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Comparison of Atmospheric Pressure Chemical Ionization, Electrospray Ionization, and Atmospheric Pressure Photoionization for the Determination of Cyclosporin A in Rat Plasma

Ganfeng Wang, Yunsheng Hsieh,* and Walter A. Korfmacher

Drug Metabolism and Pharmacokinetics Department, Schering-Plough Research Institute, Kenilworth, New Jersey 07033

Atmospheric pressure chemical ionization was compared with electrospray ionization and atmospheric pressure photoionization (APPI) as an interface of high-performance liquid chromatography (HPLC)−tandem mass spectrometry (MS/MS) for the determination of cyclosporin A (CsA) in biological fluids in support of in vivo pharmacodynamic studies. These ion sources were investigated in terms of their suitability and sensitivity for the detection of CsA. The effects of the eluent flow rate and composition as well as the nebulizer temperatures on the photoionization efficiency of CsA in the positive ion mode under normal-phase HPLC conditions were explored. The ionization mechanism in the APPI environment with and without the use of the dopant was studied using two test compounds and a few solvent systems employed for normal-phase chromatography. The test compounds were observed to be ionized mainly by proton transfer with the self-protonated solvent molecules produced through photon irradiation. Furthermore, ion suppression due to sample matrix interference in the normal-phase HPLC-APPI-MS/MS system was monitored by the postcolumn infusion technique. The applicability of these proposed HPLC-API-MS/MS approaches for the determination of CsA at low nanogram per milliliter levels in rat plasma was examined. These proposed methods were then compared with respect to specificity, linearity, detection limit, and accuracy.

Bioanalytical method development is an essential element in the process of finding new drugs for people. For example, most of the pharmacokinetic (PK) parameters such as mean residence time, volume of distribution, and bioavailability are strictly derived from the concentrations of the drug in plasma samples collected at specific time points following an oral or intravenous administration. Due to the use of combinatorial chemistry and high-throughput parallel organic synthesis, today's pharmaceutical industry has continued to increase the number of new chemical entities tested in the discovery arena each year. Consequently, there continues to be a demand for testing new leads for their drug metabolism and PK properties. Therefore, the capability of establishing rapid, sensitive, and reliable bioanalytical assays for the determination of drug components at a nanogram per milliliter concentration range or lower from a small number of biological samples is an important step in new drug discovery research.[1]

Atmospheric pressure ionization (API) coupled with a tandem mass spectrometer has replaced older HPLC-MS interface techniques such as thermospray, particle beam, and continuous-flow fast atom bombardment as the method of choice for small-molecule determination owing to its robustness and sensitivity.[2] API includes all ionization techniques where the ions are formed at atmospheric pressure, such as pneumatic-assisted sonic spray ionization (SSI),[3] electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI).[4] ESI and APCI are the most common API sources for qualitative or quantitative HPLC-MS/MS analyses of ionic, polar, and neutral substances. The majority of drug candidates can be readily ionized by both ionization methods and further optimized for better ionization efficiency by adjusting the eluent composition,[5,6] mass spectrometric parameters, and ion source conditions. For comprehensive screening,[7−8] there is a need for alternative ionization systems such as coupling electrochemistry (EC),[9] the APPI interface[10] or the SSI interface[11] to MS to extend the application range of HPLC-API-MS/MS to nonpolar molecules that are not readily amenable to either ESI or APCI.

Previously, we demonstrated the potential of using dopant-assisted APPI as an alternative ionization technique to APCI and showed its applicability to the quantitative analyses of drug

* To whom correspondence should be addressed. Tel: 908-740585. E-mail: yunsheng.hsieh@spcorp.com.

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Table 1. Major Experimental Operation Conditions for Different Analytical Methods

	APCI (A)	APCI (B)	ESI	APPI (A)	APPI (B)
chromatographic mode	reversed-phase	reversed-phase	reversed-phase	reversed-phase	normal phase
elution mode	gradient	gradient	gradient	gradient	isocratic
probe temperature (°C)	450	450	450	450	420
limit of detection (ng/mL)	1	2.5	0.25	2.5	2.5
spray voltage (kV)	5	5	5	1.3	1.3
transition (m/z)	1203 → 1203	1220 → 1203	1220 → 1203	1203 → 1185	1203 → 1185
ion mode	positive	positive	positive	positive	positive
HPLC flow rates (mL/min)	1.0	1.0	1.0−0.35	1.0−0.35	0.5
concentration range (ng/mL)	5−1000	5−1000	5−1000	5−1000	5−1000
regression coefficient (r^2)	0.995	0.998	0.995	0.996	0.999
collision energy (eV)	30	20	20	55	55

discovery compounds.[7] In this work, we compare the performance of different ionization methods, ESI, APCI, and APPI for the determination of cyclosporin A (CsA), the calcineurin inhibitor,[12−17] in rat plasma samples in either reversed-phase or normal-phase chromatography modes. The ionization efficiency of CsA was found to be ~1 order of magnitude higher with the addition of dopant solvent when assayed using reversed-phase conditions. However, the effect of the dopant on the ionization efficiency of CsA was found to be marginal under normal-phase conditions. Therefore, the ionization mechanism for CsA was investigated in more detail with APPI in order to understand this observation by comparing a few solvent systems used for normal-phase chromatography. Several factors such as the composition of mobile phase and the eluent flow rate were also explored using the flow injection analysis (FIA) technique. Matrix ionization suppression is a common concern when any new HPLC-API-MS/MS method is developed. In this work, the postcolumn infusion technique was adapted for the study of the matrix effects on the normal-phase HPLC-APPI-MS/MS system. The suitability of all API techniques for the determination of CsA was further confirmed through correlation of rat PK results obtained by APCI, ESI, and APPI methods in terms of analytical accuracy.

