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Research Article

Strategies for ascertaining the interference of phase II metabolites co-eluting with parent compounds using LC–MS/MS

LC-MS/MS is currently the most selective and efficient tool for the quantitative analysis of drugs and metabolites in the pharmaceutical industry and in clinical assays. However, phase II metabolites sometimes negatively affect the selectivity and efficiency of the LC-MS/MS method, especially for the metabolites that possess similar physicochemical characteristics and generate the same precursor ions as their parent compounds due to the in-source collision-induced dissociation during the ionization process. This paper proposes some strategies for examining co-eluting metabolites existing in real samples, and further assuring whether these metabolites could affect the selectivity and accuracy of the analytical methods. Strategies using precursor-ion scans and product-ion scans were applied in this study. An example drug, namely, caffeic acid phenethyl ester, which can generate many endogenous phase II metabolites, was selected to conduct this work. These metabolites, generated during the *in vivo* metabolic processes, can be in-source-dissociated to the precursor ions of their parent compounds. If these metabolites are not separated from their parent compounds, the quantification of the target analytes (parent compounds) would be influenced. Some metabolites were eluted closely to caffeic acid phenethyl ester on LC columns, although long columns and relatively long elution programs were used. The strategies can be utilized in quantitative methodologies that apply LC-MS/MS to assure the performance of selectivity, thus enhancing the reliability of the experimental data.

Keywords: Bioanalytical method validation / Interference / Parent compounds / Phase II metabolites / Precursor ion scan / Product ion scan
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1 Introduction

LC-MS/MS is a highly efficient tool for drug metabolism and pharmacokinetics (DMPK) studies in the pharmaceutical industry and clinical chemistry [1, 2]. Because of LC-MS/MS, rapid and batched analysis for pharmaceutics has become available. LC-MS/MS facilitates drug discovery and development [3]. Currently, it is the most efficient, accurate, and reliable instrument in the quantitative analysis of drugs in both clinical and preclinical studies [4–6]. In large pharmaceutical enterprises, rapid and bathed analysis is crucial for enhancing work efficiency and promoting the progress of drug discovery, accordingly boosting the profit. For single-drug analysis, the common analysis time for one sample is around 3–4 min. Recently developed ultra HPLC makes it possible to analyze one sample within a minute [7].

However, high-speed analysis does not always benefit DMPK studies, because inadequate chromatographic separation sometimes occurs. Although highly selective LC-MS/MS in the multiple reaction monitoring (MRM) mode can eliminate most interference caused by exogenous and endogenous compounds [8], some interferents could still negatively affect the analysis. Some endogenous compounds, especially phase II metabolites of dosed drugs, may negatively influence drug analysis although the MRM mode is utilized [9, 10]. The phase II metabolites are generated during *in vivo* metabolism processes, e.g. glucuronidation [11–13], sulfation [14, 15], methylation [16], glutathione conjugation [17], and acetylation [18]. These metabolism processes, also called phase II reactions, are usually known as conjunction reactions of the dosed drugs with endogenous bonding agents including glucuronic acid, sulfonates (sulfation), glutathione, or amino

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Abbreviations: CA, caffeic acid; **CAPE**, caffeic acid phenethyl ester; **DMPK**, drug metabolism and pharmacokinetics; **DP**, declustering potential; **MRM**, multiple reaction monitoring; **OH–PAHs**, hydroxylated polycyclic aromatic hydrocarbons

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acids [19–21]. The phase II reactions are usually detoxification processes that involve conjugation reactions of the bonding agents with polar functional groups such as carboxyl (–COOH), hydroxyl (–OH), amino (–NH_2), and sulfhydryl (–SH) groups [22–24]. Adducts generated in these conjugation reactions usually possess increased molecular weight and changed polar properties [25]. Most phase II metabolites, e.g. the products of glucuronidation, sulfation, and glutathione conjugation, are of higher polarity than their parent compounds [21, 26, 27]. However, some metabolites generated in acetylation and methylation are less polar than their parent compounds [21, 28]. Sometimes more than one type of reaction may occur on the different functional groups of one drug molecule [29], therefore, it is hard to predict the final polarity of the metabolites. It is possible that the overall polarity of the metabolites is close to or extremely similar to that of the parent compounds. Therefore, the separation of the metabolites from the parent compounds may be very difficult to achieve on an LC column with a relatively short elution program. In the ionization process, these metabolites can be dissociated into the adduct parts and pre-metabolized parts, which can give rise to precursor ions of the parent compounds [30, 31]. Because the proportion of phase II metabolites of some drugs is relatively high in real samples [32–34], these metabolites can generate a very high signal response in MS and present a high signal response in the same MRM transitions of the parent compounds [35, 36]. Hence, if sufficient chromatographic separation cannot be achieved, these metabolites may negatively influence the accuracy and reliability of the method.

