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TUTORIAL

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Pitfalls in drug testing by hyphenated low- and high-resolution mass spectrometry

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Abstract

This paper reviews various pitfalls observed during developing, validation, application, and interpretation of drug testing approaches using GC–MS and low- and high-resolution LC–MS. They include sampling and storage of body samples, sample adulteration and contamination, analyte stability, sample preparation without or with cleavage of conjugates, extraction, derivatization, internal standardization, false negative and positive results by GC–MS or LC–MS screening and/or confirmation procedures including artifact formation, ion suppression or enhancement by electrospray ionization, and finally pitfalls in data interpretation. Conclusions and prospects close the Tutorial.

KEYWORDS

Drug testing, Mass spectrometry, Pitfall, Screening

1 | INTRODUCTION

Drug testing consists of screening for and confirmation of the presence of drugs of abuse or therapeutic drugs in body samples such as urine, blood, oral fluid, sweat, meconium, hair, or nails. It plays a major role in clinical toxicology (e.g. in drug dependence treatment, pain management, or adherence testing), in forensic toxicology (e.g. in driving impairment or criminal responsibility testing), in workplace drug testing, and in doping control.

Although many old or new psychoactive substances (NPS) cannot be monitored by immunoassays (IA), conventional drug of abuse testing still includes IA prescreening and confirmation by gas chromatography–mass spectrometry (GC–MS) or liquid chromatography-mass spectrometry (LC–MS).[1] Many pitfalls in IA drug testing have been described such as interferences with other drugs, metabolites, or biomolecules leading to false positive results.[2,3] This is one reason for the need of confirmation. Another big issue is the risk of adulterations leading to false negative results. In case of IA testing, they can be caused by denaturation of the antibodies and/or enzymes e.g. by acids, bases, or salts as well as by disturbance of the measurement e.g. by fluorescents or detergents. Further adulterations also affecting MS-based assays are described below. In the following, pitfalls relevant for MS-based drug testing are discussed.

2 | SAMPLING AND STORAGE OF BODY SAMPLES

2.1 | Sampling

Urine sampling should be performed under supervision. Blood must not be sampled via an indwelling catheter for infusions, but from contralateral veins avoiding dilution of the sample. Postmortem blood should be taken from peripheral veins to avoid contamination e.g. by gastric content.

Time of sampling is of relevance if blood levels should be monitored over a particular time, e.g. in therapeutic drug monitoring (TDM) where the trough level is determined at the time of minimal concentration, the time before next drug administration. Another example is an acute paracetamol poisoning. For assessing the severity and thus

the need of antidote treatment, the paracetamol concentration should be measured in a blood sample taken four hours after ingestion when absorption and distribution are almost completed.[4]

Pitfalls concerning TDM in dried blood spots (DBS) are reviewed by Antunes et al.[5] with special focus on influence of the hematocrit on the plasma concentrations. They discussed besides sensitivity, validation, and standardization issues, the influence of the blood sample hematocrit on translating DBS concentrations to reference plasma levels. The lack of comprehensive clinical validation of published drug assays using DBS have been considered as the major pitfall to its implementation in the routine of TDM.[6] Capiau et al.[6] published recently guidelines on dried blood analysis in TDM discussing also the hematocrit effects. Pitfalls are also described in oral fluid drug testing[7,8] or hair testing.[9-11]

