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Porous graphitic carbon chromatography/tandem mass spectrometric determination of cytarabine in mouse plasma

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A high-performance liquid chromatography (HPLC) system using a porous graphitic carbon (PGC) stationary phase interfaced with an electrospray ionization (ESI) source and a tandem mass spectrometer (MS/MS) for the analysis of cytarabine (ara-C) in mouse plasma samples has been developed in support of a pharmacodynamic study. The graphitized carbon column was adopted for the separation of ara-C and endogenous peaks from mouse plasma samples under the reversed-phase phase mode in liquid chromatography. The retention characteristics of the PGC column and the ionization efficiencies of all analytes based on the experimental factors such as the composition of mobile phases were investigated. The potential of ionization suppression resulting from the endogenous biological matrices on the PGC column during HPLC/ESI-MS/MS was investigated using post-column infusion. The concentrations of ara-C in mouse plasma obtained by using PGC-HPLC/MS/MS and ion-pairing HPLC/MS/MS were found to be in good agreement in terms of analytical accuracy. Copyright © 2007 John Wiley & Sons, Ltd.

Reversed-phase chromatography is the most commonly used chromatographic method for pharmaceutical analysis. However, it is very challenging to develop high-performance liquid chromatography/tandem mass spectrometric (HPLC/MS/MS) methods for the quantitation of small polar compounds due to the lack of retention on traditional silica-based reversed-phase stationary phases. Maintaining appropriate retention and separation power for the determination of small drug components in biological fluids is highly recommended to avoid ion suppression or endogenous interferences for MS-based methods.[1–3] HPLC columns containing polar-endcapped and polar-enhanced stationary phases under highly aqueous conditions have been utilized to enhance the retention capability for small molecules with lower octanol/water partition coefficient values.[4] However, substantial retention of very polar compounds using these modified reversed-phase chromatography columns is normally not achievable. Also, in general, the ionization efficiency of the analytes is unfavorably affected by an increase in the content of aqueous mobile phase.[5,6]

The goal of this work was to develop a fast and straightforward HPLC/MS/MS method using a porous graphitic carbon (PGC) column for the quantitative determination of cytarabine in mouse plasma samples to support *in vivo* pharmacodynamic studies. Cytarabine (ara-C), a chemotherapy drug and a polar compound ($\log P = -2.712$),[7] was used as a model compound administered in mice. The PGC stationary phase was previously reported to retain highly polar analytes.[8–13] The column effluent was directly connected to the electrospray ionization (ESI) source as part of the tandem mass spectrometer system. Simultaneous selective reaction monitoring (SRM) of both ara-C and nicotinic acid, (NiAc) ($\log P = 0.225$) as the internal standard (ISTD), was used for the quantitative determination of the analyte. The influence of mobile phase composition on the ionization efficiencies and the separation power for all the test compounds was investigated. The need for chemical separation between the endogenous compound and the analyte due to mass spectrometric interference was demonstrated. The matrix ionization suppression effect for the PGC-HPLC/MS/MS system was investigated using a post-column infusion technique.[2] Furthermore, a direct comparison of the analytical results for plasma exposures of ara-C in mice obtained by the PGC-HPLC/MS/MS method and the ion-pairing HPLC/MS/MS method was performed to demonstrate the assay applicability.

EXPERIMENTAL

Reagents and chemicals

Cytarabine and nicotinic acid were purchased from Sigma (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Ammonium acetate and formic acid (FA) (99.999%) were purchased from Aldrich (Milwaukee, WI, USA). Nonafluoropentanoic acid (NFPA) as an ion-pairing

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reagent was purchased from Sigma. Deionized water was generated using a Milli-Q water-purifying system purchased from Millipore Corp. (Bedford, MA, USA) and in-house high-purity nitrogen (99.999%) was used. Drug-free mouse plasma samples (with heparin) were purchased from Bioreclamation Inc. (Hicksville, NY, USA).

