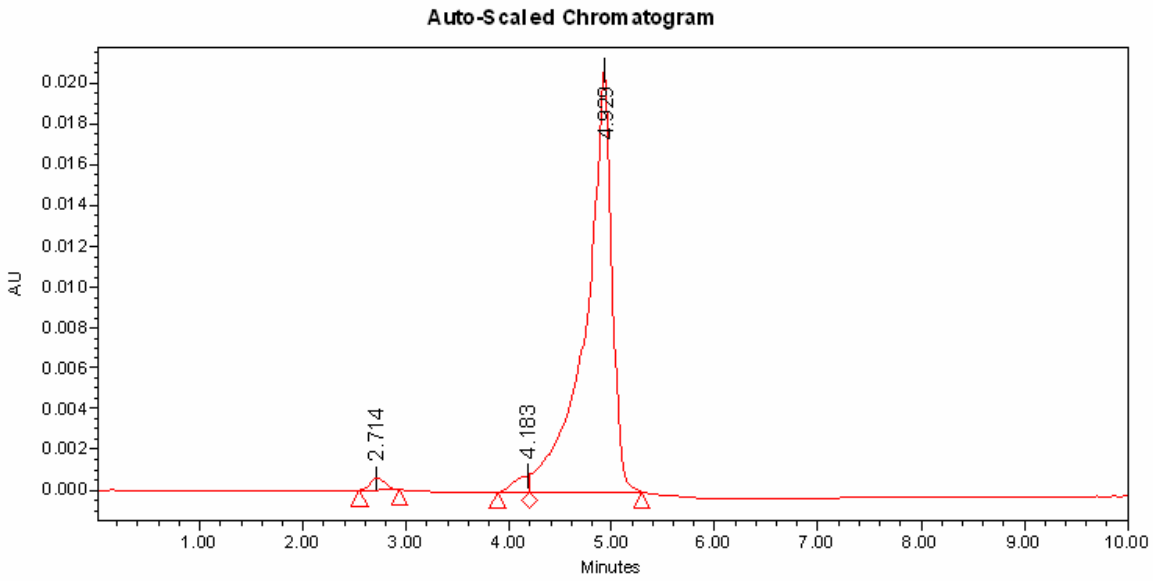

Figure 5: Example of chromatogram of test solution of azelaic acid cream.

Robustness

The saturation of the column in the mobile phase with at least ten times the column dead volume was found to be an essential step to obtain robust results from this method. This time was required to produce a stable equilibrium between the adsorbed molecules of 1,10-phenanthroline in the stationary phase and the dissolved molecules of it in the mobile phase. Robustness was evaluated by producing deliberate changes in the column temperature, wavelength of detection, concentration of 1,10-phenanthroline and organic modifier in the mobile phase. Changes of these parameters with extents less than ± 3% of each produced precise results with RSD values less than 2.5%.

Recovery Study

A recovery study was performed by the standard addition method to pre-analyzed azelaic acid cream. Standard materials were added at three concentrations of 50, 100 and 200% of the cream labeled amount of azelaic acid; each concentration was injected in triplicate. The percentages of recovery were in the range of 97.0 – 103.5% of the added amount with a standard deviation below 2.18%. The results confirm the accuracy of the method.

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تطبيق تحليل جهاز عامود الفصل عالي الكفاءة المربوط بالأشعة فوق البنفسجية على مركبات التي تفتقر إلى خصائص لونية، حامض الأزيلك نموذجاً

محمد زويري ويوسف حياري وطلال ابو رجيع واسامة عبد العظيم

كلية الصيدلة، الجامعة الأردنية.

ملخـص

تقدم هذه الورقة البحثية حلا لطريقة الكشف عن المواد التي تفتقر إلى خصائص لونيه (مثل حامض الازيلك) من خلال تطبيق التحليل بجهاز عامود الفصل عالي الكفاءة المربوط بالأشعة فوق البنفسجية وذلك من خلال تعديل يجري على المادة المحلله داخل عامود الفصل. إن حامض الازيليك قد تم استخدامه هنا من اجل اختبار جدوى الطريقة على طول موجه 265 نانوميتر وقد تبين أن التحليل الكوماتوغرافي كان خطيا (R=.999) وانه متكرر (RSD <2.0%) وان الطريقة صحيحة (97-103.50%) وعلاوة على ذلك تم التبين على كون الطريقة انتقائية لحامض الازيليك في مستحضرات تحوي على مواد أخرى مثل مادة تراي ايثانولامين، فازلين، وحامض الستيريك. وقد تبين أيضا أن الحد الأدنى للقياس النوعي والحد الأدنى للقياس الكمي لحامض الازيليك هما 9 و30 مايكروغرام لكل مل على التوالي

الكلمـات الدالـة: حامض الأزيلك، جهاز عامود الفصل، الأشعة فوق البنفسجيةز.

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