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A mixed-mode liquid chromatography-tandem mass spectrometric method for the determination of cytarabine in mouse plasma

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Abstract

A novel mixed-mode high performance liquid chromatographic system (HPLC) interfaced with an atmospheric pressure chemical ionization (APCI) source and a tandem mass spectrometer (MS/MS) was developed for the determination of cytarabine (ara-C) in mouse plasma to support pharmacodynamic studies. The mixed-mode reversed-phase ion-exchange chromatography column was adapted for sufficient retention and separation of a small and polar analyte. The impact of the mobile phase composition on both chromatographic separation and the ionization efficiency of the test compound in the positive mode was investigated. The potential of ionization suppression from endogenous biological matrices on the mixed-mode LC–APCI/MS/MS method was evaluated using the post-column infusion technique. Furthermore, the feasibility of using the mixed-mode HPLC–MS/MS method for the determination of the plasma concentrations of cytarabine in mice was demonstrated by comparing those obtained by the ion-pairing HPLC–MS/MS method.

Keywords: Mixed-mode liquid chromatography; Tandem mass spectrometry; Cytarabine; Matrix ionization suppression

1. Introduction

The greater numbers of samples derived from various drug discovery experiments have yielded challenges for developing reliable and higher throughput bioassays for quantitation of a large diversity of new chemical entities (NCEs) during the lead optimization process [1–3]. The high performance liquid chromatography (HPLC) coupled with a tandem mass spectrometer (MS/MS) provides excellent sensitivity and selectivity for monitoring drug compounds and has become a standard equipment in modern pharmaceutical industries [4,5]. While fast liquid chromatography technologies using a micro-column [6,7], monolithic silica column [8,9] or ultra-performance [10,11] linked with a tandem mass spectrometer have been utilized to shorten run cycle times, it is important to maintain appropriate separation power to avoid ion suppression issues or endogenous interferences [6,7,12]. In this work, a mixed-mode liquid chromatographic approach was developed to help retain and promote separation of a highly polar compound, cytarabine (ara-C), an anticancer agent, [13] as the model compound which normally could not be retained with a high carbon load column under typical reversed-phase chromatographic conditions. The column effluent was directly connected to an atmospheric pressure chemical ionization (APCI) source as part of the tandem mass spectrometer (MS/MS) system. Selective reaction monitoring (SRM) of the analyte and the internal standards (ISTD) in the positive ion mode was used for the quantitative determination of the analytes. The effect of mobile phase composition on the ionization efficiencies of the analyte was investigated. The relationship between retention factors of the analyte and mobile phase composition on a mixed-mode reversed-phase/weak anion-exchange type stationary phase was explored. The matrix ionization suppression potential for the mixed-mode HPLC–APCI/MS/MS system was examined using a post-column infusion technique where a simple sample treatment procedure based on the use of the protein precipitation technique with acetonitrile/methanol (90/10) was tested. Furthermore, a direct comparison of the analytical results obtained by the mixed-mode HPLC–APCI/MS/MS method and the ion-pairing HPLC–APCI/MS/MS method for determining

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the mouse plasma exposures of cytarabine was performed to demonstrate the assay feasibility in support of pharmacodynamic studies.

2. Experimental

2.1. Reagents and chemicals

Ara-C as the probe drug and clofazimine as the internal standard (ISTD) were purchased from Sigma (St. Louis, MO, USA). The chemical structures of all test compounds were shown in Fig. 1. Methanol and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Trifluoroacetic acid (TFA) and formic acid (FA) were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). The volatile perfluorinated carboxylic acid ion-pairing reagent, nonafluoropentanoic acid (NFPA), was purchased from Sigma (St. Louis, MO, USA). Deionized water was generated from a Milli-Q water purifying system purchased from Millipore Corporation (Bedford, MA, USA) and house high-purity nitrogen (99.999%) was used. Drug-free mouse plasma samples (with EDTA) were purchased from Bioreclamation Inc. (Hicksville, NY, USA).