2.2 | Sample adulteration and contamination

Besides simple urine exchange (e.g. urine of a drug-free person), urine adulterations are common such as dilution by drinking a lot of water before sampling or by simple addition of water to the urine sample or addition of adulterants disturbing the measurement. Commercial adulterants promising negative drug tests are available. In addition to simple pH and temperature measurement, adulteration check kits are sold for testing e.g. specific gravity and creatinine as marker for internal or external urine dilution, oxidative/reductive chemicals or detergents. However, such tests cannot detect synthetic urine ("fake urine") having normal pH and creatinine values. Krug et al. published recently systematic investigations of novel validity parameters in urine drug testing and prevalence of urine adulteration.[12] Steuer et al.[13-16] described interesting metabolomic strategies for detection of sample manipulations using liquid chromatography-high resolution mass spectrometry (LC-HRMS). In their recent review article,[13] they discussed different applications of metabolomic strategies in biomarker research for indirect identification of drug consumption and sample manipulation. Such strategies may allow detecting unexpected metabolites in metabolism studies as well as indirect detection of urine manipulation attempts by chemical adulteration or replacement with artificial urine samples. For example, nitrite-based adulteration was studied focusing on uric acid, histidine, methylhistidine, and their oxidation products, for example 5-hydroxyisourate as potential biomarkers for urine adulteration using potassium nitrite.[16] They found that the higher the adulterant concentration, the lower the concentrations of histidine, methylhistidine, and uric acid are and that amounts of their oxidation products increased. 5-Hydroxyisourate and uric acid proved suitable markers to identify urine adulteration using potassium nitrite.

In addition to intentional sample manipulation, various interferences and contaminants contained e.g. in used chemicals, solvents, or plastic materials may affect GC–MS and LC–MS approaches. Diisooctylphthalate for example can be the major or even only peak in GC–MS when analyzing an analyte in low concentration and/or with poor GC properties and thus maybe falsely identified e.g. as drug.[17] LC–MS approaches may also be affected by interfering and contaminating compounds of proteinaceous and non-proteinaceous nature.[18] The supplementary data to this paper by Keller et al. contain a huge list of such compounds of proteinaceous and non-proteinaceous nature with the monoisotopic ion mass, ion type, empirical formula of compound, subunit, or sequence, and possible origin to help in identifying such contaminants, particularly using HRMS.

2.3 | Analyte stability

Analyte instability has a major impact on quantitative case interpretation. Stability of classic drugs of abuse, therapeutic drugs, and their metabolites in blood, plasma, or serum has been discussed elsewhere.[19-21] Although the majority of drugs are stable under common analytical conditions, instability occurs for drugs carrying ester moieties, sulfur atoms, or other easily oxidized or reduced structures. To prevent e.g. ester hydrolysis of cocaine or heroin/6-monoacetylmorphine, addition of e.g. sodium fluoride inhibiting esterases is recommended.[22-24] Wu et al.[25] studied the stability of cocaine, opioids, and benzodiazepines in meconium and concluded that some of them may not be stable for a longer time. Also NPS can be affected by instability e.g. cathinones,[26,27] piperazines,[27,28] and synthetic cannabinoids.[29] Martin et al.[30] could overcome the psilocin instability in plasma by freezing. In contrast, psilocin glucuronide, the main psilocin metabolite, was stable in serum and urine and should thus be included in the approach for monitoring psilocin intake.[31] Instability of ethyl glucuronide (EtG) caused by bacterial degradation in contaminated urine samples could be overcome e.g. by using dried urine spots.[32] Another issue is the (postmortem) instability of peptide-based analytes such as insulin as shown by Wunder et al.[33] Therefore, Thevis and Thomas[34] recommended immediate asservation and preservation of dried blood spots, even prior to autopsies.

For all these reasons, stability tests are mandatory under different storage conditions and time periods depending on the procedures finally used. Ideally, the following stability tests should be evaluated[35]: stability of the stock solution and working solutions of the analyte and internal standard, freeze and thaw stability of the analyte in the matrix from freezer storage conditions to room temperature or sample processing temperature, short term stability of the analyte in matrix at room temperature or sample processing temperature, and long term stability of the analyte in matrix stored in the freezer. Depending on the application, stability studies should include stability of the processed sample at room temperature or under the storage conditions and on-instrument/autosampler stability of the processed sample at injector or autosampler temperature.

