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Ultra-performance hydrophilic interaction liquid chromatography/tandem mass spectrometry for the determination of everolimus in mouse plasma

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Ultra-performance hydrophilic interaction liquid chromatography (UPHILIC) interfaced with the electrospray ionization (ESI) source of a tandem mass spectrometer (MS/MS) was developed for the simultaneous determination of everolimus in mouse plasma samples. UPHILIC was performed on a sub-2 μm bare silica particle packing with the column pressure under traditional high-performance liquid chromatography (HPLC) to allow fast separation of pharmaceutical compounds within a chromatographic analysis time of 1 min. This UPHILIC technology is comparable with reversed-phase ultra-performance liquid chromatography (RPUPLC) in terms of chromatographic efficiency but demands neither expensive ultra-high-pressure instrumentation nor new laboratory protocols. With the ESI source, multiple reaction monitoring (MRM) of the ammoniated adduct ions of the analyte was used for tandem mass spectrometric detection. The retention mechanism profiles of the test compounds 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 the test compounds in positive ion mode were investigated. A UPHILIC/MS/MS approach following a protein precipitation procedure was applied for the quantitative determination of everolimus at the low ng/mL region in support of a pharmacodynamic study. The analytical results obtained by the UPHILIC/MS/MS approach were fond to be in good agreement with those obtained by the RPUPLC/MS/MS method in terms of assay sample throughput, sensitivity and accuracy.

High-performance liquid chromatography/tandem mass spectrometry (HPLC/MS/MS) approaches have evolved to become the mainstream techniques in the analysis of drug-related molecules in biological samples.[1–6] One of the common goals in the bioanalytical areas is to develop a generic HPLC/MS/MS method to determine a wide range of drug candidates in either *in vitro* or *in vivo* samples. In practice, reversed-phase liquid chromatography (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 phases is well known as a valuable supplement to RPLC for the retention of polar analytes.[8] 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 investigated the feasibility of applying an ultra-performance hydrophilic interaction liquid chromatography/tandem mass spectrometry (UPHILIC/MS/MS) method for the quantitative determination of a neutral compound, everolimus, in mouse plasma samples to support a pharmacodynamic study. UPHILIC was run on a sub-2 μm unmodified silica stationary phase 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 the electrospray ionization (ESI) source as part of an integrated tandem mass spectrometry (MS/MS) system. A multiple reaction monitoring (MRM) approach for 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 the test compounds were investigated. Furthermore, a direct comparison of the analytical results obtained from both UPHILIC/MS/MS and RPUPLC/MS/MS methods for the mouse plasma levels of the analyte in study samples was performed to demonstrate the assay feasibility.