Equipment

HPLC/MS/MS analysis was performed using a PE Sciex (Concord, ON, Canada) model API 5000 triple quadrupole mass spectrometer equipped with either an atmospheric chemical ionization (APCI) or an electrospray ionization (ESI) interface. The chromatographic system consisted of a Leap autosampler with a refrigerated sample compartment (set to 10°C) from LEAP Technologies (Carrboro, NC, USA), and a Shimadzu on-line degasser, LC-10AD VP pump and LC-10A VP controller (Columbia, MD, USA). The PGC column, Hypercarb (50 × 2.0 mm, 5 μm), was purchased from Thermo Electron Corporation (Waltham, MA, USA). For ion-pairing chromatography using NFPA as an ion-pairing reagent, a MetaSil Basic C18 column (4.6 × 100 mm, 3 μm) from MetaChem Technology (Torrance, CA, USA) was used as the analytical column. The Quadra 96 (Tomtec, Hamden, CT) system was used for semi-automated sample preparation with the protein precipitation technique.

HPLC methodologies

For the analysis of ara-C in mouse plasma using the PGC column, mobile phases A and B, consisting of water and acetonitrile containing 0.1% formic acid, respectively, were employed as follows: 0.3 min (10% B), 1.1 min (30% B), 1.2 min (100% B), 1.6 min (100% B), 1.61 min (10% B) and finished at 2.0 min with a constant flow rate of 0.8 mL/min to achieve satisfactory resolution among interferences. The retention times for ara-C and nicotinic acid were 1.11 and 0.77 min, respectively. For the ion-pairing (IP)-HPLC method, mobile phase A, consisting of water containing 0.1% NFPA, and mobile phase B, consisting of acetonitrile containing 0.1% formic acid and 4 mM ammonium acetate, were employed as follows: 0.3 min (2% B), 3.6 min (30% B), 4.0 min (100% B), 4.8 min (100% B), 4.9 min (2% B) and finished at 5.0 min at a constant flow rate of 1.3 mL/min. The retention times for ara-C and nicotinic acid were 3.77 and 2.43 min, respectively. The effluent from the PGC column was connected directly to either the ESI or the APCI source and the effluent from the C18 column was connected to the APCI source prior to a tandem mass spectrometer.

For the matrix effect studies, a mixture of ara-C and NiAc solution was continuously infused into PEEK tubing between a PGC column and the mass spectrometer through a tee-piece using a model 2400 syringe pump (Harvard Apparatus, South Natick, MA, USA). Either a protein precipitation extract of the blank mouse plasma samples or mobile phase B (5 μL) was injected onto the PGC column. Effluent from the PGC column was mixed with the infused compounds and entered the ESI source.

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 and doses following intraperitoneal administration to individual mice. After being clotted on ice, plasma was isolated by centrifugation and stored frozen (−20°C) until analysis.

Standard and sample preparation

Stock solutions of ara-C and NiAc 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 the analyte in mouse plasma was 50–25 000 ng/mL. The mouse plasma samples were prepared using the protein precipitation technique. A 300-μL aliquot of a methanol/acetonitrile (90/10) solution containing 1 ng/mL internal standard 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 into the HPLC/MS/MS systems for quantitative analysis.

Mass spectrometric conditions

The mass spectrometer was operated in the positive ion mode. Our generic mass spectrometric conditions regularly used for the screening of new chemical entities in our lab were employed without optimization, providing sufficient sensitivity for quantitation. The ESI instrumental settings for temperature, ion gas 1, ion gas 2, ionspray voltage, collision gas, curtain gas, declustering potential, entrance potential and collision cell exit potential were: 480°C, 50, 10, 5000 V, 6, 12, 120 V, 15 V and 10 V, respectively. The APCI instrumental settings for temperature, ion gas 1, nebulizer current, collision gas, curtain gas, declustering potential, entrance potential and collision cell exit potential were: 500°C, 50, 5, 6, 12, 100 V, 15 V and 10 V. For both ionization sources, the numbers without units are arbitrary values set by the Analyst software. The MS/MS reaction selected to monitor ara-C was the transition from *m/z* 244, $[M+H]^+$ ion, to a product ion at *m/z* 112 (Fig. 1). The ISTD was monitored using the transition from the $[M+H]^+$ ion at *m/z* 124 to *m/z* 80, as reported previously.[14] The protonated molecules were fragmented by collision-activated dissociation with nitrogen as the collision gas. The collision offset voltages were set at 20 and 25 V for the analyte and the ISTD, respectively.