In conclusion, clinical and especially forensic specimens should generally be stored at least in the refrigerator and preferably at −20° C or lower as long as analyte stability was not confirmed. If analytes are identified as unstable during analysis their concentrations should be interpreted with great care particularly when stored at room temperature for a longer time. At least partial degradation should always be considered.[19]

3 | SAMPLE PREPARATION

3.1 | Cleavage of conjugates

False workup may lead to analytical pitfalls. For example, if a compound and/or its metabolites are excreted into urine in a completely conjugated form, GC–MS analysis would lead to false negative results if no cleavage of the polar conjugates was performed. However, use of acid hydrolysis approved for fast conjugate cleavage[36] can lead to various artifacts such as cleavage of ethers (e.g. diphenhydramine or norfluoxetine) or formation of benzophenones from benzodiazepines some followed by rearrangements.[36,37] It must be considered that different drugs may form the same artifact such as oxazepam, chlordiazepoxide, clorazepate, cyprazepam, nordazepam, oxazolam, and prazepam. However, as long as the artifacts are formed reproducibly and are included in the GC–MS approach, they can be used for detection.[36,38] Norfluoxetine can be transformed by hydrolysis (see above) followed by water elimination to an artifact with a GC–MS spectrum almost similar to that of tranylcypromine, both after acetylation.[39] Another example for GC–MS pitfalls is the formation of a dihydroergotamine artifact, a cyclo dipeptide of phenylalanine and proline also called cyclo (Phe-Pro), which is also formed during roast processes and hence contained in several foodstuffs.[40] This study showed that cyclo (Phe-Pro) is also contained in cocoa powder and may be excreted unchanged in urine after ingestion of cocoa powder. It further showed that cyclo (Phe-Pro) was present in or formed from ingredients of a glucuronidase/arylsulfatase enzyme preparation.

Enzymatic cleavage by glucuronidase is selective for 1-O-substituted beta-D-glucopyranosiduronic acids. In case of acyl (ester) glucuronides, acyl migration (intramolecular transesterification) may lead to ester binding at another hydroxy group of the glucuronic acid.[41-43] These ester glucuronides can then not be cleaved by glucuronidase as demonstrated e.g. for valproic acid glucuronides.[42] Again, this may lead to false negative GC–MS results under the conditions discussed above.

3.2 | Extraction

A variety of selective or universal isolation approaches are in use.[37] It is trivial that a compound cannot be detected if not extracted. Therefore, recovery studies are recommended in method development and validation particularly in multi-analyte approaches. The extent of recovery plays a minor role as long as it is reproducible and the sensitivity of the assay sufficient. Pitfalls may occur if artifacts are formed by oxidation such as N- or S-oxides during extraction e.g. with diethyl ether containing traces of peroxides.

3.3 | Derivatization

Sarris et al.[44] demonstrated that demoxepam produces artifacts after trimethylsilylation, which could be identified as nordiazepam and oxazepam. Thus, this pitfall must be considered when interpreting analytical data from demoxepam cases and also form chlordiazepoxide cases being a metabolite of them.

Artificial methylation of carboxy derivatives solved in methanol during GC–MS is advantageous but must be considered in MS data interpretation.[36] It should also be noted that compounds can be acetylated by acetylsalicylic acid in the gastric contents if acetylsalicylic acid was simultaneously ingested.[36]

3.4 | Internal standardization

Internal standards allow compensation of analytical errors, particularly in quantitative assays. Use of non-deuterated structural analogues is not recommended as they may have different extraction behavior, stability, and chemical reactivity. Therapeutic drugs should never be used as they can occur in the sample leading to false results. Stable-isotope-labeled analogues are the gold standard, but pitfalls can also occur. They can contain impurities of the unlabeled compound, cause cross contribution, especially if number of labels is below 4, show (slightly) different chromatographic behavior, especially for highly deuterated compounds, and finally, they can cause ion suppression/enhancement in LC–MS.[45,46]

4 | GC–MS SCREENING AND/OR CONFIRMATION PROCEDURES

4.1 | False negatives

Insufficient GC and/or MS performance can lead to false negative results. Therefore, for system suitability test, the apparatus performance should be checked daily before starting the analyses by analyzing a mixture of analytes covering a wide range of physicochemical properties and relevant retention times.[36,37] The quality acceptance criteria are that all compounds must be chromatographically resolved and the peaks should be sharp and of sufficient abundance to be clearly identifiable. If these criteria are not fulfilled, the injection port liner should be removed, and the GC column either be shortened (10–20 cm), or replaced. Last but not least, the ion source should be cleaned. In addition, the current performance should be tested in an analysis series using certified control samples to prevent false negative results.

