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Major signal suppression from metal ion clusters in SFC/ESI-MS - Cause and effects

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ABSTRACT

The widening application area of SFC-MS with polar analytes and water-containing samples facilitates the use of quick and simple sample preparation techniques such as "dilute and shoot" and protein precipitation. This has also introduced new polar interfering components such as alkali metal ions naturally abundant in e.g. blood plasma and urine, which have shown to be retained using screening conditions in SFC/ESI-TOF-MS and causing areas of major ion suppression. Analytes co-eluting with these clusters will have a decreased signal intensity, which might have a major effect on both quantification and identification. When investigating the composition of the alkali metal clusters using accurate mass and isotopic pattern, it could be concluded that they were previously not described in the literature. Using NaCl and KCl standards and different chromatographic conditions, varying e.g. column and modifier, the clusters proved to be formed from the alkali metal ions in combination with the alcohol modifier and make-up solvent. Their compositions were $[(XOCH_3)_n + X]^+$, $[(XOH)_n + X]^+$, $[(X_2CO_3)_n + X]^+$ and $[(XOOCOCH_3)_n + X]^+$ for X = Na^+ or K^+ in ESI+. In ESI−, the clusters depended more on modifier, with $[(XCl)_n + Cl]^-$ and $[(XOCH_3)_n + OCH_3]^-$ mainly formed in pure methanol and $[(XOOCH)_n + OOCH]^-$ when 20 mM NH_4Fa was added.

To prevent the formation of the clusters by avoiding methanol as modifier might be difficult, as this is a widely used modifier providing good solubility when analyzing polar compounds in SFC. A sample preparation with e.g. LLE would remove the alkali ions, however also introducing a time consuming and discriminating step into the method. Since the alkali metal ions were retained and affected by chromatographic adjustments as e.g. mobile phase modifications, a way to avoid them could therefore be chromatographic tuning, when analyzing samples containing them.

1. Introduction

Currently, there is an ongoing transformation of supercritical fluid chromatography (SFC), from a niche technique used for e.g. chiral and preparative scale separations, to a more widely accepted analytical tool [1]. Several vendors have in recent years launched new improved instruments on the market, with increased robustness and precision. The use of CO_2 in combination with polar organic solvents as methanol, which in turn allows a higher addition of water [2] has increased the solubility of polar compounds, and broadened the area of application. This also gives the possibility to inject water-containing samples, which recently has been both evaluated [3,4] and applied in several methods [5–7]. Even if the addition of water to the sample medium does not provide the optimal chromatographic conditions, it enables a "quick and easy" sample preparation for e.g. urine (dilute and shoot) and blood plasma/serum (injection directly after protein precipitation). Recently published methods include the analyses of polar urinary metabolites in metabolomics studies [8], amino acids in blood serum [7], urine samples for doping control [6], vitamins in blood plasma [9] and cannabinoids in wastewater [10]. Together, these examples show the widening application field for SFC, often with polar analytes and complex matrices.

With modern instrumentation, the hyphenation to mass spectrometry (MS) has also grown in importance. The interface to SFC for the two dominating ionization techniques electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were recently evaluated [11,12]. However, both these ionization techniques are known to be influenced by co-eluting interferences, altering the signal intensity of

Abbreviations: ACN, acetonitrile; ME, matrix effect; MeOH, methanol; PCIMP, post column infusion matrix profile

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the analyte. This alteration is known as matrix effects (ME) and has so far been studied using different ionization sources coupled to LC, finding ESI as the more affected one of them [13,14].

In a recent study by the authors, comparing ME for LC/ESI-MS and SFC/ESI-MS, several metal ion clusters were identified as the source of major ion suppression using SFC/ESI-MS and common screening conditions analyzing blood plasma and urine samples after a quick and simple sample preparation [15]. Although the existence of e.g. $[(NaCl)_n + Na]^+$ clusters in ESI/MS has been known for a long time [16], a description of the clusters matching our observations [15] could not be found in present literature. The alkali metal ions observed in our previous study did also have different retention times, demonstrating an interaction with the chromatographic system. Even if sodium and potassium ions are naturally abundant in many biological sample matrices, alkali metal ions have in general not been introduced into the SFC instruments historically, as organic solvents have normally been used as the injection medium, with some exceptions [17]. Separation of alkali metal ions using SFC with CO_2 and methanol has been described previously, but to the authors' knowledge only through the use of added chelators and other compounds forming complexes [18,19].

The aim of this study was to investigate the alkali metal clusters causing ion suppression in SFC/ESI-MS: how they arise, what they consist of, their impact on the MS detection and how to possibly avoid them.

To the authors' knowledge, this is the first time these clusters are described, both the source of their components and their impact on the MS signal. It is also shown that the alkali metal ions can be separated using SFC without chelators or similar complex binders.

2. Materials and methods

2.1. Chemicals and reagents

Ammonia solution (4.0 M in methanol), methanol (Fluka Chromasolv, LC-MS grade), sodium chloride (≥99.5), amiloride hydrochloride-hydrate (≥98%), atenolol (≥98%), enalapril maleate (≥98%), metoprolol tartrate (≥99%), meloxicam-sodium hydride (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ammonia chloride (pro analysis) and potassium chloride (pro analysis) were obtained from Merck (Darmstadt, Germany). Acetonitrile (LC-MS grade), ethyl acetate (HPLC grade) and sodium hydroxide pellets (laboratory reagent grade) were purchased from Fischer scientific (Pittsburgh, PA, USA). Ethanol (99.7% European Pharmacopeia) was obtained from Solveco (Rosersberg, Sweden). Water was obtained from a Milli-Q Q-POD-system from Millipore (Billerica, MA, USA). Carbon dioxide (purity 99.99%) was obtained from Air Liquide (Paris, France). Metanol-d4 (D: 99.8%) and deuterium oxide (D 99.9%) were purchase from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2. Standards and sample preparation

Stock solutions of NaCl and KCl were prepared by dissolving the salts using the appropriate sample solvent, generally acetonitrile:water 3:1 (v/v), to obtain 100 mM solutions.

