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Original article

Signal interference between drugs and metabolites in LC-ESI-MS quantitative analysis and its evaluation strategy

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ABSTRACT

Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS) is a widely utilized technique for in vivo pharmaceutical analysis. Ionization interference within electrospray ion source, occurring between drugs and metabolites, can lead to signal variations, potentially compromising quantitative accuracy. Currently, method validation often overlooks this type of signal interference, which may result in systematic errors in quantitative results without matrix-matched calibration. In this study, we conducted an investigation using ten different groups of drugs and their corresponding metabolites across three LC-ESI-MS systems to assess the prevalence of signal interference. Such interferences can potentially cause or enhance nonlinearity in the calibration curves of drugs and metabolites, thereby altering the relationship between analyte response and concentration for quantification. Finally, we established an evaluation scheme through a step-by-step dilution assay and employed three resolution methods: chromatographic separation, dilution, and stable labeled isotope internal standards correction. The above strategies were integrated into the method establishment process to improve quantitative accuracy.

1. Introduction

Liquid chromatography-tandem mass spectrometry (LC-MS) has become a powerful tool for quantitative analysis of drugs, metabolites, and biomarkers in the complex matrix due to its excellent sensitivity and selectivity [1]. In the most commonly used electrospray ionization (ESI) source equipped for mass spectrometry, the LC effluent forms charged droplets, which are then converted to gas phase ions after continuous solvent evaporation and repeated droplet fission, and finally delivered to the mass analyzer. However, co-effluents in the matrix can interfere with the ionization efficiency of the analyte, which is referred to as matrix effects, manifested as suppression or enhancement of the analyte signal. Especially in the preclinical or clinical LC-MS analysis, the analysis of generic methods is typically 1–5 min [1,2], and the improvement of analysis speed may bring the sacrifice of separation ability. Matrix effects are a widespread concern as they are very common in the analysis of biological samples and may affect the accuracy, sensitivity, or reproducibility of analytical methods [3,4].

Guidelines for the quantitative analysis of biological samples indicate the need for evaluation of matrix effects, the combined effect of all components of the sample other than the analyte [5,6]. The post-extraction addition method is commonly used for quantitative assessment of matrix effects. This method involves the pretreatment of a blank matrix (e.g., plasma, urine, and tissue), and mixing the resulting clear solution with the standard as a way to simulate possible effects caused by the matrix components. Matrix-matched calibration is the most commonly used method to compensate for matrix effect [4]. The impracticality of matrix calibration perfectly matched samples has been recognized. However, how to evaluate and avoid the potential quantitative harm caused by matrix mismatch still needs further understanding and exploration. In LC-MS-based pharmaceutical analysis, a class of endogenous substances, drug metabolites, can cause ionization interference. The blank matrix used for current method validation does not contain drug metabolites, so signal interference caused by

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metabolites is easily overlooked. Therefore, there is a risk of inaccurate quantification without matrix-matched calibration. Three key features characterize these substances: (1) the prevalence of metabolites for drugs in living organisms, (2) the structural similarity of the drug and its metabolites, which often results in their simultaneous elution during fast and generic chromatography, and (3) the individual differences in the concentrations of drugs and metabolites. Currently, in the study of drug-metabolite interference in mass spectrometry, more attention has been paid to the damage of isobaric metabolite or metabolite decomposition to quantitative results [7]. Ionization interference between drugs and metabolites and its effect on quantitative analysis has not been fully understood.

Troubleshooting and identifying these interferences after the quantitative results abnormally increase the workload. It is therefore advantageous to assess these interfering substances as early as possible. Using test samples to investigate the interference could overcome the matrix mismatch. Sample dilution has been applied in the investigation of matrix effect [8–10]. In some studies, the existence of matrix effect or specific substance-induced interference is judged by the signal change of analyte after the standard solution is mixed with the sample [11,12]. Unfortunately, these methods do not yet have a standard of operation and judgment. The well-known compensation or elimination strategies for matrix effects include blank matrix matching, isotope internal standard correction, chromatographic separation, dilution, etc. In addition, some literature has proposed new solutions to matrix effect or specific substance-induced interference. For signal suppression between isotope internal standard and unlabeled homolog, a component equation has been developed to improve the accuracy of the quantitative method [13]. Tisler et al. [14] combined post extraction spike, post column infusion, and quantitative structure-property relationship models to correct matrix effects. There are also some improvements in the instrument, such as improving the fixed-voltage to step-voltage nano-electrospray to prevent matrix effect [15]. However, effective methods for correcting ionization interference between drugs and metabolites still need to be studied and validated.

In this study, we investigated the main factors influencing the ionization interference between drugs and metabolites and the effect of their signal interference on the quantitative analysis by LC-ESI-MS. Our results showed that the most severe signal interference between drug and metabolite can reduce the signal of the analyte by 90%. In quantitative analysis, metabolite concentrations can be exaggerated by 30% due to enhanced signals from the parent drug, which could lead to unreliable pharmacokinetic data. We then developed an effective assessment method based on dilution and explored three problem-solving approaches. Overall, our work confirmed the prevalence of signal interference between drugs and metabolites and its hazard for quantitative LC-ESI-MS analysis and provided a systematic process for assessing and resolving such interference.

2. Experimental

2.1. Chemicals and reagents

Ten pairs of drugs and metabolites were included in this study based on the main physicochemical properties of the drug (molecular weight, pKa, LogP, LogD), clinical indications, and types of metabolites (oxidation, reduction, hydroxylation) (Figs. S1 and S2). The suppliers and purity of all standards are shown in Table S1. Methanol and acetonitrile were of high-performance liquid chromatography (HPLC) grade and purchased from Merck (Darmstadt, Germany). Ammonium formate and formic acid were purchased from Macklin (Shanghai, China). Ultrapure water was freshly prepared using the Milli-Q Advantage A10 system (Milli-Q Reference, Millipore, Boston, MA, USA).

The stock solution of each analyte and internal standard was prepared in methanol at a concentration of 1 mg/mL, and all were stored at −80 °C. The working solution was prepared by serial dilution with methanol-water (1:1, *V/V*).

2.2. Chromatographic and mass spectrometric conditions

Three LC-ESI-MS systems commonly applied in bioanalysis were used in this study. Two of these were identical LC-ESI-MS systems (TSQ-1 and TSQ-2), both including a UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) and a TSQ Quantum Access Max API mass spectrometer (Thermo Fisher Scientific) with an electrospray ionization (H-ESI) interface. Tune Plus software 2.4 was used to control the instruments above. The third system, API 4000, consists of a SHIMADZU LC-20 UHPLC (SHIMADZU, Kyoto, Japan) and an API 4000 mass spectrometer (AB Sciex, Framingham, MA, USA) and was controlled by Analyst Software 1.6.2. All target analytes were quantified in selection reaction monitoring mode under positive ion mode. One-to-one optimization was applied to the main MS parameters, of which the ion pair and collision energy can be seen in Table S1, and other parameters were uniformly set as a relatively optimal combination (Table S2).