4.2 | False positives

False positive results can be obtained even using GC–MS when the selected ion monitoring (SIM) mode is used and the ions and their relative abundance are not specific enough and the retention time does not differ between the target analyte and a potential interference. A minimum of three diagnostic ions per analyte with defined relative abundances is requested. If possible, besides the molecular ion, ions

from different part of the molecule should be selected. Ions derived from derivatized fragments should not be used in addition to the underivatized fragments as they do not increase selectivity. Of course, ions resulting from the derivatization reagent must be avoided. Pitfalls arising from MS/MS modes will be discussed in the LC–MS section as they are used more frequently for LC–MS. In any case, intensive selectivity test should be performed with several blank samples as well as blank samples spiked with drugs typically co-administered in the corresponding field.[47]

Another cause of false positives is analyte carryover, a major problem in trace analysis. Sufficient cleaning steps of the injector depending on the analyte concentration (e.g. urine sample after acute poisoning) helps to overcome it. Negative control samples between any analyses run prevent false positive results.

4.3 | Artifacts formed during GC

Various artifacts can be formed by thermolysis during GC such as decarboxylation of carboxylic acids, Cope elimination of N-oxides, or elimination of various residues (e.g. hydrochloric acid or methyl bromide). Methylation of carboxylic acids in methanolic solution can also occur, but this can be advantageous in order to improve their volatility. Primary amines (e.g. amphetamine), beta-blockers and some local anesthetics (e.g. flecainide) injected in methanolic solution are altered during GC by reaction with formaldehyde, which is probably formed by thermal dehydrogenation of methanol in the injection port of the GC. This was confirmed using deuteromethanol.[48,49] Further artifacts can be formed by thermolysis during GC such as dehydration of alcohols, or decarbamoylation of carbamates.

5 | LC–MS SCREENING AND/OR CONFIRMATION PROCEDURES

Various pitfalls are associated with the use of LC–MS, LC–MS/MS, LC–MS^n, and LC-HRMS (thereafter shortly mentioned as LC–MS) in clinical and forensic toxicology,[50,51] which may result in false negative or positive results or inaccurate quantification.

5.1 | False negatives

As already discussed for GC–MS, insufficient LC and/or MS performance can lead to false negative results. Again, the apparatus performance should be checked daily before starting the analyses by analyzing a mixture of analytes covering a wide range of physicochemical properties and relevant retention times.[37,52,53] The analyte concentrations, the injection volume, and the separation have to be adopted to the apparatus used. To ensure adequate instrument performance, all peaks in the test mixture must be chromatographically resolved and of sufficient abundance to give clearly identifiable peaks. If these criteria are not fulfilled, the ion source should be cleaned and the instrument recalibrated.

In case of HR apparatus, mass deviations between exact mass and measured mass should be lower than ±5 ppm for each precursor according to the author's experience. If one is higher, the apparatus should be recalibrated.[53] In addition, the current performance should be tested in an analysis series using certified control samples to prevent false negative results.

Another important source of false negative results is the high risk of ion suppression caused by co-eluting matrix compounds (so-called matrix effect),[54] contaminants, analytes, and/or internal standards. This will be discussed in separate section below. In urine drug testing by LC–MS, there is a high risk of even unexpected ion suppression because of the varying matrix content. Therefore, LC–MS approaches should include metabolites as additional target to avoid false negative results.[55-57] Of course, the metabolite data must first be identified and included in the procedure and reference libraries.[52,53]

False negative results can occur in case of bromine containing compounds if the precursor selection is based on triggering the most intense ions in the full scan. As both bromine isotopes have almost the same abundance it may happen that the ion with bromine 81 is triggered and the fragments and/or full MS/MS spectra cannot be detected if only those of the bromine 79 isotope are selected and/or in the reference MS/MS library. Therefore, the data of both isotopes should be included.[53]