Moreover, as this kind of interferent is in possession of very similar chromatographic behavior and can generate chromatographic peaks in the same MRM transitions of the parent compounds, it is very difficult to observe them based on the chromatograms. So, it may be unreliable to use this data for DMPK studies without checking and ascertaining the co-eluting phase II metabolites.

This was a complementary study based on one of our previous studies that focused on method development for the quantitative analysis of caffeic acid phenethyl ester (CAPE) and caffeic acid (CA) in dog plasma [37]. When doing the method development, we found many phase II metabolites present in the dosed dog plasma at some collection time points, and the signal response of some metabolites in ESI MS was relatively high. Some peaks generated by the phase II metabolites could not be sufficiently separated from that of the parent compound with the previously developed and optimized LC-MS/MS method, which had been validated with spiked samples only. In addition, the elimination or existence of the totally co-eluting phase II metabolites cannot be confirmed, which could affect the selectivity of the quantitative analysis for CAPE and CA.

In this study, we propose some strategies for checking the existence of the co-eluting phase II metabolites to enhance the reliability, selectivity, and accuracy of the quantitative methods with LC-MS/MS. This proposal can be extended to the application of most drug analysis methods employing LC-MS/MS, for enhancing and assuring the reliability of the methods and experimental data.

Table 1. Mass spectrometric parameters for precursor ion scan and product ion scan modes

Scan mode	Fixed ion (*m/z*)	Scan range (*m/z*)	Declustering potential (*V*)	Collision energy (*eV*)	Dwell time (*ms*)
	135	150∼700	−90	−33	300
Precursor ion	179	150∼700	−90	−33	300
	283	285∼1000	−90	−33	300
Product ion	359	100∼380	−90	−33	300

2 Materials and methods

Considering the space limit, we present some additional information, in terms of *chemicals and materials, instrumentation, sampling procedures and sample pretreatment* in the Supporting Information.

2.1 LC-MS/MS conditions and parameters

Mobile phase A was 0.5% formic acid in water containing 1 mM ammonium acetate, and mobile phase B was 0.5% formic acid in methanol/acetonitrile (1:1, v/v) with 1 mM ammonium acetate. The flow rate was 0.30 mL/min, and the injection volume was 10 μL. The auto sampler and the column thermostatic oven were kept at 4 and 20°C, respectively.

The working conditions and parameters of the MS were optimized as follows: the ion source was ESI operated in negative mode; resolution of Q-1 and Q-3 was unity (0.7 ± 0.1 amu); the collision-activated dissociation gas, curtain gas, gas 1, and gas 2 were set at 8, 25, 50, and 55 psi, respectively; the source temperature was 550°C; the ionization voltage was −4200 V; the entrance potential and the collision cell exit potential were −10 V; dwell time was 80 ms for each MRM transition. All the gases used were high-purity nitrogen. MS acquisition was performed with MRM, precursor-ion scan and product-ion scan modes. The MRM conditions and parameters including ion transitions, declustering potential (DP) and relative collision energy are provided in Supporting Information Table S1. When conducting the experiments with precursor-ion scan and product-ion scan, the working conditions of the LC and ionization source were the same as those of the method using the MRM mode. The relative collision energy and declustering potential used in the precursor-ion scan and product-ion scan were the same as those used for the corresponding MRM transitions (Table 1). The data acquisition and processing were conducted with Analyst 1.5.0 (Applied Systems).

