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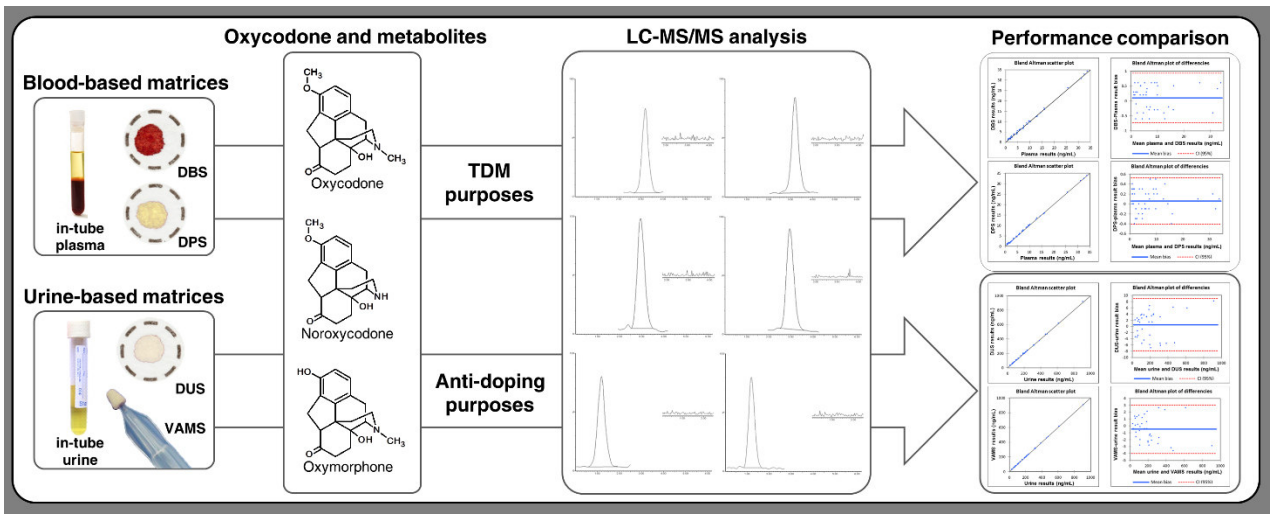

ABSTRACT

Oxycodone is a widely prescribed, full agonist opioid analgesic. As such, it is used clinically to treat different kinds of painful conditions, with a relatively high potential for doping practices in athletes. In this paper, different classic and innovative miniaturised matrices from blood and urine have been studied and compared, to evaluate their relative merits and drawbacks within therapeutic drug monitoring (TDM) and to implement new protocols for anti-doping analysis. Plasma, dried blood spots (DBS) and dried plasma spots (DPS) have been studied for TDM purposes, while urine, dried urine spots (DUS) and volumetric absorptive microsamples (VAMS) from urine for anti-doping.

These sampling techniques were coupled to an original bioanalytical method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for the evaluation and monitoring of the levels of oxycodone and its major metabolites (noroxycodone and oxymorphone) in patients under pain management and in athletes. The method was validated according to international guidelines, with good results in terms of precision, extraction yield and accuracy for all considered micromatrices. Thus, the proposed sampling, pre-treatment and analysis are attractive strategies for oxycodone determination in human blood and urine, with advanced options for application to derived micromatrices. Microsampling procedures have significant advantages over classic biological matrices like simplified sampling, storage and processing, but also in terms of precision (< 9.0% for DBS, < 7.7 for DPS, < 7.1 for DUS, < 5.3 for VAMS) and accuracy (> 73% for DBS, > 78% for DPS, > 74% for DUS, > 78% for VAMS). As regards extraction yield, traditional and miniaturised sampling approaches are comparable (> 67% for DBS, > 74% for DPS, > 75% for DUS, > 75% for VAMS). All dried matrices have very low volumes, which leads to a significant advantage in terms of analysis feasibility. On the other hand, this also leads to a corresponding decrease in the overall sensitivity.

Keywords:

Oxycodone; Bioanalysis; Volumetric absorptive microsampling; Blood and urine microsamples; Therapeutic drug monitoring; Anti-doping analysis.

1. INTRODUCTION

Opioids represent a highly effective class of drugs widely prescribed for the treatment of moderate to severe pain [1]. They include natural (e.g. morphine), semi-synthetic (e.g. oxycodone, OXC) and synthetic (e.g. fentanyl) compounds. Regarding semi-synthetic opioids, one of the most important representatives is certainly OXC ((5*R*,9*R*,13*S*,14*S*)-4,5α-epoxy-14-hydroxy-3-methoxy-17-methylmorphinan-6-one, Figure 1a), which is synthesised from thebaine [2]. The first clinical use of OXC dates back to the early 20th century, but in the last few years it has become the most prescribed narcotic medication for treating moderate to severe pain [3]. OXC is *N*-demethylated to noroxycodone ((1S,5R,13R,17S)-17-hydroxy-12-oxa-4-azapentacycloocatadeca-7(18),8,10-trien-14-one, NOC, Figure 1b) and *O*-demethylated to oxymorphone (4,5α-epoxy-3,14-dihroxy-17methylmorphinan-6-one, OMR, Figure 1c) [4]. *N*-demethylation is carried out by cytochrome P450 subtype 3A4 (CYP3A4), while CYP2D6 catalyses the *O*-demethylation pathway [5] (Figure 1). Both NOC and OMR have antinociceptive properties, but NOC activity is considerably lower than that of OXC. OMR potency is similar to that of OXC; however, its plasma levels are usually much lower and its contribution to the overall analgesic effects is still debated [4]. Due to its full opioid agonist activity, OXC can potentially lead to severe side effects, addiction, misuse and withdrawal upon discontinuation [6]. For these reasons, two main areas of OXC use monitoring and control can be individuated. Firstly, chronic pain patients treated with OXC can greatly benefit from Therapeutic Drug Monitoring (TDM), with the determination of blood or plasma levels and their correlation to side, toxic and therapeutic effects [7, 8]. Unfortunately, in these last few years several public health problems occurred, particularly in the USA: skyrocketing numbers of side effects, with many fatal cases; correspondingly increasing numbers of misuse cases; thousands of patients in need of addiction treatment [9]. Monitoring the use and potential abuse of OXC during therapy is one of the main actions taken to minimise the risk of addiction and is now becoming a pressing necessity [10], which has often to be carried out in less than optimal settings and conditions.