RESULTS AND DISCUSSION

Development of a PGC-HPLC/MS/MS method

One of the common goals in the pharmacokinetic area is to develop a reliable bioanalytical method to measure the concentrations of a wide range of pharmaceuticals in biological fluids. Due to the inherent selectivity of tandem mass spectrometers, the hyphenated MS methods normally do not require extensive chromatographic separation for the analyte and the ISTD prior to detection. However, sufficient chromatographic retention of the drug components in the complex biological samples is highly recommended to avoid possible interferences from drug-related biotransformation

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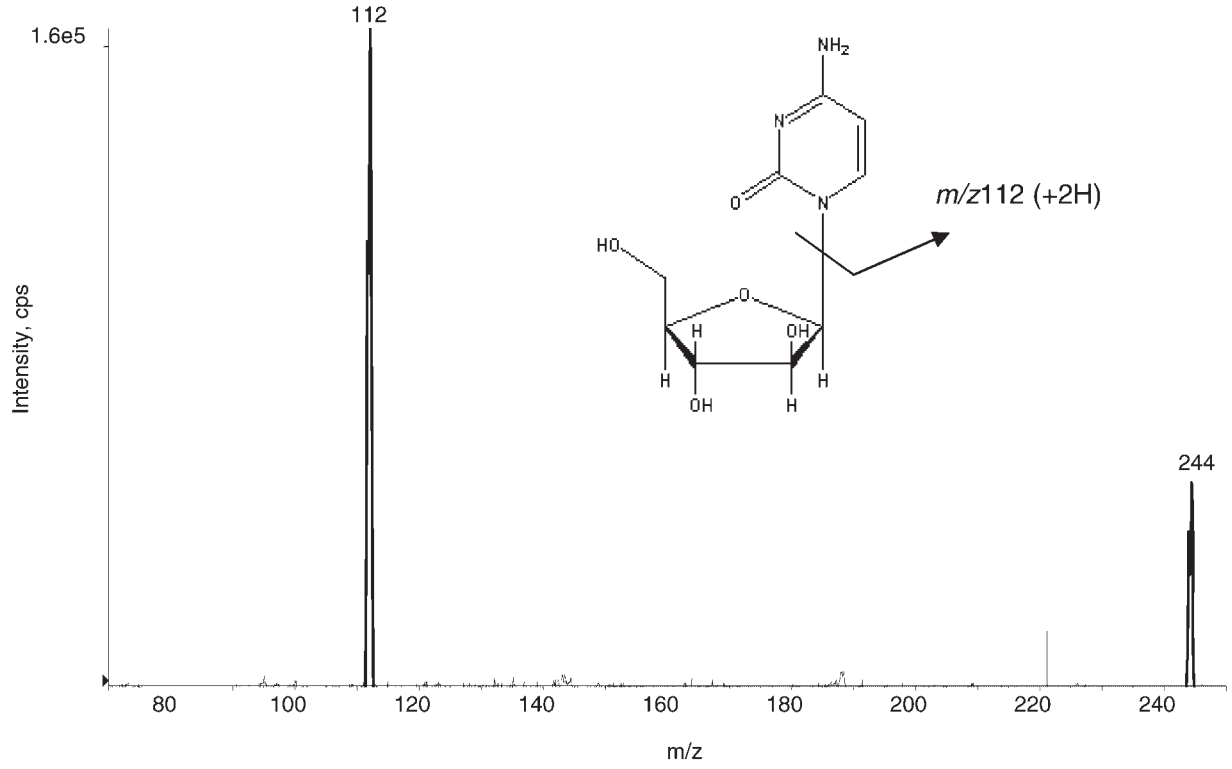

