RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2009; **23**: 1364–1370
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.4010

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Hydrophilic interaction liquid chromatography/tandem mass spectrometry for the simultaneous determination of dasatinib, imatinib and nilotinib in mouse plasma

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Received 12 November 2008; Revised 1 March 2009; Accepted 2 March 2009

Hydrophilic interaction liquid chromatography (HILIC) interfaced with atmospheric pressure ionization (API) sources and a tandem mass spectrometer (MS/MS) was developed for the simultaneous determination of dasatinib, imatinib and nilotinib in mouse plasma samples. The retention profiles of all analytes on several silica stationary phases under HILIC conditions were explored. The influences of experimental factors such as the compositions of mobile phases on the chromatographic performance and the ionization efficiency of all analytes in positive ion mode were investigated. The applicability of the proposed HILIC/MS/MS approach following a protein precipitation procedure for the quantitative determination of dasatinib, imatinib and nilotinib at low nano-mole levels was examined with respect to assay specificity and linearity. The analytical results obtained by various HILIC/MS/MS approaches were found to be in good agreement with those obtained by reversed-phase liquid chromatography/tandem mass spectrometry (RPLC/MS/MS) methods in terms of assay sample throughputs, sensitivity and accuracy. Furthermore, the potential of matrix ionization suppression on the proposed HILIC/MS/MS systems was investigated using the post-column infusion technique. Copyright © 2009 John Wiley & Sons, Ltd.

Compound-specific analytical methods are the fundamental criteria in supporting various absorption, distribution, metabolism, excretion and toxicology (ADMET) studies to select lead compounds for drug development.[1–3] Among these methods, mass spectrometry (MS)-based approaches have evolved to become irreplaceable techniques in the analysis of drug- related molecules in biological samples.[4–6] One of the common goals in the ADMET areas is to develop a generic reversed-phase liquid chromatography/tandem mass spectrometry (RPLC/MS/MS) method to simultaneously determine a wide range of drug candidates and their metabolites in either *in vitro* or *in vivo* samples. RPLC is the most widely employed technique in pharmaceutical analysis due to its extensive application to most small drug molecules which are separated by their degree of hydrophobic interaction with the stationary phase.[7] Hydrophilic interaction liquid chromatography (HILIC) with low-aqueous/high-organic mobile phase is well known as a valuable supplement to RPLC for the retention of polar analytes.[8] HILIC separates compounds by eluting with a strong organic mobile phase against a hydrophilic stationary phase where elution is driven by increasing the water content in the mobile phase. The highly volatile organic mobile phases such as methanol and acetonitrile used in HILIC provide not only low column back-pressure, but also an increased atmospheric pressure ionization (API) efficiency for MS detection.[8]

In this work, we have extended applications of using HILIC/MS/MS methods for the quantitative determination of nonpolar compounds, dasatinib, imatinib and nilotinib, novel Bcr-Abl tyrosine kinase inhibitors for the treatment of leukemias,[9,10] in mouse plasma samples to support *in vivo* studies. HILIC was run on various polar stationary phases using a high-organic and low-aqueous mobile phase for the separation of the analytes following a simple protein precipitation technique. The column effluent was directly connected to either an atmospheric pressure chemical ionization (APCI) or an electrospray ionization (ESI) source as part of an integrated tandem mass spectrometry (MS/MS) system. A multiple reaction monitoring (MRM) approach of all analytes and the internal standard (ISTD) was used for the quantitation. The influences of mobile phase compositions on both the separation power and the ionization efficiencies of all analytes were investigated. An inter-instrumental comparison of two popular triple quadrupole mass spectrometers integrated with the described chromatographic systems was investigated on their susceptibility to the analytical accuracy and the matrix ionization effect from the endogenous compounds of mouse plasma samples. Furthermore, a direct comparison of the analytical results obtained from both HILIC/MS/MS and RPLC/MS/MS methods for the mouse plasma levels of all analytes in study samples was performed to demonstrate the feasibility of the assay.

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EXPERIMENTAL

Reagents and chemicals

Dasatinib, imatinib and nilotinib used as the analytes and clofazimine used as the ISTD were purchased from Sigma (St. Louis, MO, USA). The chemical structures of the analytes are shown in Fig. 1. Acetonitrile (HPLC grade) and ammonium acetate were purchased from Fisher Scientific (Pittsburgh, PA, USA) and formic acid (99.999%) was purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI, USA). Deionized water was generated from a Milli-Q water-purifying system (Millipore Corp., Bedford, MA, USA) and house high-purity nitrogen (99.999%) was used. Drug-free mouse plasma samples were purchased from Bioreclamation Inc. (Hicksville, NY, USA). Mobile phases A and B were water and acetonitrile containing both 0.1% formic acid and 4 mM ammonium acetate, respectively.