Secondly, reliable methods to detect this drug use are very important in the field of anti-doping control, in order to reveal possible frauds carried out by professional or amateur athletes.

Since both these two application fields are currently dominated by traditional sampling methods and procedures, aim of this study was the development and application of innovative collection methods for OXC, NOC and OMR determination in biological microsamples, to be compared to classic ones. In particular, TDM is normally carried out on blood samples drawn from patients, from which plasma or serum is then obtained and finally analysed [11]. We have implemented two original sampling procedures based on dried blood spots (DBS) and dried plasma spots (DPS): they sample microamounts of hematic matrix and facilitate pre-treatment, also allowing the easy handling, storage and transportation of many samples with reduced requirements for safety, refrigeration and space [12].

Regarding illicit uses of OXC, doping is one of the areas of concern, where the large majority of assays are carried out in urine. Thus, two different microsampling strategies have been devised and tested for this purpose: dried urine spots (DUS) and volumetric absorptive microsamples (VAMS). DUS provide advantages over classic urine samples that are similar to those of DBS or DPS in comparison to plasma [13]. On the other hand, VAMS are obtained by means of innovative samplers that can produce highly-reproducible dried specimens, overcoming some specific disadvantages of dried sample spots, such as area bias and homogeneity issues [14, 15]. Although VAMS approach has been proposed for blood, information on its application to other biological matrices is rather sparse [16]. This study, in fact, represents one of the first attempts to explore VAMS suitability as an approach to urine sampling for anti-doping purposes.

Both blood-based and urine-based samples were analysed using an original, fully validated LC-MS/MS method, providing high sensitivity and selectivity.

Numerous analytical methods have been published for the determination of OXC in biological fluids (among the most recent ones: [17-24]. However, methods suitable for both human blood- and urine-based matrices are very few [18], as are those dealing with micromatrices [17]. None of them includes the comparison of different, original microsampling procedures with significant advantages over traditional ones, such as those proposed in this paper.

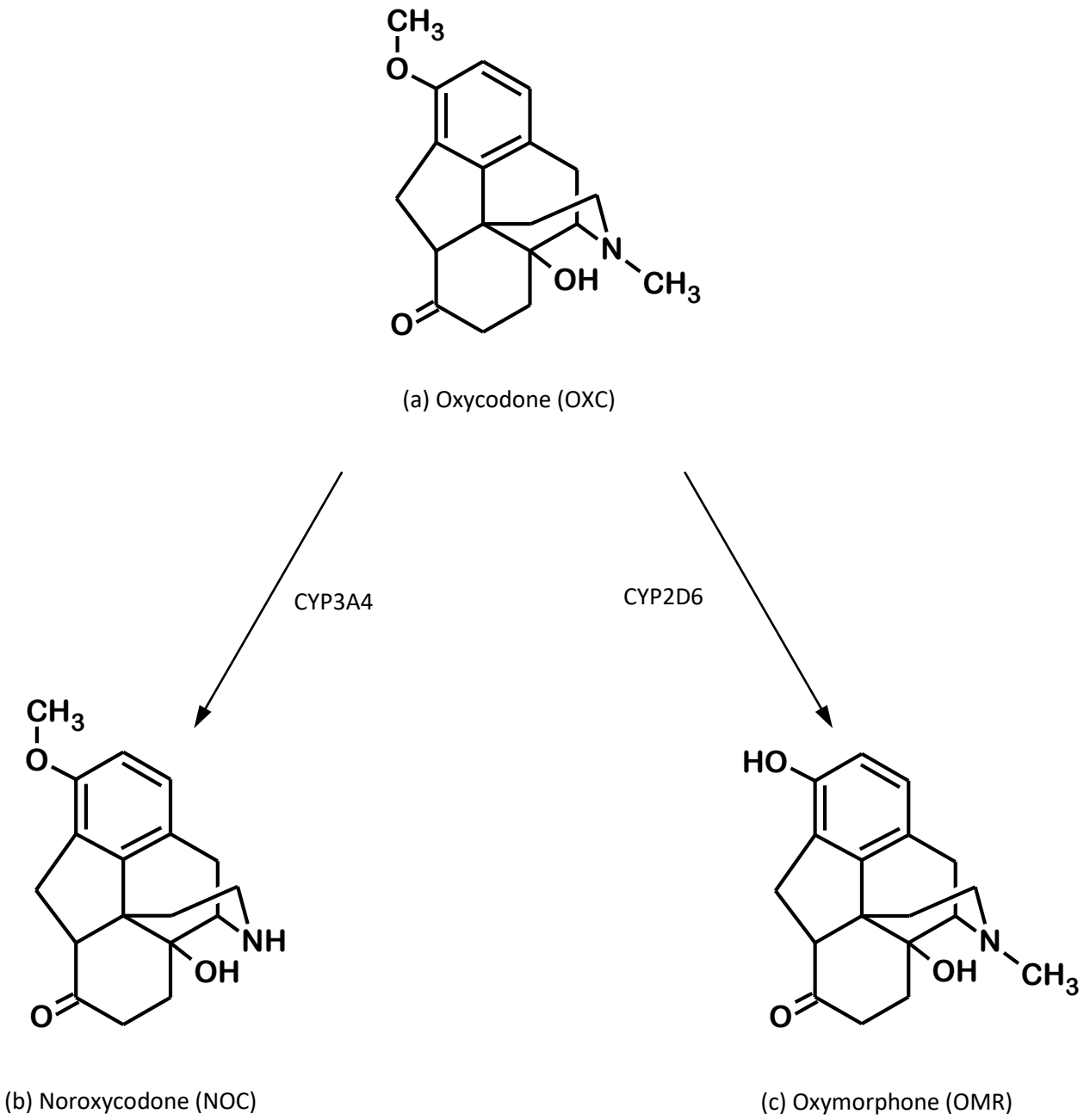

Figure 1. Chemical structures of (a) OXC, (b) NOC, (c) OMR.