The LC condition was set according to the routine analytical methods. The mobile phase consisted of 0.1% (*V/V*) aqueous formic acid solution (phase A) and methanol (phase B). Unless otherwise specified, the mobile phase used for flow injection analysis (FIA) was the aqueous phase and methanol (20:80, *V/V*) at a flow rate of 200 μL/min and an analysis time of 2 min. The gradient elution procedure used in the LC analysis was set as follows. The proportion of phase B from 0 to 0.05 min was 25%. The proportion of phase B rose to 95% from 0.05 to 0.5 min, and was maintained at 95% for 1.5 min. Then, the proportion of phase B decreased to 25% from 2.0 to 2.2 min and was maintained until 3.5 min. The flow rate was 365 μL/min and the chromatographic separation was performed on a Thermo Hypersil GOLD C_{18} (50 mm × 2.1 mm, 3 μm) column(Thermo Fisher Scientific). The injection volume was 10 μL.

2.3. Signal interference on three LC-ESI-MS systems

Signal interference between the drug and corresponding metabolite in ten groups (Table S1) was studied on three LC-ESI-MS systems and was analyzed at concentrations of 10, 100, 1000, and 10000 nM of each analyte. Working solutions were diluted and kept the same solvent composition as the mobile phase. Through FIA, two signals were obtained, one was when the drug and the metabolite were injected at the same time, and the other was when the drug (or metabolite) was injected alone, respectively. The difference between the two signals was calculated to obtain the signal change rate of the drug (or metabolite). Signal interference is considered to exist when the signal increases or decreases by more than 15% compared to the signal when detected alone.

2.4. Influencing factors for signal interference

Four groups of drugs and metabolites were used to investigate factors contributing to signal interference on the API 4000 system with FIA procedures. The conditions were changed as follows: ion spray voltage 3.5 kV, source temperature 100 °C, spray needle height 5 (2.86 in the original condition), and mobile phase aqueous phase containing 1 mM ammonium formate. Only one condition was changed at a time to obtain signal changes in drugs or metabolites. The effect of organic phase percentage in the mobile phase was studied at four methanol percentages (100%, 80%, 60%,

and 40%), and the flow rate effects were studied at 100, 200, 300, and 400 μL/min. In addition, to investigate the influence of using the chromatographic column on signal interference, isocratic elution, aqueous phase and methanol (20:80, *V/V*) at a flow rate of 200 μL/min, were used in LC analysis. The analysis time was 2.5 min. Some data points are not shown in the result plot because the signal of the analytes was too low.

2.5. Signal interference experiments between oxcarbazepine (OXC) and its monohydroxy derivative metabolite (MHD)

The signal interference between OXC and its metabolite 10,11-dihydro-10-hydroxycarbamazepine (MHD), was analyzed at 100, 200, 400, 800, 1600, 3200, 6400, 12800, 25600, and 51200 nM of each analyte. Samples were analyzed under FIA and LC conditions on the API 4000 system to obtain the signal change rate. The gradient elution was used in the LC analysis to closely approximate the real analysis scenario.

2.6. Simulated pharmacokinetic samples for OXC and MHD

Calibration standards were prepared by adding 5 μL working solution to 45 μL water at the concentrations of 20, 40, 80, 160, 320, 640, 1,280, and 2,560 ng/mL for OXC, 200, 400, 800, 1,600, 3,200, and 6,400 ng/mL for MHD (the first linear range), and 6,400, 9,600, 12,800, 16,000, 19,200, 22,600, and 25,600 ng/mL for MHD (the second linear range). The lower limit of quantification (LLOQ), low quality control (LQC), medium QC (MQC), and high QC (HQC) were prepared with the same procedure at concentrations of 20, 50, 500, and 2,000 ng/mL for OXC, 200, 500, 1,500, and 4,500 ng/mL for MHD (the first linear range), and 6,400, 9,000, 18,000, and 24,000 ng/mL for MHD (the second linear range) (Tables S3 and S4). The concentration of the loratadine (internal standard) working solution was 250 ng/mL, and that of the MHD-d_4 (stable isotope-labeled internal standard (SIL-IS)) working solution was 2,000 ng/mL.

Simulated biological samples for pharmacokinetic studies in this study were obtained by adding 5 μL working solution to 45 μL water. Then, 50 μL simulated biological samples, 10 μL internal standard working solution, and 450 μL acetonitrile were added to a 1.5 mL centrifuge tube and mixed well. The supernatant was collected for LC-MS analysis with gradient elution. Therefore, the samples were diluted 10-fold for analysis. Concentration and blood collection time settings for the simulated biological samples were based on previous pharmacokinetic studies of OXC and MHD after a single oral dose [16–18], as shown in Table S5.

To test whether dilution could solve the signal interference between OXC and MHD, the samples were diluted 20-fold for analysis. 25 μL simulated biological samples, 10 μL internal standard working solution, and 475 μL acetonitrile were added to a 1.5 mL centrifuge tube and mixed well. The supernatant was collected for LC-MS analysis.

2.7. Stable-isotope-labeled internal standard correction

To test whether SIL-IS could correct the signal interference between OXC and MHD, MHD-d_4 was added to the samples. The signal interference between OXC and MHD was analyzed at 100, 800, 6,400, and 51,200 nM of each analyte. Meanwhile, four concentrations of MHD-d_4 (100, 800, 6,400, and 51,200 nM) were added to each sample respectively, and the ratio of the signal change rate of MHD to that of MHD-d4 was calculated. The ratio of 85%–115% indicates that MHD-d_4 is capable of correcting the signal interference of MHD by OXC.

2.8. Evaluation algorithms

The samples were diluted step by step at a certain multiplicity, and the samples obtained at each concentration were measured and the series concentration was calculated using the calibration curve. This study used 2-fold dilution to obtain a series of samples. Two calculation methods were used to investigate changes in the analyte signal. The first calculation method is to calculate whether the concentration changes by a factor of 2 before and after a 2-fold dilution of the sample and the calculation formula is given in Equation (1). We also calculated the values at 4-fold dilution for evaluating the analysis. The second calculation method used the published calculation formula of Oldekop [10]. An absolute value of Diff greater than 20% suggests that the analyte signal in the sample is interfered with by other substances.

$$Diff_1 = \frac{C/2 - C_{1/2}}{C_{1/2}} \quad \text{Equation 1}$$

$$Diff_2 = \frac{\sqrt{\left(\left(C - C_{mean}\right)^2 + \left(2 \times C_{1/2} - C_{mean}\right)^2 + \left(4 \times C_{1/4} - C_{mean}\right)^2\right) \Big/ 3}}{C_{mean}} \quad \text{Equation 2}$$

In the equation, C, $C_{1/2}$ and $C_{1/4}$ refer to the calculated concentrations of analytes in the sample before dilution, after 2-fold dilution, and after 4-fold dilution, respectively.

