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Comparison of fast liquid chromatography/tandem mass spectrometric methods for simultaneous determination of cladribine and clofarabine in mouse plasma

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Abstract

Several fast high performance liquid chromatography/atmospheric pressure ionization/tandem mass spectrometric (HPLC-API/MS/MS) methods were evaluated for the simultaneous determination of cladribine and clofarabine in mouse plasma samples. The chemical separation for analytes under reversed-phase conditions were achieved by using either ultra-performance liquid chromatography (UPLC) or micro-column HPLC coupled to either a quadrupole linear ion trap mass spectrometer (QTrap MS) or a triple quadrupole mass spectrometer. Atmospheric pressure chemical ionization (APCI) or atmospheric pressure photoionization (APPI) interfaces in the positive mode were employed prior to mass spectrometric detection. The effects of various dopant solvents on the APPI sensitivities of analytes and the internal standard were investigated. The matrix ionization suppression potential for the test compounds in plasma samples on fast HPLC-MS/MS methods was examined by a post-column infusion technique. In this work, these proposed approaches were successfully employed to determine the concentrations of cladribine and clofarabine in mouse plasma in the low ng/ml region. The mouse plasma levels of all analytes obtained by these fast HPLC-MS/MS methods were compared and found to be well correlated in terms of analytical accuracy.

Keywords: Ultra performance liquid chromatography; Atmospheric pressure chemical ionization; Photoionization; Cladribine; Clofarabine

1. Introduction

Due to its inherent selectivity and sensitivity, liquid chromatography coupled to tandem mass spectrometry offers higher sample throughput for pharmaceutical analysis as compared with the traditional HPLC methods with other detectors [1–7]. Therefore, the HPLC–MS/MS systems have become the instrument of choice for drug assays in the modern pharmaceutical industry. In order to keep pace with the speed of drug discovery, we are continuously challenged to find faster ways of delivering quality analytical results across a range of project-driven demands. A number of approaches such as direct plasma injection [5–7], and fast chromatography [8–13] are integrated to HPLC–MS/MS systems in this laboratory to increase sample throughput for the determination of small molecules in biological samples. In this work, the performance of fast chromatography approaches using micro-columns packed with 3 μm and 1.7 μm materials for the simultaneous determination of two anti-cancer agents, cladribine [14] and clofarabine [15], in mouse plasma was investigated. Cladribine and clofarabine were chosen randomly as the test compounds. Separation of the analytes from endogenous compounds which might suppress the ionization efficiency was successfully achieved on the reversed phase conditions using a short gradient with run times less than 2 min.

The column effluent was directly connected either to the atmospheric pressure chemical ionization (APCI) source or the atmospheric pressure photoionization (APPI) source as part of the tandem mass spectrometer (MS/MS) system. The impact of dopant solvents and flow rates on the APPI ionization efficiencies of the test compounds was explored. Simultaneous selective reaction monitoring (SRM) of multi-analytes and the internal standard were used for the quantitative determination of the analytes. The matrix effect of ionization suppression in mouse plasma samples for quantitative fast micro-column HPLC–MS/MS and UPLC–MS/MS analyses was investigated

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using a post-column infusion technique. Furthermore, the assay accuracy was demonstrated by a direct comparison of the mouse plasma concentrations of cladribine and clofarabine obtained by these proposed fast hyphenated-MS methods. The performances of the proposed method in terms of chromatographic resolution, matrix ionization suppression and sample throughputs were evaluated using both drug-treated and spiked plasma samples.

2. Experimental

2.1. Reagents and chemicals

Cladribine and clofarabine as the analytes, (Fig. 1) as well as ketoconazole as the internal standards (ISTD), were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA) and ammonium acetate (99.999%) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Deionized water was generated from 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 were purchased from Bioreclamation Inc. (Hicksville, NY, USA). The chemical structures of all analytes were reported elsewhere [14–15].