EXPERIMENTAL METHODS

Reagents and Chemicals. Lonafarnib, a drug candidate for antitumor therapy,[18] was produced by Schering-Plough Research Institute. Acetonitrile, ethanol, isooctane, and toluene (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). CsA, ammonium acetate (99.999%), and ethyl alcohol-D (99.5+ atom % D) were purchase from Sigma-Aldrich Chemical Co., Inc. (St. Louis, MO). Deionized water was generated from a Milli-Q water purifying system purchased from Millipore Corp. (Bedford, MA), and house high-purity nitrogen (99.999%) was used. Drug-free rat plasma samples were purchased from Bioreclamation Inc. (Hicksville, NY). For reversed-phase chromatography, mobile phases A and B consisted of water/acetonitrile (90:10) and (10:90) supplemented with ammonium acetate solution containing 0.02% acetic acid to achieve a final concentration of 4 mM, respectively. For normal-phase separation, mobile-phase composition was isocratic isooctane (80%)/ethanol (20%).

Equipment. HPLC-API-MS/MS analysis was performed using a PE Sciex (Concord, ON, Canada) model API 3000 triple quadrupole mass spectrometer equipped with heated nebulizer (APCI), Turboionspray (ESI), or PhotoSpray (APPI) probes. The APPI system is composed 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 syringe pump for dopant delivery. The toluene solution used as a dopant was continuously introduced into the heated nebulizer through a fused-silica capillary at 20 μL/min for the determination of CsA when the reversed-phase chromatographic conditions were employed. The major mass spectrometric parameters for all analytical approaches used in this work are summarized in Table 1.

The HPLC system consisted of a Leap autosampler with a refrigerated sample compartment (set to 10 °C) from LEAP Technologies (Carrboro, NC), a Shimadzu on-line degasser, LC-10AD VP pump, and LC-10A VP controller (Columbia, MD). A Synergi Luna CN column (2.0 × 30 mm, 5 μm) from Phenomenex Inc (Torrance, CA) was used as the analytical column for the reversed-phase mode. A Hypersil Silica (50 × 3.0 mm, 5 μm) from Thermo-Hypersil-Keystone (Bellefonte, PA) was used as the analytical column for normal-phase mode. The Quadra 96 (Tomtec, Hamden, CT) system was used for semiautomated sample preparation with the protein precipitation method.

A schematic diagram of the postcolumn infusion system for the matrix effect studies in rat plasma was shown previously.[7] CsA and lonafarnib dissolved in mobile-phase solvent, used for normal-phase chromatography, were continuously infused into PEEK tubing between the silica column and mass spectrometer through a tee using a Harvard Apparatus model 2400 (South Natick, MA) syringe pump. Either a reconstituted extract of blank rat plasma or mobile phase (10 μL) (as a reference signal) was injected into silica column for comparison of ionization responses. Effluent from the silica column mixed with the infused compounds and then entered APPI interface.

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Standard and Sample Preparation. Stock solutions of CsA and lonafarnib were prepared as 1 mg/mL solutions in methanol. Standard solutions were prepared by serial dilution in methanol/water and isooctane for reversed-phase and normal-phase separation, respectively. Analytical standard samples for rat PK studies were prepared by spiking known quantities of the standard solutions to blank plasma from 5 to 1000 ng/mL levels.

The rat plasma samples were processed using a protein precipitation technique. For the reversed-phase chromatography, 150 μL of acetonitrile solution containing 1 ng/μL lonafarnib was added to 50 μL of the study plasma samples and standard plasma samples in a 96-well plate. After vortexing and centrifugation, the supernatant was transferred to a new 96-well plate using the Tomtec Quadra 96 system. Aliquots of 10 μL of supernatant were then injected onto the CN column. For the normal-phase chromatography, the supernatant was further evaporated to dryness and the dried residues were reconstituted in 200 μL of mobile phase. Aliquots of 10 μL of extract were injected onto a silica column prior to mass spectrometric detection.

To quantify plasma CsA concentrations, blood was collected from individual rats at certain time points following an oral administration at a dose of 30 (group 3) and 50 mg/kg (group 4). CsA concentrations in rat plasma samples were measured using various proposed HPLC-API-MS/MS assays. Study plasma samples were found to be above the quantitation limit for CsA for all the bioanalytical approaches.