The stock solution of meloxicam was diluted with acetonitrile:water 3:1 (v/v), while atenolol, amiloride, enalapril and metoprolol were diluted in methanol. Solutions containing drug compounds were kept in freezer (−26 °C) when not used, whereas alkali-salts were kept at room temperature.

The horse urine (surplus blank horse urine from National Veterinary Institute, Uppsala, Sweden) was kept in a freezer (−26 °C) until use. The samples were prepared using the dilute-and-shoot approach: after thawing in a water bath, 100 µL were transferred to a 1.5 mL Eppendorf tube and diluted with 900 µL acetonitrile:water 3:1 (v/v), followed by centrifugation 10 min (12,100*g*). After centrifugation, 200 µL of the supernatant were transferred to vials for analysis. When comparing dilution solvents, the urine was diluted with 1:1, 1:3 (v/v) acetonitrile:water or pure acetonitrile, in triplicates for each treatment.

The liquid-liquid extraction was performed using the following method: Triplicates of 100 µL horse urine were mixed with 100 µL 0.1% NaOH (w/v) and then extracted with 1.4 mL ethyl acetate by vortexing 30 s. After 5 min centrifugation (2504*g*), the organic layer were transferred to new glass sample tubes, and the solvent was evaporated at 30 °C under a gentle stream of nitrogen gas. When dry, the samples were re-dissolved in 1.0 mL of acetonitrile:water 3:1 (v/v).

2.3. Instrumentation and SFC-MS conditions

The SFC/ESI-Q-ToF instrument consisted of an Acquity UPC^2 connected to a Synapt G2-S Q-ToF both from Waters Corporation (Milford, MA, USA). A 515 HPLC-pump (Waters) added a post column flow of methanol (if not stated otherwise) at a flowrate of 0.2 mL/min through a T-connection, prior to a second T-connection splitting the flow between the back-pressure regulator and the MS, see supplemental, Fig. S-1. Three different analytical columns were used: a Torus 2-PIC (2-picolyl-amine) 1.7 µm, 100 × 3.0 mm, an Acquity UPC^2 BEH 1.7 µm, 3.0 × 100 mm and a C18 XTerra RP18, 3.5 µm, 3.9 × 100 mm, all three from Waters. The column temperature was kept at 50 °C, the flow-rate was generally 1.0 mL/min except for the C18 column, where 1.5 mL/min was used in some experiments to shorten the analysis time. The back-pressure was 150 bar and 4 µL was generally injected using partial loop with needle overfill-mode. As sample manager weak wash, 500 µL of 2-propanol were used, with 1500 µL 4:1 (v/v) methanol:water as strong wash to reduce carry-over of the injected salts. The injection solvent used was acetonitrile:water 3:1 (v/v).

Several different mobile phases were used, all with CO_2 in combination with an organic solvent as modifier, generally methanol with the addition of 20 mM ammonium formate (NH_4Fa) and 2% (v/v) H_2O. When this modifier was used, the ammonium formate additive was prepared by dissolving the salt in pure water (1 M), and thereafter diluting to the desired concentration in methanol. The modifier was altered between the use of methanol, ethanol and acetonitrile, pure or in combinations, with or without the addition of 20 mM ammonium formate or ammonia, or different percent of water.

The gradient was occasionally varied, but if not stated otherwise it started with 10% modifier, and went to 45% from 0 to 6 min linearly, holding at 45% between 6 and 8.5 min before equilibration at the starting condition of 10% modifier 8.5–10 min.

The Q-ToF/ESI MS system was used in both positive and negative ionization mode, scanning from *m/z* 50 to 1200 using MS^E. Using this scan-mode, the scans were altered between the use of low collision energy and every second scan with high collision energy (20 eV in transfer together with a ramp of 20–40 eV in the trap) for the Q-ToF. The scan time was set to 0.2 s, data were collected in resolution mode, and stored as centroid data. In ESI+, the capillary voltage was set to 0.8 kV, the cone voltage to 40 V, the source temperature to 120 °C, and the nitrogen desolvation gas flow to 800 L/h with a temperature of 650 °C. In ESI−, a capillary voltage of −2.0 kV was used, with remaining settings as for ESI+.

Instrument control and data evaluation for all instruments were performed using the Mass-Lynx software (version 4.1, from Waters).

2.4. Matrix effect determination

The matrix effect (ME) was evaluated using post-column infusions and thereafter generation of infusion matrix profiles, as first described by Stahnke et al. [20]. Amiloride, atenolol, metoprolol and enalapril were added to a concentration of 15 nM into the make-up solution to perform the post-column infusion. Three injections of sample and blank were then analyzed, and the *m/z* corresponding to $[M + H]^+$ of the four compounds were extracted using a 0.05 Da window. Thereafter, the extracted chromatograms were smoothed to gain the mean intensity

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