2.2. Equipment

Micro-column HPLC–MS/MS analysis was performed using an Applied Biosystems/MDS Sciex (Ontario, Canada) Model API 5000 triple quadrupole mass spectrometer equipped with either APCI or APPI interfaces. The chromatographic system consisted of a Leap autosampler with a refrigerated sample compartment (set to 10 °C) from LEAP Technologies (Carrboro, NC), Shimadzu on-line degasser, LC-10AD VP pump and LC-10A VP controller (Columbia, MD, USA). A Gemini C18 column (2.0 mm × 30 mm, 3 μm) from Phenomenex (Torrance, CA) was used as the analytical column. The UPLC–APCI/MS/MS system consisted of a Waters Acquity system, and an Acquity C18 column (50 mm × 2.1 mm, 1.7 μm) coupled to an Applied Biosystems/MDS Sciex (Ontario, Canada) Model API 4000 hybrid quadrupole/linear ion trap mass spectrometer and equipped with the APCI source.

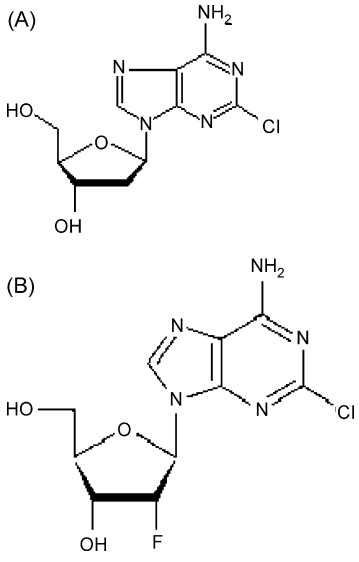

Fig. 1. Chemical structures of cladribine and clofarabine.

The Quadra 96 (Tomtec, Hamden, CT) system was used for semi-automated sample preparation via the protein precipitation procedure. As a standard procedure to investigate the matrix ionization suppression with plasma samples, a mixture of cladribine, clofarabine and ketoconazole 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 plasma samples or methanol (5 μl) was injected into the analytical column. Effluent from the analytical columns mixed with the infused compounds and then entered the ionization 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 oral administration to individual mice at certain doses and chilled on crushed ice. Plasma was then separated by centrifugation and stored frozen (−20 °C) until analysis.

2.4. Standard and sample preparation

Stock solutions of the analytes 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 2.5–500 ng/ml level. The mouse plasma samples were prepared using the protein precipitation technique. A 150-μl aliquot of an acetonitrile solution containing 1000 ng/ml of internal standard was added to 50 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. For the comparison of assay accuracy, 10-μl aliquots of the extract were injected by the Leap autosampler to the proposed micro-column HPLC–MS/MS and UPLC–MS/MS systems for quantitative analysis.

2.5. Chromatographic conditions

Mobile phase A consisted of water containing 4 mM ammonium acetate and 0.1% of formic acid and mobile phase B consisted of acetonitrile containing 4 mM ammonium acetate and 0.1% of formic acid. For micro-column HPLC method, gradient chromatographic separation using mobile phases A and B at a constant flow rate of 1.2 ml/min was as follows: 0 min (5% B), 0.2 min (5% B), 1 min (15% B), 1.3 min (100% B), 1.8 min (100% B), 1.9 min (5% B), and finished at 2 min. The retention times for cladribine, clofarabine and the ISTD with the micro-

column HPLC method were 0.78 min, 0.88 min and 1.24 min, respectively. For the UPLC method, gradient chromatographic separation using mobile phases A and B at constant flow rate of 0.8 ml/min was as follows: 0 min (5% B), 0.2 min (5% B), 0.5 min (95% B), 0.8 min (95% B), 0.9 min (5% B), and finished at 1 min. The retention times for cladribine, clofarabine and the ISTD with the UPLC method were 0.63 min, 0.63 min and 0.66 min, respectively.