Chromatographic and mass spectrometric conditions

Chromatographic separation was achieved using mobile phases A and B and was carried out on a Waters ACQUITY system using either a 50×2.1 mm HALO^{TM} C_{18} 2.7 μm column (MAC-MOD Analytical, Inc., Chadds Ford, PA, USA) or 50×2.1 mm 3 μm HILIC columns with a series of chemistries of weak acidic, neutral, and basic stationary phases (Sepax Technologies, Inc., Newark, DE, USA) as a separation media operated at 45°C. The ACQUITY system is capable of pumping mobile phase at pressures up to 15 000 psi and includes an autosampler that can hold ten 96-well plates. For RPLC, a ballistic gradient chromatographic separation was employed for the simultaneous determination of three analytes in mouse plasma samples as follows: 0.2 min (5% B), 0.5 min (95% B), 0.9 min (95% B), 0.91 min (5% B) and finished at 1 min at a constant flow rate of 0.8 mL/min. For HILIC, either a 1 min gradient run (0.1 min (95% B), 0.3 min (60% B), 05 min (60% B), 0.9 min (95% B), 0.91 min (95% B) and finished at 1 min or a 1.2 min isocratic run (78% B) was employed with a 1.2 mL/min flow rate. Identical mobile phases were used for all chromatographic systems using both ESI and APCI modes for all instruments. The HPLC eluant passed through a divert valve and was introduced into the API interfaces prior to the mass spectrometers.

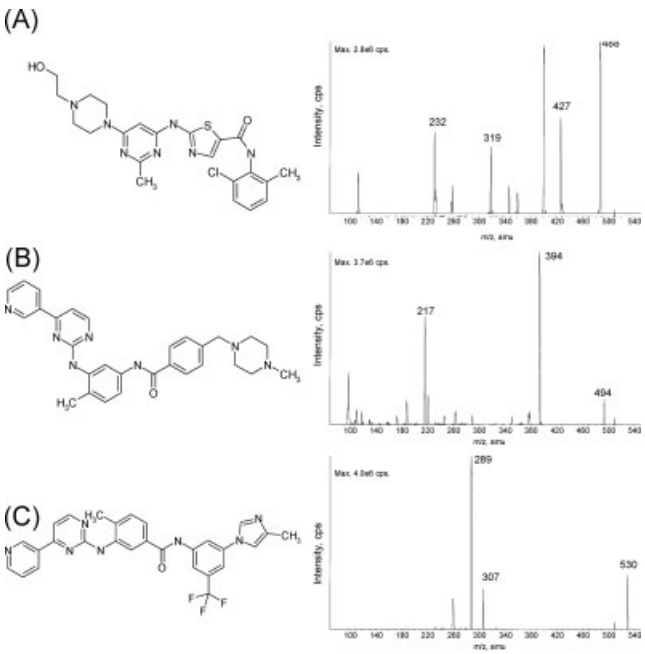

Figure 1. Chemical structures and tandem mass spectra of (A) dasatinib, (B) imatinib, and (C) nilotinib.

Two triple quadrupole MS systems operated in positive ion mode were compared: a TSQ Quantum Ultra (Finnigan, San Jose, CA, USA) and a 4000 Q TRAP mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) with both vendor-supplied ESI and APCI sources. The Quantum triple quadrupole mass spectrometer was operated with a spray voltage of 4500 V, capillary temperature of 270°C, sheath gas 50 psi, and auxiliary gas 23 psi. For the Applied Biosystems 4000 Q TRAP linear tandem mass spectrometer, the APCI instrumental settings for probe temperature, ion gas 1, nebulizer current, collision gas, curtain gas, declustering potential, entrance potential and collision cell exit potential were as follows: 500°C, 50, 5, 6, 12, 60 V, 11 V and 20 V, respectively and the ESI instrumental settings for probe temperature, ion gas 1, ion gas 2, ion spray potential, collision gas, curtain gas, declustering potential, entrance potential and collision cell exit potential were as follows: 500°C, 50, 20, 5500 V, 6, 12, 60 V, 11 V and 20 V, respectively. (The numbers without units are arbitrary values set by the Analyst software.) The protonated molecules were fragmented by collision-activated dissociation with nitrogen as collision gas at a pressure of instrument setting 5. The collision offset voltage was set at 40 V and 45 V for the analytes and the ISTD, respectively. The MS/MS transitions selected to monitor dasatinib, imatinib, nilotinib and clofazimine used their protonated molecules ([M + H] + ions) as precursors at *m/z* 488, 494, 530, 473 and the product ions at *m/z* 401, 394, 289, 431, respectively (Fig. 1).