2.9. Method validation

The assay method validation was assessed in terms of linearity, precision, and accuracy according to the bioanalytical method validation guidance of the US Food and Drug Administration [5].

2.10. Statistical analysis and software

The raw data was sorted using Microsoft Excel. GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA) and MATLAB R2022a v9.12.0 (MathWorks Inc., Natick, MA, USA) were used for plotting. Statistical analysis was performed with the IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA). The Analyst Software 1.6.2 was used to establish the calibration curves fitted with weighted ($1/x^2$) and to calculate the accuracy and precision of the QC samples ($n = 6$). The physicochemical properties of compounds were obtained from the ChemSpider database (http://www.chemspider.com), the DrugBank database (https://go.

drugbank.com/), and an online prediction platform (http://pka.luoszgroup.com/) [19].

3. Results and discussion

3.1. Signal interference between drugs and metabolites

Signal changes for ten groups of drugs and corresponding metabolites combined at four concentration levels were investigated on three instrument systems. The four concentration settings (10, 100, 1,000, 10,000 nM) were based on the common ranges of blood drug concentrations of clinical drugs. The results showed a widespread phenomenon of signal variation due to ionization interference between drugs and metabolites. A signal variation of more than ±15% was observed for 85% of the compounds (drugs or metabolites) in a total of 60 analytical events (Figs. 1A, S3, and S4). These signal changes included both enhancement and suppression, with suppression predominating.

Signal changes not only varied by different drugs but were also inconsistent between drugs and their corresponding metabolites. Signal interference was seen in 76.7% of drug analysis batches compared to 93.9% of the metabolite ones, and the results combining the degree of interference suggest that metabolites are more inclined to be affected by the interference between them. This may be due to the enhanced polarity of these metabolites following hydroxylation, demethylation, or other metabolic pathways. The equilibrium partitioning model assumes that the ESI droplet is divided into an electrically neutral interior and an overcharged surface [20]. Prototype drugs have a stronger affinity for the droplet surface due to their weaker polarity, which leads to easier charge acquisition during charge competition and easier suppression of metabolite signal. In addition, the results of signal interference between several drugs and metabolites exhibit suppression on one side and enhancement on the other. Oxcarbazepine is a typical example. All results obtained by the three instruments showed that the oxcarbazepine signal was suppressed, while its metabolite signal was enhanced (Figs. 1A and B).

Another remarkable phenomenon is that both drug and metabolite concentrations can affect the degree of signal interference (Fig. 1A). The degree of interference with drug (e.g., dextromethorphan and oxcarbazepine) can be significantly different when the concentrations of metabolites differ. This phenomenon is more obvious when several metabolites (2-hydroxy atorvastatin, dextrorphan, and 10,11-dihydro-10-hydroxycarbamazepine) are interfered with. In a nutshell, there is a general signal interference between drugs and metabolites in electrospray mass spectrometry.

3.2. Influencing factors for signal interference

Ionization interference in ESI is considered to be a function of the relative concentration, ionization efficiency, and solvation energy of analytes within the electrospray ionization droplet. The ionization efficiency of the analyte is influenced by the eluent [21] and the instrumentation [22]. Here, we analyzed the main influences of signal interference between drugs and metabolites, including substance concentration, MS parameters, and LC conditions.

3.2.1. Concentration of drugs and metabolites

Ten groups of drugs and metabolites with four concentrations (10, 100, 1,000, and 10,000 nM) were subjected to signal interference experiments on three LC-ESI-MS systems. The results of 16 concentration ratios were analyzed to find the pattern of concentration effects on the degree of signal interference (Fig. 1C). Each concentration ratio grouping contained 60 events. The results show a significant increase in the degree of signal interference (predominantly suppression) of the analytes as the concentration of co-analytes increased. Moreover, the proportion of signal interference events also increased with increasing co-analyte concentrations across the 60 analyte events, with the highest incidence being 60% for the 10/10,000 group. In addition, the incidence of signal interference differed between the drug and metabolite. When both concentrations were 10,000 nM, only 16.7% of the drug was interfered with, compared to 50% of the metabolite.

3.2.2. Mass spectrometry parameters

Source temperature affects the ionization efficiency of analytes. Higher source temperatures result in faster evaporation of solvent from ESI droplets, higher droplet generation rates in sub-droplets, and higher analyte ionization efficiencies [22]. Our results also presented that most compounds showed better response intensities at 350 °C than at 100 °C. However, the increase in response may tend to be accompanied by an increase in the degree of signal interference (Figs. 2A and S5), such as atorvastatin, dextromethorphan, and their metabolites. The possible reason is that there is no significant increase in surface excess charge, while the increased source temperature enhances the charge competition between the more daughter droplets formed in the spray.

The effects of spray voltage and spray needle position on signal interference were also investigated (Figs. 2A, S6, and S7). In this study, a small increase in spray voltage from 3.5 kV to 4.5 kV can improve sensitivity without enhancing signal interference, suggesting that increasing the spray voltage may be a feasible way to balance sensitivity and signal interference. The effect of needle height on signal interference and sensitivity was not significant at the range examined.