5.2 | False positives

Again, false positive results can be obtained when the selected ions for SIM or the selected transitions in the selected reaction monitoring (SRM) are not specific enough and the retention time does not differ. In any case, intensive selectivity test with several blank samples and blank samples spiked with drugs typically co-administered in the corresponding field are mandatory.[47] A minimum of two diagnostic transitions (e.g. one target ion, two qualifier ions) per analyte is requested. They may derive from the same precursor ion if the product ions are sufficiently different. The importance of more than one transition was demonstrated years ago with the differentiation of tramadol and O-demethyl-venlafaxine.[58] Both have the same elemental composition and thus the same precursor ion. The prominent fragment ion has also the same elemental composition and thus mass.

HRMS would not allow to distinguish them, either. Only unique fragments with different masses and/or different retention times allow distinguishing. Isomeric parent drugs such as the NPS beta-keto-4-bromo-2,5-dimethoxyphenethylamine (bk-2C-B) and 2,5-dimethoxy-4-bromoamphetamine (DOB) can also lead to similar pitfalls. In LC-low-resolution-MS, the nominal mass of the precursor and the fragment ions are the same, but with HRMS, they can be differentiated as the exact mass of the precursor and, with one exception, the exact mass of the fragment ions are different.[59]

HRMS single stage apparatus can be used for screening measuring only the accurate (protonated or deprotonated) molecular masses

of the detected compounds. For identification, the measured masses are compared with reference lists of potential drugs or poisons sorted according to increasing exact masses.[60] If more than one compound has the same exact mass, false positives can occur as only isobaric but not isomeric compounds can be differentiated. For example, the elemental composition of morphine and the pepper ingredient chavicine are identical, but the structure and toxicological effect are quite different. This limitation can be overcome by fragmentation e.g. in-source in single-stage apparatus or in collision cells in conventional MS/MS, Q-TOF or Q-Orbitrap equipment.[55,57,61,62]

Another example for pitfalls in LC–MS testing was published by Toennes et al.[51] They detected a previously unrecognized interfering compound in their LC–MS/MS assay for delta-9-tetrahydrocannabinol (THC) and of its two metabolites in serum. This has the same elemental composition and fragments as the carboxy metabolite, except for relative fragment intensities and should be an isomer.

Finally, the risk of analyte carryover and thus false positives increases with the sensitivity of modern apparatus. It should be tested during method development and validation to assess and finally prevent the risk. Again, negative control samples between any analyses run prevent false positive results.

5.3 | Ion suppression or enhancement

LC–MS approaches particularly using electrospray ionization (ESI) are affected by mutual ion suppression or enhancement caused by co-eluting matrix compounds (so-called matrix effect), contaminants, analytes, and/or internal standards.[54,63,64] It may influence assay sensitivity, reproducibility, accuracy, and linearity in quantitative LC–MS. Ion suppression results from the presence of less volatile compounds that can change the efficiency of droplet formation or droplet evaporation in ESI, which in turn affects the amount of charged ions in the gas phase that ultimately reaches the detector.[65] Salts, ion-pairing agents, endogenous compounds, drugs, metabolites, and even isotope-labelled internal standards were shown to be responsible for ion suppression.[45,46] Dams et al.[54] were the first who evaluated systematically the synergistic effect of ionization type, sample preparation technique (direct injection, dilution, protein precipitation, solid-phase extraction), and bio-fluid (urine, oral fluid, and plasma) on the presence of matrix effects. The remaining matrix components were specific to each bio-fluid and interfered at different time points in the chromatogram. Simple dilution was sufficient for urine, acetonitrile protein precipitation for clean-up and concentrating of oral fluid, but solid-phase extraction was necessary for plasma. Both, ESI and atmospheric pressure chemical ionization (APCI) showed matrix effects, but ESI was more affected. Such studies should include an approach using post column infusion of the analyte to detect protracted ionization effects at which retention time.[54,63] Particularly for quantitative assessment, the strategy described by Matuszewski et al.[66] is recommended, which includes also the determination of recovery and process efficiency. If relevant ion suppression or enhancement occur, sample dilution, specimen clean-up, chromatographic changes, reagent modifications, use of APCI instead of ESI, and effective internal standardization should be done.[64,67] However, it should be kept in mind that co-eluting isotope-labelled internal standards can also cause or be affected by ion suppression/enhancement.[45,46] Therefore, a systematic investigation of their ion suppression and enhancement effects by their native analogues is recommended particularly in quantitative multi-analyte assays.[45]