Chromatographic Conditions. For reversed-phase chromatography, separation was achieved using a two-solvent gradient system with mobile phases A and B. For the APCI interface, a ballistic gradient from 10 to 100% mobile phase B was run over 0.3 min, held for 0.7 min, and reequilibrated to 10% B over 0.1 min at a constant flow rate of 1.0 mL/min. For both ESI and APPI interfaces, the flow programming technique was employed for better sensitivity; for this work, the flow rate was reduced to 0.35 from 1 mL/min at 100% mobile phase B. The run cycle times were less than 2.5 min under the reversed-phase HPLC conditions. For the normal-phase chromatography, the reconstituted samples were injected onto the silica column and eluted using an isocratic method. The chromatographic modes for individual ionization sources are given in Table 1. The effluent from the HPLC systems was connected directly to the mass spectrometer without splitting.

Mass Spectrometric Conditions. The tandem mass spectrometer was operated in positive ion mode. The ion spray voltage for photospray probe was set at 1.3 kV. The rest of the mass spectrometric parameters for both APCI and APPI interfaces were set to be identical. The MS/MS reactions and the collision offset voltage for all interfaces selected to monitor CsA depending on the stability of the precursor ions are shown in Table 1. The MS/MS reaction selected to monitor lonafarnib was the transition from m/z 639, the $[M + H]^+$ ion, to a product ion at m/z 471. The protonated molecules were fragmented by collision-activated dissociation with nitrogen as the collision gas at a pressure of instrument setting 5. Data were acquired and calculated using Analyst 1.1 software (PE Sciex). Solvent ion spectra were recorded using a focusing potential of 50 V and a declustering potential of 0 V at a probe temperature of 350 °C and a total flow rate of 40 μL/min. The scan range was m/z 40−200 (1 s/scan).

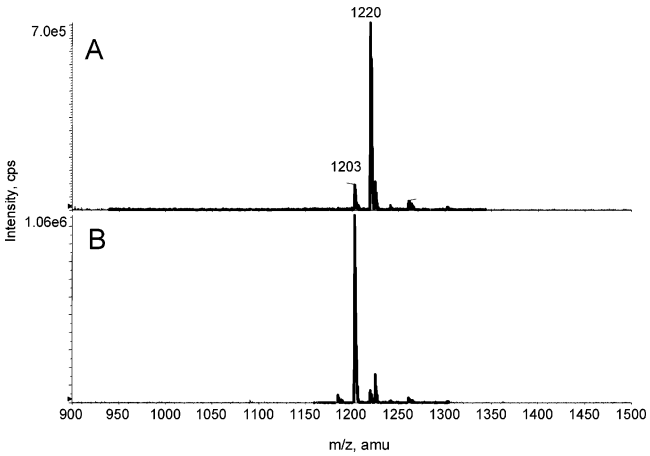

Figure 1. Mass spectra of CsA at (A) high and (B) low declustering potential conditions.

RESULTS AND DISCUSSION

Development of HPLC-API-MS/MS Methods. HPLC-API-MS/MS systems combined with sample extraction via the protein precipitation technique are the standard procedure used to qualitatively and quantitatively determine drug components in biological samples in our laboratory.[19−23] Although the ESI source covers a wider application range, the APCI source is frequently employed as an ionization interface for PK screening of new chemical entities at the lead identification stage. The ESI and APPI sources are available as alternative interfaces when the ionization efficiencies of the test compounds with the APCI are poor. The calcineurin inhibitor, CsA, is a potent immunosuppressive agent.[12−15] To obtain a pharmacokinetic/pharmacodynamic relationship between the amount of the dosed drug and its activity, routine therapeutic measurements for the exposure of CsA in biological fluids are required. In general, it is often a greater analytical challenge to develop quantitative methods for compounds with a molecular mass above 1000 Da using HPLC-API-MS/MS systems than for those with a molecular mass in the 300−800 Da range. In this work, we have described several rapid, reliable, and sensitive assay methods for monitoring CsA in rat plasma samples.

The chemical structure of CsA was shown elsewhere.[16] Typical mass spectra of CsA in the m/z 900−1500 range are shown in Figure 1 in the presence of ammonium ions. The intensity of the ammonium adduct ion, $[M + NH_4]^+$, of CsA at m/z 1220, was found to be either more or less abundant than that of the protonated molecule, $[M + H]^+$, at m/z 1203, depending on the selected ion source declustering potential. By increasing the declustering potential the ammonium adduct ion was reduced relative to the protonated molecule as shown in Figure 1B. These ammonium adduct ions were found to be readily fragmented to the protonated molecule, $[M + H]^+$, as shown in Figure 2A. At

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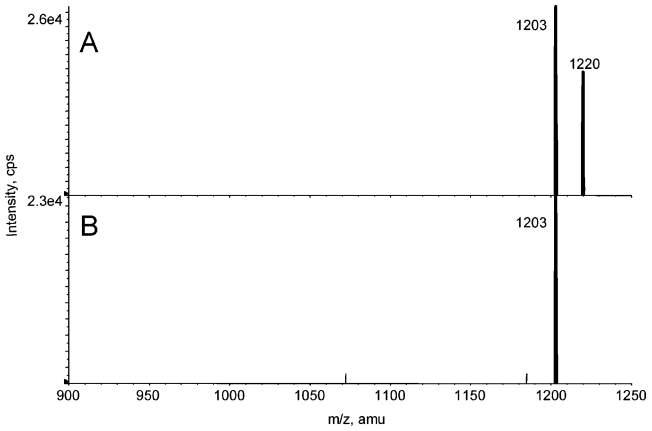

