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Supercritical fluid chromatography/tandem mass spectrometric method for analysis of pharmaceutical compounds in metabolic stability samples

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Abstract

Packed-column supercritical fluid chromatography (pSFC) coupled to an atmospheric pressure chemical ionization (APCI) source and a tandem mass spectrometer (MS/MS) for rapid and simultaneous determination of clozapine, ondansetron, tolbutamide and primidone in in vitro samples was developed in support of metabolic stability experiments. The effects of the eluent flow-rate and composition as well as the nebulizer temperatures on the ionization efficiency of the analytes in positive ion mode under normal phase pSFC conditions were studied. The metabolic stability of the test drug components through microsomal incubation by the proposed pSFC–APCI/MS/MS approaches requiring approximately 1 min per samples were evaluated with respect to specificity, durability and accuracy. These metabolic stability results obtained by pSFC–MS/MS methods are in a good agreement with those obtained by fast high-performance liquid chromatography/tandem mass spectrometry (HPLC–MS/MS) method.

Keywords: Supercritical fluid chromatography; Tandem mass spectrometry; Pharmaceuticals; Metabolic stability study

Both combinatorial chemistry and parallel synthesis provide a valuable means for production of large numbers and diversity of molecular architectures available for evaluation of various drug-like properties. Regardless of lead optimization or lead selection stages, one requirement throughout all processes is bioanalytical support. It is always desirable to develop high throughput methodologies for pharmacokinetic screens with a series of drug leads. Conventionally, most bioanalytical methods for drug assays were developed using HPLC coupled with UV or fluorescent detection. However, there is a trend toward interfacing various separation technologies with more sensitive tandem mass spectrometry (MS/MS)-based systems [1–5]. Normally, the MS/MS detection offers the complete resolution of the parent compounds from their first pass metabolites to avoid extra efforts for separation and sample clean-up procedures resulting in shorter run times.

In this work, we investigate the analytical potential of using the higher throughput pSFC–APCI/MS/MS method as a complimentary coverage to HPLC–APCI/MS/MS method for the quantitation of drug molecules in metabolic stability samples. Two kinds of supercritical fluid packed-columns were adapted for separation of analytes prior to tandem mass spectrometric detection. Several factors such as the compositions of mobile phase, eluent flow-rate used for the normal phase mode, which might affect both the chromatographic performances and ionization efficiencies of the test components, were explored. The time courses for the determination of the clearance of four pharmaceuticals (clozapine, ondansetron, tolbutamide and primidone) in rat microsomes were studied. The suitability of the proposed pSFC–APCI/MS/MS assays for the simultaneous determination of the test compounds in in vitro samples was evaluated

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by comparing the analytical results with those obtained by fast HPLC–APCI/MS/MS method.

1. Experimental

1.1. Reagents and materials

Carbon dioxide (SFC size) was obtained from Airgas (Cheshire, CT, USA). Methanol, and isopropylamine (IPA) (HPLC grade) were purchased from Sigma–Aldrich Chemical Company, Inc. (Saint Louis, MO, USA). Clozapine, ondansetron, tolbutamide and primidone were purchased from Sigma–Aldrich Chemical Company, Inc. and used as standard materials. Ketoconazole and trihydroxylflavanone were purchased from Sigma–Aldrich Chemical Company, Inc. and used as internal standards (ISTD). Formic acid (ACS >88%) was purchased from ACROS Organics (Morris, NJ, 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. Liver microsomes from untreated male Sprague–Dawley rats were purchased from In Vitro Technologies (Baltimore, MD). β-Nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (β-NADPH) and all other chemicals (reagent grade) were from Sigma–Aldrich.

