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Using a postcolumn-infused internal standard for correcting the matrix effects of urine specimens in liquid chromatography–electrospray ionization mass spectrometry

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ABSTRACT

Matrix effects (MEs) are a major problem affecting the quantitative accuracy of liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) when analyzing complicated samples. While analyzing urine specimens, the wide diversity of endogenous materials and different urine concentrations may result in inaccurate quantification. In this study, we propose a postcolumn-infused internal standard (PCI-IS) strategy for universal correction of MEs in urine specimens. MEs can be effectively corrected by dividing the target analyte signal intensity by the PCI-IS intensity. To evaluate the performance of PCI-IS, we used 6 benzodiazepine (BZD) drugs in 5 different concentrations of urine matrixes as a test model. The divergence of the BZD drug signal responses in 5 different urine matrixes was expressed using their respective coefficients of variation (CV) to evaluate the efficiency of using PCI-IS in correcting matrix effects. The CV of the BZD drug signal intensities in these 5 different concentrations of the urine matrixes were reduced from 10 to 30% to less than 10% when the PCI-IS correction method was employed. When the PCI-IS method was used to correct the 6 BZDs in 25 real human urine samples, over 90% of the test results exhibited quantification errors of less than 20%, and all of the test results had quantification errors of less than 30%. These results demonstrate that the PCI-IS method can resolve the problem of inaccurate quantification that arises from the diversity of urine specimens. The PCI-IS method is particularly useful for clinical analysis or forensic toxicology to improve the quantification accuracy in an economical way.

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1. Introduction

Over the past decade liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS) has become a versatile analytical tool. The high sensitivity and selectivity of LC–MS render it a powerful tool for analyzing complicated samples. Chromatographic separation and sample preparation are often minimized to meet the demands of high-throughput analysis [1]. Matrix effects (MEs) are a major problem affecting the quantitative accuracy of the analysis of complicated samples, especially in fast analysis with little separation. Compounds that coelute during LC may cause ion enhancement or ion suppression in ESI and may thus result in quantification errors [2,3]. Because MEs can seriously impact the limit of detection, limit of quantification, linearity, accuracy, and precision, they have been recently discussed in several review articles [4,5].

Urine specimens are one of the most frequently encountered biological fluids in the bioanalytical lab. One of the main challenges in the quantification of chemicals in urine specimens originates from the great diversity of urine concentrations between individuals. Urine concentration is significantly affected by an individual's diet, water uptake, and also the time at which the sample is taken. The large differences in individual urine concentrations result in high variations in matrix effects encountered in different urine specimens. Several approaches have been proposed to reduce MEs, such as diluting samples, employing sample purification procedures, and improving LC separation [2,5]. These approaches suffer from reduced sensitivity or increased analytical time. Another approach for decreasing MEs is the use of ionization techniques that are less affected by MEs, such as atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) [6,7]. However, sensitivity may be sacrificed, and alternative ionization sources may not always be available. Internal standards (ISs) are regularly used to correct the signal alteration arising from MEs. Structural analogs of the target analyte are generally selected as internal standards because they possess similar physicochemical properties to the target analyte. However, because of the different retention times of target analytes and ISs, different coeluting

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compounds may be encountered, leading to poorly corrected results. A stable isotopically labeled internal standard (SIL-IS) that coelutes with the analyte can overcome this problem and is considered to be an ideal internal standard [8]. Even though SIL-ISs have been used extensively, it have been noticed that SIL-IS may have different retention times than the target analyte due to the deuterium isotope effect [7,9,10]. Other problems, such as the isotopic purity of the SIL-IS and cross-talk between MS/MS channels, should also be noted [11]. Most importantly, SIL-ISs are generally expensive and may not be commercially available. Thus, a more economical and effective method is required for overcoming MEs.

Postcolumn introduction of an internal standard in LC–MS/MS has been originally proposed by Bernard et al. to correct quantitative errors associated with matrix signal suppression [12,13]. Stahnke et al. later modified this method for analyzing pesticides. Each sample must be analyzed twice when applying their method (with or without post-column infusing these target analytes) to calculate the MEs at each retention time [14]. In addition, direct using the target analytes to calculate MEs may face problems if true blank extract is unavailable.

Benzodiazepines are a class of CNS-depressants that are frequently used to treat sleeping disorders, anxiety, increased muscle tone and epilepsy [15]. However, these drugs have been increasingly abused and have caused drug-facilitated sexual assault and traffic accidents over the past few years [15–17]. Therefore, BZD drugs are currently categorized as controlled drugs in many countries. In this study we propose a postcolumn-infused internal standard (PCI-IS) method for correcting the MEs of urine specimens in LC–ESI-MS. We selected BZD drugs as our test chemicals. The MEs of urine specimens were corrected by dividing the target analyte signal intensity by the PCI-IS signal intensity. The most important parameters for improving quantification accuracy were investigated. The performance of the PCI-IS method was compared with the traditional IS and SIL-IS methods. Then, the quantification accuracy of PCI-IS correction was determined by spiking 6 BZD drugs into 25 real human urine samples for evaluating the applicability of the PCI-IS method.