2. EXPERIMENTAL

2.1 Chemicals, solutions and equipment

Stock methanolic solutions of OXC, NOC and OMR (1 mg/mL), OXC-D_3, NOC-D_3 and OMR-D_3 (0.1 mg/mL, used as the Internal Standards, ISs) were purchased from Cerilliant Corporation (Round Rock, TX, USA). HPLC-grade (> 99.8%) methanol, acetonitrile, >98% formic acid (FA), Whatman 903 protein saver and Whatman FTA™ DMPK-B IND cards were purchased from Sigma-Aldrich (St. Louis, USA). Ultrapure water (18.2 MΩ cm) was obtained by means of a MilliQ apparatus by Millipore (Milford, MA, USA). Analyte and IS standard solutions were obtained by diluting stock solutions with a 5:95 (V/V%) mixture of 0.1% FA in acetonitrile and 0.1% FA in water. Stock solutions were stable for two months when stored at -20°C (as assessed by HPLC assays); standard solutions were prepared fresh every day. All solutions were stored in Waters (Milford, MA, USA) amber glass vials during all procedures. A Whatman (Maidstone, MA, USA) Harris Uni-Core Punch, 7 mm was used for punching the dried matrix spot (DMS) discs out of the spotting cards. Mitra® VAMS™ microsamplers (10 µL) were provided by Neoteryx (Torrance, CA, USA).

2.2 Sample collection and pre-treatment

2.2.1 Blood-based matrices

2.2.1.1 Plasma

Blood was collected in glass tubes containing anticoagulant and then centrifuged at 1400×*g* for 15 min at 4°C; the supernatant (plasma) was then transferred to polypropylene tubes and stored at -20 °C until analysis. These samples were obtained from drug-free healthy volunteers ("blank" samples), while real samples were from patients receiving OXC for pain management therapy.

A 5-µL aliquot of analyte standard and/or IS mixtures at known concentrations and 100 µL of methanol were added to 100 µL of plasma; the vial was then vortex-mixed and centrifuged at 1400×*g* for 5 min. The supernatant was collected and a 10-µL aliquot was directly analysed in LC-MS/MS.

2.2.1.2 Dried blood spots (DBS)

A hand fingertip was punctured with a sterile, disposable finger pricker and blood drops were collected on a Whatman 903 card. As soon as the blood was completely absorbed, 5 µL of analyte standard and/or IS mixtures at known concentrations were carefully transferred onto each spot. The spots were dried by microwave-assisted drying (MAD) treatment at 700 W for 1.5 minutes then stored in the dark in sealed plastic bags containing silica gel, until analysis.

To extract the analytes and the ISs, a 7-mm diameter circle was punched out from the card with a punching tool and transferred into a vial with 500 µL of methanol. The vial was then subjected to ultrasound-assisted extraction (UAE) for 1 min and centrifuged at 1400×*g* for 1 min. The supernatant was brought to dryness, re-dissolved in 50 µL of 5:95 (V/V%) mixture of 0.1% FA in acetonitrile and 0.1% FA in water and a 10-µL aliquot was injected into the LC-MS/MS system.

2.2.1.3 Dried plasma spots (DPS)

Aliquots of 10 µL of plasma (either blank or from patients) were transferred onto a Whatman FTA™ DMPK-B IND card by means of micropipetting. All the subsequent procedures were the same described for DBS, except the whole spots were completely punched out with a punching tool before extraction.

2.2.2 Urine-based matrices

Urine samples, used as blank matrices, were obtained from drug-free healthy volunteers, while real ones from volunteer athletes, positive to OXC. Urine aliquots of 200 µL were spiked with 10 µL of analyte standard and/or IS mixtures at known concentrations and incubated with 200 µL of 0.1 M sodium acetate buffer (with 5000 UI/mL β-glucuronidase, pH 5.0) at 60°C for 2.5 h, according to the hydrolysis protocol developed by C. Aurand and K. Brown [25] with minor modifications. Then, urine mixtures were subjected to different pre-treatment procedures according to the desired matrix: urine, DUS, VAMS.

2.2.2.1 Urine

The mixture was alkalinised with 100 µL of 2 M NaOH, resulting in final pH values in the 11-13 range. It was extracted three times with 2 mL of an ethyl acetate/n-hexane (20:80, V/V%) mixture. The organic layers were merged and evaporated to dryness under a nitrogen stream, then re-dissolved with 50 µL of 5:95 (V/V%) mixture of 0.1% FA in acetonitrile and 0.1% FA in water and a 10-µL aliquot was processed by LC-MS/MS.

2.2.2.2 Dried urine spots (DUS)

Aliquots of 20 µL of mixture were transferred onto a Whatman FTA™ DMPK-B IND card by means of micropipetting, the obtained spots were dried by MAD at 700 W for 1.5 minutes, then stored in the dark with suitable desiccant until analysis. To extract the analytes and the ISs, a whole DUS was punched out from the card, extracted with 250 µL of 0.1% FA in methanol by UAE (15 min) and microwave-assisted extraction (MAE) at 210 W for 20 s. The solution was brought to dryness under a nitrogen stream, re-dissolved with 50 µL of 5:95 (V/V%) mixture of 0.1% FA in acetonitrile and 0.1% FA in water and a 10-µL aliquot was injected into the LC-MS/MS system.