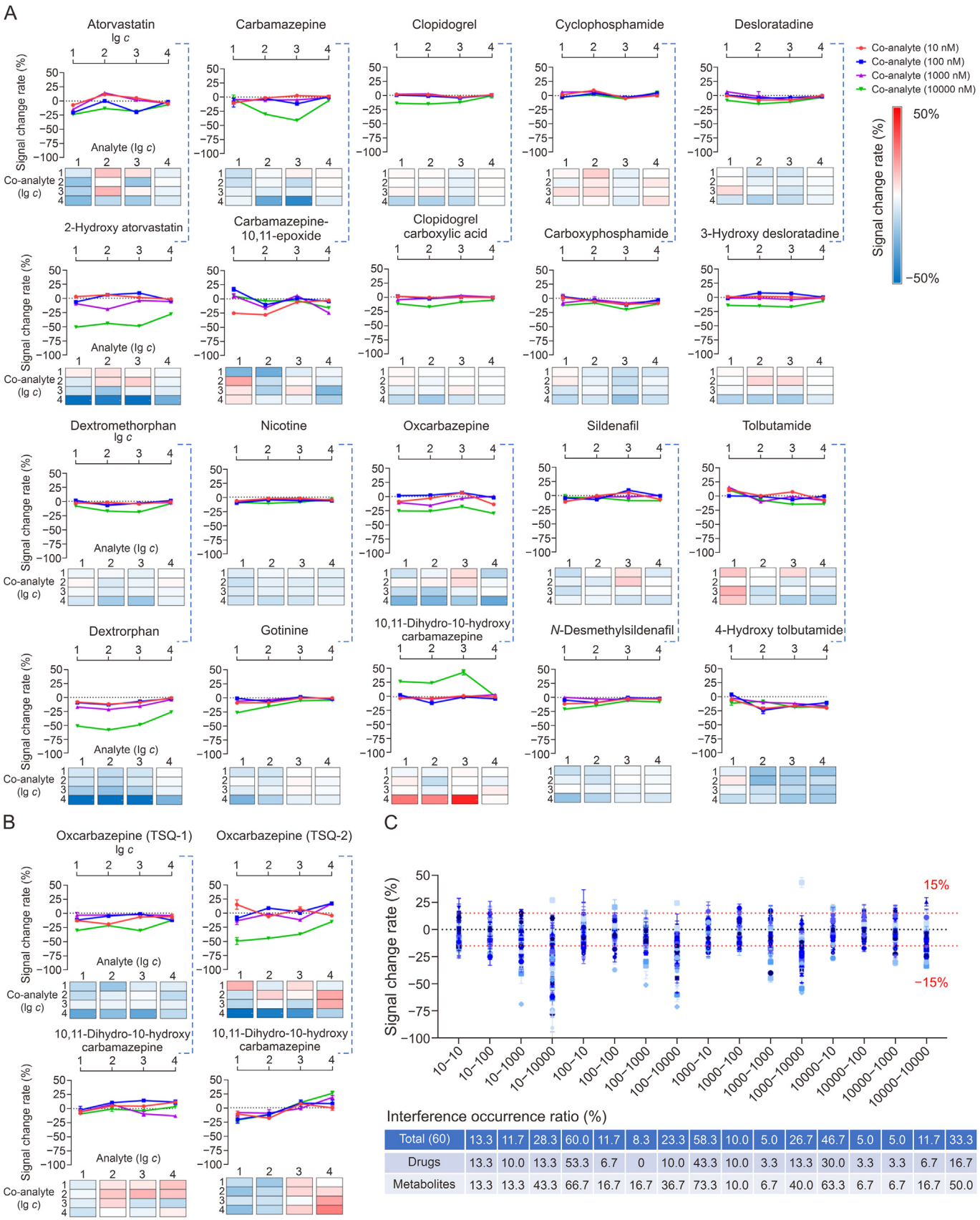
3.2.3. Liquid chromatography conditions

The composition of the mobile phase and the elution procedure under liquid phase conditions determine the composition of the droplets in ESI and are therefore important for the ionization of the analytes [21,23]. In this study, we compared the effect of 0.1% formic acid and 1 mM ammonium formate as aqueous phase additives on the degree of signal interference between drugs and metabolites, as shown in Figs. 2A and S8. Ammonium formate has a slightly greater effect on the level of interference than formic acid. The probable reason for this is that ammonium formate solutions are closer to neutral and the reduction of protons intensifies the competition.

In reversed-phase chromatography, a high percentage of the organic phase is usually required to elute the analytes. Therefore, we set four methanol ratios of 100%, 80%, 60%, and 40% for isocratic elution of the FIA. Fig. S9 shows that the changes in signal interference between drugs and metabolites can vary from compound to compound as the proportion of the organic phase increases. The perturbation of the organic phase ratio on the level of signal interference is evident in the overall picture. We speculate that the organic phase ratio affects the charge competition by combining droplet formation and evaporation, droplet surface charge distribution, etc. It is worth mentioning that the signal of oxcarbazepine metabolite was predominantly enhanced and was enhanced by more than 200% at 40% methanol, which suggests the existence of interference mechanisms other than charge competition.

In addition, the effect of flow rate on the signal interference between drug and metabolite was also investigated. Figs. 2A and S10 show that the effect of flow rate is slight and depends on these substances. However, the possible effect of flow rate is still of interest. Reducing the flow rate to nanoliters per minute can simultaneously improve sensitivity and reduce matrix effects [24].

The chromatographic column is the core of the liquid



Interference occurrence ratio (%)

Total (60)	13.3	11.7	28.3	60.0	11.7	8.3	23.3	58.3	10.0	5.0	26.7	46.7	5.0	5.0	11.7	33.3
Drugs	13.3	10.0	13.3	53.3	6.7	0	10.0	43.3	10.0	3.3	13.3	30.0	3.3	3.3	6.7	16.7
Metabolites	13.3	13.3	43.3	66.7	16.7	16.7	36.7	73.3	10.0	6.7	40.0	63.3	6.7	6.7	16.7	50.0

Fig. 1. Signal suppression/enhancement between ten groups of drugs and corresponding metabolites ($n = 3$). (A) Signal suppression/enhancement between ten groups of drugs and corresponding metabolites on API 4000 mass spectrometry. (B) Signal suppression/enhancement between oxcarbazepine and its metabolites on TSQ-1 and TSQ-2 mass spectrometry. (C) The degree of signal suppression/enhancement and frequency of occurrence of all analytes grouped according to concentration ratio on the three instrument systems.

chromatography system that performs the separation role. Five columns commonly used in the laboratory were selected to study the separation of drugs and metabolites by isocratic elution. The results (Fig. 2B) show that all three groups of drugs and metabolites except the nicotine group present different degrees of column overlap under the five-column conditions. This indicates that similar chromatographic behavior due to the structural similarity of drugs and metabolites increases the probability of signal interference. In the dextromethorphan and oxcarbazepine groups, the degree of signal interference is more severe in the chromatographic analysis than in the FIA (Fig. S11). A possible reason for this is that the analytes eluted by chromatography result in concentration changes due to the diffusion effect of chromatography, which affects the degree of signal interference. These results show that signal interference between drugs and metabolites can also occur in analysis employing chromatography, and even that the degree of interference increases as a result (Fig. 2A).

In short, the concentrations of the drug and metabolite play an important role in their interference. The proportion of organic phase and columns perturbed the level of interference relatively strongly. In addition, the standardized regression coefficients of the factors under multiple linear regression also vary partly by compound. Therefore, the effect of physicochemical properties also deserves further analysis.