2. Experimental

2.1. Chemicals

Nitrazepam, flurazepam, diazepam, estazolam, temazepam, flunitrazepam, flunitrazepam-d7, and nordiazepam were purchased from Cerilliant (Round Rock, TX, USA). Hexakis(1H, 1H, 3H-perfluoropropoxy)phosphazene (HKP) was purchased from Apollo Chemical (Graham, NC). Acetonitrile (ACN) was obtained from J.T. Baker (Phillipsburg, NJ). MS-grade methanol was purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain). Tetrakis(decyl)ammonium bromide (TKDA), tetramethylammonium iodide (TMA), and formic acid solution were purchased from Sigma (St. Louis, MO, USA). All reagents and solvents were of either analytical or chromatographic grade.

2.2. Sample preparation

Protein was precipitated by mixing 40 μL of a urine sample with 160 μL of methanol. The deproteinized sample was centrifuged at 10,000 × g for 15 min, and the supernatant was then filtered through a 0.22-μm PP membrane (RC-4, Sartorius, Göttingen, Germany) before UPLC–ESI-MS analysis. To prepare the urine used to construct test models, pooled urine samples were diluted with deionized water to generate 20, 40, 60, 80, and 100% urine solutions. All of the BZD drugs were spiked at 10, 50, 150, 250, 500 ng mL^{-1}.

The 25 real urine samples used to test the quantification accuracy of the developed adjustment method were collected from 4 healthy volunteers at different time points.

2.3. UHPLC–ESI-MS system

LC analyses were performed using an Agilent 1290 UHPLC system equipped with a binary solvent pump, an autosampler, a sample reservoir, and a column oven (Agilent Technologies, Waldbronn, Germany). Postcolumn infusions were accomplished with an Agilent 1260 quaternary solvent pump. The mass spectrometric analysis was performed using an Agilent 6460 triple quadrupole system (Agilent Technologies, Waldbronn, Germany). A Kinetex C18 2.1 × 50 mm (2.6 μm) column (Phenomenex, Torrance, USA) was employed for separations.

The mobile phase consisted of 0.1% aqueous formic acid (solvent A) and 0.1% formic acid in ACN (solvent B). A 0.3 mL min^{-1} linear gradient elution was used: 0–2 min, 20–95% B; 2–3 min, 95% B; and 3–4.5 min, column re-equilibration with 20% B. The sample reservoir and column oven were maintained at 4 °C and 40 °C, respectively. The injection volume was 5 μL. Positive electrospray ionization mode was utilized with the following parameters: a 325 °C dry gas temperature, a 7 L min^{-1} dry gas flow rate, a 45 psi nebulizer pressure, a 325 °C sheath gas temperature, an 11 L min^{-1} sheath gas flow rate, a 3500 V capillary voltage, and a 500 V nozzle voltage. MS acquisition was executed in multiple reaction monitoring (MRM) mode. The transitions for HKP, TKDA, TMA, flurazepam, flunitrazepam-d7, flunitrazepam, temazepam, estazolam, diazepam, nitrazepam, and nordiazepam were m/z 922.0 → 790.1, 578.7 → 310.3, 74.1 → 59.1, 388.2 → 134, 321.1 → 246.1, 314.1 → 183, 301.1 → 106, 295.1 → 205.1, 285.1 → 193, 282.1 → 152, and 271.1 → 140, respectively.

PCI-ISs were dissolved in ACN at 100 ng mL^{-1} and introduced into the ESI interface at a 0.1 mL min^{-1} flow rate.

2.4. PCI-IS method

The PCI-IS method used a postcolumn infusion strategy to correct MEs in ESI-MS. The MEs at each time point were measured using a postcolumn-infused internal standard (PCI-IS). Using the PCI-IS response changes, the degree of ion suppression (or ion enhancement) at each time point allowed MEs to be calculated. The basic concept of the PCI-IS method is described by Eq. (1).

$$\frac{R_{\text{analyte},x}}{R_{\text{PCI-OS},x}} = \frac{A_{\text{analyte},x} * C_{\text{analyte},x}}{A_{\text{PCI-IS},x} * C_{\text{PCI-IS},x}} \quad (1)$$

where A represents the ability of the analyte to generate signals, which is determined by the physicochemical properties (e.g., pK_a, proton affinity, hydrophobicity, and hydrophilicity) of the analyte. A is influenced by the surrounding ionization conditions (e.g., mobile phase viscosity, surface tension, and nonvolatile components in the coeluent). $R_{\text{analyte},x}$ and $R_{\text{PCI-IS},x}$ represent the responses of the analyte and the PCI-IS at time point x. C represents the concentration.

When Eq. (1) was applied, $C_{\text{PCI-IS},x}$ was held constant. If a suitable PCI-IS is selected to ensure similar $A_{\text{analyte},x}$ and $A_{\text{PCI-IS},x}$, the analyte to PCI-IS response ratios should be proportional to the analyte concentration (Eq. (2)):

$$\frac{R_{\text{analyte},x}}{R_{\text{PCI-IS},x}} \propto (C_{\text{analyte},x}) \quad (2)$$

We used Eq. (2) to adjust the analyte signal intensity and calculate the analyte concentration in LC–ESI-MS. For each chromatogram, the analyte to PCI-IS signal intensity ratio at each time point was utilized to generate a new corrected chromatogram.

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