2.2. Equipment

HPLC–MS/MS analysis was performed using an Applied Biosystems/ MDS Sciex (Concord, Ontario, Canada) Model API 4000 triple quadrupole mass spectrometer equipped with the APCI interface. The chromatographic system consisted of a Leap autosampler with a refrigerated sample compartment (set to 10 °C) from LEAP Technologies (Carrboro, NC, USA), Shimadzu on-line degasser, LC-10AD VP pump and LC-10A VP controller (Columbia, MD, USA). For the mixed-mode HPLC method, a Primesep A column (3.2 mm × 50 mm) from SIELC

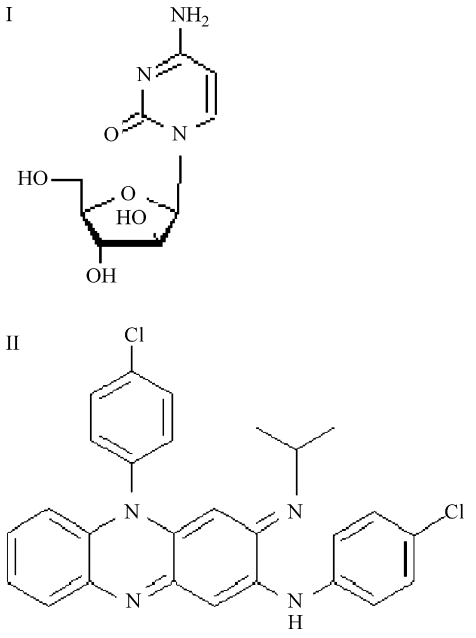

Fig. 1. Chemical structures of (I) ara-C and (II) clofazimine.

(Holland, MI, USA) was used as the analytical column. For ion-pairing HPLC using NFPA as an ion-pairing reagent, a CAPECELL PAK C18 column (2.0 mm × 35 mm, 5 μm) from Phenomenex (Torrance, CA, USA) was used as the analytical column. The Quadra 96 (Tomtec, Hamden, CT, USA) system was used for semi-automated sample preparation via the protein precipitation procedure. The experimental mass spectrometric conditions were determined without optimization for the APCI source.

For the matrix ionization suppression studies with mouse plasma samples, a mixture of ara-C and clofazimine solution was continuously infused into PEEK tubing in between an analytical column and a mass spectrometer through a tee using a Harvard Apparatus Model 2400 (South Natick, MA, USA) syringe pump. Either a protein precipitation extract of the blank mouse plasma samples or methanol (5 μL) was injected into the mixed-mode analytical column. Effluent from the analytical columns mixed with the infused test compounds and then entered the APCI source.

2.3. Sample collection

The animal dosing experiments were carried out in accordance to the National Institutes of Health Guide to the Care and Use of Laboratory Animals and the Animal Welfare Act. Study blood samples were collected at specified time-points up to 24-h following a single intraperitoneal administration to individual mice. After clotting on ice, serum was isolated by centrifugation and stored frozen (−20 °C) until analysis.

2.4. Standard and sample preparation

Stock solutions of ara-C and clofazimine were prepared as 1 mg/mL solutions in methanol. Analytical standard samples were prepared by spiking known quantities of the standard solutions into blank mouse plasma. The concentration range for ara-C in mouse plasma was 50–10,000 ng/mL level. The mouse plasma samples were prepared using the protein precipitation technique. A 300-μL aliquot of methanol solution containing 1 ng/mL of clofazimine was added to 10 μL of plasma located in a 96-well plate. After mixing and centrifugation the supernatant was automatically transferred to a second 96-well plate by the Quadra 96 instrument. A 5-μL aliquot of the extract was injected by the Leap autosampler to the mixed-mode HPLC–APCI–MS/MS and the ion-pairing HPLC–APCI–MS/MS systems for quantitative analysis.

2.5. Chromatographic and mass spectrometric conditions

Mobile phases A and B consisted of water and methanol containing 0.1% TFA, respectively. Gradient chromatographic separation using mobile phases A and B was as follows: 0 min (35% B), 1.2 min (35% B), 1.21 min (100% B), 2.5 min (100% B), 2.6 min (35% B) and finished at 3.0 min to achieve satisfactory resolution among interferences. The retention times for ara-C and clofazimine were 1.05 and 2.56 min, respectively. For the ion-pairing HPLC procedure, mobile phases A and B were

composed of water and acetonitrile containing 0.1% NFPA and 0.1% FA as mobile phase additives, respectively. Gradient chromatographic separation using mobile phases A and B was as follows: 0.3 min (0% B), 3.1 min (12% B), 3.2 min (100% B), 3.7 min (100% B), 3.8 min (0% B), and finished at 4.0 min at a constant flow rate of 1 mL/min to achieve satisfactory resolution between ara-C and the endogenous compound from the spiked standard and study mouse plasma samples. The retention times for ara-C and clofazimine were 2.2 and 3.37 min, respectively, when NFPA was employed as an ion-pairing reagent [13]. The effluent from both HPLC systems was connected directly to the mass spectrometer.