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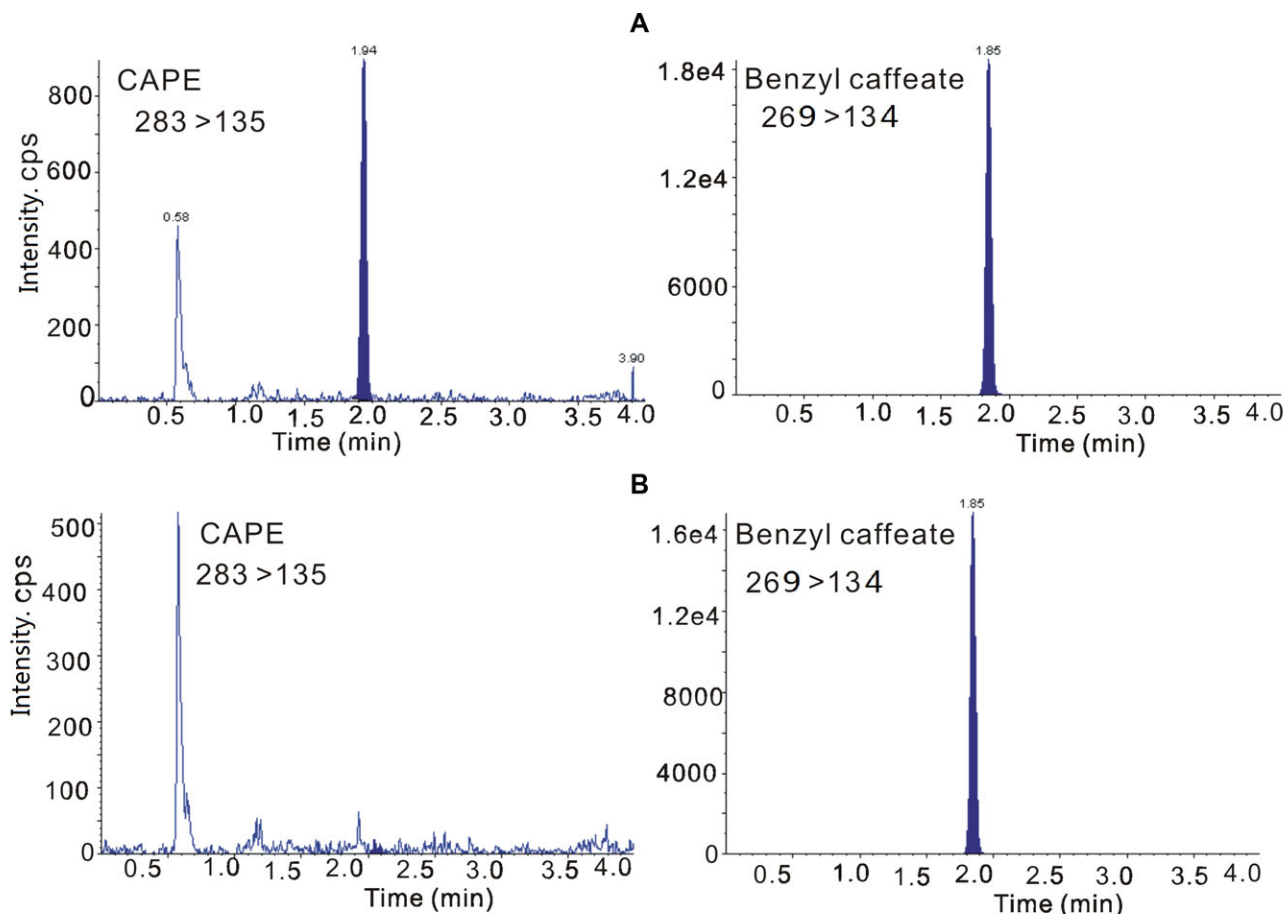

Figure 1. MRM chromatograms of CAPE and benzyl caffeate in lower limit of quantification (LLOQ, 10 ng/mL) sample (A) and control blank (blank sample spiked with internal standards only) sample (B) with a short elution program. The chromatographic conditions were optimized before application to the analysis of real samples.

3 Results and discussion

3.1 Sampling procedures and sample pretreatment

As reported previously [35, 38], phase II metabolites and the parent compounds can be mutually converted to the samples during sampling, storing, and pretreatment procedures. This is because the enzymes manipulating the conversion and back-conversion of the metabolites in the real samples are active at room temperature. To inactivate the enzymes, methanol was added into the tube and mixed with the plasma immediately after harvesting the plasma sample. The temperature of both the centrifugation procedure and the storage were relatively low, which could suppress the activities of the enzymes, and thus reduce the mutual conversion and degradation.

3.2 LC-MS/MS conditions

During the earlier stage of the method development, due to the lack of real samples, we developed an LC-MS/MS method with a 4 min elution program using some short columns including Zorbax-C_{18} (2.0 × 50.0 mm, 5 μm, Agilent), Luna-C_{18} (2.0 × 50.0 mm, 5 μm, Phenomenex), and CAPCELL PAK C_{18} (2.0 × 50 mm, 5 μm, Shiseido). Satisfactory chromatograms can be achieved with all these columns (Fig. 1) and very few impure peaks were observed in the MRM chromatograms. Yet, when this established analytical method was applied to the analysis of real samples, many impure peaks generated by the metabolites were present in the MRM chromatograms, and the separation between the interference peaks and the analyte peaks was not satisfactory. Therefore, we replaced the short columns with a relatively long one and lengthened the elution program to 6.3 min. With these chromatographic conditions, acceptable separation of the metabolites and the analytes was achieved (Fig. 2), and the chromatograms showed no inseparable interference peaks for the analytes.