Figure 2. Product ion spectra of CsA of (A) ammonium adduct ion and (B) the protonated molecule with a collision energy of 20 and 30 eV, respectively.

collision energy below 35 eV, the protonated CsA molecule was resistant to fragmentation by the collision-induced dissociation mechanism as shown in Figure 2B. Several product ions of the protonated CsA molecule, including $[MH - 18]^+$, become observable at collision energy higher than 50 eV (data not shown). For comparison, three transitions ($[M + NH_4]^+$ to $[M + H]^+$, $[M + H]^+$ to $[M + H]^+$, or $[M + H]^+$ to $[MH - 18]^+$) were chosen for the quantitative determination of CsA as given in Table 1. Of these transitions, $[M + H]^+$ to $[MH - 18]^+$ provided the least signal but the lowest background noise for CsA measurement. Although the $[M + H]^+$ to $[M + H]^+$ transition offered the maximum response, it gave a poorer assay selectivity. To provide the maximum responses, the mass spectrometer was then tuned on the ammonium adduct ion, $[M + NH_4]^+$, or the protonated molecule, $[M + H]^+$, depending upon the selected transition ranges.

The detection sensitivity for analytes with all API interfaces under reversed-phase conditions is well recognized to be a function of many experimental factors such as solvent composition, flow rate, and probe temperature.[7,19] However, much less is known regarding the effects of experimental conditions on the APPI source when performed under normal-phase conditions.[24] In this work, the FIA technique was employed to study the influence of normal-phase conditions on the photoionization efficiency using a mixture of CsA and lonafarnib in solution. The effects of the ethanol/isooctane ratios on the abundance of the peak areas of the extracted ion chromatograms of CsA and lonafarnib are shown in Figure 3. Figure 3 indicates that the photoionization efficiency for the test compounds changed minimally as the content of ethanol increased up to 30%. The influences of the probe temperatures and mobile-phase flow rate on the photoionization efficiency of the test compounds in the reversed-phase conditions were observed to be similar to those obtained in the normal-phase conditions. The relation of solvent eluent flow rate versus the photoionization responses of CsA and lonafarnib can be seen in Figure 4. As indicated in Figure 4, the relative sensitivities of both test compounds were reduced gradually as the flow rate increased from 0.2 to 0.5 mL/min. This could be due to less effective heat-

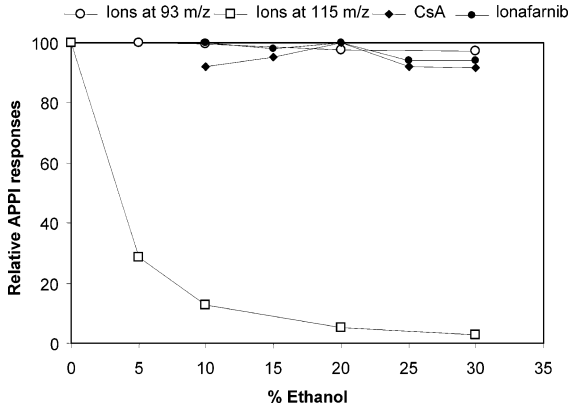

Figure 3. Relative APPI responses of CsA, lonafarnib, ion at *m*/*z* 93 and ion at *m*/*z* 115 as a function of the ethanol/isooctane ratios of the solvent.

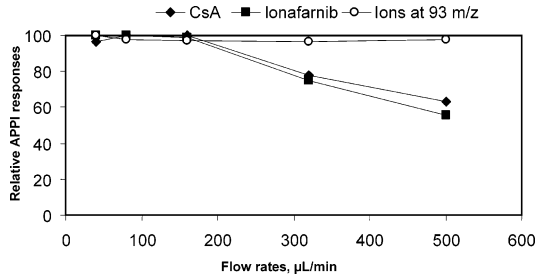

Figure 4. Effects of eluent flow rates on photoionization efficiencies of CsA, lonafarnib, and ion at *m*/*z* 93.

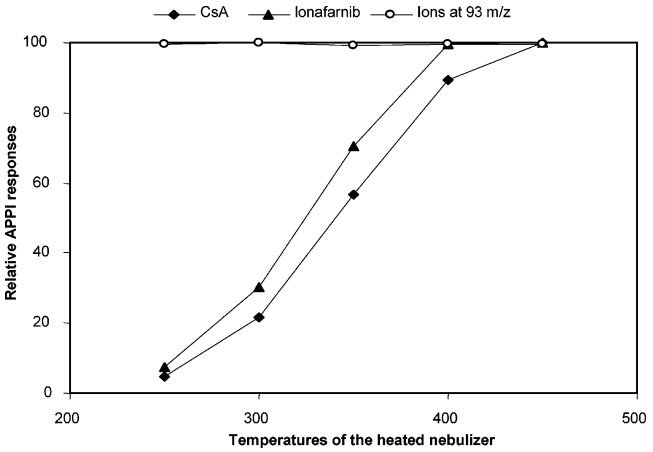

Figure 5. Effects of heated nebulizer temperatures on photoionization efficiencies of CsA, lonafarnib, and ion at *m*/*z* 93.

transfer processes as the solvent flow rate increases. Figure 5 indicates that the APPI responses of both test compounds increased linearly as the probe temperature increased. This phenomenon was assumed to be likely due to incomplete vaporization at lower probe temperatures. It is well known that the evaporation process to remove the solvent and to leave the ionized analytes in the gas phase plays an important role in all API interfaces. Interestingly, we also observed that the production of the protonated CsA and lonafarnib molecules in the normal-phase HPLC-APPI system was found to be unchanged with or without the addition of toluene as the dopant as demonstrated in Figure 6; therefore, the introduction of dopant solvent made no appreciable impact on the photoionization efficiency of either compound under normal-phase chromatography.