The mass spectrometer was operated in the positive ion mode. The instrumental settings for the temperature of the heated pneumatic nebulizer probe, ion source gas 1, ion source gas 2, declustering potential, entrance potential, collision cell exit potential were as follows: 480 °C, 50, 10, 80 V, 10 V and 15 V, respectively (The numbers without units are arbitrary values set by the Analyst software). The MS/MS reactions selected to monitor ara-C and clofazimine were the transitions from *m/z* 244 → *m/z* 112 and *m/z* 473 → *m/z* 431 with collision energy of 20 and 45 eV, respectively. The protonated molecules were fragmented by collision-activated dissociation with nitrogen as collision gas at a pressure of instrument setting 5.

3. Results and discussion

3.1. Development of the mixed-mode HPLC–MS/MS method

One of the common goals in the pharmacokinetic area is to develop a reliable bioanalytical method to simultaneously monitor a wide range of drug candidates in biological samples. Our initial attempt for the HPLC separation of ara-C was to employ traditional C1, C8 and C18 columns, but the analyte was not retainable under gradient elution starting at 100% aqueous mobile phase by the traditional reversed-phase stationary phases routinely employed in our laboratory (data not shown). Sufficient chromatographic retention in the quantitative determination of the drug components in biological samples using mass spectrometric detection is highly recommended to avoid possible interferences from drug-related biotransformation products or ionization suppression due to co-eluted endogenous materials [7]. HPLC columns containing polar-endcapped and polar-enhanced stationary phases have been utilized to retain polar small molecules under highly aqueous conditions [13]. However, no substantial retention of ara-C using these reversed-phase chromatography columns under aqueous mobile phase conditions was achieved.

Hydrophilic interaction chromatography (HILIC) has been demonstrated to be a powerful technique for the retention of polar analytes offering a difference in selectivity compared to traditional reversed-phase chromatography [14–19]. HILIC separates compounds by eluting them with a strong organic mobile phase against a neutral hydrophilic stationary phase resulting in solutes that are retained in order of increasing hydrophilicity. However, it normally requires complex sample clean-up procedures that are not desirable for higher throughput assays. Ion-pairing chromatography has been widely used as an alternative way for obtaining satisfactory retention of polar analytes [20–26]. The ion-pairing reagents added into mobile phase are used to improve chromatographic retention of analytes on the lipophilic stationary phase through the formation of neutral ion pairs. Volatile ion-pairing reagents are normally required for liquid chromatography–mass spectrometry systems [27]. However, it was reported that inorganic salts and other competing ion-pairing reagents might decrease the ion signals of the target compounds regardless of mass analyzers or instrument geometries [28].

In this work, one of our objectives was to develop a reliable HPLC–MS/MS assay in conjunction with a mixed-mode stationary phase for retaining a highly polar compound, ara-C, in mouse plasma samples following a simple protein precipitation procedure. With an embedded ion-pairing group in the reversed-phase stationary, the mixed-mode column providing the capability for ion-exchange and hydrophobic interactions requires no ion-pairing reagent in the mobile phase to retain and to separate ionizable polar compounds. With a carbon load stationary phase as a basis for interaction with analytes, the mixed-mode column offers a typical reversed-phase retention profile for neutral compounds. As shown in Fig. 2, ara-C was sufficiently retained on the mixed-mode column using the same mobile phases employed for the conventional C18 column.

In general, the composition of the eluent affects not only the chromatographic resolution, but also the ionization efficiency of the analytes in various atmospheric pressure ionization sources. The greater organic content in the mobile phase normally generate higher ionization efficiencies for small molecules in most atmospheric pressure ionization interfaces. As expected, Fig. 3 shows that the APCI responses of ara-C measured by flow injection analysis at a consistent flow rate of 1 mL/min increase