2.2.2.3 Volumetric absorptive microsampling (VAMS)

A VAMS microsampler is a polypropylene rod (about 4 cm of length) with a small globous tip of a proprietary polymeric porous material (about 2-mm diameter). The surface of the sample mixture was touched with 10-µL VAMS microsamplers for 5 s, dried at room temperature (RT) for 1h and stored at RT, in the dark for 2 months at most. To extract the analytes and the ISs, the microsampler tip was detached from the handler and subjected to UAE for 20 min in 100 µL of MeOH. The resulting solution was brought to dryness under a nitrogen stream, re-dissolved with 25 µL of 5:95 (V/V%) mixture of 0.1% FA in acetonitrile and 0.1% FA in water and a 10-µL aliquot was analysed by LC-MS/MS.

2.3 LC-MS/MS analysis

All plasma- and urine-based samples were processed with the same original LC-MS/MS method, developed on a Waters Alliance e2695 system coupled to a Waters Micromass Quattro Micro triple quadrupole mass analyser.

2.3.1 LC conditions

The chromatographic separation was carried out on a Waters Sunfire C18 (2.1 mm × 50 mm, 3.5 µm) reversed-phase column, maintained at RT and equipped with a guard column. The mobile phase was a mixture of 0.1% FA in acetonitrile (solvent A) and 0.1% FA in water (solvent B) flowing at a 0.4 mL/min rate in gradient mode. The gradient was programmed as follows:

- from 0.0 to 2.0 min, isocratic elution at 2% of solvent A;
- from 2.0 to 3.0 min, linear gradient from 2% to 10% of solvent A;
- from 3.0 to 5.5 min, isocratic elution at 10% of solvent A;
- from 5.5 to 8.0 min, linear gradient from 10% to 2% of solvent A;
- from 8.0 to 10.0 min, column equilibration with isocratic elution at 2% of solvent A.

2.3.2 MS/MS conditions

Tandem mass spectrometry acquisition was carried out in multiple reaction monitoring (MRM), using an electrospray ionisation source operating in positive mode (ESI+). The optimised parameters were as follows: ion source voltage 3.8 kV, ion source temperature 125°C, desolvation gas temperature 200°C; desolvation gas flow rate 250 L/h. Nitrogen was used as the desolvation gas and was generated from pressurised air by an N2 LC-MS (Claind, Lenno, Italy) nitrogen generator; collision gas was 99.995% argon (SIAD, Bergamo, Italy). The precursor ion and the product ions, with dwell time, cone voltage and collision energy, were optimised by analyte and IS solution infusion at 10 µL/min. Cone voltage was set to 27 V for OXC, NOC, OXC-D_3 and NOC-D_3; 29 V for OMR and OMR-D_3. Collision energy was 27 eV for OXC and OXC-D_3, 23 eV for NOC and NOC-D_3, 25 eV for OMR and OMR-D_3. For all analytes and ISs, the quasi-molecular $[M+H]^+$ ion was selected and acquired and the chosen transitions were: m/z 316.6 → 242.1 for OXC, m/z 302.6 → 227.8 for NOC and OMR, m/z 319.6 → 245.1 for OXC-D_3, m/z 305.6 → 230.8 for NOC-D_3 and OMR-D_3. The dwell times per channel were set at 300 ms for each analyte and ISs. Data were acquired and processed by using Waters MassLynx 4.1 software.

2.4 Method validation

According to official guidelines [26] the following validation parameters were tested: linearity, absolute recovery, precision, matrix effect, selectivity, stability and accuracy.

2.4.1 Linearity range, LOQ and LOD

Calibration samples were prepared by adding different known concentrations of analyte and IS to blank matrices. The resulting samples were subjected to sample preparation and analysis carried out in triplicate for each concentration. The obtained analyte/IS peak area ratios were plotted against the corresponding nominal concentrations and the calibration curves set up by least-square method. Verification of the quality of fit to the calibration curves was evaluated by comparing back-calculated concentrations to the nominal ones and by using both coefficient of determination (r^2) and lack of fit test significance ($p \leq 0.05$). Limit of quantitation (LOQ) is defined as the lowest concentration of analyte in a sample which can be reliably quantified with an acceptable accuracy and precision, while limit of detection (LOD) is the lowest concentration that can be detected. LOQ and LOD values for each analyte were determined by analysing 7 samples at different concentrations and assessed as the concentrations which gave rise to peaks whose heights are 10 and 3 times the baseline noise, respectively.

2.4.2 Absolute recovery and precision

Absolute recovery and precision were evaluated by adding known amounts of the analytes (at three different concentrations, corresponding to the lower limit, a middle point and a high value of each calibration curve) and of ISs (at constant concentration) to blank samples, then subjecting them to sample pre-treatment and LC–MS/MS. The analyte and IS absolute peak areas were compared to those of injected standard solutions at the same concentrations and the absolute recovery was calculated. The assays described above were repeated six times within the same day to obtain intraday precision and six times over six different days to evaluate interday precision, in both cases expressed as percentage relative standard deviation (RSD%).

2.4.3 Matrix effect

IS-corrected matrix effect of dried microsamples was evaluated on blank sample extracts from 6 different sources, fortified post-extraction by adding known amounts of the analytes. Mean analyte/IS peak area ratios of each extract were then compared with analyte/IS peak area ratio from analyte solution prepared in mobile phase and the resulting percentage ratios were calculated. Moreover, angular coefficients (slopes) of the calibration curves for each analyte obtained from standard solutions were statistically compared (significance level $p \leq 0.05$) with those obtained from each spiked matrix extract.

2.4.4 Selectivity

Method selectivity was assessed by individually analysing blank samples from 6 different healthy volunteers not subjected to any pharmacological therapy. The resulting chromatograms were checked for possible endogenous interference and the acceptance criterion was no peak whose signal exceeded the LOD for the analytes, or 5% of the signal/noise ratio for the ISs.