Figure 1. The product ion spectrum of the $[M+H]^+$ ion of ara-C at *m/z* 244.

products or ionization suppression due to co-eluted endogenous materials when HPLC/MS/MS methodologies are employed.[15,16] Our initial attempt to separate ara-C was to employ traditional silica-based C18 columns. However, ara-C was found not to be retained on various reversed-phase stationary phases even under high aqueous mobile phase conditions. The PGC column was able to provide more retention than other reversed-phase columns designed to trap and separate very polar compounds.[17] The surface of PGC is composed of flat sheets of hexagonally arranged carbon atoms. It is stable throughout the entire pH range and chemically inert to aggressive solvent systems to enable separation of extensive polarities within a chromatographic run.

PGC chromatography commonly employs water, methanol and acetonitrile as the mobile phase for the elution of the polar compounds but requires much stronger organic solvents such as dichloromethane and tetrahydrofuran for the elution of non-polar analytes. The ratio of organic solvent in the mobile phase normally has a substantial impact on retention in reversed-phase chromatographic modes. The influence of the composition of the mobile phase on the retention factor, k', of ara-C and NiAc on a PGC column under isocratic conditions was determined in this work. The relationship between log k' of ara-C and of NiAc and the concentration of organic solvent in the mobile phase on a PGC column is shown in Figs 2 and 3. The overall retention on a PGC column involves two major mechanisms: (1) dispersive interaction between analyte-mobile phase and analyte-graphite surface and (2) dipolar and ionic interaction of a polar analyte with the polarizable graphite.[17,18] Figure 2 indicates that the log k' values of ara-C decrease linearly at two different concentration intervals of organic solvent in the mobile phase. For methanol as the organic solvent, the values of log k' in the first interval (15–30% fraction) decrease faster than those in the second interval (30–60% fraction). A similar pattern for acetonitrile as the organic solvent was observed, where the values of log k' in the first interval (5–25% fraction) decrease faster than those in the second interval (25–50% fraction). The dependence of log k' values of NiAc on the ratios of organic solvent in the mobile phase is given in Fig. 3. Likewise, the results indicated that the log k' values of NiAc also decrease linearly at two different concentration intervals of organic solvent in the mobile phase. For methanol as the

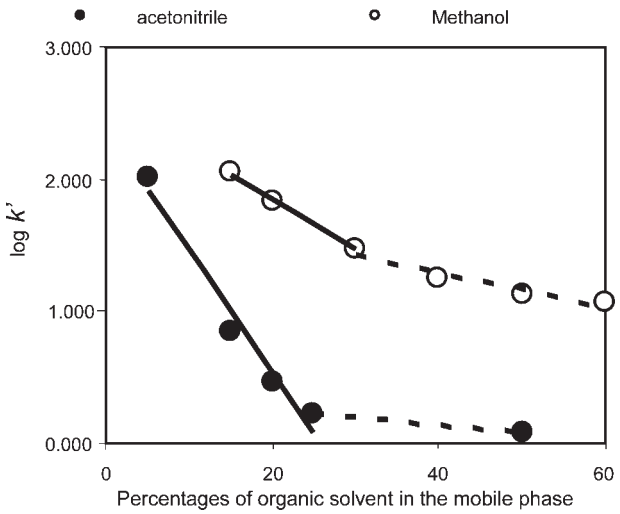

Figure 2. Plots of the capacity factor log k' of ara-C against the percentage of organic solvents in the mobile phase at a constant flow rate of 0.6 and 0.5 mL/min for acetonitrile and methanol, respectively.