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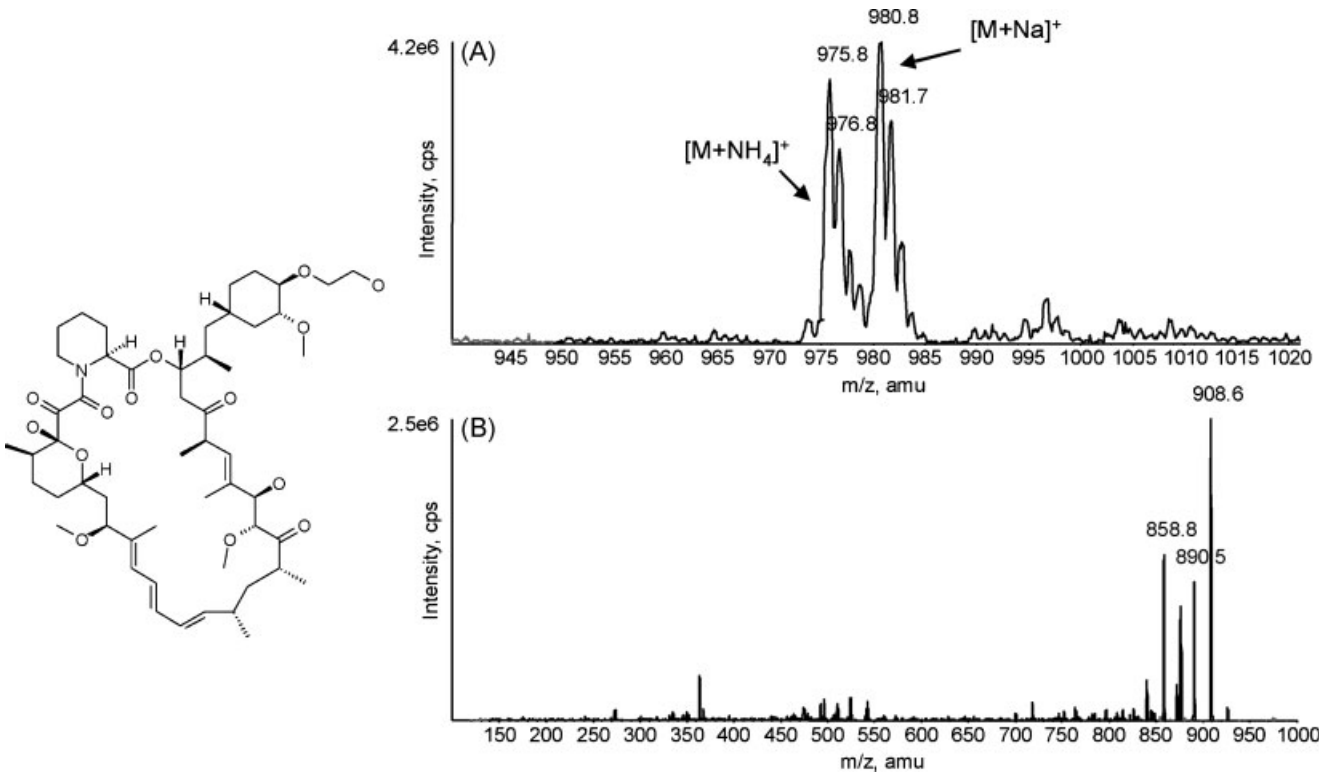

Figure 1. Chemical structure and (A) a full scan spectrum and (B) a tandem mass spectrum of everolimus.

EXPERIMENTAL

Reagents and chemicals

Everolimus used as the analyte was purchased from Sequoia Research Products Ltd. (Pangbourne, UK) and clofazimine used as the ISTD was purchased from Sigma (St. Louis, MO, USA). The chemical structure of the analyte is shown in Fig. 1. Acetonitrile (HPLC grade) and ammonium acetate were purchased from Fisher Scientific (Pittsburgh, PA, USA) and formic acid (FA) (99.999%) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Deionized water was generated from a Milli-Q water purifying system (Millipore Corporation, 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 A1 and B1 were water and acetonitrile containing both 0.1% FA, respectively. Mobile phases A2 and B2 were water and 95% acetonitrile containing both 0.1% FA and 4 mM ammonium acetate, respectively.

Chromatographic and mass spectrometric conditions

Unless otherwise indicated, all binary step-gradient chromatographic systems were achieved using the same mobile phases A1 and B1 and were carried out on a Waters ACQUITY system without column switching. RPUPLC conditions employed in this study consisted of a 50×2.1 mm Waters ACQUITY UHPLC BEH (bridged ethyl hybrid) C_{18} 1.7 μm column (Waters Corporation, Milford, MA, USA). HILIC and UPHILIC were operated on 50×2.1 mm 3 μm and 50×2.1 mm 1.8 μm columns, respectively, packed with an underivatized silica stationary phase (Sepax Technologies, Inc., Newark, DE, USA) as a separation media. The column temperature for both RPLC and HILIC was set 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 the RPUPLC/MS/MS method, a ballistic gradient chromatographic separation using mobile phases A1 and B1 was employed for the determination of everolimus in mouse plasma samples as follows: 0 min (2% B1), 0.2 min (2% B1), 0.4 min (95% B1), 0.8 min (95% B1), 0.9 min (2% B1) and finished at 1.0 min at a flow rate of 0.8 mL/min. The retention times for the analyte and the ISTD were 0.69 and 0.59 min, respectively (Fig. 2). For the UPHILIC/MS/MS method,