2.6. Mass spectrometric conditions

The mass spectrometer was operated in the positive ion mode. The experimental mass spectrometric conditions were determined using generic state files without optimization for ionization sources. The heated pneumatic nebulizer probe conditions were as follows: 450 °C temperature setting, 80 psi nebulizing gas pressure, 1.0 l/min auxiliary gas flow, 0.9 l/min curtain gas flow-rate. The APPI system comprised of a heated nebulizer to vaporize the sample prior to inducing ionization, a power supply for the krypton lamp for photoionization, a nitrogen supply for cooling the lamp and a HPLC pump for dopant delivery at a constant flow rate of 0.2 ml/min. Cladribine, clofarabine and the ISTD were monitored using the transitions from *m/z* 286 → *m/z* 170, *m/z* 304 → *m/z* 170 and *m/z* 531 → *m/z* 489, respectively. The protonated molecules were fragmented by collision-activated dissociation with nitrogen as collision gas at a pressure of instrument setting 6. The collision offset-voltage was set at 35 V and 50 V for the analytes and the ISTD, respectively.

3. Results and discussion

3.1. Development of fast gradient LC–MS methods

Faster chromatographic separation for determination of multiple components in biological samples is a common goal in the pharmaceutical analysis area. In general, our strategies for achieving rapid chromatography include: the utilization of shorter columns with small particles size and inner diameter [8], using a monolithic column with the reduced column backpressure [13] and using the elevated column temperature chromatography with the reduced viscosity of the mobile phases [12]. According to the van Deemter equation, one effective way to improve the HPLC column efficiency and analysis time is to reduce the particle size. Decreasing in particle size causes a linear improvement in separation efficiency per unit column length. However, the pressure required to perform such an exchange is proportional to the particle diameter cubed. Therefore, the enhanced column efficiency gained from using small particles is accompanied with a tremendous increase in the column pressure which is prohibitive for traditional HPLC hardware to pressures below 6000 psi. This allows users to adopt a shorter (less than 5 cm length) narrow-bore (~2 mm i.d.) column with small particles operated at higher than optimal flow rates to provide for fast chromatographic separations while still maintaining satisfactory chromatographic resolution [8]. Microcolumn technique offers analytical chemists several attractive benefits over standard analytical columns including reduction of solvent consumption and reduced solvent waste disposal. So far, we have successfully assayed more than fifty thousand in vivo and in vitro samples containing more than three thousand different new chemical entities and their metabolites on micro-column HPLC–MS/MS systems showing reproducible peak shapes and the retention times. The assay accuracy obtained by the micro-column HPLC–MS/MS method compares well to standard (4 mm ID) column HPLC–MS/MS methods for the quantitative analysis of drug candidates in plasma samples. These analytical results demonstrated that the micro-column HPLC–MS/MS methods were equivalent to the standard HPLC–MS/MS methods in terms of analytical accuracy but provided results four times faster in terms of sample throughput for the HPLC–MS/MS assay [8].

UPLC was pioneered in the late 1990s by the Jorgenson and Lee research group [16,17]. UPLC utilizes sub-2 μm particles at higher pressures (up to 100,000 psi) than traditional HPLC instruments. It was demonstrated that the use of analytical columns packed with sub-2 μm particles at high flow rates allows a higher sample throughput without severe effect on column efficiency. Recently, a commercial instrument capable of handling pressures up to 15,000 psi combined with columns packed with 1.7 μm particles in the form of the Waters® ACQUITY UPLC system has become available. In this work, our goal was to develop a UPLC–MS/MS method and to compare quantitative screen-type micro-column HPLC–MS/MS methods for simultaneously monitoring cladribine and clofarabine in mouse plasma samples. Fig. 2 shows the micro-column HPLC–APCI/MS/MS chromatograms of cladribine, clofarabine and the ISTD with 2 min run time. The plate heights for cladribine, clofarabine and the ISTD by micro-column HPLC were calculated as 7.1, 8.2 and 5.9 μm, respectively. Fig. 3 shows the UPLC–APPI/MS/MS chromatograms of cladribine, clofarabine and the ISTD with 1 min run time where cladribine and clofarabine required stronger organic mobile phase for elution as compared to micro-column HPLC method with the same mobile phases. The plate heights for cladribine, clofarabine and the ISTD by UPLC method were calculated as 1.8, 1.8 and 1.4 μm, respectively. The micro-column HPLC method yielded weaker column efficiency for cladribine, clofarabine and the ISTD than UPLC method under the reversed-phase conditions. Also, the observed signals for all analytes by the UPLC method were found to be two times greater than those by micro-column method using the APCI source (data not shown).