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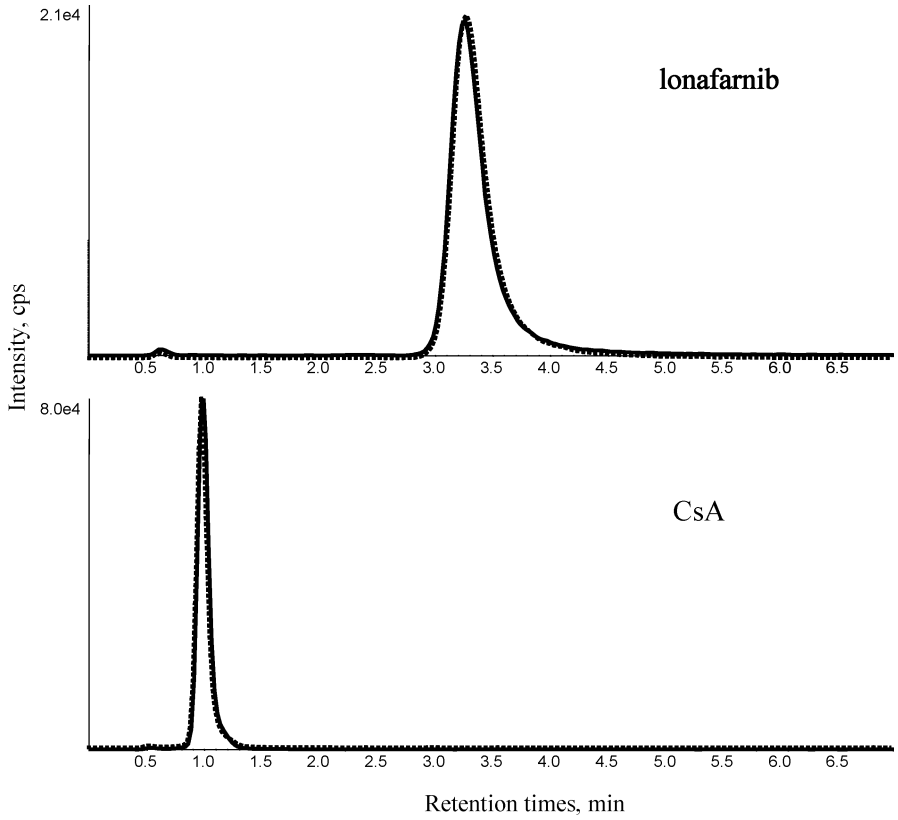

Figure 6. Normal-phase HPLC-APPI-MS/MS chromatograms of CsA and lonafarnib with (solid line) and without (dot line) the use of a dopant.

Ionization Mechanism in Normal-Phase Chromatographic Mode. To improve APPI performance, a better understanding of fundamental processes of its ionization mechanism is always desirable. For an HPLC-APPI-MS/MS system, the HPLC eluent is first vaporized and subjected to photoionization prior to mass spectrometric detection. Ionization is mainly based on either charge or 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.[7] For reversed-phase chromatography, the ionization potentials (IP) of most common solvents are greater than the photon energy emitted from the krypton discharge lamp (water, IP = 12.6 eV; methanol, IP =10.8 eV; acetonitrile, IP = 12.2 eV). In principle, the photon energy should ionize only the drug molecules and dopant (toluene, IP = 8.83 eV) molecules resulting in the formation of dopant photoions. These abundant photoions react in turn with vaporized solvent molecules through collision in the gas-phase, producing an intermediate protonated charged cluster. The production of analytical ions is by the proton-transfer process if the proton affinity of the analytes is higher than that of solvent molecule.[13−14] These findings indicate that the proton-transfer reaction is one of the rate-limiting steps in the ionization process and the presence of the dopant is essential for maximum photoionization detection with the typical mobile-phase solvents used for reversed-phase chromatography. Therefore, the use of dopant frequently plays a significant role in the APPI process.