2.4.5 Stability

The stability of the analytes in the different matrices was assessed as follows. For the "wet" matrices (plasma, urine), blank samples were spiked at a 20 ng/mL level, then stored at -80°C for 2 months. At regular intervals (1 week), a different sample vial was thawed, subjected to pre-treatment and analysed. For dried matrices (DBS, DPS, DUS, VAMS), blank samples were spiked at a 20 ng/mL level, then stored at RT for 2 months. At regular intervals (1 week), a different sample was subjected to pre-treatment and analysed. The measured analyte concentrations were compared to those of the same samples extracted and analysed immediately after biosampling (wet matrices), or after biosampling and drying (dried matrices).

2.4.6 Accuracy

Known amounts of the analytes at three levels (lower limit, middle point and high value of each calibration curve) and of ISs at constant levels were added to real samples whose analyte content was already known. The spiked samples were prepared and analysed to obtain the mean recovery

(%) and repeated three times during the same day to calculate the corresponding standard deviation (SD).

2.5 Data comparisons

All results obtained on real samples from OXC-treated patients (blood-based matrices) and OXC-taking subjects (urine-based matrices) were compared by plotting the results for each micromatrix (DBS or DPS, DUS or VAMS) as a function of the results for the corresponding classic matrix (plasma, urine). Then, the least-square method was applied and linearity correlation coefficient, slope and variance of each line were calculated. Moreover, Bland Altman analysis was performed to compare data sets. Statistical analysis was carried out using Data Analysis ToolPak and XLSTAT for Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1 LC-MS/MS analysis

Optimised mass spectrometry conditions have been obtained by syringe infusion of analyte solutions directly into the ESI source, thus obtaining MS and MS/MS spectra whose parameters are reported in Section 2.3.2. A suitable chromatographic setup was investigated to obtain good resolution and short retention times (while avoiding co-elution with the residual matrix components): a reversed phase column (C18, 50 mm × 2.1 mm, 3.5 µm) was chosen while a gradient elution was applied, starting at 2% organic modifier and going up to 10% over 1 min and flowing at a constant rate of 0.4 mL/min. Baseline separation was achieved in less than 8 min, considering that chromatographic resolution of NOC and OMR is mandatory as both exhibit the same transition mode.

3.2 Pre-treatment of blood-based matrices

Different procedures were tested in order to compare the performance and suitability of the three blood-based matrices (plasma, DBS, DPS). For plasma, liquid-liquid extraction (LLE), plasma protein precipitation (PPP) and solid phase extraction (SPE) were tried, using different solvents/reagents (for LLE and PPP) and different kinds of cartridges and procedures (for SPE). Complete comparison data is not reported, however the best results, in terms of speed-cost-purification balance were obtained using PPP. Pure methanol in a 1:1 volume ratio allowed to obtain the highest extraction yields and a reasonably low matrix effect. The effect of vortex mixing time on extraction yields was also tested: 1 min was chosen since longer times did not lead to significant improvements. Pre-treatment optimisation on DBS and DPS is usually simpler and more straightforward than on plasma. In fact, the simple, unmanaged transfer, absorption and drying of the fluid sample onto the card is in itself a pre-treatment of sort. Each DMS techniques has different pros and cons: DBS are easy to produce from a minimally invasive fingerprick and do not need any matrix handling before absorption on the card; however, they also suffer from volume and spot size variability due to haematocrit, and also from a more complex matrix (and thus potentially higher matrix effect/interference) when compared to DPS.

Drying time was the first tested parameter, both at RT and using MAD under different conditions. At RT, complete drying (i.e., constant weight) was achieved in under 1 h for both DMS; however, MAD drastically reduced drying times, with best results obtained using a 700-W power for 1.5 min. Higher power values produced significant analyte degradation.

Regarding DBS, the problem of the blood volume corresponding to specific spot dimensions arises. As reported in previous works, it is possible to calculate this value, knowing that the area / blood volume ratio is relatively constant [27], and corresponds to about 3.5 $mm^2/\mu L$ (some variability is caused by differences in viscosity). Based on this calculation, 10 µL of blood correspond to a 7-mm diameter spot.

Typically, the analyte extraction step is carried out on both DMS types with a suitable solvent and the resulting sample can be injected as such. Alternatively, the extract can be subjected to further treatment procedures (LLE, SPE). In this case, also thanks to the high sensitivity and selectivity of the LC-MS/MS method, solvent extraction was the only step needed and the solvent type and volume the only tested parameters. The obtained results were comparable: acetonitrile, acidified methanol and acetonitrile, and organic solvent/water mixtures were unsuitable, since acetonitrile provided low extraction yields, while acidified solvents and mixtures caused a relatively high matrix effect. Pure methanol, on the other hand, proved to be a very efficient extractor, and also provided the cleanest solutions.

Different extraction volumes were also tried and it was ascertained that extraction yields did not improve when the volume exceeded 500 µL; this volume was used for all subsequent assays. As regards the extraction mode, UAE, MAE and a sequential combination of both modes [28] were tested: while UAE gave better results with respect to MAE, the combination of UAE and MAE did not appreciably improve extraction performances when compared to UAE alone, thus the latter was chosen.

The extracted solution was then dried under vacuum and re-dissolved in 50 µL of an acidified water/acetonitrile mixture to avoid excessive dilution.

3.3 Pre-treatment of urine-based matrices

After glucuronidase hydrolysis, urine samples were simply alkalinised to keep the basic analytes uncharged, then extracted with a plain organic solvent mixture. Despite the strong basicity of the mixture (pH 11-13), no unwanted analyte degradation or the formation of emulsions were observed. More detailed studies were carried out on the other two urine-based matrices.