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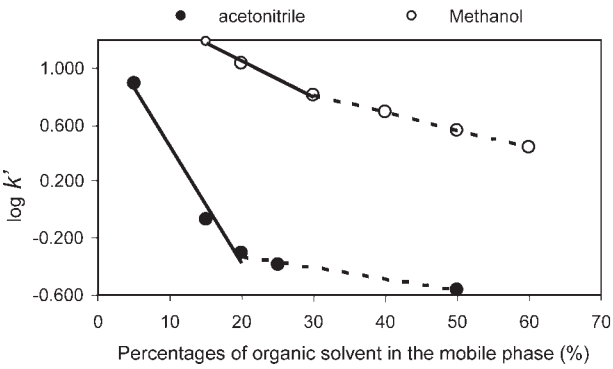

Figure 3. Plots of the capacity factor log k' of NiAc against the percentage of organic solvents in the mobile phase at a constant flow rate of 0.6 and 0.5 mL/min for acetonitrile and methanol, respectively.

modifier, the log k' values in the first interval (15–30% fraction) decrease faster than those in the second interval (30–60% fraction). Similarly, the log k' values in the first interval (5–20% fraction) decrease faster than those in the second interval (20–50% fraction) when methanol was replaced with acetonitrile. The reduction in log k' values of polar analytes indicated that the overall molecular interaction between the solutes and the graphite surface is decreasing. The linear relationship in each instance between the logarithm of capacity factor and the concentration of organic solvent in eluent implied that the elution order of polar analytes on a given PGC column might be governed by at least two specific retention mechanisms of interaction. This again demonstrates the unique utility of the PGC column for the analysis of polar compounds. The strength of organic solvents might be solute-dependent to allow selectivity adjustment for a PGC column. In this work, acetonitrile appeared to have stronger elution strength than methanol on a PGC column for the analyte and the ISTD which were eluted in order of decreasing polarity (NiAc then ara-C) as in reversed-phase chromatography.

The composition of the eluent may have a strong impact not only on the chromatographic performance, but also on the ionization efficiency of the analytes when hyphenating HPLC to various atmospheric pressure ionization sources.[19–21] Organic modifiers for the PGC column such as methanol and acetonitrile are MS-friendly. As shown in Fig. 4, the higher aqueous content decreases the ion signals of the test compounds in both the water/methanol and the water/acetonitrile solvent systems. The PGC-HPLC system requires higher concentrations of organic solvent in the mobile phase for the elution of polar molecules than does the silica-based reversed-phase HPLC system, and this will improve the effectiveness of nebulization in the ESI source resulting in better sensitivity of the analysis with MS detection.

Matrix ionization suppression studies

Matrix ionization suppression becomes a major concern when using the protein precipitation method for sample preparation.[2,22,23] In order to observe the matrix ionization suppression effects on plasma protein precipitation extracts

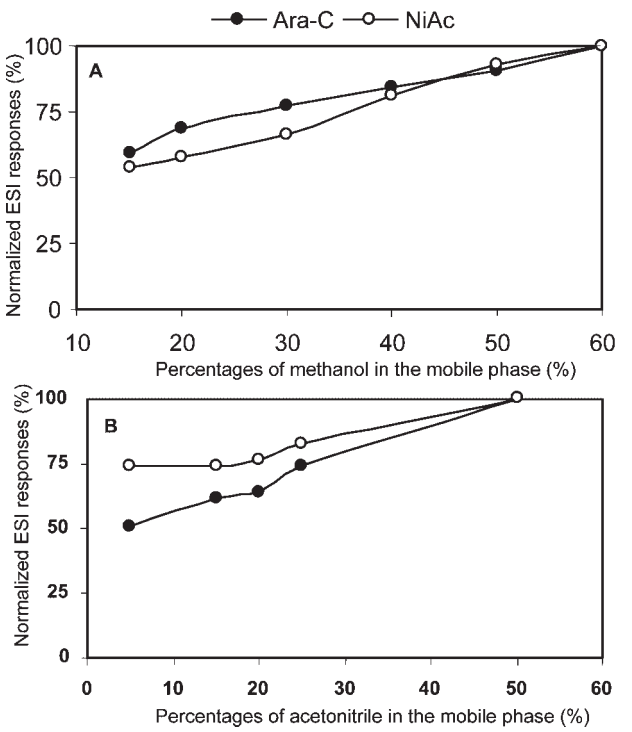

Figure 4. The normalized ESI responses of ara-C and NiAc against the percentage of (A) methanol and (B) acetonitrile in the mobile phase.