The precursor ion was found with Q-1 scan mode. Then, a product-ion scan was conducted to find the product ions. The ion fragmentation patterns of the analytes and the internal standards are provided in Supporting Information Fig. S1.

For CAPE and CA, the most abundant product ion was *m/z* 135, which is in accordance with previous studies [39–41].

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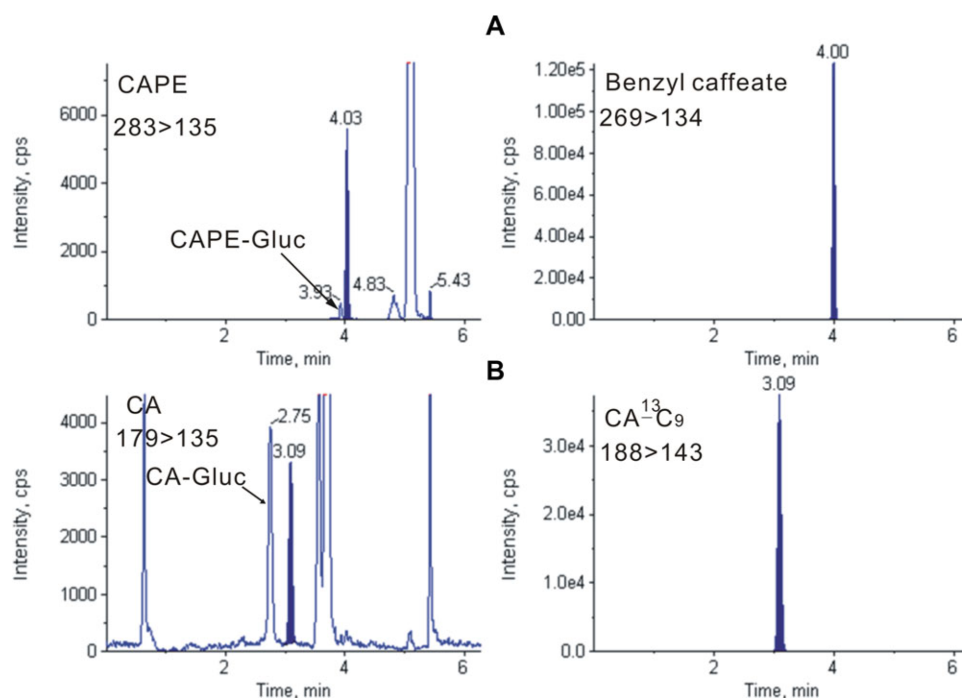

Figure 2. MRM chromatograms of CAPE and CA in a real plasma sample.

This ion (*m/z* 135) was the decarboxylated product from the caffeate ion (*m/z* 179), which was the precursor ion of CA and the ester-cleaved product ion of CAPE. The other major product ion *m/z* 161 may be generated by the dehydration from the caffeate ion (*m/z* 179).

3.3 Ascertaining the interference

Figure 3 illustrates the mechanism and processes of the influence caused by the co-eluting phase II metabolites to the quantification of the parent compounds. Metabolites 1, 2, and 3 can negatively affect the quantification of the parent compounds. Amid those metabolites, it is easy to observe that metabolites 1 and 3 can influence the quantification of the parent compound. Those metabolites therefore can be further separated from their parent compounds with a longer LC column (higher column effect) and an optimized longer elution program. The more critical issue is the influence caused by metabolite 2, the chromatographic peak of which is merged into that of the parent compound and very hard to observe. Therefore, metabolite 2 is the most "dangerous" interferent for the analytical method for the determination of the parent compounds, which can generate high-proportion phase II metabolites *in vivo*. Hence, it is necessary to ascertain whether these metabolites are present or not, accordingly confirming the target chromatographic peaks are generated by the analytes only.

As shown in Fig. 2, there are several metabolites present in the real samples. Some possible metabolism pathways of CAPE have been tentatively confirmed (Supporting Information Fig. S2). One peak of the metabolite was quite close to that of CAPE. This metabolite has been identified to be one of the glucuronide metabolites of CAPE [37]. The signal response of some metabolites is much higher than that of the parent compounds. As so many metabolite peaks appeared in the chromatograms, we could not confirm that there was any interference peak hiding in the peaks of the parent compounds. Therefore, further experiments were conducted to confirm that the target peaks are purely generated by the target analytes.