For the matrix effect studies, a mixture of dasatinib, imatinib, nilotinib and clofazimine solution was continuously infused into PEEK tubing in between the analytical column and the mass spectrometer through a tee 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 (3 μL) was injected into the analytical column. Effluent from the HPLC columns mixed with the infused compounds and entered either the APCI or the ESI interface.

Sample collection

The animal-dosing experiments were carried out in accordance with 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 oral administration to individual mice. After clotting on ice, plasma was isolated by centrifugation and stored frozen (−20°C) until analysis.

DOI: 10.1002/rcm

Standard and sample preparation

Stock solutions of dasatinib, imatinib, nilotinib and the ISTD 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 both analytes in mouse plasma was 10–10 000 ng/mL. To examine the susceptibility of the method for lot-to-lot matrix variations, low, medium, and high quality control (QC) samples at 50, 500 and 5000 ng/mL were prepared from the same spiking solution but using different lots of mouse plasma for each run. The mouse plasma samples were prepared using the protein precipitation technique. A 200-μL aliquot of acetonitrile solution containing 1 ng/mL of ISTD was added to 20 μL of plasma located in a 96-well plate. After mixing and centrifugation the supernatant was automatically transferred to a second 96-well plate using a Quadra 96 instrument (Tomtec, Hamden, CT, USA). An aliquot (3 μL) of the extract was injected by the ACQUITY autosampler to either the HILIC/MS/MS or RPLC/MS/MS system for quantitative analysis.

RESULTS AND DISCUSSION

Development of HILIC/MS/MS methods

The fate of drug candidates is frequently based on their pharmacokinetic parameters, which are mainly calculated from the plasma concentrations of the dosed compounds over a certain post-dose time period. Due to its inherent selectivity, chromatographic techniques coupled to mass spectrometric systems normally require neither labor-intensive sample preparation procedures nor extensive chromatographic run times in order to avoid matrix interference. This allows researchers to shorten chromatographic times and therefore to increase sample throughput. RPLC is the most widely employed chromatographic technique for pharmaceutical analysis due to its extensive application to most small drug molecules which are separated by their degree of hydrophobic interaction with the stationary phase. HILIC is emerging as a supplement to RPLC for the retention of highly hydrophilic, ionic, and polar compounds.[8,11–14] In this work, our goal was to investigate the feasibility of extending the application range of HILIC to the analysis of nonpolar pharmaceuticals in biological samples. For normal-phase LC, the elution is generally promoted by the use of a polar organic mobile phase. In HILIC, an appropriate amount of water in the mobile phase is suggested for maintaining a stagnant enriched water layer on the surface of the polar stationary phase where the analytes partition. HILIC separates compounds by eluting with a strong organic mobile phase against a hydrophilic stationary phase where elution is driven by increasing the water content in the mobile phase (Fig. 2). Figure 2 shows that under organic solvent-rich conditions (water is less than 50%), water becomes an elution solvent on an underivatized silica column for all test compounds. An increase in water content (15% to 30%) significantly decreases the retention factors (ln *k*) of the nonpolar compounds tested. Similar to the retention behavior of the polar compounds on the HILIC columns previously reported,[15] an increased retention for

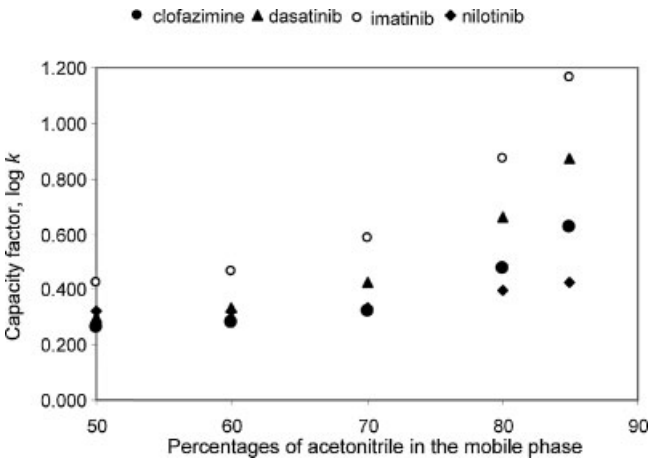

Figure 2. The effect of water contents on the retention factors of clofazimine, dasatinib, imatinib and nilotinib.

greater hydrophilic analytes was observed. As shown in Fig. 2, a nonlinear relationship between the retention factors and water ratios in the mobile phase was observed which implied multiple modes of the retention mechanisms such as ion exchange, hydrogen-bonding, hydrophobic and hydrophilic interaction.[15] In HILIC, the use of the organic-rich mobile phases providing low column back-pressure is advantageous to achieve fast chromatography.