1.2. Equipment and instrumentation

SFC was performed on a Berger Instruments (Newark, DE, USA) equipped with a SFC pump (pump A), a modifier pump (pump B) and a column oven (Fig. 1). Tandem mass spectrometric detection was performed using a PE Sciex (Concord, Ont., Canada) Model API 4000 triple quadrupole mass spectrometer equipped with a heated nebulizer (APCI) probe. The pSFC–APCI/MS/MS system consisted of a Leap autosampler with a refrigerated sample compartment (set to 10 °C) from LEAP Technologies (Carrboro, NC, USA). The pSFC columns were obtained from Princeton Chromatography (Cranbury, NJ, USA). For the separation of clozapine and ondansetron, a Diol column (250 mm × 4.6 mm, 5 μm) was used. For the separation of tolbutamide and primidone, a Cyano column (150 mm × 4.6 mm, 5 μm) was used. All pSFC columns were maintained at 45 °C. The effluent from the pSFC systems was connected directly to the mass spectrometer without splitting and make-up flow. Pumps A and B were used for neat carbon dioxide and modifier, respectively. Outlet pressure was regulated to 100 bar by a pressure-regulator. The amount of modifier that was added into the carbon dioxide mobile phase was expressed in volume.

HPLC–MS/MS analysis was performed using a PE Sciex Model API 3000 triple quadrupole mass spectrometer equipped with a heated nebulizer interface. The HPLC system consisted of a Leap autosampler from LEAP Technologies, Shimadzu on-line degasser, LC-10AD VP pump and LC-10A VP controller (Columbia, MD, USA). For the fast gradient method, a Synergi C18 column (2.0 mm × 30 mm, 4 μ) from Phenomenex, Inc. (Torrance, CA, USA) was used as the analytical column for all analytes and internal standards as described previously [6,7]. Chromatographic separation was achieved using mobile phases A and B. Mobile phases A and B are 0.1% formic acid in water and 0.1% formic acid in methanol, respectively.

1.3. Microsomal stability

Substrates (tolbutamide, clozapine, primidone or ondansetron) were dissolved in methanol and diluted such that the final concentration in the incubations was 1 μM and 1% methanol. Reactions were performed in duplicate in 1.5 ml microtubes in a final volume of 200 μl in a buffer containing 50 mM Tris–acetic acid, pH 7.4, 150 mM potassium chloride and 1 mg/ml rat liver microsomal protein. The tubes were warmed at 37 °C for 5 min and the reactions were started by the addition of 1 mM β-NADPH. The reactions were terminated at various times by the addition of 400 μl of ice-cold methanol containing an internal standard. The tubes were stored on ice for 10 min and then centrifuged at 16,000 × *g*. The supernatant was transferred to a new 96-well injection plate. The plates were refrigerated at 4 °C prior to analysis. Aliquots of 10 μl were injected for both pSFC–APCI/MS/MS and HPLC–APCI/MS/MS assays.

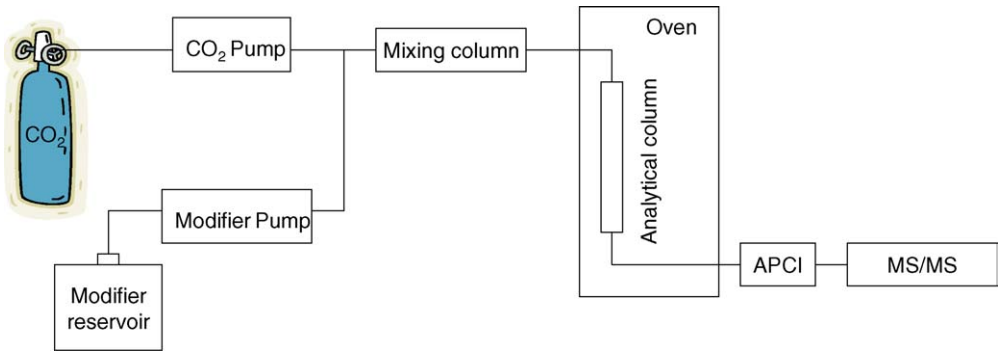

Fig. 1. A simplified schematic diagram of the pSFC–MS/MS system with an APCI interface.

