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Systematic evaluation of matrix effects in hydrophilic interaction chromatography *versus* reversed phase liquid chromatography coupled to mass spectrometry

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ABSTRACT

Reversed phase liquid chromatography (RPLC) coupled to mass spectrometry (MS) is the gold standard technique in bioanalysis. However, hydrophilic interaction chromatography (HILIC) could represent a viable alternative to RPLC for the analysis of polar and/or ionizable compounds, as it often provides higher MS sensitivity and alternative selectivity. Nevertheless, this technique can be also prone to matrix effects (ME). ME are one of the major issues in quantitative LC–MS bioanalysis. To ensure acceptable method performance (*i.e.*, trueness and precision), a careful evaluation and minimization of ME is required. In the present study, the incidence of ME in HILIC–MS/MS and RPLC–MS/MS was compared for plasma and urine samples using two representative sets of 38 pharmaceutical compounds and 40 doping agents, respectively. The optimal generic chromatographic conditions in terms of selectivity with respect to interfering compounds were established in both chromatographic modes by testing three different stationary phases in each mode with different mobile phase pH. A second step involved the assessment of ME in RPLC and HILIC under the best generic conditions, using the post-extraction addition method.

Biological samples were prepared using two different sample pre-treatments, *i.e.*, a non-selective sample clean-up procedure (protein precipitation and simple dilution for plasma and urine samples, respectively) and a selective sample preparation, *i.e.*, solid phase extraction for both matrices. The non-selective pretreatments led to significantly less ME in RPLC *vs.* HILIC conditions regardless of the matrix. On the contrary, HILIC appeared as a valuable alternative to RPLC for plasma and urine samples treated by a selective sample preparation. Indeed, in the case of selective sample preparation, the compounds influenced by ME were different in HILIC and RPLC, and lower and similar ME occurrence was generally observed in RPLC *vs.* HILIC for urine and plasma samples, respectively. The complementary of both chromatographic modes was also demonstrated, as ME was observed only scarcely for urine and plasma samples when selecting the most appropriate chromatographic mode.

1. Introduction

Liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) belongs to the state-of-the-art analytical techniques widely used for quantitative approaches in bioanalysis due to its high selectivity and sensitivity, as well as its high throughput. However, when using electrospray ionization (ESI), LC–MS/MS can be prone to significant matrix effects (ME). The latter is defined as signal modification of a chromatographic peak height/area (*i.e.*, suppression or enhancement) caused by the presence in the biological matrix of endogenous compounds, such as proteins, phospholipids, and salts, and/or of exogenous substances that may be introduced during sample preparation [1–4]. These compounds may coelute with the analytes of interest and thus affect their ionization, leading to a possible detrimental effect on the overall method precision, trueness, as well as sensitivity [5,6]. ME depend on the nature of the biofluid (*i.e.*, urine, plasma, saliva, tissue, *etc.*), the physico-chemical properties of the analytes of interest, the

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sample clean-up procedure, the separation conditions as well as the ionization source and its geometry [3,5,7,8]. The ME issue concerns not only biological samples, but almost all type of complex matrixes analyzed by LC–MS, such as food and environmental samples [9,10].

Two approaches are commonly used to assess ME, namely the post-column infusion and the post-extraction addition. The post-column infusion is a qualitative approach first proposed by Bonfiglio et al. in 1999 [7], which consists in infusing the compound of interest between the separation column and the ionization source *via* a tee connection, leading to a constant MS baseline signal. A prepared blank matrix is then injected into the column. In case of ME, the MS signal is altered in given regions of the chromatogram where ion suppression or enhancement occurs, affecting the performance of the quantitative method. Since ME are analyte-dependent, post-column infusion experiments have to be performed individually for targeted compounds, which remains tedious in case of multi-target approaches.

The post-extraction addition approach has been first proposed by Matuszewski et al. [6] and is based on a quantitative signal comparison between a blank matrix spiked with compound(s) of interest after the sample clean-up *vs.* a neat standard.

It is commonly assumed that the use of a systematic correction with stable isotopically labeled internal standards (IS) represents the first choice to correct ME, provided that the repeatability between matrix batches is ≤15% [11]. However, these standards may not be available for all targeted compounds or too expensive, especially in case of multi-target quantitation approaches. Therefore, different approaches can be considered to circumvent ME, *i.e.*, (i) an improved and more selective sample clean-up procedure, (ii) the use of another ionization technique such as atmospheric pressure chemical ionization, and (iii) the modification of the chromatographic conditions or the use of an alternative chromatographic mode.