3.3.1 Precursor-ion scan

In this scan mode, MS-2 is fixed to measure the occurrence of a particular fragment ion, and the MS-1 scans the parent ion or ions that can generate this product ion [42, 43]. The result of this scan mode is a spectrum of precursor ions giving rise to the particular product ion, which can ascertain which precursor ions can generate the product ion. In this study, we first fixed the product ion of *m/z* 179 to conduct the precursor ion scan with scan range of *m/z* 150–700. Figure 4 shows that, around the retention time of CAPE (4.00 min), several precursor ions were detected, including *m/z* 252, 269, 283, 450, 632, and 654.

Second, the product ion *m/z* 135 was set to perform a precursor-ion scan and the scan range was set from *m/z* 150–700. As shown in Fig. 4, a similar mass spectrum can be observed at the retention time around 4.00 min, compared with that of the precursor-ion scan with the fixed ion of *m/z* 179. The ions *m/z* 283 and 269 are precursor ions of CAPE and its internal standard benzyl caffeate, respectively. Other observed precursor ions are possible metabolites of CAPE. We assumed the ion *m/z* 450 was probably a phase II metabolite of CAPE because it gave rise to the product ions *m/z* 135 and 179, and its *m/z* value was higher than that of CAPE. The

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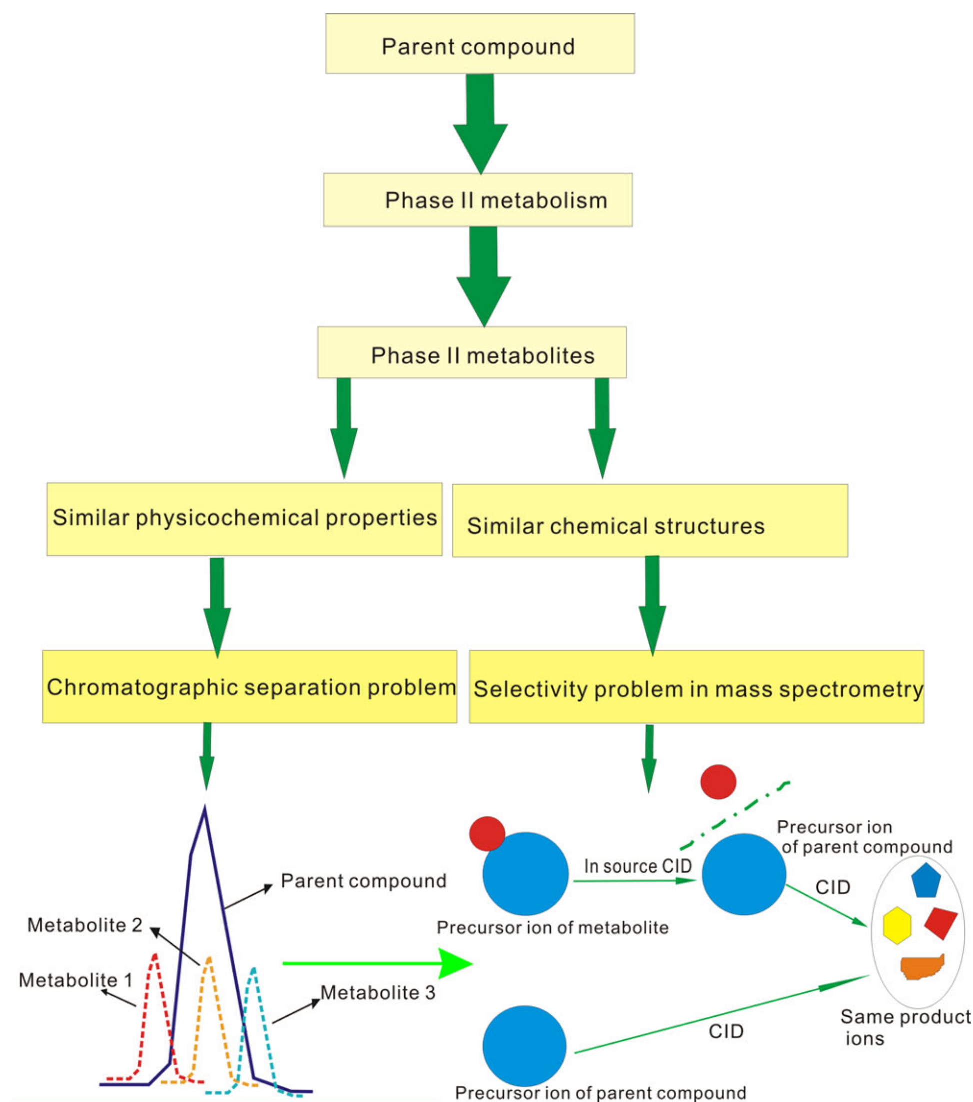