1.4. Analysis of clozapine, ondansetron, tolbutamide and primidone

For the pSFC–APCI/MS/MS methods, a Diol column was used for separation of clozapine, tolbutamide and ketoconazole (ISTD) under isocratic condition of 30% modifier containing 0.3% IPA, and a Cyano column was used for separation of tolbutamide, primidone and trihydroxylflavanone (ISTD) under isocratic condition of 40% modifier containing 0.1% formic acid at a constant flow-rate of 4 ml/min. For the fast HPLC–APCI/MS/MS method, a ballistic gradient from 20 to 100% mobile phase B was run over 0.3 min, held for 0.6 min and re-equilibrated to 20% B over 0.1 min at a constant flow-rate of 1.0 ml/min for all analytes as described elsewhere [6,7]. The run cycle times with both pSFC and fast HPLC methods were around 1 min. The mass spectrometer was operated in positive ion mode. 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 MS/MS reaction selected to monitor clozapine, ondansetron, tolbutamide, primidone ketoconazole and trihydroxylflavanone were the transitions from *m*/*z* 327, 294, 271, 219, 533 and 273, the $[M+H]^+$ ions, to the product ions at *m*/*z* 269, 170, 90, 90, 491 and 152, respectively. The protonated molecules were fragmented by collision-activated dissociation (CAD) with nitrogen as the collision gas at a pressure of instrument setting 5. Data were acquired and calculated using Analyst 1.4.1 software (PE Sciex).

2. Results and discussion

Due to the varieties of chemical properties of new chemical entities, exploring new chromatographic methodologies to perform accurately analysis is playing an important role to efficiently provide drug metabolism and pharmacokinetic (DMPK) information. Today, the high-resolving power HPLC coupled with tandem mass spectrometry providing straightforward method development with unique analytical sensitivity and selectivity for monitoring therapeutic drugs has become the standard analytical tool in modern pharmaceutical industries. Several efforts such as direct plasma injection [8–11], and fast chromatography [12–14] techniques linked to HPLC–MS/MS methodologies to enhance sample throughput have been explored in our laboratory.

2.1. Development of pSFC–APCI/MS/MS method

SFC is related to HPLC. In SFC, the majority of the mobile phase is substituted with liquid carbon dioxide, which possesses low viscosity and high diffusivity to allow for the fast separations at higher flow-rates. In general, packed-column SFC is a normal phase chromatography ideally suited to the isolation of polar solutes that are challenging to separate by other chromatographic techniques. Although the UV is the most common detector for pSFC, there have been a few literature reports on interfacing pSFC to a single mass spectrometer for higher throughput screening [3,15–20]. In this work, coupling pSFC directly to the APCI source and a tandem mass spectrometer for the simultaneous determination of clozapine, ondansetron, tolbutamide and primidone in metabolic stability samples was investigated. The electrospray source (ESI) and the APCI source are the most popular interfaces for the hyphenated systems for qualitative or quantitative analysis of small molecules over the last decade. For the compounds tested in this work, APCI was observed to be superior to ESI in terms of sensitivity (data not shown).

In general, the normal phase conditions for HPLC are not completely amenable to the APCI source. The primary challenge is the possible explosion hazard when a high flow of flammable solvents such as hexane (typically 1 ml/min) is introduced into the APCI source at elevated temperature in the presence of a corona discharge. To circumvent this problem, two approaches have been explored. The first approach is to lower the heated nebulizer temperature [21]. The disadvantage of this approach is the cause of the incomplete desolvation of the column eluent resulting in poorer sensitivity. For the second approach, a make-up flow technique was adapted to reduce the hexane concentration prior to the APCI source [22]. A principle drawback of this approach is the dilution of the analyte. Supercritical fluid mobile phases ease solvent removal and disposal concerns. Also, the unique CO_2/methanol mobile phase with the addition of acidic or basic additives makes the hyphenation of pSFC to the APCI sources compatible.

The SFC retention is primarily governed by the polarity of the mobile phase. The polarity of the mobile phase increases as the content of modifier increases. The higher the polarity of the mobile phase yields the smaller retention factor, *k*. The reconstructed pSFC–APCI/MS/MS chromatograms of tolbutamide and primidone on a Diol column under isocratic conditions phases as a function of the ratios of liquid methanol–CO_2 mixtures containing a low concentration of IPA were shown in Fig. 2A and B, respectively. For clozapine, ondansetron and ketoconazole, the reconstructed pSFC–APCI/MS/MS chromatograms on a Cyano column under isocratic conditions phases as a function of the ratios of liquid methanol–CO_2 mixtures containing a low concentration of formic acid were shown in Fig. 2B–D, respectively. As demonstrated in Fig. 2, the lower modifier content would increase the retention of both analytes and ISTD. Here, methanol is a major polar elution solvent under pSFC conditions. Altering the methanol concentration was an effective way for significantly changing retention. The decrease of retention factor of the analytes by increasing modifier concentration is due to the increase of solvent strength of the mobile phase and the deactivation of the stationary phase [23].