Regarding DUS, the same accelerated drying procedure already tested for DBS and DPS was applied; it was ascertained that the same conditions (i.e., 1.5 min at 700 W) were suitable for complete drying without significant analyte loss. Then, three main parameters were studied: extraction solvent, extraction volume and extraction mode. Pure methanol and acetonitrile, different concentrations of FA in methanol or acetonitrile and water/organic solvent mixtures were tested as possible extractors. The best results in terms of extraction yields were obtained using 0.1% FA in methanol. Similarly, solvent volumes in the 50-350 µL range were tested and extraction yields did not increase when volumes exceeded 250 µL. Finally, three extraction modes were compared: UAE, MAE and a sequential combination of both modes. While the two single modes gave similar results, the combined mode provided significantly better yields.

Regarding VAMS, the first optimisation step for this innovative procedure was the evaluation of the sampling volume and its variability. In a previous paper, gravimetric experiments were carried out to establish the volume absorbed after VAMS tip exposure to biological matrices other than blood, showing how the collected volumes (9.68 ± 0.84 µL) are statistically indistinguishable from those pipetted (10.12 ± 0.26 µL), thus demonstrating a good sampling accuracy by means of VAMS devices also for urine [16]. Sampling time was tested in the 2-10 s range. It was verified that no significant differences exist within this time range, and this probably means that the VAMS tip reaches saturation within a very short time (< 2 s), without further changes in the next few seconds. To keep times at a minimum while avoiding measurement errors, a 5-s sampling time was set for VAMS. Drying time can also be a significant source of time consumption; tests verified that constant weight (i.e., complete water evaporation) is reached in slightly less than 1 h; the latter was chosen as the standard drying time. Then, extraction solvent and volume were also optimised; 100 µL of methanol gave the best results, although pure acetonitrile produced almost identical extraction yields (but a slightly higher matrix effect), while acidified solvents and water/organic solvent mixtures provided lower extraction yields and/or unacceptable matrix effect. Finally, as regards extraction method,

UAE was chosen over MAE and a combination of the two techniques, as in the latter cases higher matrix effect was observed, although extraction yields were similar. It can be noted that urine extraction by LLE required the use of a water-immiscible solvent mixture and three extraction steps, while dried micromatrix extraction was carried out simply with water-miscible solvents (methanol, FA in methanol) in just one step. This is, at least in part, due to the matrix absorption process on the supports, which can be considered as a pre-treatment step in itself.

3.4 Method validation

3.4.1 Linearity on spiked matrices

Standard solutions of the analytes at different concentrations and ISs at constant concentrations of 30 ng/mL were added to each blank matrix and subjected to the respective pre-treatment procedure. Satisfactory linearity ($r^2 \geq 0.9991$) and non-significant lack of fit test results ($p > 0.05$) were obtained for all analytes over wide concentration ranges. The detailed results are reported in Table 1.

3.4.2 Extraction yield and precision

Extraction yield and precision were tested on blank matrices spiked with three different analyte concentrations (ISs at constant concentration). The resulting data were satisfactory, being always higher than 67% for both the analytes and ISs. The methodology granted also good precision, with RSD values always lower than 9.0% (Table 2).

3.4.3 Matrix effect

Possible ion suppression or matrix effects were also investigated by comparing mean analyte/IS peak area ratios of each extract with analyte/IS peak area ratio from analyte solution prepared in mobile phase. Moreover, the angular coefficients of the calibration curves for each analyte obtained from standard solutions were compared with those obtained from each spiked matrix extract. The IS-corrected matrix effect results obtained were good, and statistically ($p > 0.05$) non-relevant. Assays results are reported in Supplementary Material (Table S1).

3.4.4 Stability

One of the most important advantages of dried micromatrices is their enhanced stability when compared to "traditional" matrices. In this case, we opted to compare the stability of the analytes in plasma and urine samples under deep-freezing conditions (-80°C) to those of the analytes in the dried matrices, but stored at RT.

Over time, all dried matrices consistently produce similar recovery values (> 93.2% after 2 months) with respect to the corresponding "wet" matrices (> 92.1% after 2 months), despite having been stored at RT. This highlights the fact that drying stops with high efficiency most enzymatic and chemical reactions that could potentially cause analyte degradation. Complete stability assays results are reported in Supplementary Material (Table S1).

3.4.5 Selectivity

Six different blank samples of each matrix, taken from volunteers not undergoing any pharmacological therapy, were analysed according to the respective experimental protocols. No analyte peak larger than the LOD was detected in any matrix (data not shown). Selectivity was thus deemed very satisfactory.

3.5 Analysis of real blood-based samples: patients under pain management therapies

A clinical observational study was carried out on eighteen patients undergoing pain management therapies, with moderate to severe pain, who took for at least seven days oral controlled-release OXC. According to the study protocol, all analyses were carried out on traditional, in-tube samples: the blood was centrifuged and the resulting plasma was pre-treated as reported in Section 2.2.1.1 and analysed with the described original LC-MS/MS method (Section 2.3). A plasma microsample was also pipetted from each sample and used to produce DPS. At the time of sampling, the patients were also fingerpricked and 2 blood drops were used to produce DBS. In this way, the three blood-based matrices were sampled at the same time and in the same exact conditions in order to grant maximum result comparability. As an example, the chromatogram of a DBS sample from a patient is shown in Figure 2 (together with the corresponding blank DBS chromatogram). Complete analysis results on the three matrices are reported in Supplementary Material (Table S2). It should be noted

that DBS samples include red blood cells (RBC), while plasma and DPS do not. For this reason, the raw results obtained from direct DBS analysis should be corrected for haematocrit and for RBC/plasma partition. The mean normal haematocrit was assumed to be 38% (V/V) in women and 48% (V/V) in men; mean analyte RBC/plasma partition coefficients have been previously found to be 1.5 for OXC and 1.7 for NOC (no value was found for OMR, it was assumed to be similar to that of NOC) [29]. The two parameters were combined to obtain different correction factors for female and male subjects, and for each analyte; these factors were applied to analyte levels found in DBS to estimate the corresponding plasma concentrations (the concentration values found in Table S2 for DBS have already been converted to equivalent plasma concentrations).