For the APPI source, ionization is mainly based on charge and proton transfer to the analytes from the protonated dopant molecules that have been ionized by the 10 eV photons produced by a vacuum-ultraviolet lamp. It was reported that the use of dopant solvents and dopant flow affected the APPI ionization efficiency of the analytes [18–20]. Toluene and acetone are the most common dopant solvents for the APPI source [21]. Recently, the possibility of using tetrahydrofuran (THF), a nontoxic, and inexpensive chemical with a photoionization energy less than 10 eV, as a dopant solvent was demonstrated [22]. As indicated in Fig. 3, the introduction of dopant solvent at a constant flow rate of 0.2 ml/min had shown the most impact on the photoionization efficiency of all test compounds. These findings indicate that the proton transfer reaction is one of the rate-

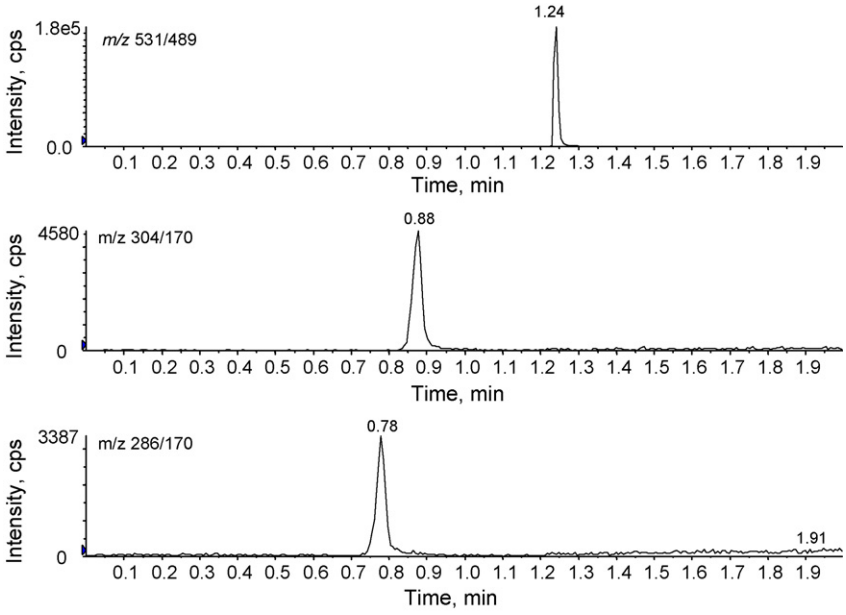

Fig. 2. HPLC–APCI/MS/MS chromatograms of (top row) the internal standard, (middle row) clofarabine, and (bottom row) cladribine in the spiked standard mouse plasma at a concentration of 10 ng/ml.

limiting steps in ionization process and the presence of dopant is essential for photoionization detection which is similar to previous report [18]. The relative APPI responses of cladribine, clofarabine and the ISTD with addition of various dopant solvent were measured by the changes in their chromatographic peaks under the same HPLC condition. Fig. 4 shows that toluene yields the better sensitivity for the analytes and the ISTD than acetone or THF. Regarding the effect of dopant flow rate, Fig. 5 shows that raising dopant flow rates up to 0.6 ml/min increase the APPI responses for all analytes and the ISTD. These results are consistent with earlier experiments with different test articles [18].