The experimental conditions, such as the compositions and the flow-rates of eluent, normally have a strong impact not only on the chromatographic performances but the ionization

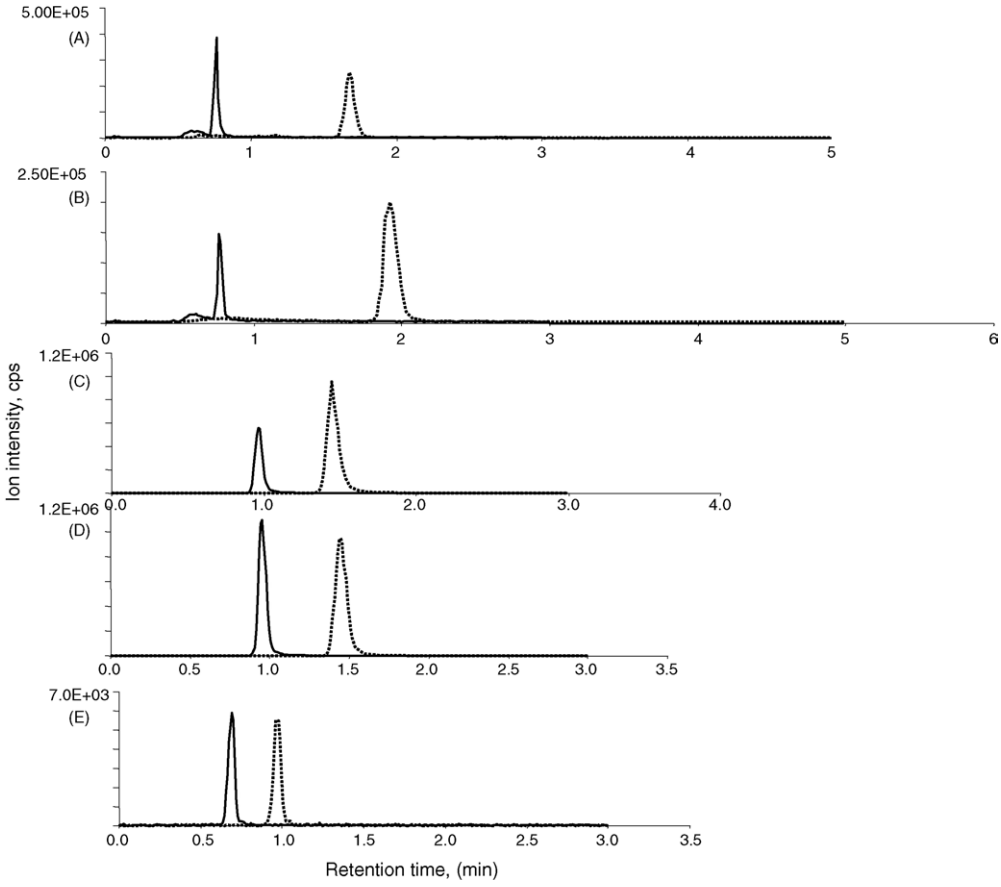

Fig. 2. The reconstructed pSFC–APCI/MS/MS chromatogragrams of (A) tolbutamide and (B) primidone using a Diol column under 30% (solid line) and 10% (dot line) modifier with an acidic additive and (C) clozapine, (D) ondansetron and (E) ketocanozole using a Cyano column under 50% (solid line) and 35% (dot line) modifier with a basic additive.

efficiencies of the analytes when hyphenating liquid-based chromatographic columns to the most ionization sources [24,25]. Supercritical liquid CO_2 and modifier (methanol) are normally MS-friendly and provide low column back pressure to allow higher flow-rate for rapid analysis. However, much less is known regarding the effects of normal phase SFC conditions on ionization efficiency. In this work, the influence of SFC conditions on the relative APCI responses using a mixture of clozapine, ondansetron, tolbutamide, primidone, ketoconazole and trihydroxylflavanone in solution was investigated.