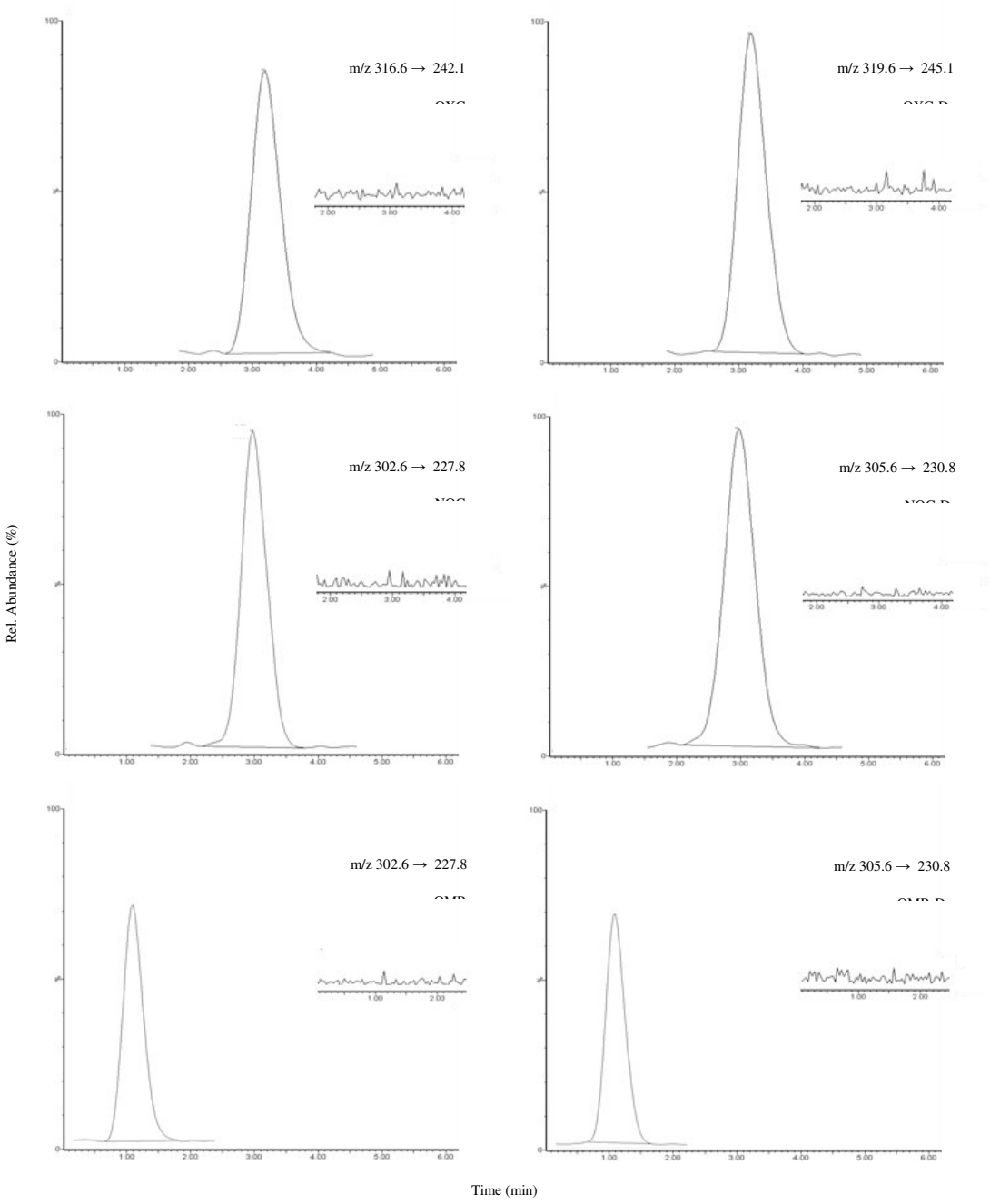

Figure 2. DBS chromatogram of a patient treated with OXC vs the corresponding blank matrix.

3.5 Analysis of real urine-based samples: anti-doping analysis

Similarly to blood-based matrices, in-tube urine samples were obtained from anonymised urine samples of volunteer athletes resulted positive to OXC. Then, the urine sample was sub-sampled for DUS and VAMS production, as described in Section 2.2.2. In this case, the three urine-based matrices were materially obtained from the same identical sample. As an example, the chromatogram of a urine VAMS sample from a subject is shown in Figure 3 (together with the corresponding blank VAMS chromatogram). Complete analysis results on the three matrices are reported in Supplementary Material (Table S3).