from blank mouse plasma samples, we monitored the variability of the ESI responses for ara-C and the ISTD using post-column infusion. Any changes in the ESI responses of the infused test compounds were assumed to be due to matrix ionization suppression caused by the endogenous materials eluting from the PGC columns. The differences in the infusion chromatograms between the methanol solvent and the plasma extract injections were considered to be caused by matrix effect. For accurate quantitative determination, it is strongly recommended that the retention times of all analytes should be in the 'safer' chromatographic regions which demonstrate little or no matrix ion suppression. The objectives of the post-column infusion experiments were to measure the extent of ionization suppression and to define the 'safer' portion of the chromatographic window. In general the ESI source is vulnerable to ionization suppression.[2,3] Some degree of signal reduction between chromatographic windows of 0.1–0.5 min and 0.1–0.3 min for ara-C and the ISTD, respectively, was 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. The ion signals of a certain given amount of ara-C and the ISTD spiked into the supernatant and methanol were found to be comparable. Also, the ion responses of the ISTD from the spiked standard plasma samples and the study plasma samples were found to be consistent.

Mouse plasma assays

As an example, the PGC-HPLC/MS/MS method was adapted for the determination of ara-C in mouse plasma

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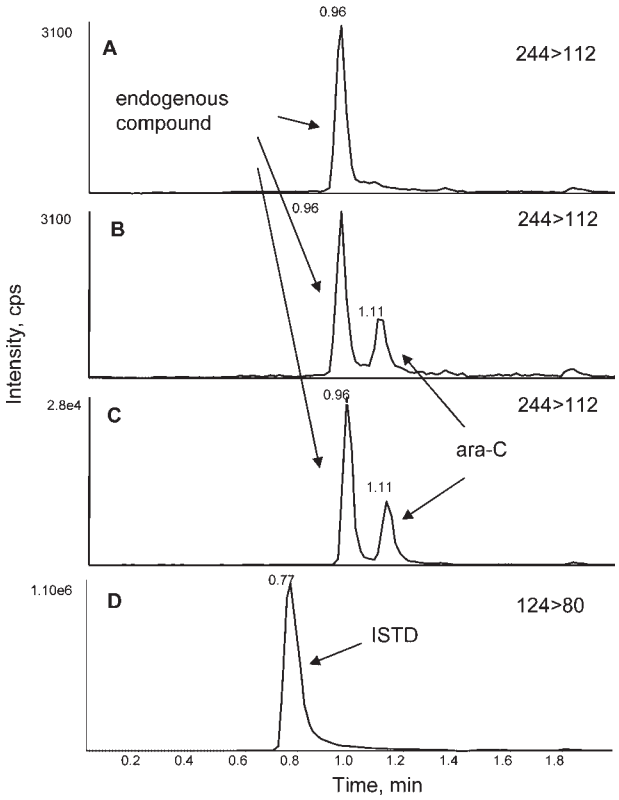

Figure 5. The extracted PGC-HPLC/ESI-MS/MS chromatograms of ara-C from (A) blank mouse plasma, (B) the spiked standard plasma of 50 ng/mL, (C) the study mouse plasma and NiAc, from (D) the study mouse plasma.