A bare silica column, a weak acidic phase, is the most popular HILIC column to separate polar small molecules.[16,17] As with RPLC, HILIC can be performed on a variety of modified silica-based adsorbents with chemically bonded ligands to resolve potential separation problems. In this work, in addition to the bare silica column, a series of chemically bonded diol, pyridine and imidazole ligands were employed as the stationary phases for HILIC separations of test nonpolar pharmaceuticals. The diol-bonded phase is neutral and pyridine- and imidazole-bonded phases are basic on the material surface. As shown in Figs. 3, 4 and 5, the strength of the polar interactions with basic analytes, imatinib, dasatinib and nilotinib, under the same LC conditions, increases in the order: silica > pyridine > imidazole > diol stationary phases. This was expected since basic compounds are normally strongly retained on silica gel by hydrogen-bonding and ion-exchange interactions with silanol groups.[17] The selection of different stationary phases could play a core role in HILIC method development depending on the chemical properties of the analytes.

The compositions of the eluent may have a strong impact on the chromatographic performance and the ionization efficiency of the analytes when hyphenating high-performance liquid chromatography (HPLC) to various atmospheric pressure ionization sources.[18,19] ESI and APCI have been the most popular API interfaces for the hyphenated MS systems for qualitative or quantitative analysis of small molecules over the last decade. In general, the greater organic contents in the mobile phase yield higher ionization efficiencies for small molecules on both ESI and APCI. A possible explanation for this improved response could be due to more effective nebulization and vaporization processes with higher organic ratios in the mobile phase. Figure 7 reveals that the sensitivity of all test compounds by ESI increased as the ratios of water in the mobile phase decreased from 50% to

DOI: 10.1002/rcm

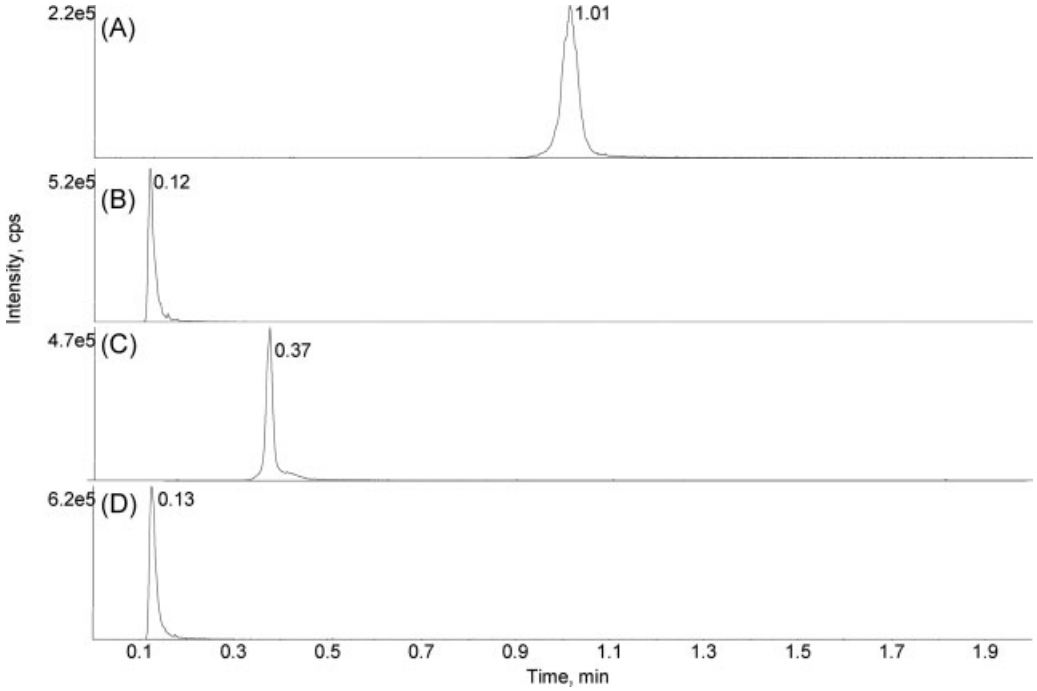

Figure 3. The extracted HILIC/MS/MS chromatograms of imatinib from (A) bare silica, (B) diol-bonded, (C) pyridine-bonded, and (D) imidazole-bonded stationary phases. Mobile phase: 4 mM ammonium acetate and 0.1% formic acid in acetonitrile/water (85:15).