The ionization mechanism in the pSFC eluent so far still remained unreported. The effects of the liquid CO_2–methanol ratios on the abundance of the peak areas of the reconstructed ion chromatograms of clozapine, ondansetron, tolbutamide, primidone, ketoconazole and trihydroxylflavanone at a flow-rate of 4 ml/min and at the APCI probe temperature of 450 °C are shown in Fig. 3A and B. Fig. 3 indicates that the APCI responses of the test compounds were stronger at higher contents of liquid CO_2 regardless of the modifier used with either a basic or an acidic additive. These results suggest that the introduction of carbon dioxide may assist the nebulization of the effluent to yield efficiently gas phase molecules from the modifier before ionization. The influences of the

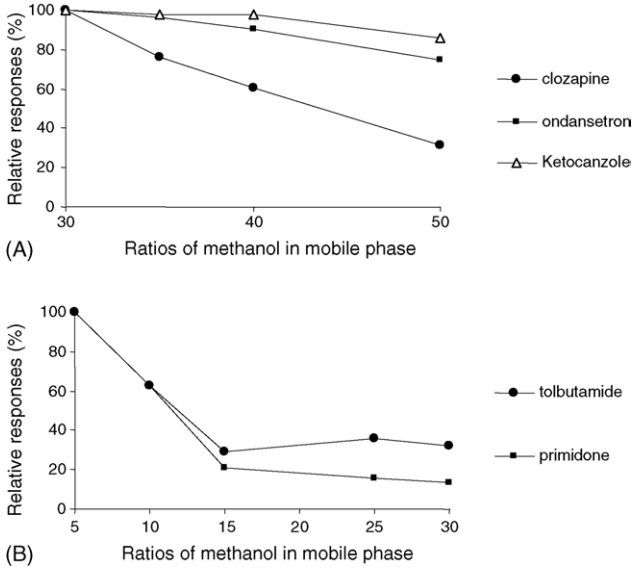

Fig. 3. The effects of eluent composition on APCI efficiencies of (A) tolbutamide and primidone and (B) clozapine, ondansetron and ketocanozole as a function of the CO_2-modifier ratios at a flow-rate of 4 ml/min.

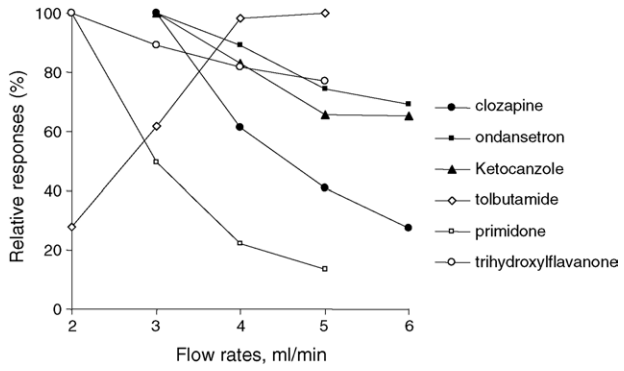

Fig. 4. The effects of eluent flow-rates on APCI efficiencies of tolbutamide, primidone, tryhydroxylflavanone, clozapine, ondansetron and ketoconazole.

mobile phase flow-rate on the APCI sensitivity of the test compounds observed in the SFC conditions at a constant temperature of the heated nebulizer were demonstrated in Fig. 4. Fig. 4 indicates that except for tolbutamide the APCI responses of the rest of remaining compounds decrease as the flow-rate increases. This phenomenon due to poorer heat transfer cross-section with greater flow-rates was found to be similar to the other heated nebulizer-based interface [24]. The relationship of the probe temperature versus the APCI responses of the test compounds is given in Fig. 5. As a general trend, the relative sensitivities of most of the test compounds are enhanced gradually from 350 to 500 °C. This was likely due to incomplete vaporization of carrier solution at lower temperature. However, the APCI signals of ketoconazole and tolbutamide decreased when the probe temperature was beyond 400 °C. This indicates that these two compounds are thermal-unstable.