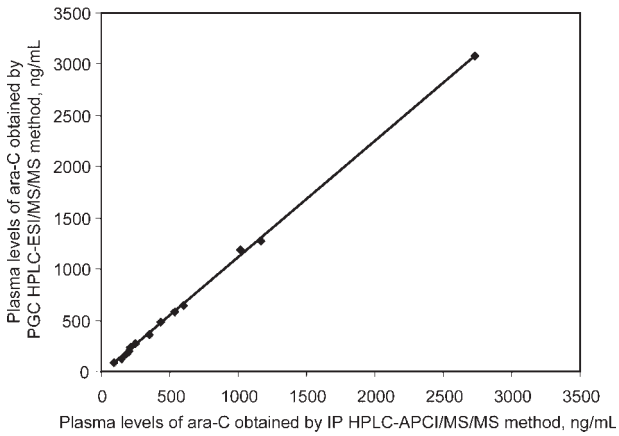

Figure 6. The correlation between the plasma concentrations of ara-C in mice obtained by the PGC-HPLC/ESI-MS/MS method and the IP-HPLC/APCI-MS/MS method.

to demonstrate its analytical applicability. The assay procedure involved a one-step protein precipitation procedure. The representative PGC-HPLC/ESI-MS/MS chromatograms of ara-C and the ISTD from the blank, spiked standard and the study mouse plasma samples are shown in Fig. 5. Gradient elution with increasing acetonitrile content in the mobile phase was used for the separation of ara-C and the endogenous compounds. As shown in Fig. 5(A), an endogenous peak ($t = 0.96$ min) sharing the same SRM transition as the analyte from the blank plasma sample was found. Therefore, it is necessary to obtain a baseline resolution between the chromatographic peaks of the endogenous compound and ara-C. As shown in Fig. 5(B), base-line separation for the endogenous peak ($t = 0.96$ min) and ara-C ($t = 1.11$ min) was achieved to avoid the mass spectrometric interference. The retention times and peak shape for ara-C were found to be reproducible during the course of the study. All stability assessments were carried out using the spiked standard mouse plasma. Ara-C was found to be stable on the benchtop for 6 h, in the autosampler for at least 24 h at 10°C, and after three freeze/thaw cycles.

The calibration curves for ara-C from the spiked standard mouse plasma samples at each concentration level were linear with a correlation coefficient, r^2, greater than 0.997 (graph is not shown). Accuracy (% bias) was less than 15% at all concentrations, 0.05 to 10 μg/mL. As shown in Fig. 6, the plasma concentrations of ara-C obtained by the PGC-HPLC/ESI-MS/MS method are found to be comparable with those obtained by the IP-HPLC/APCI-MS/MS method. The ESI source normally provides greater ionization capability than the APCI source for polar chemical species and produces little fragmentation for the conjugated metabolites. As expected, the ESI source yields greater ionization efficiencies for ara-C than the APCI source. However, the PGC-HPLC/MS/MS methods using either the ESI or the APCI source were equivalent for the determination of ara-C in mouse plasma samples in terms of analytical accuracy.

CONCLUSIONS

A porous graphitic carbon (PGC) column, a complementary stationary phase to reversed-phase chromatography, has been proven to be a useful analytical tool for the retention of small polar molecules. The retention of polar analytes was found to be substantially greater using the PGC column than the traditional C18 column. The content of organic solvent in the mobile phase plays an important role not only in the retention mechanism of the PGC stationary phase, but also in the ionization efficiencies of the test compounds. Because polar compounds are well retained on the PGC column it is necessary to use a high percentage of organic solvent to elute them and this is beneficial for sensitivity in the ESI source. The matrix effect on the PGC-HPLC/ESI-MS/MS system was observed and avoidable. The use of the PGC column under the reversed-phase conditions for the separation of ara-C from the endogenous compounds in mouse plasma samples to avoid mass spectrometric interference was demonstrated. Two HPLC/MS/MS assays using either the PGC column or a C18 column in conjunction with an ion-pairing reagent were successfully applied for the determination of ara-C in study mouse plasma samples from *in vivo* experiments. It is more challenging to develop a generic run for the analysis of samples containing multiple analytes with a wide range of polarities or unknown using the PGC-HPLC method than using an IP-HPLC method. However, the PGC-HPLC approach avoids the use of ion-pairing reagents which might decrease the ESI signal of the target compounds regardless of mass analyzers and instrument geometries.[24] These two hyphenated-MS methods in combination with a simple sample pre-treatment

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procedure showed equivalent accuracy to the analytical results and have been proven to be reliable in support of pharmacodynamic studies.