15%. As indicated in Fig. 7, the ESI response of clofazimine increases nearly three-fold as the acetonitrile content in the mobile phase increases from 50% to 85%. For the APCI source, except for dasatinib, all other analytes and the ISTD had the same degree of the influence of ionization efficiency (data not shown). The APCI response of dasatinib was found to be consistent regardless of the water content in the mobile phase ranging from 50% to 15%. Here, the ion signals of all analytes and the ISTD were calculated based on their chromatographic peak areas. Unlike RPLC, in HILIC mode separations, analytes are typically eluted and introduced into the API sources under lower dielectric and higher organic

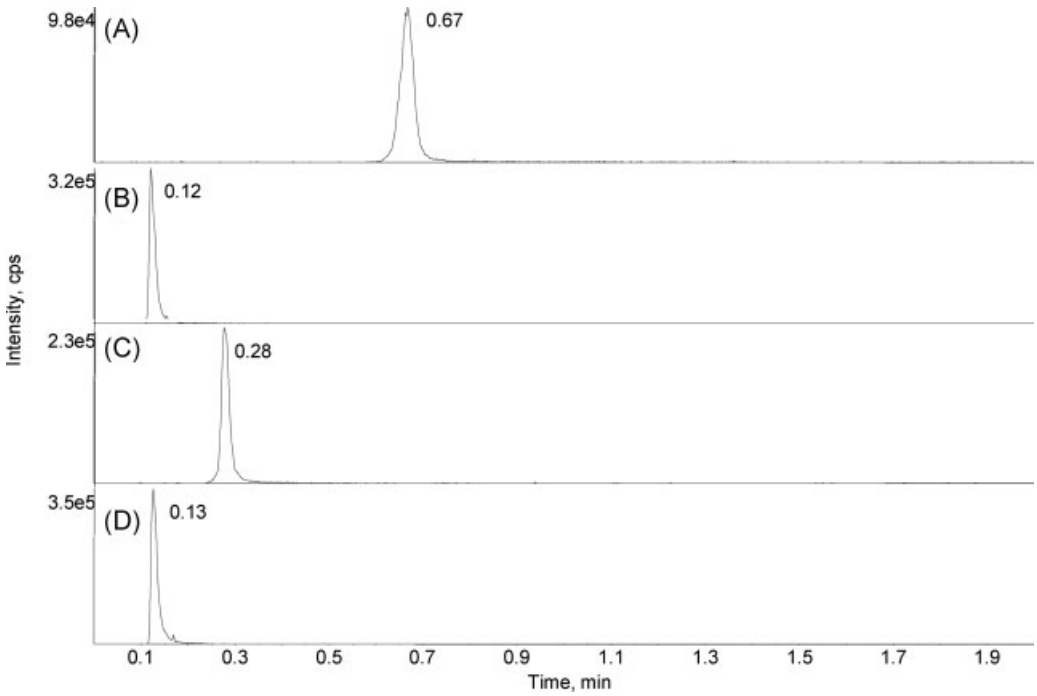

Figure 4. The extracted HILIC/MS/MS chromatograms of dasatinib from (A) bare silica, (B) diol-bonded, (C) pyridine-bonded, and (D) imidazole-bonded stationary phases. Mobile phase: 4 mM ammonium acetate and 0.1% formic acid in acetonitrile/water (85:15).