Figure 3. The mechanism and processes of the negative influence caused by phase II metabolites on quantification using LC-MS/MS with MRM mode for drug analysis.

metabolites with those precursor ions as *m/z* 450 were very critical in our method development. If they were confirmed to be the phase II metabolites of CAPE, the present elution program should be optimized again, and a longer column with longer elution time was probably needed. Therefore, it was essential to confirm whether the compound with the precursor ion of *m/z* 450 was a phase II metabolite of CAPE.

Finally, *m/z* 283 was fixed as the product ion to carry out the precursor-ion scan. The scan range was set from *m/z* 285 to 1000. Fortunately, the ion *m/z* 450 was not found in the precursor ion scan with the fixed ion of *m/z* 283. Only two precursor ions *m/z* 500 and 569 with relatively low signal response were found (Fig. 4). Accordingly, we confirmed the precursor ion *m/z* 450 was not the phase II metabolite of CAPE. Actually, it was confirmed to be a phase II metabolite of CA. This metabolite was identified to possess a $–HSO_4$ and a glucuronide group. When the two adducted groups were split during the in-source collision-induced dissociation process, the metabolite gave rise to the precursor ion of CA (*m/z* 179), followed by generation of the product ion *m/z* 135. Therefore, the ultimately optimized LC elution program was found with no need of further changes.

3.3.2 Product-ion scan

In this mode, the precursor ion is focused in MS-1 and transferred into the collision cell, where the precursor ion interacts with the collision gas and gives rise to fragment ions. The fragments (product ions) are then detected by the MS-2 scan. This mode results in a typical product ion mass spectrum, which can indicate the fragmental pathways of the analytes and always be applied to the elucidation of the chemical structure [44].

In some batches of dosed dog plasma, when the precursor ion scan with fixed product ion of *m/z* 135 was conducted, an inseparable peak was found closely following the peak of CAPE (Fig. 5). This peak was confirmed to be produced by the compound with precursor ion *m/z* 359. For further

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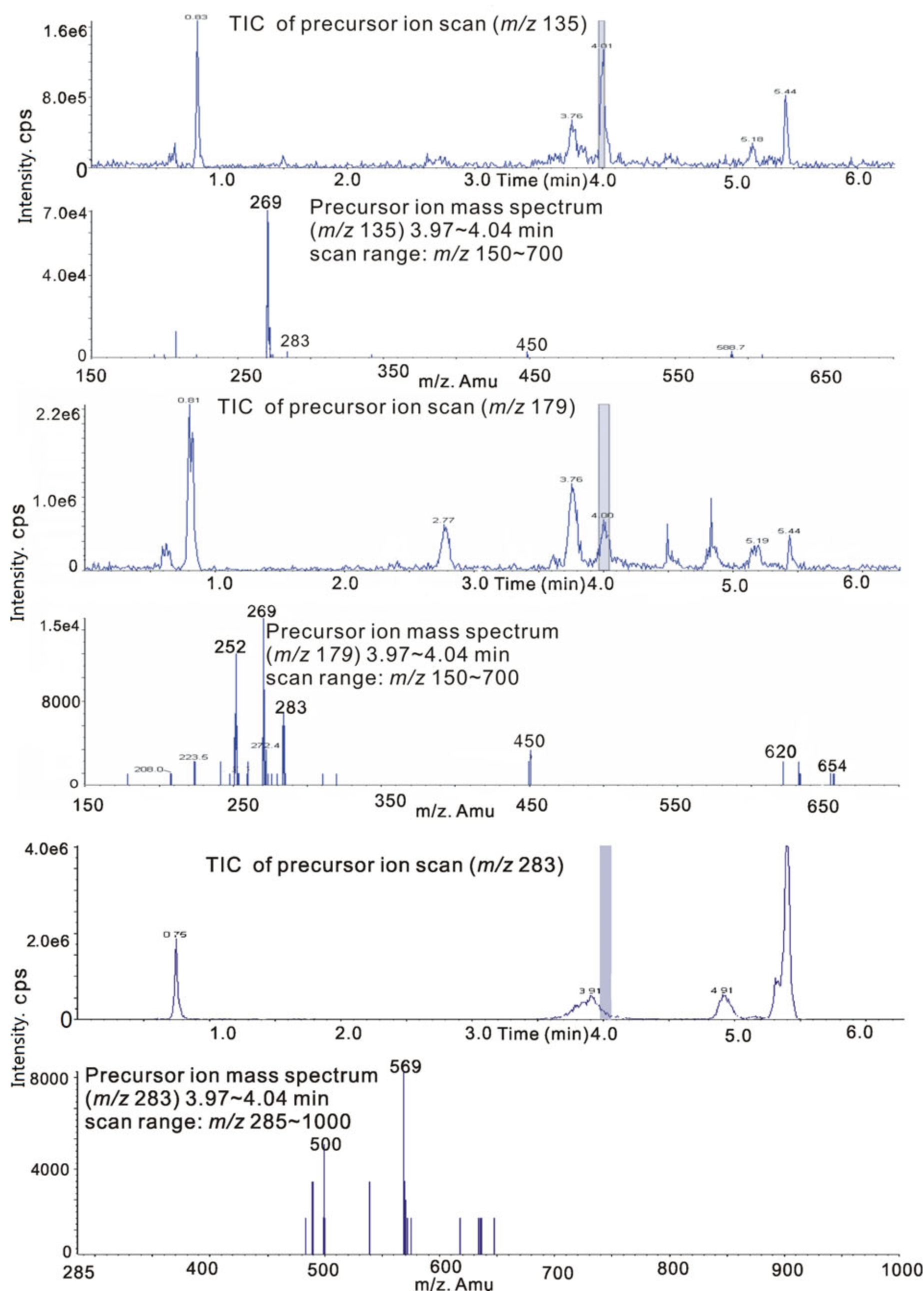