Sufficient chromatographic resolution in the quantitative determination of the dosed compound in biological samples using HPLC–MS/MS is recommended to avoid possible interferences from drug-related biotransformation products [8]. Gradient elution is our first choice for HPLC conditions because it provides better chromatographic resolution of mixtures and loading capacity than isocratic elution, although it requires longer analysis time for column re-equilibration. In this work, we employed a ballistic gradient approach to eliminate interferences from the salts such as sulfates and phosphates and polar nonvolatile compounds in plasma extracts, which are likely to be contributors to ionization suppression.

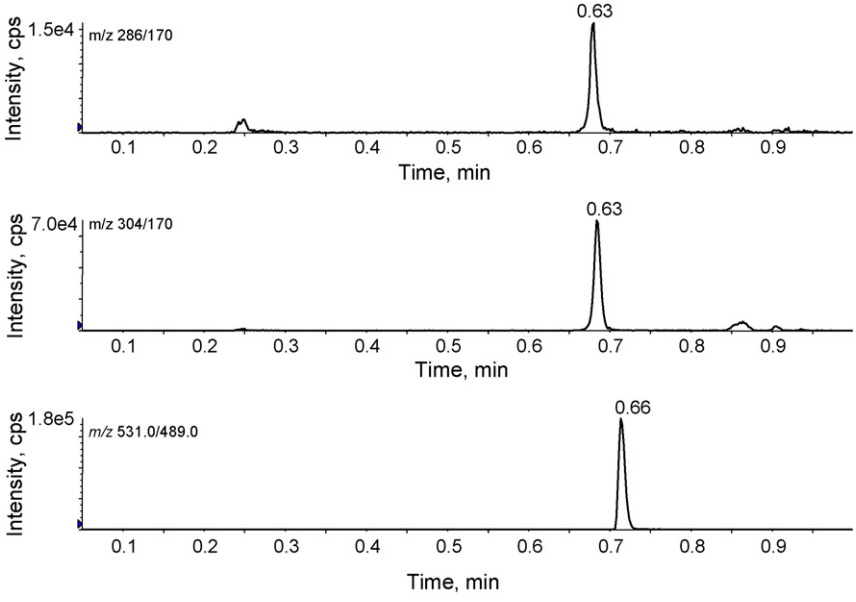

Fig. 3. UPLC–APCI/MS/MS chromatograms of (top panel) cladribine, (middle panel) clofarabine and (bottom panel) the internal standard in the spiked standard mouse plasma at a concentration of 25 ng/ml.

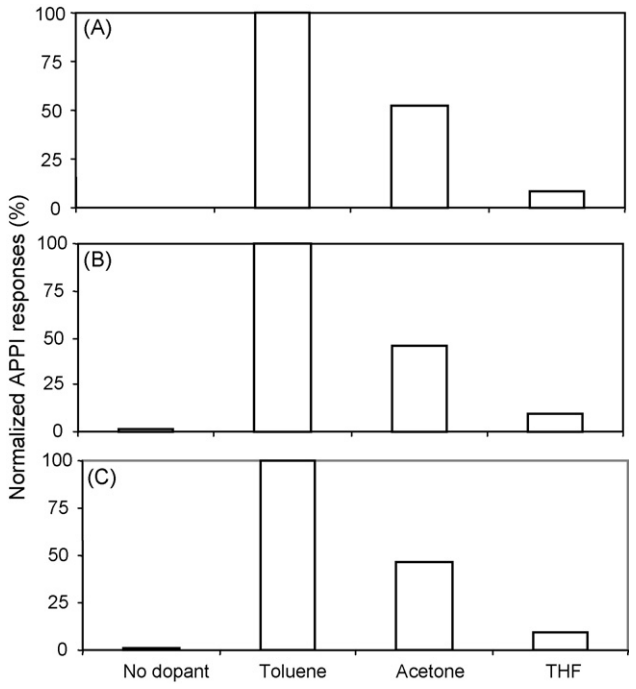

Fig. 4. Normalized APPI responses of (A) clofarabine, (B) cladribine and (C) the internal standard as a function of dopant solvents used.