REFERENCES

1. Hsieh Y, Brisson JM, Wang G, Ng K, Korfmacher WA. *J. Pharm. Biomed. Anal.* 2003; **33**: 251.
2. Hsieh Y, Chintala M, Mei H, Agans J, Brisson JM, Ng K, Korfmacher WA. *Rapid Commun. Mass Spectrom.* 2001; **15**: 2481.
3. Mei H, Hsieh Y, Nardo C, Xu X, Wang S, Ng K, Korfmacher WA. *Rapid Commun. Mass Spectrom.* 2003; **17**: 97.
4. Majors RE, Przybyciel M. *LC/GC Eur.* 2002; **15**: 2.
5. Hsieh Y, Merkle K, Wang G, Brisson JM, Korfmacher WA. *Anal. Chem.* 2003; **75**: 3122.
6. Dams R, Benijts T, Gunther W, Lambert W, De Leenheer A. *Rapid Commun. Mass Spectrom.* 2002; **16**: 1072.
7. Kern W, Estey EH. *Cancer* 2006; **107**: 116.
8. Bohnstedt KC, Karlberg B, Wahlund LO, Jonhagen ME, Basun H, Schmidt S. *J. Chromatogr B* 2003; **796**: 11.
9. Xing J, Apedo A, Tymiak A, Zhao N. *Rapid Commun. Mass Spectrom.* 2004; **18**: 1599.
10. Reepmeyer JC, Brower JF, Ye H. *J. Chromatogr A* 2005; **1083**: 42.
11. Roy S, Delobel A, Gaudin K, Touboul D, Germain DP, Baillet A, Prognon P, Laprevote O, Chaminade P. *J. Chromatogr A* 2006; **1117**: 154.
12. Xia YQ, Jemal M, Zheng N, Shen X. *Rapid Commun. Mass Spectrom.* 2006; **20**: 1831.
13. Elfakir C, Dreux M. *J. Chromatogr A* 1996; **727**: 71.
14. Hsieh Y, Chen C. *Rapid Commun. Mass Spectrom.* 2005; **19**: 3031.
15. Wang G, Hsieh Y, Cui X, Cheng KC, Korfmacher WA. *Rapid Commun. Mass Spectrom.* 2006; **20**: 2215.
16. Wu Y, Farrell JT, Lynn K, Euler D, Kwei G, Hwang TL, Qin XZ. *Anal. Chem.* 2003; **75**: 426.
17. Thiebaut D, Vial J, Michel M, Hennion MC, Greibrokk T. *J. Chromatogr. A* 2006; **1122**: 97.
18. Holmgren E, Carlsson H, Goede P, Crescenzi C. *J. Chromatogr. A* 2005; **1099**: 127.
19. Wang G, Hsieh Y, Korfmacher WA. *Anal. Chem.* 2005; **77**: 541.
20. Rauha JP, Vuorela H, Kostiainen R. *J. Mass Spectrom.* 2001; **36**: 1269.
21. Gao S, Zhang ZP, Karnes HT. *J. Chromatogr. B* 2005; **825**: 98.
22. Matuszewski BK, Constanzer ML, Chavez-Eng CM. *Anal. Chem.* 1998; **70**: 882.
23. King R, Bonfiglio R, Fernandez-Metzler C, Miller-Stein C, Olah T. *J. Am. Soc. Mass Spectrom.* 2000; **11**: 942.
24. Holcapek M, Volna K, Jandera P, Kolarova L, Lemr K, Exner M, Cirkva A. *J. Mass Spectrom.* 2004; **39**: 3.

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