Figure 4. Chromatograms and mass spectra of dosed dog-plasma using a precursor-ion scan mode with fixed product ions at *m/z* 135, 179, and 283.

confirmation, the ion *m/z* 359 was set as the precursor ion to carry out the product ion scan. Product ions *m/z* 135, 161, and 179 were found (Fig. 5), while *m/z* 283 was absolutely undetectable. Therefore, we confirmed this ion *m/z* 359 was produce by a phase II metabolite of CA instead of CAPE, because it can generate the product ions *m/z* 135, 161, and 179, which are the product ions or precursor ion of CA.

3.3.3 Confirmation of selectivity

Before lengthening the elution program and using a longer LC column, we tentatively conducted a precursor-ion scan experiment with fixed ion of *m/z* 135. An interfering compound with *m/z* 379 was observed. This interferent eluted at the same retention time as that of CAPE, and it was probably a phase II metabolite of CAPE. We therefore optimized the chromatographic working conditions again and found that another phase II metabolite was separated from the parent drug CAPE. This metabolite was a glucuronide metabolite of CAPE. Considering the utilization of the precursor-ion scan was useful to detect the suspicious co-eluting phase II metabolites, we thus conducted sufficient precursor-ion scan experiment for examining the co-eluting phase II metabolites, and further developed the product-ion scan experiment to achieve the same objective.