3.2. Matrix ionization suppression studies

Although matrix ionization suppression is considered to be more likely a problem when using the protein precipitation method for sample preparation [23–25], the protein precipitation method has been chosen as the major sample preparation procedure for LC–MS/MS assays in our laboratory due to its simplicity. In order to observe the matrix ionization suppression effects using HPLC–MS/MS techniques on plasma protein precipitation extracts from plasma samples, we monitored the variability of the ion responses for the test compound using the post-column infusion scheme. Any changes in the ion responses of the infused compounds were assumed to be due to matrix ionization suppression caused by the sample-related materials eluting from the HPLC columns. The differences in the infusion chromatograms between the mobile phase injection and the plasma extract injection were considered to be caused by ionization suppression due to plasma sample extract constituents eluting from the column. For accurate quantitative determination, it is strongly recommended that the retention times of all analytes should be in the region of 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 assay time window yielding little or no ionization suppression when using various HPLC–MS/MS systems. Some degree of matrix effects for cladribine and clofarabine within a chromatographic window between 0 and 0.2 min were observed for the micro-column HPLC–APCI/MS/MS and UPLC–APCI/MS/MS methods (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.

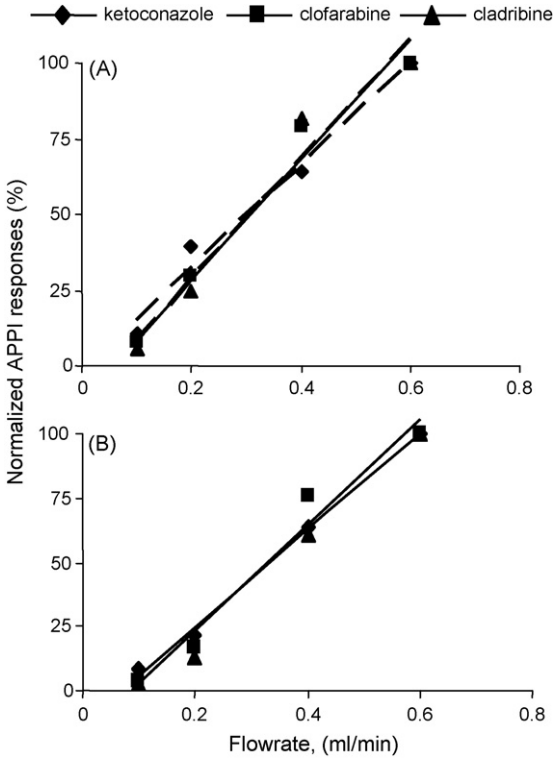

Fig. 5. Normalized APPI responses of clofarabine, cladribine and ketoconazole as a function of dopant flow rates using (A) acetone and (B) THF.

3.3. Analysis of mouse plasma samples

As an example, all proposed fast HPLC–MS/MS methods were applied for the simultaneous determination of the dosed compounds in mouse plasma to demonstrate the realistic suitability of fast analyses. Fig. 2 shows the micro-column HPLC–APCI/MS/MS chromatograms for the cladribine, clofarabine and the ISTD from a spiked mouse plasma sample, respectively. Fig. 3 shows the UPLC–APCI/MS/MS chromatograms for the cladribine, clofarabine and the ISTD from a spiked mouse plasma sample, respectively. The retention times and peak areas for the ISTD observed in the spiked standard plasma and study plasma were found to be consistent through the experiments with all of the proposed fast LC–MS methods. The calibration curves for both cladribine and clofarabine obtained from duplicate standard mouse plasma samples at each concentrations 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, from 2.5 to 500 ng/ml. The same mouse plasma standard and study samples were independently analyzed using all proposed assay methods for the analytes.

