A straightforward, validated liquid chromatography coupled to tandem mass spectrometry method for the simultaneous detection of nine drugs of abuse and their metabolites in hair and nails

A straightforward, validated LC-MS/MS method for the simultaneous detection of seven drugs of abuse and their metabolites in hair and nails

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Abstract

Hair and nails allow for stable accumulation of compounds over time and retrospective investigation of past exposure and/or consumption. Owing to their long window of detection (weeks to months), analysis of these matrices can provide information complementary to blood and urine analysis or can be used in standalone when e.g. elimination from the body has already occurred. Drugs of abuse are often used together and, therefore, multi-analyte methods capable of detecting several substances and their metabolites in a single run are of importance. This paper presents the development and validation of a method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the simultaneous detection of seven drugs of abuse and their metabolites in hair and nails. We focused on a simple and straightforward sample preparation to reduce costs and allow application in routine laboratory practice. Chromatographic and mass spectrometric parameters, such as column type, mobile phase, and multiple reaction monitoring transitions were optimized. The method was validated according to the European Medicine Agency (EMA) guidelines with assessment of specificity, limit of quantification (LOQ), linearity, accuracy, precision, carry-over, matrix effects, recovery, and process efficiency. Linearity ranged from 25 to 20 000 pg mg^{-1} hair and from 50 to 20 000 pg mg^{-1} nails and the lowest calibration point achieved the requirements for the LOQ (25 pg mg^{-1} for hair and 50 pg mg^{-1} for nails). The applicability of the method was proven through successful participation in a proficiency test and by investigation of authentic hair and nail samples from regular polydrug users. In the future, the method should allow comparison between the two matrices to acquire knowledge on nail analysis and to define cutoff levels for nail analysis, as they exist for hair.

Keywords: hair, nails, LC-MS/MS, validation, drugs of abuse, metabolites

1. Introduction

Keratinized matrices, such as hair and nails, are of considerable importance in forensic and clinical toxicology as they allow for a stable accumulation of compounds over time [1, 2]. Thus, these matrices can provide information about exposure to and consumption of substances, such as pharmaceuticals and illicit drugs. In contrast to the more traditional matrices such as blood and urine, hair and nails have a longer window of detection of weeks to months, compared to hours to days for blood and urine [3]. As a result, the analysis of hair and nails allows for a retrospective investigation of previous drug consumption after their elimination from the body. Combined analysis of classical and keratinized matrices gives complementary information on substance use over an extended timeframe. Nevertheless, keratinized matrices can also be used in standalone analysis, when collection relates to an event that occurred several weeks or months earlier, or when retrospective information over a large period of time is required. Other advantages include the easy, non-invasive collection of samples which does not require qualified medical staff and can be performed under close supervision to avoid adulteration, as well as the transport and storage at room temperature. On the other hand, the complexity of these matrices and the low concentrations of compounds present, necessitate an optimized sample preparation (i.e., decontamination, pulverization, extraction and clean-up), and a sensitive and specific quantification method [1, 2].

Despite the emergence of new psychoactive substances, amphetamine-like stimulants, cocaine, and opioids continue to be in high demand and supply, with major impacts on public health [4]. According to the European Drug Report 2016, cocaine remains the most commonly used illicit stimulant drug in Europe. The combined consumption of several drugs of abuse, i.e. polysubstance abuse, is a prevalent pattern of substance use and represents a significant health concern. The monitoring of these patients within a therapeutic or forensic/legal procedure, represents a particular challenge that requires multi-analyte methods capable of detecting several compounds in a single run.