The formation of major reactant ions recorded from the mass spectra of different solvent systems commonly used in the normal-phase chromatography is shown in Table 2. These data should provide a fundamental evidence for proposing ionization mechanisms in APPI. The APPI mass spectrum of toluene indicated the production of the radical cation of toluene ($C_7H_8^{\bullet+}$, m/z 92) which is in a good agreement with a previous report.[25] The APPI mass spectrum of isooctane without the combination of toluene suggests the existence of protonated isooctane ($[C_8H_{18} + H]^+$, m/z 115) in the gas phase. The protonated isooctane molecules are proposed to be mainly generated through the protonated conversion reaction between neutral isooctane molecules and the radical cation of isooctane ($C_8H_{18}^{\bullet+} + C_8H_{18} \rightarrow [C_8H_{18} + H]^+ + [C_8H_{18} - H]$). Here, the formation of radical cations of isooctane in the gas phase is expected due to the lower ionization potential of isooctane (IP = 9.89 eV[25]) compared to the average photon energy (10 eV). In the presence of toluene, both the radical cation of toluene and the protonated isooctane molecules coexisted in the gas phase as shown in Table 2. This indicated that the radical cation of toluene might not be involved with the formation of the radical cations of isooctane in the system. Further analyses were performed on isooctane mixed with ethanol. For the combined solvents containing ethanol, the intensity of the ethanol−dimer cluster ion, $[(C_2H_5OH)_2 + H]^+$, m/z 93] became dominant over the protonated isooctane molecules, $[C_8H_{18} + H]^+$. In the gas phase, the proton-transfer reaction occurs when the proton affinity (PA) of the neutral solvent molecules is greater than that of the donors. The formation of the ethanol−dimer cluster ions indicated that the ethanol−dimer cluster has a higher PA than the radical cations of isooctane. For the mixture of ethanol and toluene, the proton transfer between $C_8H_{18}^{\bullet+}$ and the ethanol molecule is not expected because the PA of the ethanol (776.4 kJ/mol) is less

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Table 2. Key Ions in the Positive Ion APPI Spectra of Different Solvents[a]

	m/z (relative abundance)		
solvents	$C_7H_8^{\bullet+}$	S_nH^+	other ions
toluene	92 (70)		95 (88), 96 (71), 101 (17), 106 (97), 107 (60), 108 (100), 109 (100), 111 (80) 112 (57), 120 (45), 123 (35)
isooctane		115 (100)	57 (68), 59 (31), 83 (35), 89 (37), 97 (100), 98 (90),106 (24), 107 (49), 109 (28), 111 (90), 112 (62), 129 (27), 135 (22)
isooctane + toluene	92 (9)	115 (69)	57 (20), 59 (12), 83 (53), 89 (37), 97 (100), 98 (92), 101 (20), 106 (46), 107 (26), 109 (89), 111 (50), 112 (44), 123 (11), 129 (17), 135 (12)
ethanol + isooctane		93 (100) $[2\times46+H]^+$	97 (25), 98 (30), 107 (88), 112 (21), 121 (21)
ethanol + toluene		93 (100) $[2\times46+H]^+$	107 (96), 108 (15), 121 (24)
ethanol-D + isooctane		93 (80) $[2\times46+H]^+$	103 (50), 124 (62), 125 (62), 126 (37), 151 (38), 152 (60), 153 (53), 175 (36)
		94 (100) $[1\times46+1\times47+H]^+$	
		95 (52) $[2\times47+H]^+$	

[a] Mass spectra were recorded by using a declustering potential of 0 V and a focusing potential of 50 V. 46 = CH_5OH; 92 = $C_7H_8^{\bullet+}$; 115 = $[C_8H_{18}+H]^+$. The values in parentheses give the relative abundance (%).

than that of the benzyl radical, $C_7H_7^{\bullet}$ (831.4 kJ/mol).[26] As indicated previously, the proton transfer between $C_7H_8^{\bullet+}$ and the methanol molecule is only thermodynamically possible to the methanol clusters that have PAs greater than that of the monomer.[25] Similar to the ionization pattern of methanol, the formation of ethanol−dimer cluster was only observed in the system containing either toluene or isooctane solvents. This suggests that the ethanol dimer has a PA significantly higher than that of the benzyl radical, the radical cations of isooctane, and the individual monomer. The proton-transfer reaction is further confirmed through a ^2H-labeling experiment by using a single deuterium-labeled ethanol solvent where the formations of $[(C_2H_5OH)_2 + H)^+]$ (*m/z* 93), $[((C_2H_5OH)(C_2H_5OD) + H)^+]$ (*m/z* 94), and $[(C_2H_5OD)_2 + H]^+$ (*m/z* 95) mixtures were observed in the APPI mass spectrum as shown in Table 2. These data suggest that the production of analytical ions is primarily through the proton-transfer process with the protonated ethanol−dimer molecules. Therefore, isooctane can serve as the mobile phase and also the dopant to assist photoionization efficiency in the normal-phase HPLC-APPI-MS/MS systems. This phenomenon is considered as the so-called self-doping effect. This explains why the APPI ionization efficiencies of both CsA and lonafarnib remained the same with or without the addition of toluene solvent as the dopant. This indicated that the major reactant ions in dopant-assisted APPI system are the protonated isooctane molecule and ethanol-dimer clusters.

