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Post-run target screening strategy for ultra high performance liquid chromatography coupled to Orbitrap based veterinary drug residue analysis in animal urine

A. Kaufmann*, S. Walker

Official Food Control Authority, Fehrenstrasse 15, 8032 Zürich, Switzerland

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ABSTRACT

The performance of liquid chromatography coupled to high resolution mass spectrometry (LC–HRMS) post-run target screening for veterinary drug residue analysis (sulfonamides, tetracyclines and quinolones) in animal urine has been critically evaluated. It was found that retention time information still remains an essential information and that accurate masses together with relative isotopic abundance data alone are not sufficient for many residue applications. Post-run target screening requires the careful setting of parameters to achieve near zero false negative (above a defined threshold level) and a manageable numbers of false positive findings. HRMS offers many possibilities for the reduction of false positives (e.g. isotopic ratio, isotopic fine structure, exact mass of fragment ions). However, the successful use of such tools requires a sufficient ion intensity. This is often not available when trace level compounds are to be detected. Nevertheless, the proposed method is sufficiently sensitive to detect the veterinary drugs at the relevant concentration levels in urine. This means that the approach is well suited to significantly reduce the number of corresponding meat samples which have to be analyzed in a final step for the regulatory relevant quantification of residue levels in meat. The semi-quantitative screening of many samples for a large number of analytes within a short period of time requires the availability of software tools which provide fast and reliable answers.

1. Introduction

The development of ultra high performance liquid chromatography (UHPLC) and modern high resolution mass spectrometry (HRMS) has opened new strategies for residue analysis in complex matrices. Such novel approaches become more and more pressing, considering the ever growing number of compounds to be covered by multiresidue methods. Modern veterinary drug methods can contain more than 100 analytes [1,2], while pesticide methods are moving towards 1000 analytes [3–6]. A very similar development can also be observed in the field of forensic analysis [7,8].

It is a logistic nightmare to maintain the integrity of mixed standard solutions containing 1000 different compounds. It will always be the most labile compound which defines the shelf life of the whole solution [9]. This forces the analyst to store the reference solutions at very low temperature. As a consequence, this approach bears the inherent risk that some poorly soluble compounds may precipitate from the mixed stock solutions. Hence, screening would be facilitated, if techniques were available which do not rely on the physical presence of reference materials. Accurate mass based measurements in combination with high mass resolutions are clearly superior over tandem mass spectrometry derived SRM (selected reaction monitoring) regarding this aspect [10,11]. Accurate masses are universal and easily calculable. On the other hand, SRM are experimentally optimized parameters and their use is still restricted to a particular MS platform [12]. This is one reason why HRMS based on a post-run target screening approach [13] received increasing attention in the recent past. Unlike in unknown screening, post-run target screening focuses on known compounds which could be present in a particular sample [13–16]. A full scan data acquisition mode is used, which serves as a data mining source. This is different to classical SRM based quantification where the user has to define compound specific transitions prior to the data acquisition. Post-run target screening allows the user to reformulate and restructure his question without having to set up an additional instrumental run. Hence, the finding of a particular compound can be confirmed by being able to extract the exact mass trace of a corresponding metabolite or degradation product [17]. Such truly orthogonal confirmations are often possible even without having physical access to a reference compound. As a matter of fact, most metabolites or degradation products of

* Corresponding author at: Kantonales Labor Zürich, Fehrenstrasse 15, 8032 Zürich, Switzerland. Tel.: +41 43 244 71 97; fax: +41 43 244 71 01.
E-mail address: anton.kaufmann@klzh.ch (A. Kaufmann).

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veterinary drugs or pesticides are not commercially available. This paper focuses on the possibilities of current UHPLC–HRMS technology to apply post-run target screening for residue analysis in complex matrices. It tries to compare this technique with the classical external standard calibration approach and discusses its possibilities and limitations. The focus of this approach is to ensure virtually no false negatives while keeping the number of false positives at a manageable level (3–10%). The term "false positive" is used throughout this paper when the use of a filtering technique (e.g. exact mass with corresponding mass window or the additional application of a criteria like isotopic ratio, or retention time deviations) produces more than one chromatographic peak within the evaluated section of the chromatogram.

The shown application focused on the fast and cost effective recognition of animals (based on residue levels in urine) where the maximum residue level of veterinary dugs in the corresponding meat, kidney or liver has likely been exceeded. It is intended to single out screened positive animals in order to limit the number of more time and cost intensive quantitative meat analysis.