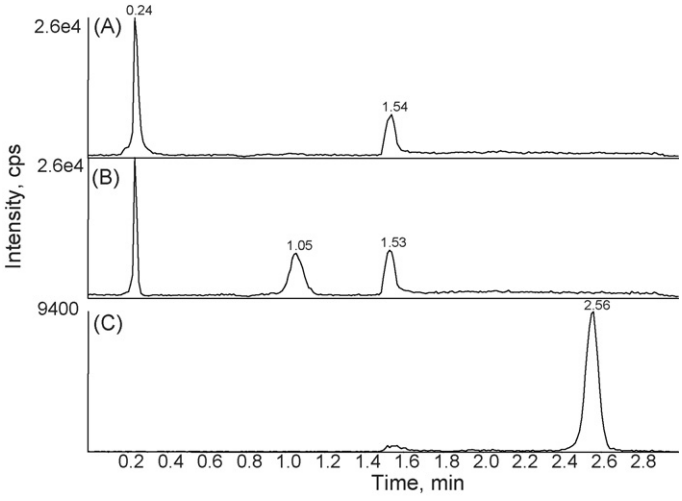

Fig. 2. The mixed-mode HPLC–APCI/MS/MS chromatograms of ara-C from (A) blank mouse plasma, (B) the spiked standard mouse plasma at a concentration of 100 ng/mL and (C) the mixed-mode HPLC–APCI/MS/MS chromatogram of clofazimine from a study mouse plasma sample at a constant flowrate of 1.3 mL/min.

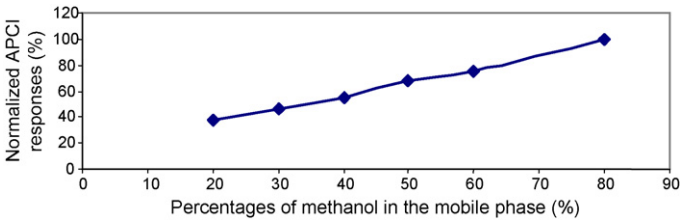

Fig. 3. Normalized APCI responses of ara-C as a function of the ratios of methanol to water using flow injection analysis.

with increasing the contents of methanol from 20 to 80%. The increased sensitivity is expected due to more effective nebulization and vaporization processes with higher percentages of methanol in the mobile phase. The mixed-mode HPLC allows retaining hydrophobic compounds by the reversed-phase mechanism and hydrophilic compounds by both the reversed-phase and ion-exchange mechanism at higher organic contents in the mobile phase. The charged functional groups in the mixed-mode column can be in an ionized form, or in a non-ionized form, depending on the pH of the mobile phase.

The influence of the mobile phase composition on retention factors (k) of ara-C with a commercial reversed-phase/ cation-exchange column under isocratic elution was determined in this work. These facts can be used to optimize the effectiveness of separation of analyte mixtures. Mobile phase variables were explored to obtain an insight into the retention and separation mechanism for a commercial mixed-mode column. According to the linear solvent strength theory for the reversed-phase retention alone [29], the values of log(k) of the analytes decrease linearly as the percent fraction of organic modifier in the mobile phase increases due to the weaker hydrophobic interaction. For the mixed-mode chromatography, the relative contribution of each mechanism includes the hydrophobicity and charge character of analytes as well as mobile phase composition within the same column. By changing the mobile phase conditions, the mode of separation might be thereby changed which allows the chromatographer to achieve the desired selectivity in the separations. The dependence of the values of log (k) of ara-C by the mixed-mode HPLC as a function of the equilibrium concentration of methanol and acetonitrile in the mobile phase is presented in Fig. 4. Fig. 4 shows that the values of log (k)

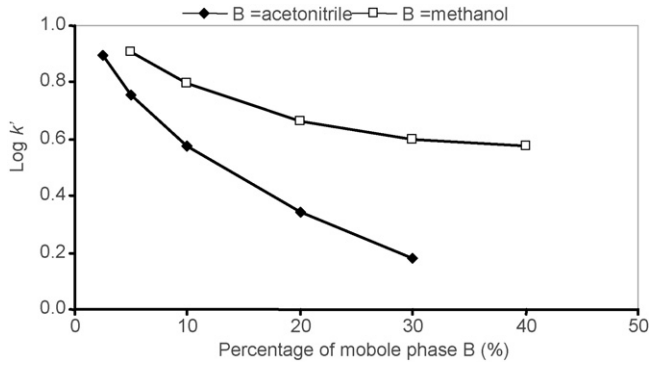

Fig. 4. Plots of the capacity factor log k vs. the concentration of methanol (open squares) and acetonitrile (solid squares) in the mobile phase.

of ara-C non-linearly decreases with an increase in the ratios of methanol and acetonitrile in the mobile phase from 5 to 40% and from 2 to 30%, respectively, indicating that the retention mechanism of ara-C in this period was governed through the combination of hydrophobic and ion-exchange interaction. Fig. 4 also indicates that the overall retention interaction of ara-C on the mixed-mode column is strongly dependent on the organic solvents.