Fig. 6 compares the values in terms of mouse plasma concentrations of both cladribine and clofarabine calculated by the response ratios of analytes over ketoconazole obtained by the UPLC–APCI/MS/MS and micro-column HPLC–APPI/MS/MS methods. The student's *t*-test results indicated no significant difference of plasma concentrations at each time point of both analytes determined by the aforementioned methods with

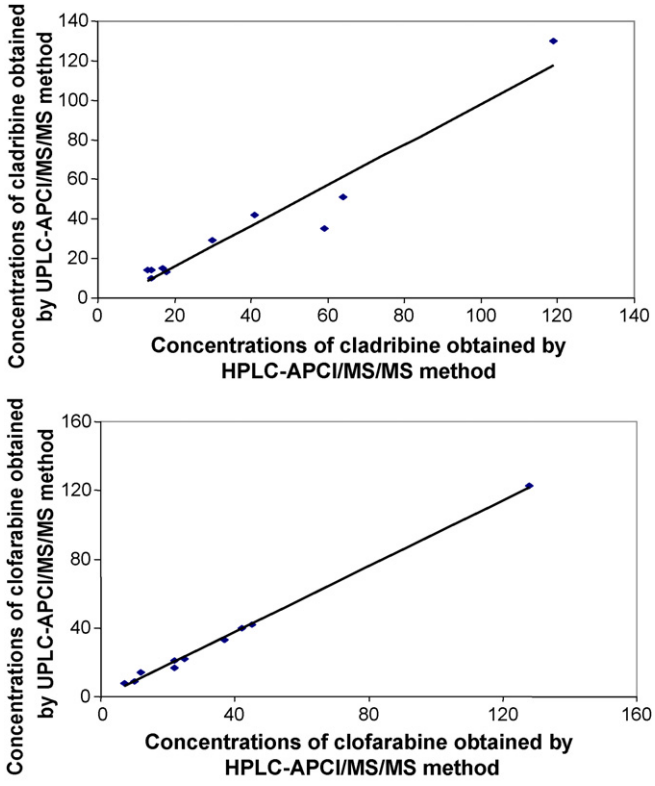

Fig. 6. Correlation of analytical results of (top panel) cladribine and (bottom panel) clofarabine in mouse plasma samples obtained by UPLC–APCI/MS/MS and micro-column HPLC–APCI/MS/MS methods.

95% confidence ($\alpha = 0.5$). Also, the results of mouse plasma concentrations of cladribine and clofarabine obtained by the micro-column HPLC–MS/MS method using either the APCI or the APPI sources were also found to be comparable (data not shown). These results concluded that the UPLC–MS/MS method provided better sensitivity and column efficiency than the micro-column HPLC–MS/MS methods for the determination of cladribine and clofarabine in mouse plasma samples. The mouse plasma concentrations of both analytes obtained by UPLC–MS/MS method and micro-column HPLC–MS/MS method were highly correlated in terms of accuracy.

4. Conclusions

Micro-column HPLC–MS/MS and UPLC–MS/MS methods were developed for the simultaneous determination of cladribine and clofarabine in mouse plasma samples. After gradient elution program, run cycle times of all analytes were achieved in less than 2 min, providing narrow peaks with good peak symmetry. The proposed UPLC–MS/MS method have been proven to be sensitive, cost-effective and reliable for high throughput PK screening and showed equivalent accuracy to the analytical results obtained using the micro-column HPLC–MS/MS method routinely employed in this laboratory. It was also demonstrated that under the UPLC conditions we are able to achieve shorter chromatographic run time and greater separation power while still avoiding a matrix ion suppression problem.

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