3.3. Explanation of signal interference between drugs and metabolites

To further investigate the pattern of signal interference between drugs and metabolites, we selected a series of concentrations of OXC and its monohydroxy derivative metabolite (MHD) to analyze their signal interference under FIA and LC analysis. The results (Fig. 3A) show that the OXC signal can be suppressed by high concentrations of MHD under FIA conditions, with a maximum suppression rate close to 80%. During LC analysis, the trend of OXC signal suppression by MHD is generally consistent with the above

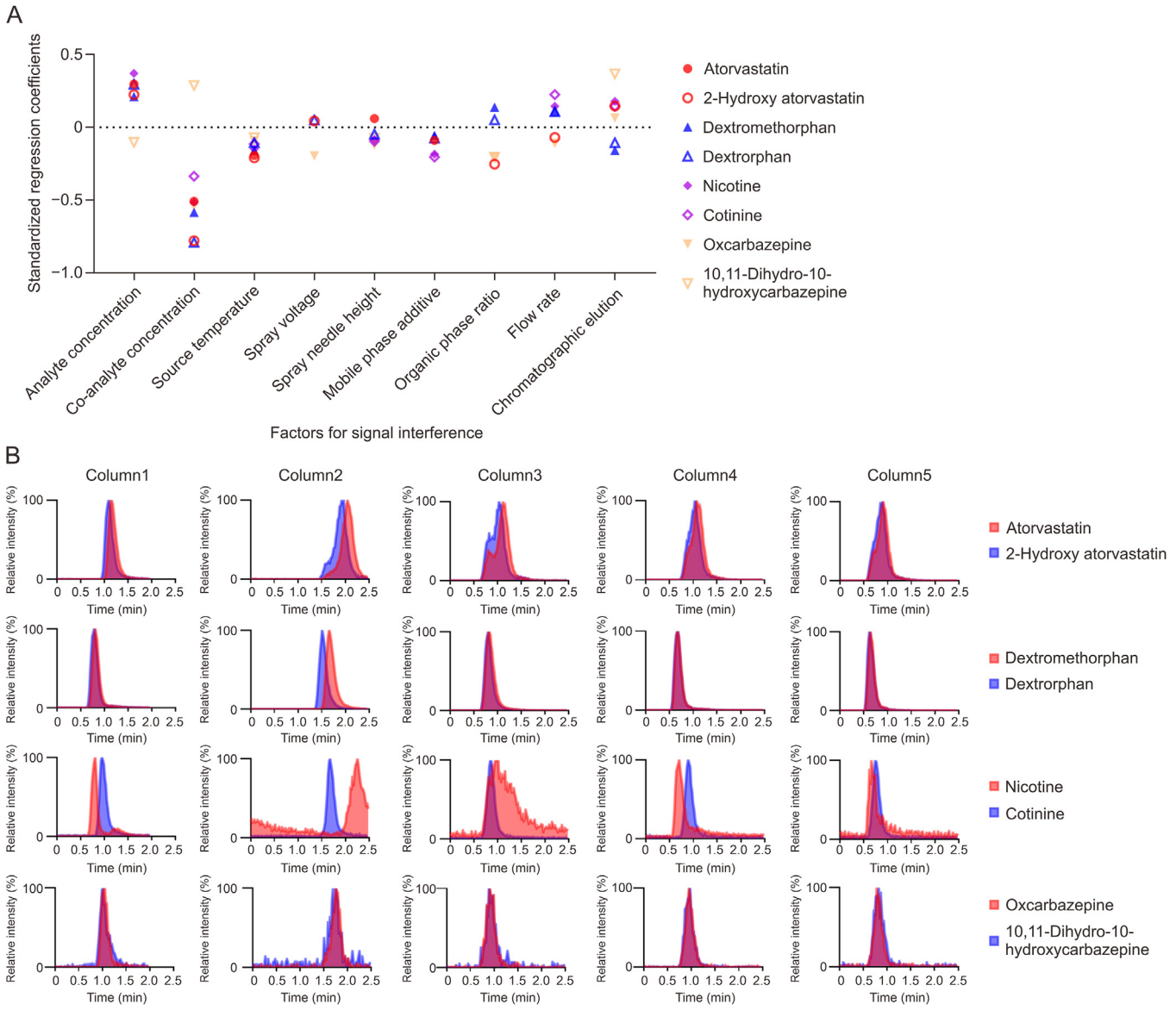


Fig. 2. Influence of liquid chromatography conditions and mass spectrometry parameters on signal suppression/enhancement between drugs and metabolites in four groups. (A) Standardized regression coefficients for each influencing factor of the eight substances under multiple linear regression. (B) Chromatographic behavior under five chromatographic Columns 1 to 5: C_{18} (50 mm × 2.1 mm, 3 μm; Thermo), C_{18} (100 mm × 2.1 mm, 3 μm; Thermo), C_{18} (50 mm × 2.1 mm, 3.5 μm; Waters), C_8 column (50 mm × 2.1 mm, 3.5 μm; Waters), and C_{18} column (50 mm × 2.1 mm, 2.6 μm; KINETEX).