Ion suppression/enhancement cannot only be caused by matrix but also by co-eluting analytes in multi-analyte approaches, again with ESI being more affected than APCI.[68] These effects may influence the drug quantification using calibrators made in presence of overlapping and thus interfering analytes. Ion suppression/enhancement effects induced by co-eluting drugs of different classes present in the patient sample may also lead to false measurements using class-specific calibrators made in absence of overlapping and thus interfering analytes.[68] Peters and Remane[63] discussed ion suppression/enhancement in methods for multi-analyte procedures or systematic toxicological analysis as well as matrix effects in alternative matrices such as meconium, hair, oral fluid, or decomposed samples in postmortem toxicology.

6 | PITFALLS IN DATA INTERPRETATION

Interpretation of postmortem blood concentrations may significantly be affected by redistribution of drugs or poisons in postmortem toxicology. For example, Gerostamoulos et al.[69] studied the effect of the postmortem interval on the redistribution of drugs by comparing drug concentration in blood samples taken at mortuary admission and at autopsy. The extent of redistribution ranged from 30 to 300%. For some drugs, significant redistribution occurred even when taking peripheral specimens irrespective of the delay in the postmortem interval. Staeheli et al.[70] developed another strategy for investigation of time-dependent redistribution using automated biopsy sampling at admission of the bodies and at autopsy (around 24 h after admission). They placed introducer needles in various organs, femoral vein, and lumbar spine using a robotic arm guided by a computed tomography scanner. Using this technique, they investigated redistribution e.g. of morphine, opioids, and entactogenic NPS.[71-75] They found redistribution relevant for interpretation for some analytes such as the tested fentanyls, but not others such as the tested entactogens, tramadol, codeine, hydrocodone and oxycodone. Thus, the authors concluded that CT-guided biopsy sampling has proved to be a valuable tool for the investigation of the extent of post-mortem redistribution in different organs or veins in a particular case.

Interpretation of MS-based drug testing results must consider that stereoisomers show identical chromatographic and mass spectral properties if not analyzed enantioselectively. For example, the N-dealkyl metabolite of the antiparkinsonian drug selegiline and methamphetamine or the antitussive dextrorphan and the opioid levorphanol cannot be differentiated. Chiral procedures using chiral derivatization or columns help in differentiation.[76-78]

Another problem arises from metabolites formed by different parent compounds, particularly if one is scheduled and the other not. For example, morphine is formed by heroin, codeine, or ethylmorphine, dihydromorphine by desomorphine, dihydrocodeine, hydrocodone, hydromorphone, or thebacone,[79,80] the designer drugs p-methoxymethamphetamine (PMMA) by the antihypotonic drug pholedrine,[81] m-chlorophenylpiperazine (mCPP) by the antidepressants trazodone and nefazodone,[82] 1-(3,4-methylenedioxybenzyl) piperazine (MDBP) by the nootropic fipexide,[83] or oxazepam by camazepam, clorazepate, diazepam, nordazepam, prazepam, temazepam.[37] Reference libraries should present all possible parent compounds, which may form the corresponding common metabolite[38,52,53] to give a hint of possible misinterpretation.

7 | CONCLUSIONS AND PROSPECTS

Various pitfalls may occur in drug testing not only by immunoassay, but also by MS-based methods. Their knowledge helps to prevent them in method development, validation, routine application, and interpretation. If new pitfalls are detected they should be published rapidly to warn other users. All-in-all, extensive method development and validation in combination with internal and external quality control performed by well-trained staff should lead to reliable results in drug testing.

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CONFLICT OF INTEREST

The author declares that there are no conflicts of interest.

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