3.2. Matrix ionization suppression studies

When developing any new HPLC–MS/MS assays, it is important to check for possible matrix ionization suppression [7,12]. Matrix ionization suppression is considered to be more problematic when using the protein precipitation method for sample preparation as compared to the liquid–liquid and the solid phase extraction methods. In order to observe the matrix ionization suppression effects on plasma protein precipitation extracts using the mixed-mode HPLC–MS/MS techniques, we monitored the variability of the APCI responses for Ara-C and the ISTD using the post-column infusion scheme. Any changes in the APCI responses of the infused test compounds between the methanol solvent and the plasma extract injections from two different batches were assumed to be due to matrix ionization suppression caused by the sample-related materials eluting from the analytical column. Some degrees of matrix effects for ara-C and the ISTD at early chromatographic window (first 0.5 min) were observed (data not shown). However, there is no impact on the assay accuracy because the retention times of all test compounds appear in the safe chromatographic window (outside of the matrix-effect window).

3.3. Analysis of mouse plasma samples

The mixed-mode HPLC–APCI–MS/MS method was applied for the determination of the dosed compound in mouse plasma to demonstrate the applicability of analyses. Several interference peaks sharing the same mass range of ara-C from the blank, the spiked standard and the study mouse plasma samples were observed shown in Fig. 2. A base line separation between the endogenous and ara-C was achieved within the extracted HPLC-MS/MS chromatogram in a single run. The retention time and the peak shape for ara-C in both the spiked standard and study mouse plasma samples were found to be reproducible during the course of the study. The calibration curves for ara-C obtained from standard mouse plasma samples at each concentration level were linear with a correlation coefficient, r^2, greater than 0.994 (graph is not shown). Accuracy (% bias) was less than 15% at all concentrations. Fig. 5 compares the plasma levels of ara-C calculated by the response ratios of analytes over the ISTD obtained by the mixed-mode HPLC–APCI/MS/MS method and those obtained by the ion-pairing HPLC–APCI/MS/MS method. The between-day precision and accuracy in the measurement of all spiked standard plasma samples in replicates of three obtained by the mixed-mode HPLC–MS/MS method was less than 15% error of the nominal values. The spiked standard mouse plasma samples were employed for all stability tests. Three freeze–thaw

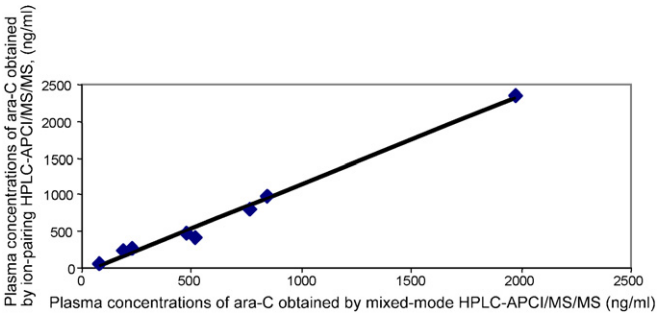

Fig. 5. Correlation of concentrations of ara-C in study mouse plasma samples obtained by the mixed-mode HPLC–APCI/MS/MS method and the ion-pairing HPLC–APCI/MS/MS method.

cycles before processing, benchtop stability for 6 h at room temperature, and the autosampler stability for 24 h at 10 °C were determined as reported elsewhere [13]. The Student's *t*-test results indicated that there were no significant differences of both values for ara-C determined by both aforementioned analytical methods with 95% confidence ($\alpha = 0.5$). These results concluded that the proposed mixed-mode HPLC–APCI–MS/MS method were equivalent with the ion-pairing HPLC–MS/MS method in terms of accuracy.

4. Conclusion

The mixed-mode stationary phase, a combination of ion-exchange and reverse-phase chemistry on the silica support in the HPLC column, has been shown to be a useful analytical tool for the retention of polar and non-polar small molecules in a single run. The use of the mixed-mode column for separation of ara-C to avoid the mass spectrometric interference was demonstrated. The relationships between the mobile phase composition and the reversed-phase/ion-exchange stationary phase and its influence on chromatographic retention factors of the analyte and ionization efficiencies with the APCI source were demonstrated to be a function of the strength of organic solvent. The described mixed-mode HPLC–APCI–MS/MS assay had been applied for the determination of ara-C in mouse plasma in combination with a simple sample treatment procedure. The analytical results for ara-C in mouse plasma samples obtained by the mixed-mode HPLC–APCI/MS/MS method and ion-pairing HPLC–APCI–MS/MS method showed equivalent accuracy.

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