The APPI ionization efficiency between the analytes and the protonated solvent molecules as a function of normal-phase chromatographic conditions was studied. The APPI responses of both CsA and lonafarnib in the ethanol−isooctane system were explored by recording the MS abundance (total ions per unit time) of $[(C_2H_5OH)_2 + H^+]$ (*m/z* 93) and $[C_8H_{18} + H]^+$ (*m/z* 115) at various probe temperatures and solvent compositions. The relative APPI responses of the protonated isooctane molecule and ethanol−dimer cluster as a function of the ratio of ethanol in the isooctane solution is given in Figure 3. As shown in Figure 3, the relative abundance of the protonated ethanol−dimer clusters remained consistent while that of the protonated isooctane molecules decreased with a reduction in the isooctane content. The decrease in the abundance of the protonated isooctane molecules had no impact on the ionization efficiencies of either CsA or lonafarnib. This indicated that the protonated ethanol−dimer cluster ions might predominate the APPI ionization process under the normal-phase system. The signals of the protonated ethanol−dimer clusters were not dependent on the eluent flow rate as shown in Figure 4. However, the APPI responses of both CsA and lonafarnib began to decrease at flow rates higher than 150 μL/min. The decrease in detection sensitivity for analytes when increasing the eluent flow rate can be explained due to poorer effective heat-transfer processes resulting in incomplete evaporation of analytes in solvent. This phenomenon was further confirmed with the results of the probe temperature effect on the ionization efficiency of the tested compounds as illustrated in Figure 4.

Matrix Effects on Normal-Phase HPLC-APPI-MS/MS. A well-understood concern about assay reliability when developing HPLC-APIMS/MS methods is the possibility of encountering matrix ionization suppression problems that strongly affect the accuracy and reproducibility of the analytical results and is often referred to as the matrix effect.[22] In general, the matrix effect is dependent on ionization sources, sample preparation and treatment, analytes, and chromatographic conditions.[22,27−29] The continuous postcolumn infusion experiments are the routine method for monitoring the ionization suppression for any new HPLC-API-MS/MS approaches developed in this laboratory.[20−21] The infusion normal-phase HPLC-APPI-MS/MS chromatograms of CsA and lonafarnib after either a 10-μL injection of mobile phase or rat plasma extract are given in Figure 7. The affected area of the chromatographic run was evaluated according to the differences in the infusion chromatograms between the mobile-phase injection and the rat plasma extract injection. The loss of APPI sensitivity was considered to be caused by the matrix ion suppression effects

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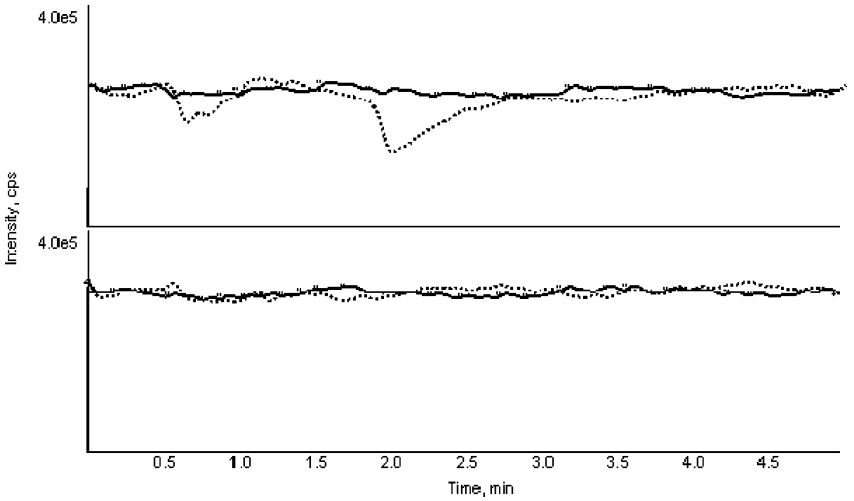

Figure 7. Infusion normal-phase HPLC-APPI-MS/MS chromatograms of CsA (top) and lonafarnib (bottom) following mobile phase (solid line) and blank plasma precipitation extract injections (dotted line). The region showing lower responses indicated the affected area of matrix ionization suppression.

due to plasma sample extract constituents eluting from the silica column. Figure 7 demonstrates that the loss of APPI response of CsA appeared at two chromatographic regions. However, for lonafarnib, the APPI responses with a given rat plasma protein precipitation extract showed no signal suppression during the entire run cycle time. For reliable quantitative determination, it is suggested that the retention times of all test compounds should not be located in the affected region of matrix ion suppression. The retention time of CsA under normal-phase chromatographic conditions (Figure 6) was located in the matrix ionization suppression-free region. The peak areas and the retention times for both CsA and lonafarnib in rat standard and study plasma samples were reproducible throughout the experiments.

Comparison of the HPLC-API-MS/MS Methods. Protein precipitation with acetonitrile gave clean chromatograms with no interfering peaks present using all transitions ranges under either the reversed-phase phase or normal-phase chromatographic modes. No interfering peaks were found throughout the study and standard plasma samples indicating good specificity of the methods. Calibration curves from individual transition ranges were constructed by plotting the CsA/internal standard peak-to-area ratios against CsA concentrations in rat plasma. These curves were linear with a good correlation over the working range as indicated in Table 1. CsA in rat plasma supernatant was stable at room temperature. All CsA levels could be detected at concentrations above 5 ng/mL in rat plasma using any of the five HPLC-API-MS/MS methods.