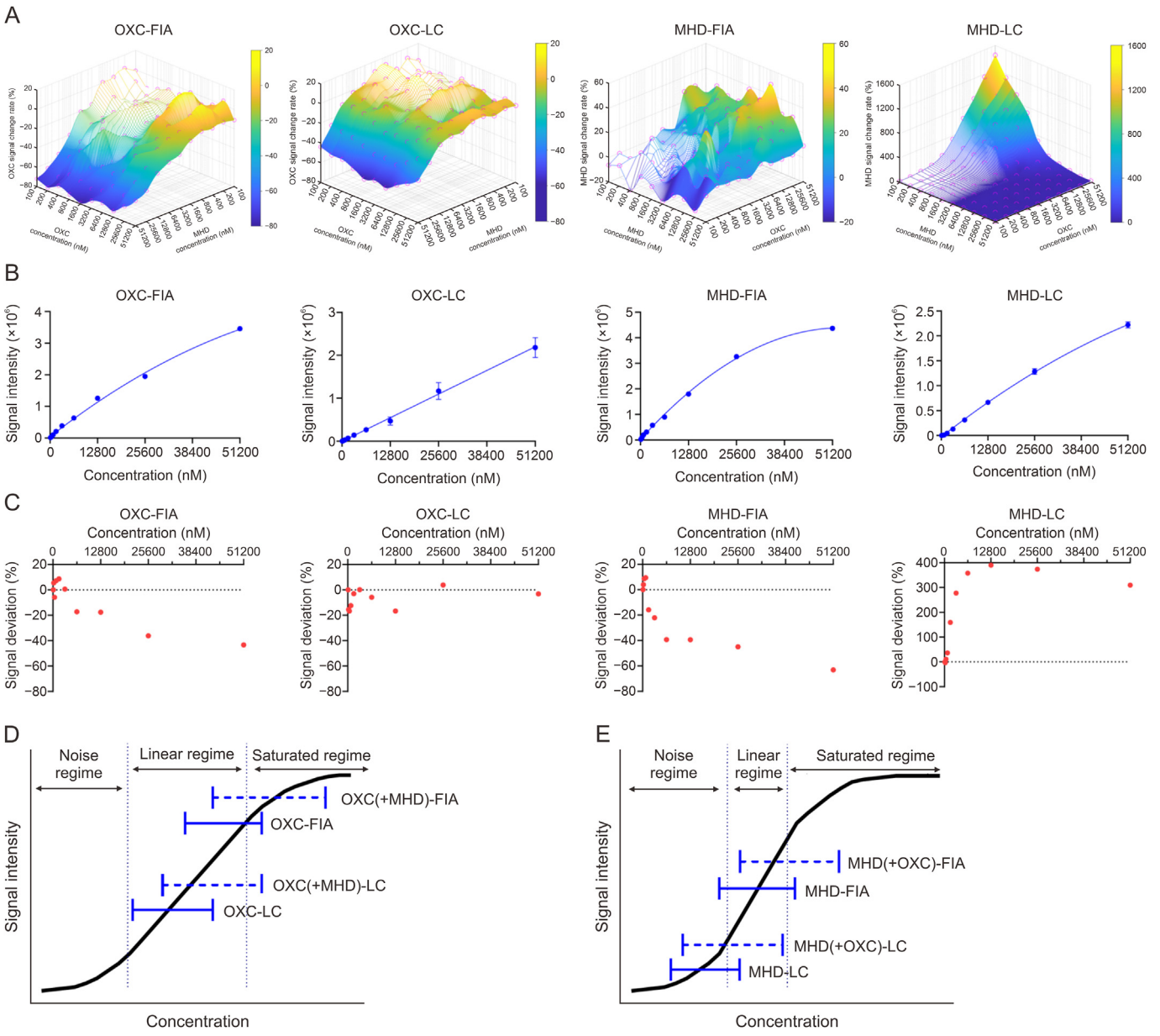


Fig. 3. Signal suppression/enhancement between oxcarbazepine (OXC) and its monohydroxy derivative metabolite (10,11-dihydro-10-hydroxycarbamazepine, MHD) at serial concentrations and their response-concentration relationships. (A) Signal suppression/enhancement between OXC and MHD in the concentration range of 100–51200 nM under flow injection analysis (FIA) and liquid chromatographic analysis (LC). (B) Response-concentration relationships for OXC and MHD in the concentration range of 100–51200 nM. (C) Deviation between response and theoretical values of OXC and MHD in the concentration range of 100–51200 nM. (D) Schematic diagram of signal interference under the response-concentration S curve of OXC. (E) Schematic diagram of signal interference under the response-concentration S curve of MHD.

results, but the degree of suppression is diminished. For MHD, the signal can be gradually enhanced with increasing OXC concentration up to 48% in FIA. When chromatography was used, the MHD signal was greatly enhanced by OXC. 100 nM of MHD can be affected by 51,200 nM of OXC resulting in a signal increase of 1,459%. In short, the interference of the OXC signal was predominantly suppressed, while the MHD signal in the same concentration range was enhanced, especially during the chromatographic analysis.

We further analyzed the signal of OXC and MHD in separate assays about concentration. When FIA was used, the OXC and MHD signals showed lower than linear theoretical values (Response also varies 2-fold) in the high concentration range of 25,600–51,200 nM and 3,200–51,200 nM, respectively. Moreover, the MHD signal in the low concentration range (100–400 nM) was slightly higher than the theoretical value. These results suggest that the linear signal of MHD is narrower in the concentration range than that of OXC. When LC analysis was employed, the signal intensity of both OXC and MHD was significantly reduced. For example, OXC and MHD responses at the lowest examined concentration (100 nM) were reduced by about 3-fold and 20-fold, respectively. The reason may be that the analytes undergo different degrees of concentration reduction due to the chromatographic elution process [25]. Moreover, the change in the response-concentration relationship also suggests that the analytes undergo concentration changes during the process (Figs. 3B and C).

According to the nonlinear signal theory of mass spectrometry, the analyte signal shows an *S*-curve about the concentration [26]. As the concentration increases, the analytes go through the signal process of noise background, linearity, and saturation in sequence

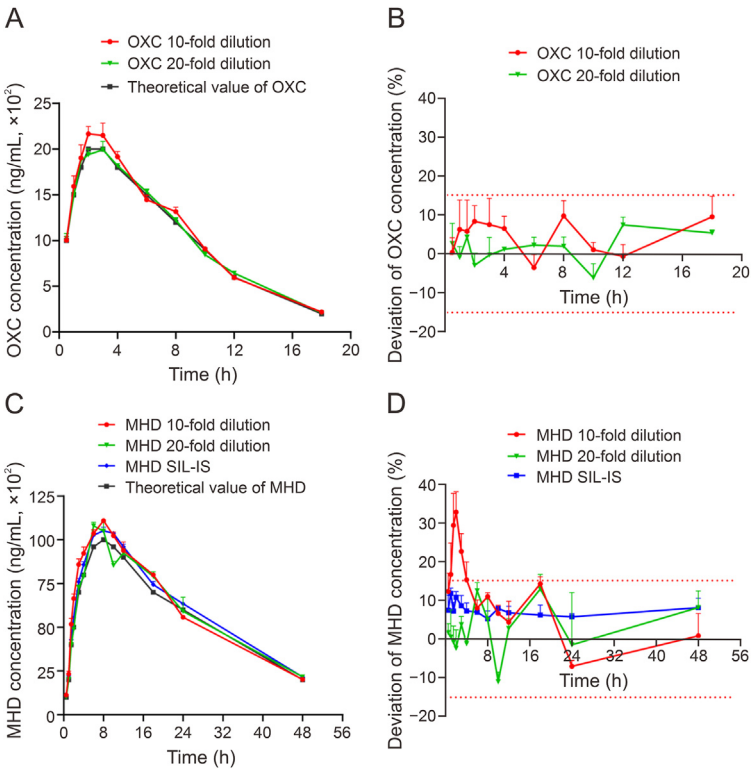


Fig. 4. Mean concentration-time curves of analytes in simulated samples obtained by different detection methods and deviations of the measured values ($n = 3$). (A) The mean concentration-time curves of oxcarbazepine (OXC) obtained by 10-fold dilution and 20-fold dilution methods. (B) The mean concentration-time curves of OXC monohydroxy derivative metabolite (10,11-dihydro-10-hydroxycarbamazepine, MHD) obtained by the stable isotope-labeled internal standard method, 10-fold dilution, and 20-fold dilution. (C) The deviation of the concentration measurement values of OXC obtained by 10-fold dilution and 20-fold dilution method. (D) The deviation of the concentration measurement values of MHD obtained by the stable isotope-labeled internal standard (SIL-IS) method, 10-fold dilution, and 20-fold dilution.