The importance of hair as a matrix for illicit drug testing has substantially increased in recent years, e.g., in driver's license regranting and in research for the causes of death [5, 6]. In this context, several analytical methods have been described mainly using gas chromatography (GC) or liquid chromatography (LC) coupled to (tandem) mass spectroscopy ((MS/)MS). Most of these methods focus on the validation of a screening method [7], and/or on a limited number of drugs [8], while quantitative multi-target methods are rather scarce [9]. Recently, two articles [10, 11] have reported upon the validation of an LC-MS/MS method for quantification of amphetamines, cocaine, opioids and metabolites in hair. However, the disadvantage of both methods is that they involve a time consuming, expensive and tough solid phase extraction step as part of their sample preparation. A possible alternative has been proposed by Chang et al. [12] in their development of a method based on microwave assisted extraction for the detection of amphetamines and opiates. Together with the increased use of hair samples, scientific organizations, such as the Society of Hair Testing (SoHT) [13] and Substance Abuse and Mental Health Services Administration (SAMHSA) (ref), have published guidelines for hair analysis and recommendation according confirmatory cutoffs to distinguish positive from negative samples. On the other hand, studies on nail analysis are limited, and they only reported on a limited number of substances, e.g. amphetamine-like stimulants or cocaine [14, 15]. Neither guidelines nor cutoff values are available for nail analysis, and therefore interpretation of

results is often complicated. Very few articles have developed methods for both hair and nails, and these methods are only applicable for one group of drugs [16, 17].

In order to close the above mentioned 'gaps', the present study aims at the development of a straightforward analytical procedure for the simultaneous detection of several drugs of abuse in keratinized matrices. The investigated drugs of abuse are presented in table 1 together with the recommended cutoff concentrations in hair. The development of a single method for the simultaneous analysis of these compounds was challenging as the drugs of abuse for which the method was validated belong to different classes (amphetamine-type stimulants, opioids, and cocaine) and have different physicochemical characteristics. The method includes a simple sample preparation, thereby reducing costs and allowing application in routine laboratory practice. The method was validated for both hair and nails, which allows comparison between the two matrices and enhances the confidence level of the results. Moreover, it extends the window of detection even more, as nails are growing slower compared to hair. In addition, as a result of their slower grow rate and increased drug accumulation, nails may enhance the method sensitivity. Finally, the presented method offers the possibility to analyze paired hair and nails samples, and to use nails as an alternative matrix whenever hair is not available (e.g., alopecia, newborn) or not reliable (e.g., bleached hair).

2. Materials and methods

2.1. Reagents and materials

Ultrapure water was prepared using an Elga Purelab Flex water purification system (Veolia Water Technologies, Tienen, Belgium). Acetone (for gas chromatography ECD and FID SupraSolv®) was acquired from Merck KGaA (Darmstadt, Germany). Methanol (MeOH; LC-MS grade) and acetonitrile (ACN; HPLC grade) were purchased from Fisher Scientific UK (Loughborough, United Kingdom). Formic acid was obtained from Merck-Schuchardt OHG (Hohenbrunn, Germany). Ammonium formiate (97%) was bought from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). The following analytical standards were acquired from Cerilliant (Round Rock, TX, USA) as solutions at concentrations of 1.0 mg mL^{-1} in MeOH, unless otherwise stated: 6-monoacetylmorphine (6-MAM; in ACN), amphetamine (AMP), benzoylecgonine (BE), cocaine (COC; in ACN), codeine (COD; 100 µg mL^{-1}), ethylenedimethyldiphenylpyrrolidine (EDDP) perchlorate, ecgonine methylester (EME; in ACN), methamphetamine (mAMP), methylenedioxyethylamphetamine (MDEA), methylenedioxymethamphetamine (MDMA), and morphine (MOR). The methadone (MTD) standard was purchased as a solution of 1.0 mg mL^{-1} in MeOH from LGC GmbH (Luckenwalde, Germany). For each analyte, the corresponding deuterated analogue was included and used for quantification. The deuterated internal standards were obtained from Cerilliant as solutions in concentrations of 1.0 mg mL^{-1} in MeOH, unless otherwise stated: 6-MAM-D_3 (in ACN), AMP-D_8, BE-D_3, COC-D_3 (in ACN), COD-D_6 (100 µg mL^{-1}), EME-D_3 (100 µg mL^{-1} in ACN), MDEA-D_6 (100 µg mL^{-1}), MDMA-D_5, MTD-D_9, mAMP-D_{11} (100 µg mL^{-1}) and MOR-D_3. The deuterated internal standard EDDP-D_3 perchlorate was bought from LGC GmbH as a solution of 1.0 mg mL^{-1}. Through participation in a proficiency testing program organized and realized by Arvecon GmbH (Walldorf, Germany), the laboratory received authentic standard hair specimens for testing. For pulverization of hair and nail samples, a ball mill Retsch MM 400 (Retsch Benelux, Aartselaar, Belgium) was employed. Incubation was performed using an Eppendorf Mixmate (Eppendorf Belgium, Rotselaar, Belgium) and centrifugation was done using a

Beckman Coulter Microfuge 18 (Analis SA, Namen, Belgium). Mini-UniPrep vials for filtration of the supernatant after incubation and centrifugation were acquired from Agilent (Diegem, Belgium).