2.2. Application for metabolic stability studies

The role of higher throughput metabolic stability studies in the drug discovery stage is to provide information for medicinal chemists to design molecules, which not only have the desired activity but also suitable duration of action. These metabolic stability experiments are based on the measurement of the substrate depletion. A general concern about assay reliability when developing mass spectrometry-based

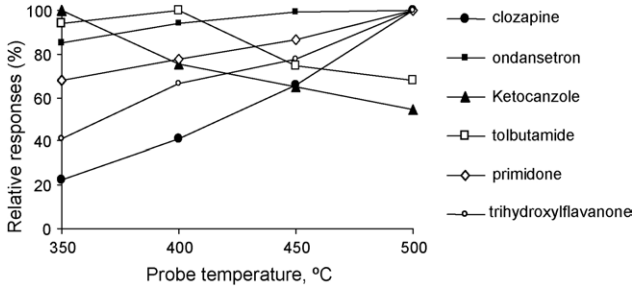

Fig. 5. The relative APCI responses of tolbutamide, primidone, trihydroxylflavanone, clozapine, ondansetron and ketoconazole as a function of the temperature of the heated nebulizer.

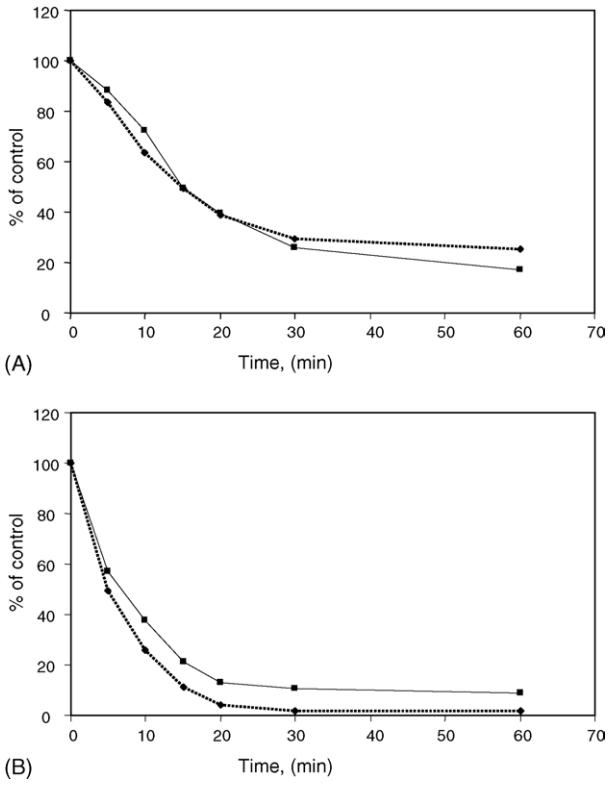

Fig. 6. Comparison of rat microsomal stabilities of (A) ondansetron and (B) clozapine obtained using both pSFC–APCI/MS/MS method (dot line) and HPLC–APCI/MS/MS method (solid line).

methods is the increased likelihood of encountering matrix ion suppression problems. 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 comparisons of APCI signals of analytes from solution and supernatant confirmed that there was no significant matrix effect from the incubation mixtures.

The microsomal stability results for the determination of in vitro intrinsic clearance of clozapine and ondansetron obtained using both pSFC–MS/MS and HPLC–MS/MS methods are comparable as shown in Fig. 6. Here, tolbutamide and primidone were stable in rat microsomal mixtures during the incubation period (data not shown). The retention times for both analytes and internal standards in metabolic stability samples were reproducible throughout the experiments. Protein precipitation with methanol gave clean chromatograms with no interfering peaks present using all transitions ranges under normal phase chromatographic modes. The conditions for pSFC–APCI/MS/MS and HPLC–APCI/MS/MS are also applicable to permeability, hepatocyte stability screening and protein binding assays.

3. Conclusions

Two rapid, reliable and sensitive pSFC–APCI/MS/MS methods were developed for simultaneously monitoring

clozapine, ondansetron, tolbutamide and primidone in metabolic stability samples. Normal phase pSFC conditions amenable to the APCI interface are able to provide a comparable sensitivity for the pharmaceutical determinations of the test compounds in positive ion mode. The eluent compositions and flow-rates and the probe temperature have substantial effects on the ionization efficiency of the analytes and chromatographic performance, which were found to be dependent on the compound of interest. The metabolic stability profiles of all analytes obtained by both pSFC–APCI/MS/MS and HPLC–APCI/MS/MS methods were in a good agreement.

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