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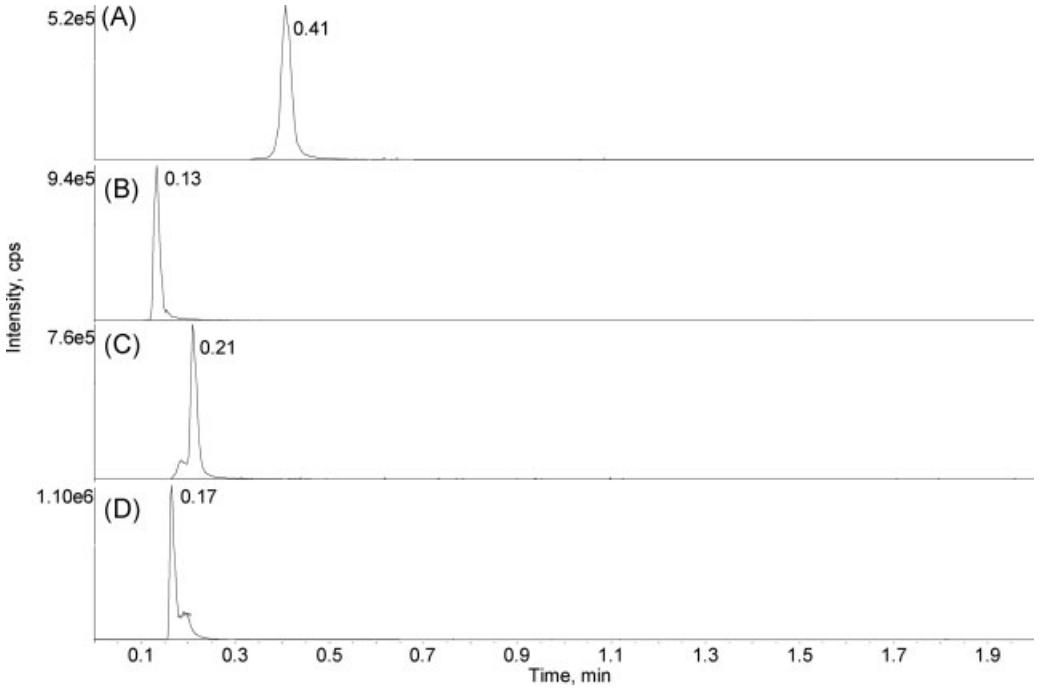

Figure 5. The extracted HILIC/MS/MS chromatograms of nilotinib from (A) bare silica, (B) diol-bonded, (C) pyridine-bonded, and (D) imidazole-bonded stationary phases. Mobile phase: 4 mM ammonium acetate and 0.1% formic acid in acetonitrile/water (85:15).

environments where a better detection limit for small molecules can be normally achieved.

Matrix ionization suppression studies

A well-recognized concern about assay reliability when developing any new MS-based methods is the increased likelihood of encountering matrix ionization suppression.[20–22] In order to observe the matrix ionization suppression effects using HILIC/MS/MS techniques on plasma protein precipitation extracts from blank plasma samples, we monitored the variability of the APCI and ESI responses for dasatinib, imatinib, nilotinib and the ISTD using the post-column infusion scheme.[21] Any differences in the infusion chromatograms between the mobile phase and the blank mouse plasma extract injections were deemed to be caused by ionization suppression due to co-elution of endogenous compounds from mouse plasma samples. For accurate quantitative determination, it is strongly recommended that the retention times of all analytes should be in the region of little 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. The results reveal that there was no impact on the HILIC or RPLC assay accuracy using either the ESI or the APCI interface because the retention times of all test compounds appeared in the safe chromatographic window (data not shown).

Mouse plasma assays

In this work, our goal was to develop fast HILIC/MS/MS methods without altering the mobile phases commonly used for RPLC/MS/MS methods routinely used in our laboratory for the simultaneous determination of dasatinib, imatinib and nilotinib in plasma samples following direct injection of supernatants after protein removal. Utilization of traditional reversed-phase C18 columns is commonly employed for chromatographic separation of small molecules for RPLC/MS/MS method development. The representative RPLC/MS/MS chromatograms of imatinib, dasatinib, nilotinib and ISTD used for analysis of study mouse plasma samples are shown in Fig. 6(A). The analytes are eluted by a ballistic gradient separation with increasing the acetonitrile contents in the mobile phase within 1 min. As shown in Fig. 6(A), an efficient separation for imatinib, dasatinib, nilotinib was achieved to avoid the possible mass spectrometric interferences. The sample preparation procedure involved a one-step protein precipitation procedure. The retention times of the analytes and ISTD were found to be reproducible throughout the experiment. RPLC/MS/MS-based methods for pharmaceutical analysis reported in the literature were frequently achieved using a variety of tandem mass spectrometers by different manufacturers. In this work, a comparison of the two tandem mass spectrometers integrated with the same chromatographic conditions was performed by analyzing the same set of study samples. The analytical results of all three analytes obtained by RPLC coupled to individual mass spectrometers were found to be comparable in terms of sensitivity and accuracy (data not shown).