Hydrophilic interaction chromatography (HILIC) is nowadays considered as a viable alternative to reverse-phase liquid chromatography (RPLC) for the analysis of polar and/or ionizable compounds in bioanalysis [12–14]. HILIC involves the use of a polar stationary phase and an aqueous-polar organic solvent mobile phase containing between 60 and 95% of an aprotic organic solvent miscible with water, usually acetonitrile (ACN). The retention mechanism is mainly based on hydrophilic partitioning of polar compounds between a water-enriched layer formed at the surface of the stationary phase and the highly organic mobile phase. Depending on the nature of both stationary and mobile phases, as well as the physico-chemical properties of the analytes, additional interaction mechanisms can occur besides hydrophilic partitioning, including hydrogen bonding, dipole–dipole interactions, and ionic interactions [15–18]. In the context of bioanalysis, HILIC–MS/MS presents two main advantages. First, the largely organic mobile phase is particularly well suited for ESI-MS detection, leading to a substantial improvement of sensitivity for a large variety of compounds [19,20]. Second, when an organic protein precipitation (PP) procedure or solid-phase extraction (SPE) is applied to the sample prior to the injection, the eluate can be directly analyzed in HILIC without any tedious and time-consuming evaporation and reconstitution in a highly aqueous solvent, unlike in RPLC mode.

The impact of ME observed in RPLC–MS/MS for biological matrices has been already well documented, while only few publications have investigated ME in HILIC–MS/MS [3,21–23], most of them using the post-column qualitative infusion method.

This comparative study consists of the systematic evaluation of ME in HILIC and RPLC conditions using two different biological matrices *i.e.*, plasma and urine. Matuszewski's quantitative approach [6] was used to assess the ME of a representative set of 38 drugs of abuse and pharmaceutical compounds in plasma samples, as well as 40 doping agents present in the Prohibited List edited by the World Anti-Doping Agency in urine samples. Firstly, the optimal generic chromatographic conditions in terms of selectivity with respect to interfering endogenous compounds were determined for both chromatographic modes using ultra-high pressure LC combined to high-resolution MS (UHPLC-HRMS) platform. For this purpose, three different stationary phases were tested in each chromatographic mode with mobile phases covering a large pH range, *i.e.*, pH 3 and 6, as well as pH 9 for BEH phases. Using the best generic chromatographic conditions for each mode, ME were then evaluated by targeted UHPLC–MS/MS analysis. Samples were subjected to two different clean-ups, *i.e.*, two procedures that are considered as non-selective (PP and simple dilution for plasma and urine samples, respectively) and a selective sample preparation (SPE for both matrices). Finally, the advantages and drawbacks of HILIC and RPLC according to the sample clean-up were critically discussed.

2. Experimental

2.1. Chemical and reagents

Water was obtained from a Milli-Q Water Purification System from Millipore (Bedford, MA, USA). Acetonitrile (ACN), methanol (MeOH), formic acid and acetic acid were of UHPLC–MS grade and purchased from Biosolve (Valkenswaald, Netherlands). Ammonium hydroxide (28%, m/v) was from Sigma–Aldrich (Buchs, Switzerland).

A 10 mM formate buffer was prepared with an adapted volume of formic acid and the pH was adjusted to 3.0 with ammonium hydroxide. A 10 mM acetate buffer was prepared with an adapted volume of acetic acid and the pH was adjusted to 6.0 with ammonium hydroxide. A 10 mM ammonium buffer 10 mM was prepared with an adapted volume of ammonium hydroxide and pH was adjusted to 9.0 with formic acid.

2.2. Compounds

2.2.1. Plasma samples

The training set used in the comparative experiments involving plasma was composed of 38 basic compounds covering a range of pK_a values between 6 and 11 with only rare exceptions in the 2–6 range, and log *P* from −0.3 to 4.7, and included the following drugs: alprazolam, alprenolol, amphetamine, atenolol, benzoylecgonine, bupropion, clonazepam, cocaine, codeine, dextromethorphan, dextropropoxyphene, dextrorphan, doxepin, fentanyl, imipramine, maproptiline, methamphetamine, 3,4-methylenedioxyamphetamine (MDA), 3,4-methylenedioxyethamphetamine (MDEA), 3,4-methylenedioxymethamphetamine (MDMA), mescaline, methadone, methylphenidate, metoprolol, mianserin, midazolam, naltrexone, norcocaine, nortriptyline, noscapine, papaverine, pethidine, prazepam, propranolol, theophylline, tramadol, trimipramine and verapamil. All compounds were purchased from Sigma–Aldrich (Steinheim, Germany) or Lipomed (Arlesheim, Switzerland). Stock solutions of each individual analyte were prepared at a concentration of 1 mg/mL in pure MeOH.

These drugs were chosen for plasma sample, as they are often analyzed in this biological fluid in toxicological analysis.

2.2.2. Urine samples

A training set of 3 neutral and 37 basic compounds was employed for the determination of ME in urine, mainly covering pK_a values between 6 and 11 with rare exceptions, and log *P* between 0.1 and 2.6, and included the following compounds: amiloride, amphetamine, benzoylecgonine, benzylpiperazine, buprenorphine, bupropion, chlorphentermine,