For the reversed-phase chromatographic separation, the retention times of CsA and lonafarnib varied within the ranges 1.5−2 and 1.4−2.0 min, respectively, resulting from differences in eluent flow rates. The HPLC-ESI-MS/MS method proved to be the most sensitive approach for measuring CsA in rat plasma with a limit of detection at 0.25 ng/mL (signal-to-noise ratio greater than 3). Parent-to-parent ion monitoring methods offered higher signals of CsA than parent-to-product transition methods but yielded less selectivity and greater background noise. In this work, all ionization sources were interfaced with a PE Sciex API 3000 triple quadrupole mass spectrometer. APPI has been reported to be better than APCI for compounds such as carbamazeoine, acridine, naphthalene,[7] dinitropyrene,[30] and benzbromarone[31] in the positive ion mode. In this study, no significant differences in the detection limits for CsA were observed between the APCI methods and the APPI methods using both reversed-phase and normal-phase modes. For normal-phase HPLC-APPI method, the introduction of dopant appeared to be not necessary to achieve optimum sensitivity.

Table 3. Concentrations of CsA in Rat Plasma Following Oral Administration Obtained by Various HPLC-API-MS/MS Approaches

	concentrations of CsA (ng/mL) analytical methods				
groups	APCI (A)	APCI (B)	ESI	APPI (A)	APPI (B)
A-1	297	298	322	300	313
A-2	203	202	226	206	234
A-3	339	339	351	353	337
B-1	479	468	483	506	458
B-2	277	285	291	297	305

The standard and study rat plasma samples were independently analyzed for CsA using the proposed methods as listed in Table 1. The concentrations of CsA at different time points measured by these proposed methods are summarized and plotted in Table 3 and Figure 8, respectively. The Student *t* test results indicated no significant difference among these values for CsA determined by individual assays with 95% confidence ($\alpha = 0.5$). The above results demonstrated that all of the proposed HPLC-API-MS/MS methods for the determination of CsA in rat plasma offered acceptable assay accuracy.

CONCLUSIONS

Five HPLC-API-MS/MS methods based on APCI, ESI, and APPI sources were developed for the analysis of CsA in rat plasma samples. The results presented in this work demonstrated that ESI, APCI, and APPI in combination with both reversed-phase

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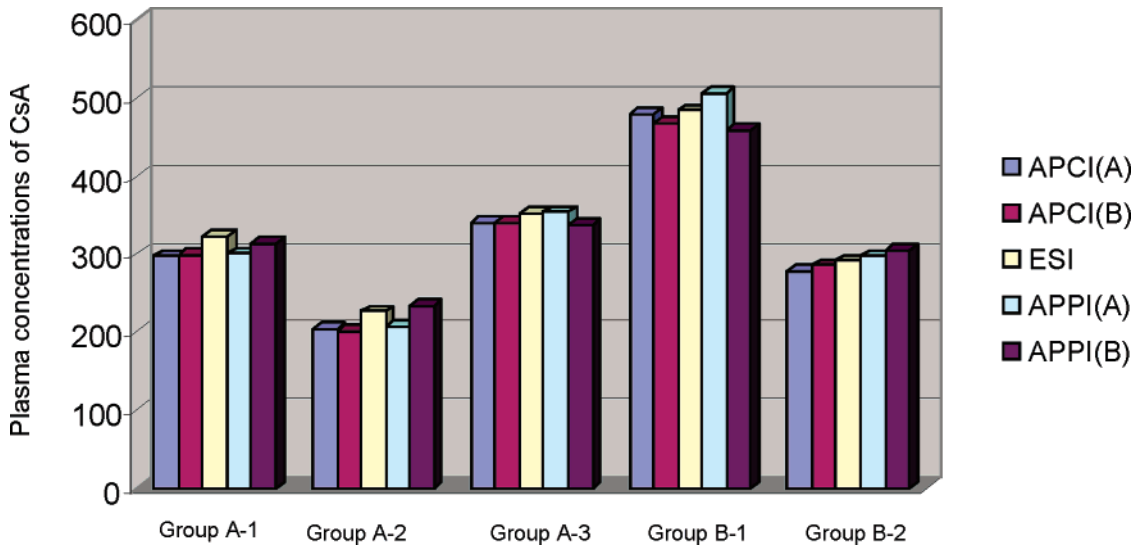

Figure 8. Plot of the CsA levels in rat plasma obtained by various HPLC-API-MS/MS approaches.

and normal-phase HPLC provide sensitive, rapid tools for high-throughput analysis of drug compounds such as CsA as a model compound in biological fluids. Normal-phase HPLC conditions amenable to the APPI interface are able to provide a comparable sensitivity for the determination of CsA over the reversed-phase HPLC mode. The coupling of normal-phase HPLC to the APPI interface showed good potential for maximum ionization efficiency of analytes without assistance from additional dopant molecules. The quantitative performance of the normal-phase HPLC-APPI-MS/MS method has been compared to a series of four reversed-phase HPLC-API-MS/MS methods and found to be equivalent in analytical accuracy.

The eluent composition, flow rate, and probe temperature have a significant effect on the ionization efficiency of CsA using all three interfaces and mobile phases. The use of dopant under normal-phase chromatographic conditions was not an essential element to achieve good ionization efficiency in the positive ion mode. In APPI, the photoionization of the mobile phases used for normal-phase separation is responsible for initiating the reactions leading to the ionization of analytes by proton transfer if the PA of an analyte is higher than that of the protonated ethanol−dimer cluster. The ionization suppression observed in the proposed normal-phase HPLC-APPI-MS/MS system was found to be dependent on the compound of interest.

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