(Figs. 3D and E). Based on this theory, we hypothesize that the interaction mechanism between OXC and MHD is similar to the "cross suppression" between analytes and stable isotope-labeled internal standards [13]. The relationship between the drug or metabolite signal and their concentrations may be better described by the higher-order function when drugs or metabolites coexist, which makes their response-concentration relationship showing a sensitivity at higher concentration points. Because of the structural differences between drugs and metabolites, their contributions to each other's signals are not simply numerical superposition, and the degree of interference varies depending on the degree of their nonlinear signal. Therefore, the signals of OXC and MHD showed sensitivity characteristics at higher concentrations. The direction and degree of signal change are influenced by their nonlinear signal characteristics, causing OXC to show a suppressed signal and the MHD signal to be enhanced. In conclusion, when a drug or metabolite has a nonlinear response-concentration relationship, interference from the other side may change the signal sensitivity of the analyte. This variation in sensitivity may increase the risk of inaccurate quantification.

3.4. Impact on LC-ESI-MS bioanalysis

To exclude unknown interferences in test results caused by individual *in vivo* variation and complex substances in the matrix such as plasma, a series of simulated samples were obtained by adding known concentrations of working solutions to the solvent to simulate sample detection scenarios in pharmacokinetic studies. We used OXC and MHD as model drugs to analyze the impact of signal interference between drugs and metabolites on quantitative studies. Based on the reported blood concentrations of OXC and MHD, we set the concentration ranges of the calibration curves for OXC and MHD to 20–2,560 ng/mL and 200–25,600 ng/mL, respectively. However, the nonlinearity of the MHD signal is very pronounced and it is difficult to establish a calibration curve with a linearity coefficient by adjusting the dilution ratio, injection volume, and LC conditions. Therefore, two linear ranges of 200–6,400 ng/mL and 6,400–25,600 ng/mL were used to establish calibration curves to quantify MHD. The validation of the assay and the detection of simulated samples were done at the level of 10-fold dilution (Tables S3 and S4). The results showed that the OXC assay was accurate, with deviations of the measured concentrations within ±15%. However, the MHD signal could be enhanced by OXC, resulting in a maximum detection deviation of 32.9% (Fig. 4 and Table S5). This indicates that even if the calibration curve and quality control samples meet the requirements, the interference in the samples will make the relationship between the mass spectrum signal and the concentration of the analyte in the samples different from that in the calibration curve, leading to inaccurate determination of analyte concentrations in biological samples. The complex relationship between the degree of signal interference and the concentration of drugs and metabolites makes matrix-matched calibration more difficult to achieve. MHD is used as an antiepileptic active metabolite in the clinic, and its efficacy and adverse effects are usually controlled by blood concentration monitoring [27]. Therefore, MHD concentrations determined based on LC-ESI-MS need to be considered for bias from co-eluting OXC. And this further verifies the potential harm of signal interference between drugs and metabolites to quantitative analysis in LC-ESI-MS.

3.5. Strategies to solve signal interference between drugs and metabolites

3.5.1. Chromatographic separation

Chromatographic separation is an effective solution for signal interference between drugs and metabolites. In commonly used reversed-phase high performance liquid chromatography, the more polar metabolite usually elutes earlier than the parent compound. However, due to the structural similarities between drugs and metabolites, their effective separation requires longer analysis times and more complex conditions involving columns and gradients, which leads to increased difficulty in method development as well as decreased analytical efficiency. Fig. 2B shows that peak overlap between drug and metabolite may still exist to varying degrees even when different columns are used in generic LC conditions. We analyzed plasma samples from epileptic patients taking OXC using the gradient elution method and detected possible *in vivo* metabolites based on the reported quantitative ion pairs [28–31]. The results showed that OXC also co-eluted with other metabolites (Fig. S12). Obviously, the chromatographic separation requires a comprehensive and time-consuming consideration of elution gradient, composition and pH of mobile phase, flow rate, and column. In addition, drugs usually have more than one metabolite in vivo. All these make the chromatographic separation strategy limited.

3.5.2. Sample dilution

Dilution is usually a simple way to address matrix effects. The concentration inflection point for the nonlinear signal of MHD was approximately 600 ng/mL. After 10-fold dilution of the samples with 2,000 ng/mL OXC and 5,000 ng/mL MHD, the signal of MHD still increased by 32.9% due to the effect from OXC, which suggests that the sensitivity of MHD is greater than that of the sample at 500 ng/mL, and even crosses the inflection point concentration (Fig. 4 and Table S5). When a 20-fold dilution assay was used, the concentrations of OXC and MHD were well below 600 ng/mL even when superimposed. The results showed that no enhancement of the MHD signal occurred and its concentration deviated from the set value within ±15%. This indicates that dilution can resolve the effect of signal interference on quantitative analysis, provided that the sensitivity is satisfied.

3.5.3. Stable isotope-labeled internal standard

SIL-ISs are very similar to the analytes in structure and physicochemical properties. Their signal ratios are usually used in calibration curves to improve quantitative accuracy. We first investigated the ability of SIL-IS (MHD-d4) to correct the interference signal of MHD at four concentration levels under FIA and LC conditions. Table S6 shows that the calibration values of the signals are almost all between 85% and 115% of the theoretical values. We then measured simulated samples using MHD-d4 as an internal standard. The results (Table S5) further verified that the SIL-IS can correct for signal interference of OXC on MHD. In addition, SIL-IS can also correct for the nonlinear response of MHD, which is consistent with previous reports [13,32]. Therefore, SIL-IS can be an effective strategy to correct the signal interference between drugs and metabolites. However, some fields still lack the feasibility of applying SIL-ISs, such as quantitative metabolomics and pharmacokinetic evaluation of active compounds at the early stage of drug development, which may be limited by technology, time, cost, etc. Moreover, reports on some problems of SIL-ISs should be considered, such as the difference between deuterated internal standards and analyte polarity [33], the interference of SIL-ISs on metabolite quantification [34], the cross-suppression of SIL-ISs and analytes [13], and unreasonable internal standard concentrations that affect the linearity of calibration curves and the accuracy of detection [35].