2.2. Standard solutions calibrators and quality control samples

Starting from the purchased stock solutions, working solutions for both standards and internal standards were prepared in order to contain all analytes in the same concentrations. Three standard working solutions were prepared in MeOH at concentrations of 1 ng μL^{-1}, 100 pg μL^{-1}, and 10 pg μL^{-1}. Calibrators and quality controls (QCs) were prepared by spiking blank matrix samples with one of those solutions. The internal standard working solution was prepared in MeOH at a concentration 100 pg μL^{-1} and 100 µL of this solution was added to each sample.

2.3. Samples

For validation blank (i.e., drug-free) hair and nails samples were obtained from healthy volunteers together with self-reports. Care was taken that cosmetic hair treatment, nail polishing, and consumption of any drug of abuse were excluded. Hair samples were collected from the vertex posterior region of the head and cut as closely to the scalp as possible. Nail samples were obtained by clipping of the distal edge of finger- and toenails. Samples were stored in aluminium foil at room temperature until analysis.

In addition, hair samples from the proficiency test DHF 3/15 organized by Arvecon GmbH were analyzed with the method to test its performance. The validated method was applied to hair and nail samples from drug-dependent individuals. The hair samples were obtained from forensic and toxicological cases, previously analyzed at ZNA Stuivenberg. The nail samples were collected from patients engaged in treatment for substance use disorders at the psychiatric Center Broeders Alexianen together with a detailed anamnesis of past drug uses. The Ethical Committee of the University hospital of Antwerp (UZA) and the local Ethical Committees of Broeders Alexianen and ZNA Stuivenberg approved the study (Belgian registration number: B30020169233).

2.4. Sample preparation

To remove external contamination, collected hair and nail samples were first decontaminated; once in water and once in acetone, both for 1 min. Samples were air dried, and subsequently pulverized for 5 min at 30 Hz using a ball mill. Approximately 20 mg of powdered sample was then accurately weighed, and transferred into a 2mL Eppendorf tube. To this Eppendorf, 100 µL of internal standard solution and 500 µL of the extraction solution [MeOH: ACN: 2 mM ammonium formiate in ultrapure water (25:25:50, v/v)] were added, and the mixture was incubated for 18 h at 500 rpm in an Eppendorf Mixmate. After centrifugation for 10 min at 13500 rpm, the supernatant was filtrated with Agilent Mini-UnipPrep vials and the vials were placed on the liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) instrument for injection.

2.5. Liquid chromatography coupled to tandem mass spectrometry

The LC-system consisted of an Agilent 1200 series binary pump and autosampler (Agilent, Diegem, Belgium). Chromatographic separation of the drugs was achieved on an EZ:faast C18 (250 mm x 2.0 mm x 4 µm) column (Phenomenex, Utrecht, the Netherlands) in the gradient mode. The mobile

phase was composed of (A) 5 mM ammonium formiate and 0.01 % formic acid in ultrapure water, and (B) 0.01% formic acid in ACN. The optimized gradient can be found in table 2. The flow rate was 0.3 mL min^{-1} and the injection volume was set at 1 µL. All compounds were eluted between 2.5 and 14 min and the total run-time including column equilibration was 21 min. The MS-system consisted of an Agilent 6410 triple quadrupole mass spectrometer equipped with an electrospray interface operating in positive ionization mode. Nitrogen was used as drying gas at a temperature of 350 °C and a flow of 10 mL min^{-1}. Quantitative analyses were performed in dynamic multiple reaction monitoring (MRM) mode and the three most abundant transitions (one quantifier and two qualifiers) were recorded for each compound. Table 3 gives an overview of the MS parameters, and the retention times for all compounds and internal standards.