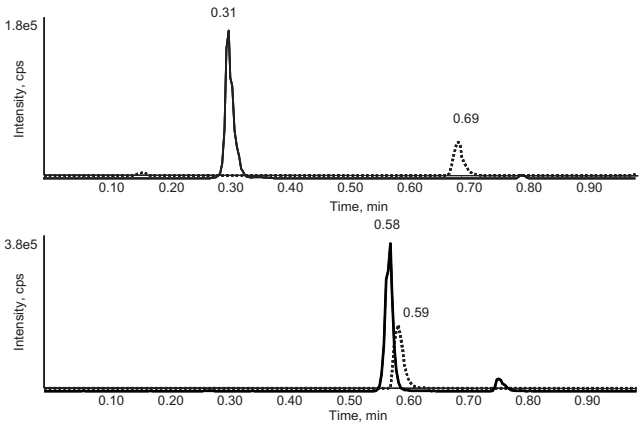

Figure 2. Top: extracted UPHILIC/MS/MS (solid line) and RPUPLC/MS/MS (dotted line) chromatograms of everolimus from a spiked mouse plasma sample of 2500 ng/mL. Bottom: extracted UPHILIC/MS/MS (solid line) and RPUPLC/MS/MS (dotted line) chromatograms of clofazimine from a spiked mouse plasma sample.

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identical mobile phases were used and a ballistic gradient chromatographic separation was employed as follows: 0 min (90% B1), 0.3 min (90% B1), 0.4 min (40% B1), 0.8 min (60% B1), 0.9 min (90% B1) and finished at 1 min at a flow rate of 1 mL/min. The retention times for the analyte and the ISTD were 0.31 and 0.58 min, respectively (Fig. 2). The eluant was introduced into the atmospheric pressure ionization (API) interfaces prior to the mass spectrometer. The total run cycle times for both RPLC and HILIC methods were around 1.5 min.

A 4000 Q TRAP mass spectrometer (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) with both vendor-supplied ESI and APCI sources operated in positive ion mode was employed for the measurement of all test compounds. For the Q TRAP mass spectrometer, 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 ammonia adducts and 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 analyte and the ISTD, respectively. The MS/MS transitions selected to monitor everolimus and clofazimine used their ammonia adducts ($[M+NH_4^+]$) and protonated molecules ($[M+H]^+$) ions as precursors at *m/z* 975.8, 473 and the product ions at *m/z* 908.6, 431, respectively (Fig. 1).

For the matrix effect studies, a mixture of everolimus and clofazimine solution was continuously infused into the 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 (2 μL) was injected into the analytical column. Effluent from the analytical columns mixed with the infused compounds and entered the ESI interface.

Sample collection

The animal dosing experiments were carried out in accordance with the US 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.

Standard and sample preparation

Stock solutions of everolimus 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 10–5000 ng/mL level. Low, medium, and high quality control (QC) samples at 25, 250 and 2500 ng/mL were prepared from different lots of blank mouse plasma for each run. The mouse plasma samples were prepared using the protein precipitation technique. A 200-μL aliquot of an 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). A 2-μL aliquot of the extract was injected by the ACQUITY autosampler to either the UPHILIC/MS/MS or the RPUPLC/MS/MS system for quantitative analysis.

RESULTS AND DISCUSSION

UPHILIC/MS/MS method development

Due to their inherent selectivity, chromatographic techniques coupled to mass spectrometric systems normally require neither labor-intensive sample preparation procedures nor extensive chromatographic run times for pharmaceutical assays in complex biological fluids. RPLC is commonly employed as a generic chromatographic technique for most weak polar, neutral and nonpolar small drug molecules. For example, everolimus, an immunosuppressive agent, is a neutral molecule with a relatively high molecular weight (~1000 Da). For the determination of everolimus in various biological samples, several RPLC/MS/MS methods using phenyl-hexyl[9] and C_{18} columns[10–12] were developed. HILIC/MS/MS is emerging as a supplement to RPLC/MS/MS for the retention and the detection of highly hydrophilic, ionic and polar compounds.[13–16] In this work, our goal was to investigate the feasibility of extending the application range of UPHILIC/MS/MS to the analysis of a neutral pharmaceutical in biological samples.