3.6. Evaluation for signal interference between drugs and metabolites

An effective assessment of signal interference between drugs and metabolites in biological samples is necessary at the early stage of method establishment. Both standard addition and dilution methods were included in our initial evaluation protocol. However, for the standard addition method, if the concentration of the added standard solution is not similar to the concentration of the analyte in the biological sample, it is likely to introduce a large error and affect the assessment [36]. The unknown concentration of analytes in biological samples limits the application of this method. Therefore, this study explored the evaluation efficiency of the two algorithms based on the dilution method (Fig. 5). We validated the reliability of both evaluation algorithms using simulated samples from pharmacokinetic studies. The samples were diluted in 2-fold steps and back-calculated concentrations were calculated using the calibration curves. Fig. 5 presents the evaluation differences obtained at 2-fold and 4-fold dilutions using Algorithm 1. The values for OXC and MHD are in general agreement with the magnitude of their signal rate of change, and thus this method provides a good indication of the presence of signal interference. Algorithm 2 reported in the literature uses the relative standard deviation as the evaluation difference value (Equation (2)) [10]. We used the measured concentration of the sample and that after diluted 2-fold and 4-fold dilution to calculate this value. This evaluation has the potential for false negatives based on the 20% evaluation criterion. Furthermore, we also diluted the samples close to the lower limit of quantification to re-validate and compare the two algorithms. The results show that Algorithm 1 has higher evaluation accuracy based on the 20% determination criterion and

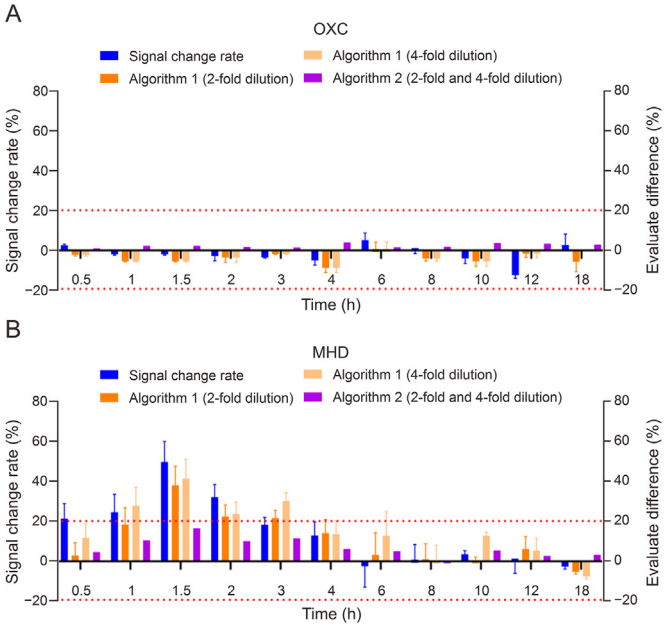


Fig. 5. Interference evaluation under a 10-fold dilution detection method for pharmacokinetic samples ($n = 3$). (A) The signal change rate of oxcarbazepine (OXC) and the corresponding evaluation difference values under the two evaluation methods. (B) The signal change rate of oxcarbazepine monohydroxy derivative metabolite (10,11-dihydro-10-hydroxycarbamazepine, MHD) and the corresponding evaluation difference values under the two evaluation methods. The samples were serially diluted twice to obtain 2-fold and 4-fold dilution samples.

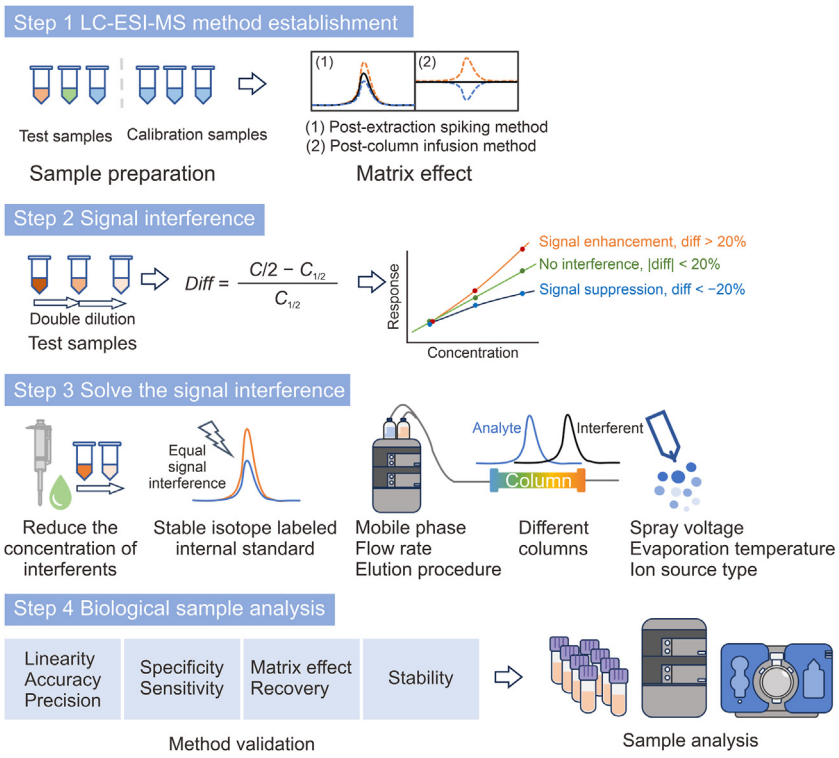


Fig. 6. Procedures for evaluating and resolving signaling interferences between drugs and metabolites in LC-ESI-MS quantitative method establishment.

Algorithm 2 requires a more suitable limit of difference values for evaluation (Fig. S13).

In summary, dilution methods can be used to assess signal interference of drugs and metabolites without knowing the concentration of the analyte in the sample, and the method is simple and easy to perform. Several studies have assessed signal interference by dilution in immunoassay [37,38] or mass spectrometry [8,39]. We compared two dilution-based algorithms and provided a solid research basis for signal interference assessment of drugs and metabolites in the early stage of method validation. A process framework was developed here to cope with such signal interferences (Fig. 6) to improve quantitative accuracy.

4. Conclusion

This study clarified the prevalence of signal interference between drugs and metabolites in LC-ESI-MS, which is due to the fact that drugs and metabolites are usually structurally similar and prone to co-elute. Based on the nonlinear theory, the concentration contribution of drugs or metabolites to each other made the response of the other more nonlinear and the signal deviated in a specific direction. Due to the structures of drugs and metabolites have slight difference, the interference effect on the response-concentration relationship of each other does not exhibit a strict summation of concentrations but rather tends to involve a multi-order function of the nonlinear response of the analyte and the concentration of the interfering substance acting together. The complex relationship makes it difficult to predict and correct the deviation of the signal under interference. This is undoubtedly harmful to the quantitative analysis of biological samples. In order to improve the quantitative accuracy, we proposed a simple and feasible evaluation method for possible interference with unknown samples diluted in appropriate proportion. Given the advantages of SIL-ISs in correcting for signal interference, we recommend this method as the preferred choice. If this is not feasible, chromatographic separation and dilution strategies can be considered to eliminate or attenuate the signal interference of drugs and metabolites. This study provided a more comprehensive consideration for the development and application of LC-ESI-MS-based methods for in vivo pharmaceutical analysis to ensure quantitative accuracy.

CRediT author statement

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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