2.6. Quantification and method validation

For each compound, the most abundant MRM transition was used for quantification (quantifier), while two other transitions (qualifiers) were used for identity confirmation. For the deuterated internal standards, only one transition was used. For confirmation of the detected compounds retention times and quantifier/qualifier ratios were evaluated. A tolerance of ± 5% deviation of compound retention time from the reference standard retention time was accepted (ref: GFTCH). The ratio quantifier/qualifier in the processed samples should not be outside the range of ± 20% of the same ratio acquired from a reference standard (ref: GFTCH).

The analytical method was validated according to the 'Guidelines on bioanalytical method validation' of the European Medicine Agency (EMA). The following parameters were assessed to evaluate the performance of the method: selectivity, limit of quantification (LOQ), linearity, accuracy, precision, carry over, matrix effects, recovery, and process efficiency.

The method selectivity was assessed using six different sources of blank hair and nails, spiked with internal standards and individually analyzed. The absence of interfering components is accepted where the response (i.e., peak area) is less than 20% of the LOQ for a given compound.

The LOQ is defined as the lowest concentration of compound that can be determined with an acceptable accuracy and precision (i.e. within 20% bias and within 20% relative standard deviation (RSD)), and is included as the lowest calibration point of the calibration curves.

Based on the expected concentration range, a seven-point calibration curve was constructed by spiking blank matrix samples at different concentrations to evaluate linearity. According to the EMA-criteria the back-calculated concentrations of the calibration standards should be within ± 15% of the nominal concentration, except at the LOQ where it should be within ± 20%.

Accuracy and precision were assessed for replicated quality control (QC) samples at three concentration levels covering the entire calibration range (low QC, mid QC, and high QC). Within-run precision and accuracy were assessed by analyzing five QC samples per concentration level prepared at the same day, and analyzed in the same analytical run. Between-run precision and accuracy at each level were calculated on QC samples prepared and analyzed in three different runs, on three separate days. Acceptance criteria were: 1) a bias less than 15%, and 2) a RSD lower than 15%.

Carry-over was assessed by injection of blank hair and nails after the highest calibration standard. The response of the analyte in the blank sample should not be higher than 20% of the response of the LOQ.

As proposed by Matuszewski et al. [18], three sets of samples (set 1, set, 2 and set 3) were prepared for the determination of matrix effects, recovery and process efficiency (see table S-1 and S-2). In the first set (set 1), samples without matrix (neat mobile phase samples) were spiked at low and high QC concentration. The second set (set 2) included blank matrix samples from six different sources spiked *after* extraction at low and high QC concentration, while in the third set (set 3) blank matrix samples from six different sources were spiked at the same concentrations but *before* extraction. For each concentration level, two replicates were included and analyte responses were normalized with the responses of the internal standards. Matrix effects could be evaluated through comparison of normalized responses obtained in set 2 with those obtained in set 1 as the only difference between both is the presence or absence of matrix. A deviation of maximum ±15% for the normalized matrix effect is tolerated.

$$matrix\ effects\ (\%) = \frac{mean\ of\ normalized\ responses\ set\ 2}{mean\ of\ normalized\ responses\ set\ 1} \times 100$$

The extraction recovery was calculated by comparison of normalized responses obtained in set 3 with those obtained in set 2 and the relative standard deviations were calculated.

$$recovery\ (\%) = \frac{mean\ of\ normalized\ responses\ set\ 3}{mean\ of\ normalized\ responses\ set\ 2} \times 100$$

Recovery and matrix effect were combined in order to calculate the overall process efficiency and the relative standard deviations were calculated.

$$process\ efficiency\ (\%) = \frac{mean\ of\ normalized\ responses\ set\ 3}{mean\ of\ normalized\ reponses\ set\ 1} \times 100$$

3. Results and discussion

3.1. Method development and optimization

As outlined in the introduction, current methods for keratinized either involve a complicated sample preparation procedure, or only include a restricted amount of compounds, or are only applicable for screening purposes. To the best of our knowledge, there are no articles that focused on the detection and quantification of a broad range of drugs of abuse in hair as well as in nails. Hence, the purpose of this study was to develop a single method for the simultaneous determination of widely used drugs of abuse in both hair and nails. Attention was paid to the ease by which the method could be applied, the possibility to quantify several common drugs of abuse in one run, and the method sensitivity. The developed method includes a simple and straightforward sample preparation allowing application in routine laboratory practice and reducing costs of consumables and personnel. Moreover it enables the sensitive and specific detection of 7 drugs of abuse and their metabolites in hair and nails.