2. Materials and methods

2.1. Reagents and standard solutions

All The reference substances (ciprofloxacin, danofloxacin, enrofloxacin, flumequin, oxolinic acid, enoxacin, lomefloxacin, nalidixic acid, norfloxacin, ofloxacin, marbofloxacin, difloxacin, sarafloxacin, sulfaguanidine, sulfanilamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethizole, sulfadimidine, sulfamethoxypyridazine, sulfachlorpyridazine, sulfadoxine, sulfachlorpyrazin, sulfadimethoxin, sulfacetamide, sulfamethoxazole, sulfisoxazole, oxytetracycline, chlortetracycline, minocycline, doxycycline and trimethoprim) were of the highest available purity and were bought from Sigma–Aldrich (Buchs, Switzerland). N4-acetylsulfadimethoxine, N4-acetylsulfadimidine, N4-acetylsulfadiazine, N4-acetylsulfamerazine, N4-acetylsulfamethizole, N4-acetylsulfanilamide, N4-acetylsulfathiazole, N4-acetylsulfadoxine, N4-acetylsulfamethoxazole, and N4-acetylsulfamethoxipyridazine were from Serva (Heidelberg, Germany).

Individual stock solutions (1000 mg l^{-1} each) were prepared by dissolving the compounds in acetonitrile.
Mixed stock solution (10 mg l^{-1}) was prepared based on the individual stock solutions and diluted with a mobile phase B.
Reference solution (0.1 mg l^{-1}) was produced by diluting the mixed stock solution with dilution solution.

The stock solutions and reference solutions are stored in plastic vessels (to prevent the adsorption of analytes on glass surfaces) in a refrigerator. Stability experiments indicated that the stock solutions are stable for six months, while the more diluted solutions should be prepared weekly. Samples were stored a −18 °C.

Formic acid 98–100% was of analytical grade and purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol and ammonium hydroxide 25% were from Scharlau (Barcelona, Spain). The purified water was made in the lab by a lab water unit from Labtec (Wohlen, Switzerland).

Dilution solution: 120 ml acetonitrile and 10 ml of formic acid were transferred into a 1 l volumetric flask and diluted to volume with purified water.

2.2. Samples and sample preparation

Urine samples from 100 animals (bovine and swine) were obtained from a local slaughtering house. No information was available regarding a possible previous treatment of these animals with veterinary drugs. The sample were frozen immediately after collection and analyzed within a single batch in the laboratory.

After thawing, 0.1 ml urine sample was transferred into a vial (well plate) and mixed with 0.9 ml dilution solution. No filtration step was performed prior to injection. One out of ten samples was spiked to test for correct recovery rates. This involved the use of 0.1 ml urine sample which was mixed with 0.4 ml dilution solution and 0.5 ml of Reference solution (0.1 mg l^{-1}).

2.3. UHPLC–HRMS parameters

The equipment consisted of an Acquity system (sample and solvent manager) from Waters (Millford, MA, USA) and a Acquity BEH C-18, 2.1 × 50 mm × 1.7 μm column with an installed prefilter, both from Waters. The column was maintained at 25 °C and the injector volume was 5 μl. The following linear gradient was used: 0–4 min: 0–30%B, 4–6 min: 30–90%B, 6.0–6.5 min: 90%B, 6.5–6.6 min: 90–0%B, 6.6–7.5 min: 0%B. The flow was set to 0.3 ml min^{-1}.

Mobile Phase A: 50 ml acetonitrile/3 ml of formic acid/947 ml purified water.
Mobile Phase B: 947 ml acetonitrile/3 ml of formic acid/50 ml purified water.

The utilized mass spectrometer was a single stage Orbitrap instrument; Exactive HCD (Thermo Fischer Scientific, Bremen, Germany) operated under Exactive Tune 1.1 and XCalibur 2.1 software.

The capillary of the ESI interface (HESI-II) was set to +3600 V. The heater temperature was adjusted to 350 °C and the capillary temperature to 200 °C. Sheath gas and auxiliary gas were set to 50, respectively 12 units. The capillary voltage was 37.5 V and the tube lens voltage 125 V. The scan range covered (m/z: 190–1000). Resolution was set to 50,000 full width at half maximum (FWHM) which provides two full scans (data points) per second. The target capacity of the C-trap was always defined at 3,000,000 charges and the maximum injection time was limited to 50 ms.

Post-run target screening was performed with a beta version of the "Exactfinder" Software (Thermo Fischer Scientific).

3. Results and discussion

3.1. Chromatographic resolution and retention time deviation

The use of modern sub-2 μm particulate columns or core shell materials of similar diameter permits a combination of speed and good chromatographic resolution. This is very relevant for this particular urine screening application, since the virtual absence of sample preparation permits the processing of a high number of samples [17]. Furthermore, a good chromatographic separation significantly improves the quality of raw data used for post-run target screening. This refers to the number of co-eluting peaks but in addition, it reduces the extent of signal suppression as caused by matrix compounds. UHPLC columns are not any more prone to clogging or fast performance degradation, as this was the case some years ago. The stated application involves the injection of diluted, unfiltered urine. Typical series contain 100 samples. There was no relevant degradation of the separation performance or the MS sensitivity (e.g. fouling of the source) to be observed within