In HILIC, as the opposite of RPLC, the strongest mobile phase has a high percentage of water. For gradient HILIC separations by increasing the aqueous component in the mobile phase, the initial mobile phase has a high percentage of organic solvents. According to the literature in LC-based assays, like RPLC, most HILIC applications using bare silica columns employ gradient elutions. As an example, the proposed HILIC/MS/MS method with the same mobile phases and sample preparation procedure used for the

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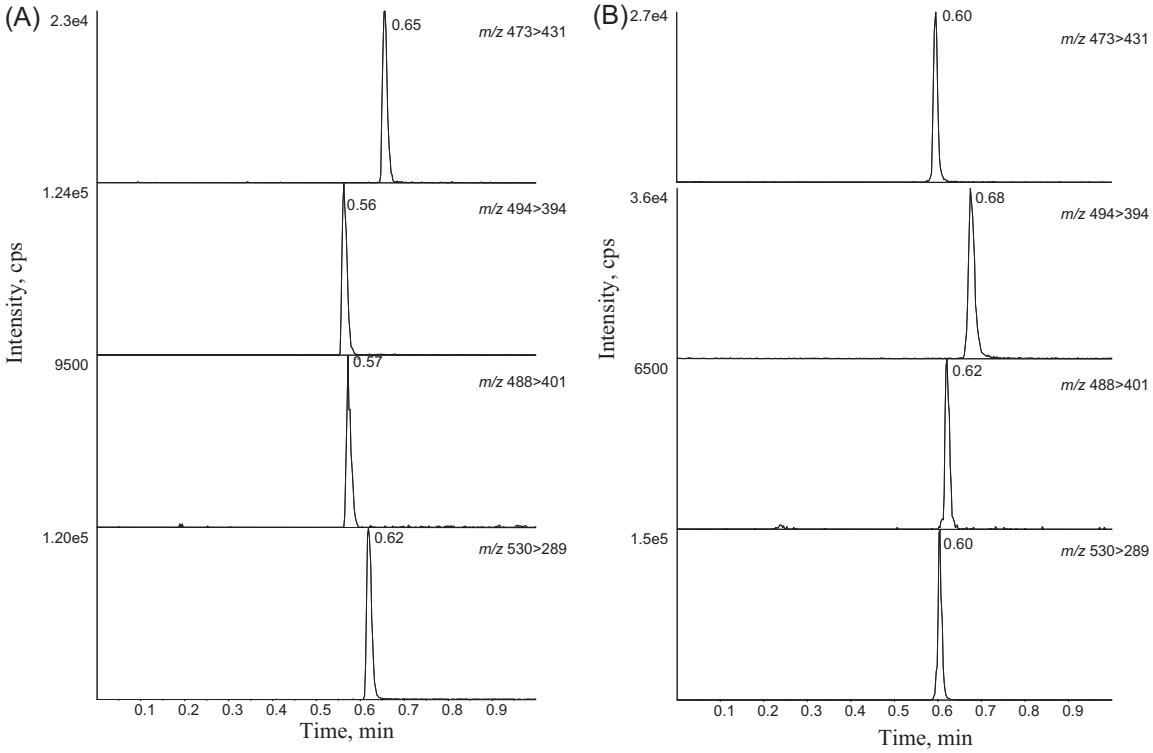

Figure 6. >(A) The extracted RPLC/MS/MS chromatograms of clofazimine, imatinib, dasatinib, and nilotinib and (B) the extracted HILIC/MS/MS chromatograms of clofazimine, imatinib, dasatinib, and nilotinib from a spiked standard mouse plasma sample of 500 ng/mL.

RPLC/MS/MS method was also applied for the simultaneous determination of the dosed compounds in mouse plasma samples to demonstrate the realistic suitability of analyses. Figure 6(B) shows the extracted mass chromatograms for all the analytes and the ISTD using the APCI interface. The analytes are eluted by a ballistic gradient separation with increasing the water contents in the mobile phase within 1 min. The retention times and peak shape, as shown in Fig. 6(B) for the analytes and the ISTD in both standard plasma and study plasma samples, were found to be reproducible during the course of the study. There was no endogenous peak from six different batches of blank mouse plasma observed to interfere with the analytes and the ISTD under experimental conditions. The calibration curves for three analytes using either the APCI or the ESI interface obtained from standard mouse plasma samples at each concentration level were linear with a correlation coefficient, r^2, greater than 0.99 (graph is not shown). Accuracy (% bias) was less than 15% at all concentrations. The interday precision and accuracy in the measurement of low, medium, and high QC samples obtained for three analytes were found to be in the range of 5–10% relative standard deviation (RSD) and in the range of 90–113% of the nominal values, respectively. The analytical results from the HILIC/MS/MS systems using either the APCI or ESI source suggest that both ionization interfaces are viable detection techniques for the measurement of imatinib, dasatinib, nilotinib in the low-ng/mL range in mouse plasma in support of a pharmacodynamic study (data not shown). In comparison with HILIC/MS/MS approaches under gradient or isocratic conditions, the plasma exposure of imatinib, dasatinib, nilotinib from the same set of study samples obtained by both systems was also found to be well correlated (data not shown). As an example, Fig. 8 compares the values in terms of mouse plasma concentrations of dasatinib calculated by the response ratios of analytes over the ISTD obtained by the gradient HILIC/MS/MS and gradient RPLC/MS/MS methods. Student's *t*-test results indicated no significant difference of plasma concentrations at each time-point of dasatinib determined by the aforementioned methods with 95% confidence ($\alpha = 0.5$). These