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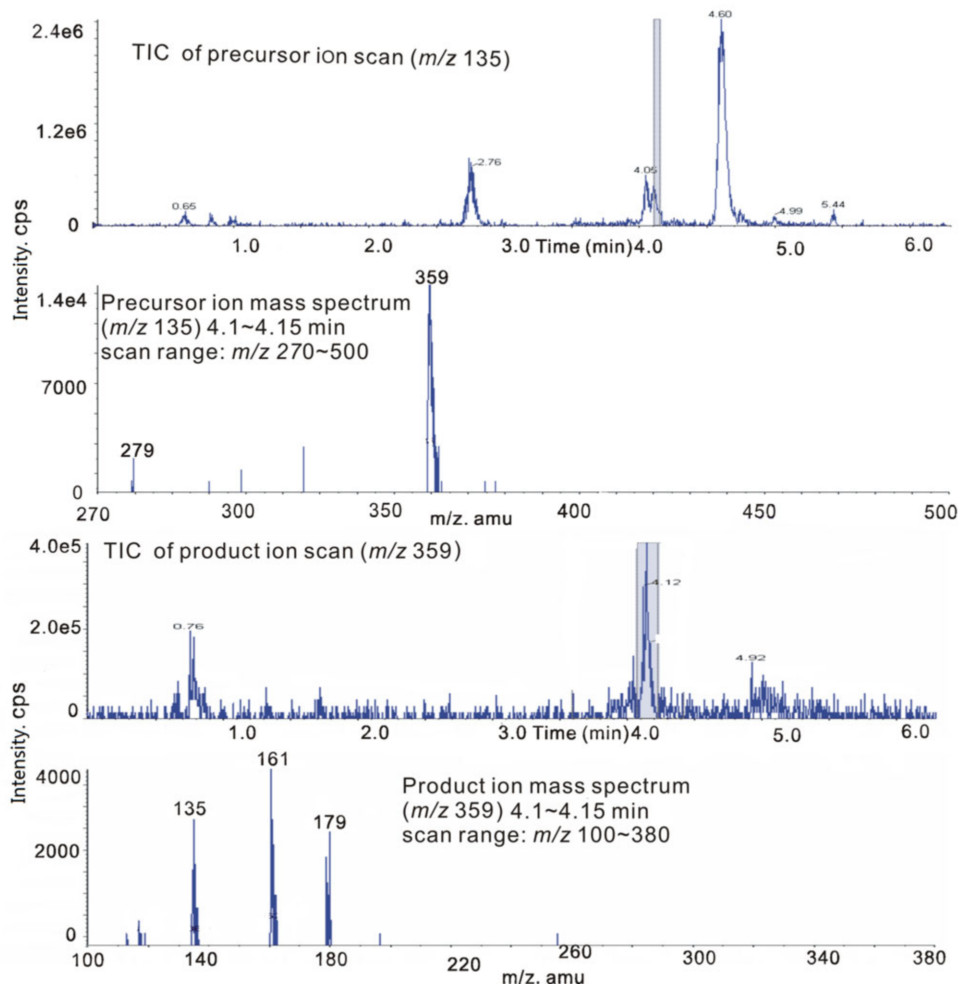

Figure 5. Chromatograms and mass spectra of real samples using precursor- and product-ion scan modes with fixed ions at *m*/*z* 135 and 359, respectively.

With the finally optimal elution program, the metabolites were separated from the analyte on a longer column. With the strategies of precursor- and product-ion scans, this quantitative method was confirmed to be free of negative influence caused by interferents for determining CAPE in dog plasma. Therefore, the selectivity of the analytical method developed in our work can fulfill the requirements in terms of the accuracy. The data indicating satisfactory quantitative and qualitative performance of the analytical method have been detailed previously [37].

3.4 Application to determination of OH–PAHs in human urine samples

Considering that the phase II metabolism is common *in vivo* for mammals and the phase II metabolites might cause interference for the determination of relevant parent compounds when using LC-MS/MS [30], the strategies proposed in this work can be applied to similar investigations, especially in analysis of compounds that can give rise to a high abundance of phase II metabolites, e.g. naloxone, ramipril, ramiprilat, and other phenolic and polyphenolic compounds [45]. The strategies were used in analytical methodology for the determination of hydroxylated polycyclic aromatic hydrocarbons (OH–PAHs) in urine samples from occupationally exposed workers to enhance the selectivity of the analysis. A great variety of phase II metabolites of OH–PAHs were found in the urine samples, which might lead to adverse effect for determination of OH–PAHs. Taking hydroxylated naphthalene for an instance, some co-eluting phase II metabolites were observed when a relatively short chromatographic column with a relatively short elution gradient program was used (Supporting Information Figs. S3 and S4). As a result, a longer column with a comparatively gentle gradient elution program was employed to eliminate the potential interference caused by the phase II metabolites, which greatly reduced interference peaks (Supporting Information Figs. S5, S6, and S7). The accuracy and the selectivity of the analysis were thus enhanced and assured.

4 Concluding remarks

In this study, two useful and necessary strategies were proposed for enhancing the selectivity and accuracy of

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quantitative methods in drug analysis. Since most drugs are metabolized into phase II metabolites *in vivo* and the conversion ratio is fairly high, therefore, the two strategies can be extended to the analytical methods for determining most drugs, especially for those drugs that can produce various phase II metabolites with high ratios. Some analytical chemists have noted the interference caused by the phase II metabolites during method development for drug analysis using LC-MS/MS and optimized the chromatographic conditions to achieve sufficient separation for eliminating the negative influence. However, no publication has mentioned the possibility that the chromatographic peaks of the phase II metabolites could totally overlap the peaks of the parent compounds. To assure the selectivity and reliability of the LC-MS/MS method for bioanalysis, the strategies proposed in this study are necessary to be conducted to confirm the existence and influence of the co-eluting metabolites.

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The authors declare no conflict of interest.

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