It is of key importance to investigate the interaction mechanisms of the analytes under different chromatographic systems during method development to control their retentions. In general, 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. Figures 3 and 4 show the influence of acetonitrile ratio in the mobile phase on capacity factor (k') of the test compounds indicating that under organic solvent-rich conditions (water is less than 50%), water becomes an elution solvent on an underivatized silica column (3 μm). An increase in water content (10% to

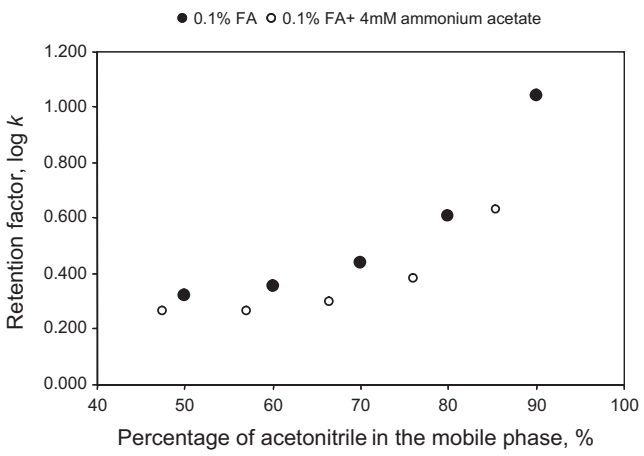

Figure 3. The effect of water ratios in the mobile phase on the retention factors of clofazimine with a 3 μm bare silica column.

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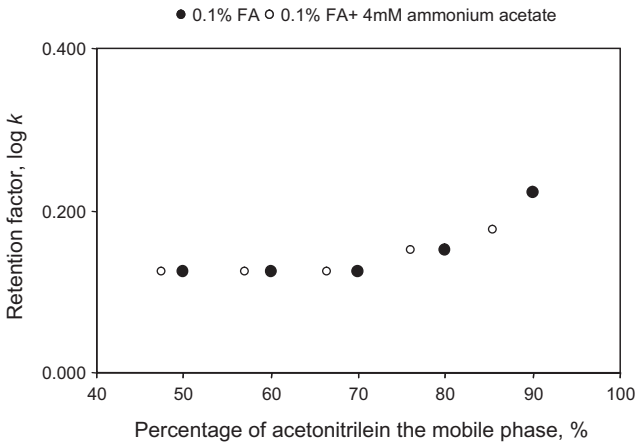

Figure 4. The effect of water ratios in the mobile phase on the retention factors of everolimus with a 3 μm bare silica column.

25%) significantly decreases the retention factors (ln *k*) of clofazimine and everolimus. As shown in Figs. 3 and 4, 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.[17] As indicated in Fig. 3, increasing ionic strength in the mobile phase by addition of certain amounts of ammonia acetate would weaken the dominant retention force leading to a decreased retention for clofazimine. However, ionic strength yields little influence on the retention for everolimus, as shown in Fig. 4. Figure 5 shows that the presence of water appeared to have stronger impact on the retention power for clofazimine than for everolimus which has greater hydrophobicity. For everolimus, the change in the respective retention time due to hydrophilic interaction is not substantial between 30% and 50% water in the mobile phase. According to the Van Deemter equation, reducing particle size of the stationary phase is one of the effective ways to directly enhance the column efficiency. For example, reducing the particle diameter from 5 μm to 1.7 μm

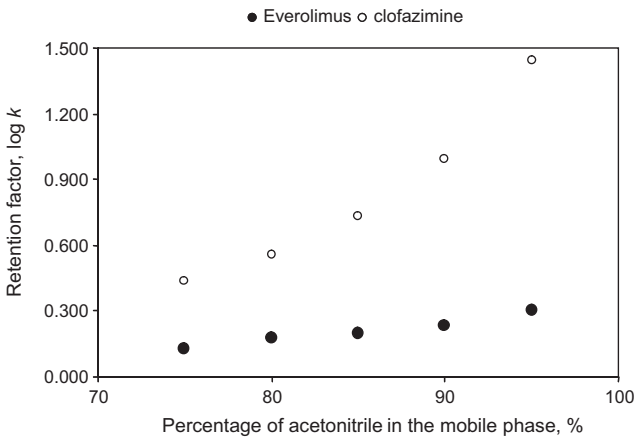

Figure 5. The effect of water ratios in the mobile phase on the retention factors of everolimus (open circles) and clofazimine (solid circles) with a 1.8 μm bare silica column.