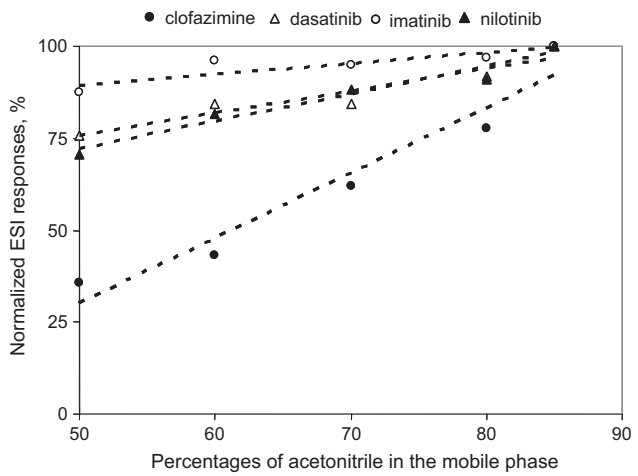

Figure 7. Normalized ESI responses of clofazimine, imatinib, dasatinib, and nilotinib as a function of the ratios of acetonitrile in the mobile phase.

DOI: 10.1002/rcm

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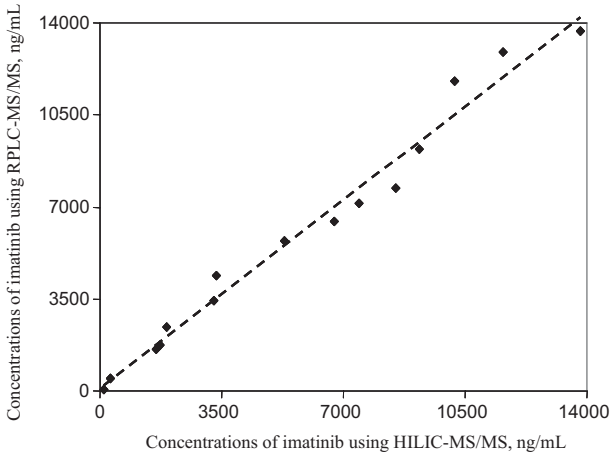

Figure 8. Correlation of the concentrations of imatinib in study mouse plasma samples obtained by the RPLC/MS/MS and the HILIC/MS/MS methods.

results concluded that the HILIC/MS/MS method proved as reliable as the RPLC/MS/MS methods for the determination of dasatinib in mouse plasma samples.

CONCLUSIONS

HILIC, known as a complementary technique to RPLC, has been proven to be a powerful analytical tool for the retention of small polar molecules but also could serve as an alternative technique to RPLC for quantitative determination of nonpolar pharmaceuticals in biological fluids. The capability of using bare silica and other modified HILIC stationary phases for the retention and separation of imatinib, dasatinib and nilotinib to avoid possible mass spectrometric interference was demonstrated. Several rapid, sensitive HILIC/MS/MS assays using two major ionization sources (APCI vs. ESI) or chromatographic elutions (gradient vs. isocratic) have been successfully developed and evaluated for the simultaneous determination of imatinib, dasatinib and nilotinib in mouse plasma. It is feasible to convert RPLC/MS/MS into HILIC.MS/MS without changing the mobile phases for nonpolar pharmaceutical analyses with an enhanced sensitivity. Both HILIC/MS/MS and RPLC/MS/MS methods in conjunction with a simple sample treatment procedure showed equivalent accuracy in the analytical results and have been proven to be reliable in support of *in vivo* pharmacodynamic studies.

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