Incubation time was based on a previous article by Nielsen *et al*., in which the extraction yield was increased up to 100% when the time of extraction increased from 2 to 18 h. In addition, 18 h is a practical timescale in the laboratory since the extraction can be performed overnight.

Mini-UniPrep vials (see figure S-1) were chosen for filtration of the supernatant, as they provide a quick (1/3 of the time), economical (40% of the cost) (ref: Agilent) and environmentally conservative way to remove particulate matter from samples. These preassembled filtration devices replace the combination of solid phase extraction cartridges, syringe filters, auto-sampler vials, septa and caps with a single disposable unit. Moreover, as filtration is accomplished by simply pressing the plunger (upper part) through the liquid placed into the chamber (lower part), the vials do not require qualified lab personnel.

Peak shapes and chromatographic separation were compared and evaluated on several types of columns with different dimensions: HILIC, biphenyl, C8 and C18. The EZ:faast C18 (250 mm x 2.0 mm x 4 µm) column was selected for its sharp and narrow peaks and sufficient chromatographic separation of the analytes (although complete separation is not necessary due to the use of specific MRM transitions for each compound). Special attention was paid to EME as this analyte shows only little retention on reversed phase columns. The gradient of the mobile phase and the flow rate were optimized for the best results in an acceptable time frame of 21 min (including column equilibration) and at an tolerable pressure (200 bar). After optimization, reproducible retention times, good peak shapes and sufficient sensitivity were obtained.

Specific MS parameters such as fragmentor voltage, collision energy and ionization mode were optimized for each compound separately by injecting standard solutions without an LC-column. Dynamic MRM was chosen over MRM as it allowed enhancing the method sensitivity with a factor three. In dynamic MRM the mass spectrometer only monitors MRM transitions of the compounds that are eluting from the column at that particular time point. As a result, fewer transitions are recorded during each MS scan which permits the mass spectrometer to use a longer dwell time and more data points per peak. In comparison to MRM, dynamic MRM provides more reliable and better quality data, and eliminates background and interferences. Consequently dynamic MRM is suitable for monitoring of many compounds at low concentrations.

3.2. Method validation

Tables 4 and 5 present an overview of the performance of the developed method, and figure 1 shows a chromatogram achieved at mid QC level (750 pg mg^{-1}) in hair.

The response in six different blank matrices, spiked with internal standard, was less than 20% of the mean response at the LOQ calculated over the three validation days for all analytes except for MDMA in hair (38%). To meet the selectivity criteria, the LOQ for MDMA in hair could be set to 50 pg mg^{-1} instead of 25 pg mg^{-1}, which is still below the recommended cutoffs of SoHT and SAMHSA, 200 pg mg^{-1} and 300 pg mg^{-1} respectively.

The LOQ in hair was 25 pg mg^{-1} (except for MDMA: 50 pg mg^{-1}) and 50 pg mg^{-1} in nails. Within- and between-run accuracy, expressed as bias, was for all compounds below 20%. For within- and between-run precision, the RSD was for all analytes lower than 20%. As presented in table 1 and 4, the LOQs of the developed method are below the cutoffs recommended by the SoHT and the

SAMHSA. By establishing specific cutoffs for hair testing, these scientific instances provide guidance to laboratories interested in hair testing. The cutoff concentration levels are set to determine whether the sample is positive or negative for the presence of a drug.

For all compounds, the seven-point calibration curves were linear in the investigated ranges in both hair (25 – 20 000 pg mg^{-1}) and nails (50 – 20 000 pg mg^{-1}). When a least-squares linear regression is used to compute a calibration curve, data at the high end of the calibration curve tend to dominate the calculation of the linear regression as the absolute variation is larger for higher concentrations. Therefore a weight factor of $1/x^2$, which weight the data inversely with the concentration to compensate for the excessive error at the lower concentration, was used. All calibrators had their back-calculated concentrations (mean over three days) of the calibration standards within ± 15% of the nominal concentration, or within ± 20% at the LOQ.