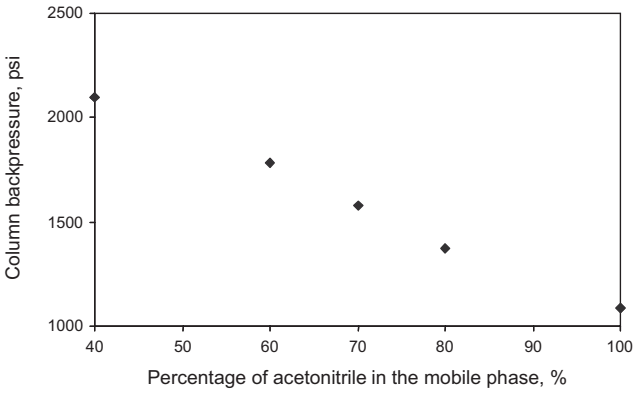

Figure 6. The column back-pressure of a 1.8 μm bare silica column as a function of acetonitrile concentrations in the mobile phase at a constant flow rate of 0.8 mL/min. The column temperature was set at 45°C.

will result in a 3-fold increase in column efficiency but 8.6-fold increase in the column back-pressure under the reversed-phase conditions. In UPHILIC, the use of organic-rich mobile phases providing low column back-pressure is advantageous to allow the use of sub 2-μm particle stationary phases under conventional HPLC operation conditions without the need for ultra-high-pressure equipment (as shown in Fig. 6).

Chromatographic performance is only part of the story during routine HPLC/MS/MS method development. The compositions of eluent may have a strong impact on the chromatographic performance but also the sensitivity of the analytes when hyphenating HPLC to various API sources.[18,19] ESI and APCI are the most popular API interfaces for the hyphenated-MS systems for qualitative or quantitative screening of small new chemical entities during lead optimization stage. In general, the greater organic contents in the mobile phase yield the higher ionization efficiencies for small molecules on both ESI and APCI. Figure 1(A) shows a full scan spectrum of everolimus in positive ion mode under the acetonitrile/water mobile phase without addition of ammonia acetate indicating that everolimus does not readily yield protonated ions, $[M+H]^+$, under ESI conditions (Fig. 1). The formation of the ammoniated ions, *m/z* 975.8, and the sodiated ions, *m/z* 980.8, is assumed due to the contribution of the ubiquitous sodium and ammonia molecules from solvents. It was reported that the addition of ammonium buffers to the mobile phase has been used to promote the formation of ammonium, $[M+NH_4]^+$ everolimus adducts[20] and the fragmentation of sodium $[M+Na]^+$ everolimus adducts for multiple reaction monitoring is poor compared with ammoniated everolimus.[21] Figure 7 reveals that the sensitivity of everolimus and clofazimine by ESI increased as the ratios of acetonitrile in the mobile phase increased from 50% to 90%. As indicated in Fig. 7(A), nearly 2-fold and 2.5-fold increases in the ESI responses of everolimus were observed as the acetonitrile ratios in the mobile phase increased from 50% to 90% with and without addition of ammonium acetate, respectively. As indicated in Fig. 7(B), 5-fold increases in the ESI responses of clofazimine were observed as the acetonitrile ratios in the mobile phase increased from 50% to 90%

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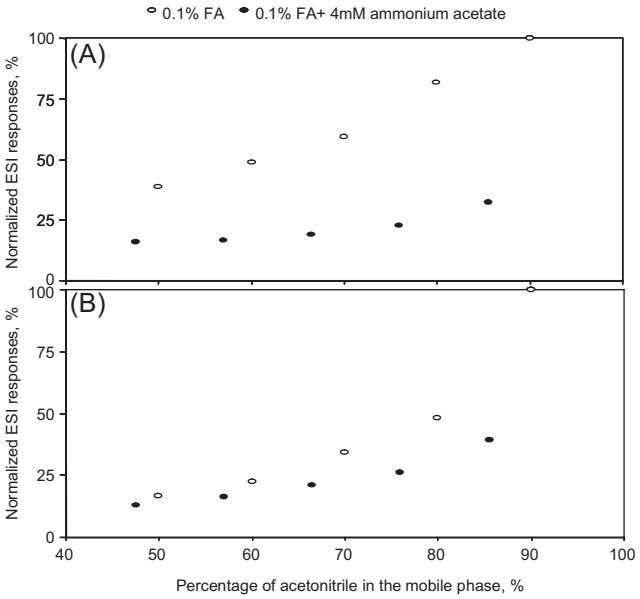

Figure 7. Normalized ESI responses of (A) everolimus (open symbols) and (B) clofazimine (solid symbols) as a function of acetonitrile concentrations in the mobile phase.

while 3-fold increases in the ESI responses of clofazimine were observed as the acetonitrile ratios in the mobile phase increased from 50% to 90% in the presence of 4 mM ammonium acetate. It was reported that high buffer concentrations might have deleterious effects in production of analyte adducts due to the formation of a solid residue or other factors and might cause background that interferes with detection of the analyte.[22,23] Figures 7(A) and 7(B) show a clear decrease in the responses of both everolimus and clofazimine by ESI with addition of 4 mM ammonia acetate to the acetonitrile/water mobile phase containing 0.1% FA. The impact of ionization suppression on the formation of the sodium $[M + Na]^+$ everolimus adducts due to the presence of excessive ammonium acetate additives in the mobile phase used in this study was found to be stronger than that for the ammoniated ions under the same experimental conditions (data not shown). As compared with RPLC, HILIC mode is advantageous where the analytes are introduced into the API sources under higher organic environments providing a better detection limit for small molecules.

Matrix ionization suppression studies

A well-recognized concern about assay reliability for any new LC/MS-based methods is the potential of matrix ionization suppression.[24,25] In order to observe the matrix ionization suppression effects using the UPHILIC/MS/MS technique on plasma protein precipitation extracts from blank mouse plasma samples, we monitored the variability of the ESI responses for everolimus and the ISTD using the post-column infusion scheme.[25] 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. The results reveal that little matrix effect was observed within the chromatographic window of all test compounds (data not shown).

Mouse plasma assays

Utilization of a carbon-rich column is frequently employed for HPLC/MS/MS method development for rapid screening of new chemical entities and their metabolites in biological samples. RPLC/MS/MS-based techniques using an ESI source in the positive ion mode were reported for the determination of everolimus in biological fluids.[9–12] In this work, UPLC using a sub-2 μm particulate C18 column was first employed to provide sufficient chromatographic resolution to avoid the possible mass spectrometric interferences and to allow times for everolimus analyses within 1 min. The sample preparation procedure involved a one-step protein precipitation procedure. The analyte and the ISTD are eluted by a ballistic gradient separation with increasing the acetonitrile contents in the mobile phase. The representative extracted RPUPLC/MS/MS chromatograms of everolimus and clofazimine are shown in Fig. 2. The retention times of the analyte and the ISTD were found to be reproducible throughout the experiment.

In this work, our primary goals were to evaluate the feasibility of developing an UPHILIC/MS/MS method without altering the mobile phases used for the RPUPLC/MS/MS method for the determination of everolimus in plasma samples following direct injection of supernatants after protein removal. In UPHILIC, as the opposite of RPUPLC, the strongest mobile phase has a high percentage of water. For gradient UPHILIC separations by increasing the aqueous component in the mobile phase, the initial mobile phase has a high percentage of organic solvents. Due to low column back-pressure generated with organic-rich mobile phases (as indicated in Fig. 6), a sub-2 μm particulate HILIC column can be operated with regular HPLC pumps at flow rates comparable with those used in UPLC. Similar to RPLC, most HILIC applications using bare silica columns employ gradient elutions. Figure 2 shows the extracted UPHILIC/MS/MS chromatograms for everolimus and clofazimine using ESI. The analyte was eluted by a ballistic gradient separation on a 1.8 μm underivatized silica column with increasing the water contents in the mobile phase within 1 min. The retention times and peak shape, as shown in Fig. 2 for the analyte and the ISTD in both standard plasma and study plasma samples, were found to be reproducible during the course of the study.