For the analysis of BE in nails, the within- and between-run accuracy at low QC exceeded 15% bias. A LOQ of 75 pg mg^{-1} instead of 50 pg mg^{-1} could therefore be proposed. For all other compounds and all other concentration levels, within- and between-run accuracy was within the limits of ±15%. Within- and between-run precision was for all compounds in hair and nails lower than 15% RSD and this for all concentration levels.

Carry-over was assessed by injection of blank samples after the highest calibration point. The carry-over in the blank sample should not be greater than 20% of the LOQ. In hair samples carry-over limits were exceeded for 6-MAM, MDMA, MDEA and COC. In nails, MDMA, BE, COC had a carry-over above 20%. Supplementary experiments showed that by successive injection of two blank samples carry-over remained well below 20%. These results underline the importance of including blank samples after samples with high concentration in a sequence.

The results obtained for matrix effects, recovery and process efficiency are outlined in tables S-1 and S-2. In hair, absolute matrix effects ranged between +5% and +12% at high QC level and RSDs were lower than 9%. Matrix effects for nails ranged between -11% and +8% at low QC level (RSDs lower than 8%). At high QC level in nails, matrix effects were between -17% and +16% (RSDs lower than 8%), and thus slightly above the ±15% limit.

Depending on the compound, recovery in hair samples was between 92% and 99% at high QC (RSDs below 6%). In nails recovery at low QC was between 94% and 115% (RSDs below 10%), and between 90% and 109% at high QC (RSDs below 8%). Recovery at low and high QC concentration was calculated as the ratio of blank matrix samples spiked *before* and *after* extraction. The EMA guidelines do not include specific recommendations for recovery, however it is important for the recovery to be high (around 100%) and reproducible (RSD below 15%). This was true for both hair and nail samples along with RSD values below 10%.

Matrix effects and recovery can be combined to calculate process efficiency. Process efficiency at low QC level ranged between 91% and 105%, and between 93% and 110%, in nails and hair, respectively. At high QC, level process efficiency was between 88% and 120% in nails, and between 99% and 105% in hair. There are no specific EMA recommendations for process efficiency, but as the percentages are around 100% and the RSDs are low (below 16%), it can be concluded that the method has an excellent process efficiency.

3.3. Method application

3.3.1. Participation to a proficiency test DHF 3/15

By means of the proficiency tests, laboratories can assess and control the accuracy of their measurements and get an idea regarding their own analytical performance in an interlaboratory comparison. Together with 77 other laboratories, our laboratory participated to the proficiency test DHF 3/15 in which two hair samples provided by Arvecon GmbH were analyzed with the optimized and validated method. The z-scores were calculated by means of the standard deviation according to Horwitz, using the following formula.

$$z - score = \frac{individual\ result - target\ value}{standard\ deviation\ according\ to\ Horwitz}$$

Concentrations of all compounds were within the acceptance range for a successful participation (|z-score|≤ 2) except for EDDP, for which the Z-score was -3.00.

3.3.2. Application to real samples

After validation, authentic hair (n = 11) and nail (n = 5) samples were analyzed with the described method to demonstrate its applicability. Table 6 presents the results obtained from the hair samples, while figure 2 shows a chromatogram from subject. The concentrations were in accordance with previous reported concentrations in regular drug users (add ref). As shown in table 7, the results of nail analysis were in agreement with data available from a comprehensive anamnesis for each nail sample.

4. Conclusions

A LC-MS/MS method for the simultaneous determination of 7 widely used drugs of abuse and their relevant metabolites in hair and nails was developed and fully validated according to the EMA guidelines. Specificity, LOQ, linearity, accuracy, precision, carry over, matrix effects, recovery, and process efficiency were assessed. Important advantages of the developed method are the easy and facile sample preparation, the simultaneous detection of several drugs with different physicochemical properties, the LOQs below the recommended cutoff concentrations, and the applicability to both hair and nails. Application of the current method to both hair and nails will allow comparison between the two matrices, achievement of knowledge on nail analysis, and may allow defining cutoff levels for nail analysis, which do not exist yet. Furthermore, the method allowed successful participation to a proficiency test and application to hair and nail samples from drug abusers.

5. References

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