On the basis of tandem mass spectrometric methods, the probability of encountering endogenous interferences for the compounds with relatively high molecular weight (>700 Da) is reduced. For everolimus and the ISTD, there was no endogenous peak from six different batches of blank mouse plasma observed under both RPUPLC and UPHILIC experimental conditions. The calibration curves for everolimus using ESI 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). The interday assay precision in the measurement of the spiked low, medium, and high QC samples obtained for everolimus was within 15% and accuracy ranged from 89% to 110%. APCI did not provide comparable sensitivity as

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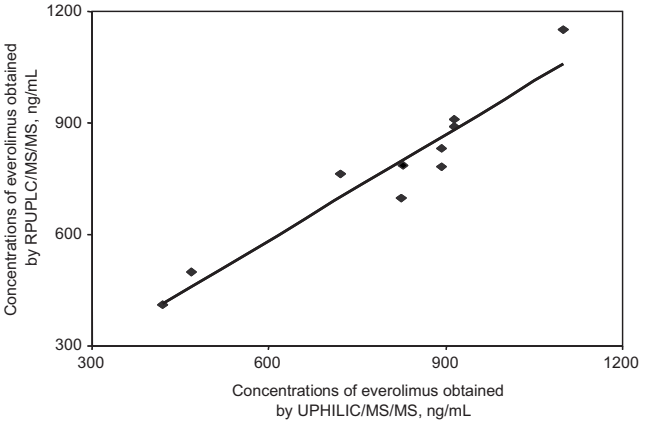

Figure 8. Correlation of the concentrations of everolimus in the study mouse plasma samples obtained by the RPUPLC/MS/MS and UPHILIC/MS/MS methods.

compared with ESI for the detection of everolimus. Accuracy (% bias) was less than 15% at all concentrations. To demonstrate the realistic suitability of analyses, the analytical results for the determination of everolimus in mouse plasma samples obtained by the proposed UPHILIC/MS/MS method were compared with those obtained by the RPUPLC/MS/MS method without altering the mobile phases, mass spectrometric conditions or sample preparation procedure. Figure 8 compares the values in terms of mouse plasma concentrations of everolimus calculated by the response ratios of analytes over the ISTD obtained by the UPHILIC/MS/MS and RPUPLC/MS/MS methods. The correlation coefficiency of the linearity curve and the slope of the regression line are calculated to be 0.958 and 0.966, respectively. Student's *t*-test results indicated no significant difference in plasma concentrations at each time-point of everolimus determined by the aforementioned methods with 95% confidence ($\alpha = 0.5$). These results concluded that the UPHILIC/MS/MS method proved as reliable as the RPUPLC/MS/MS method for the determination of everolimus in mouse plasma samples.

CONCLUSIONS

HILIC has been proven to be a useful analytical tool for the retention of small polar molecules but also could serve as an alternative technique to RPLC for quantitative determination of neutral pharmaceuticals in biological fluids. The capability of using bare silica HILIC stationary phases for the retention and separation of everolimus was demonstrated. A sensitive UPHILIC/MS/MS assay using ESI in positive ion mode has been successfully developed and evaluated for the simultaneous determination of everolimus in mouse plasma. It is feasible and convenient to convert RPUPLC/MS/MS into UPHILIC/MS/MS by simply changing the analytical column without altering the mobile phases for everolimus analyses with an enhanced sensitivity. Both UPHILIC/MS/MS and RPUPLC/MS/MS methods in conjunction with a simple sample treatment procedure showed equivalent accuracy to the analytical results and have been demonstrated to be reliable in support of an *in vivo* pharmacodynamic study.

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