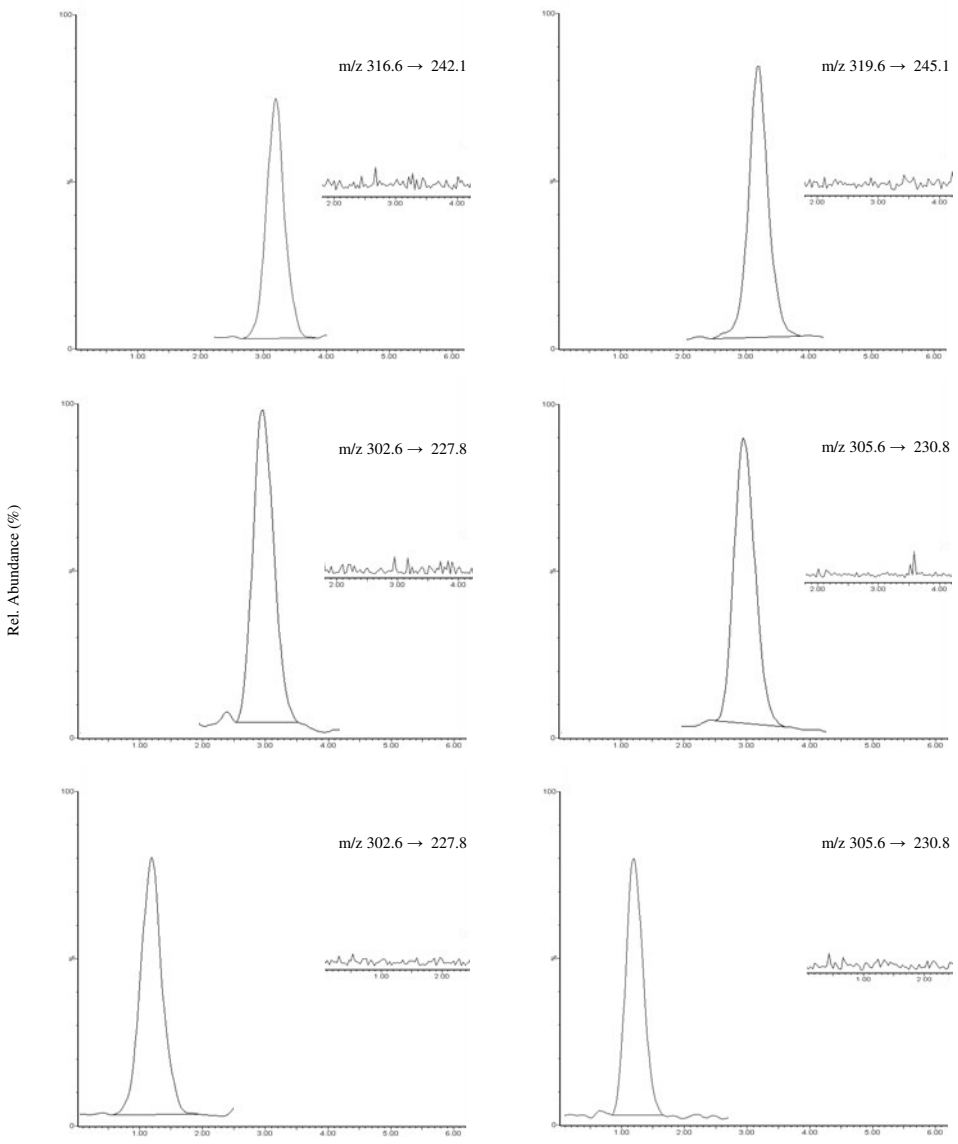

Figure 3. Urine VAMS chromatogram from an athlete taking OXC vs the corresponding blank matrix.

3.6 Accuracy

Method accuracy was generally higher for DPS (> 78%) when compared to DBS (> 73%) as regards blood-based micromatrices and for VAMS (> 78%) when compared to DUS (> 74%) as regards urine-based micromatrices. The complete results are detailed in Supplementary Material (Table S4).

3.7 Comparison between the "in-tube" and "dried" approach results

The results (analyte concentrations ± RSD%) obtained using the classic in-tube approach were compared to those obtained with each innovative dried micromatrix approach. The comparison was carried out by Bland Altman analysis and very good linear relationships were found for all dried matrices when compared to "wet" ones ($0.812 \leq$ slope ≤ 1.007, $r^2 \geq 0.858$ for DBS and DPS; $0.980 \leq$ slope ≤ 1.002, $r^2 \geq 0.999$ for DUS and urine VAMS). All detailed linearity parameters are reported in Supplementary Material (Table S5), as are the corresponding graphs (Figure S1a-d).

Consequently, all dried matrices seem to be suitable for the proposed purpose (TDM for blood-based ones, anti-doping tests for urine-based ones). The best correspondence with the reference classic matrix was observed for DPS as regards blood-based micromatrices and for VAMS as regards urine-based micromatrices.

Considering the mode and characteristics of each sampling approach, the following can be observed.

All dried matrices have very low volumes (10-20 µL), which leads to a significant advantage in terms of analysis feasibility, since it can be carried out even when just sample traces are available. On the other hand, this also leads to a corresponding decrease in the overall quantitation and detection limits of the method.

No clear winner has emerged when considering precision and accuracy; the sampling methods can be considered roughly equivalent in this regard.

Analyte stability is better for dried matrices, even when comparing different storage temperatures.

The dried matrix approaches are clearly superior in terms of practicality and reliability, due to the "solid state" of the sample that makes it more easily and safely manageable than liquid samples.

No clear winner can be declared, at this point, between DBS and DPS, nor between DUS and VAMS. Thus, the "best" sampling procedure is the one, which best conforms to the specific needs and

characteristics of the analytes and the laboratory in the situation at hand. For example, when time sparing is paramount, a matrix amenable to MAD (e.g., DUS) could be preferred over another, although the latter VAMS is less labour-intensive and leading to slightly better results with regard to result precision and accuracy.

4. Conclusion

Rapid, sensitive and selective microsampling procedures for the measurement of OXC and its main metabolites in blood- and urine-based matrices by LC-MS/MS were developed. These methodologies were compared to the classic in-tube collection procedures and proved to be cheaper, faster and also requiring lower matrix volume from subjects. Moreover, enhanced stability has been demonstrated for all analytes in the dried micromatrices, when stored at RT for 2 months. The analytical method presented herein has been fully validated, obtaining good results: good sensitivity, with LOQ values in the order of a few ng/mL, precision was also satisfactory, with RSD values always lower than 9.0%. After validation, the microsampling procedures were successfully applied for the analysis of blood-based specimens from patient treated with OXC, and of urine-based specimens from sport amateurs who took OXC for different reasons. The data obtained with the micromatrix approaches were always in good agreement with those found with the classic in-tube approach.

In conclusion, the multiple approach developed in this work allows detecting plasma and urine levels of OXC and its main metabolites in a reliable way, benefiting of different classic and microsampling procedures with specific advantages and drawbacks. The most suitable collection and treatment procedure can thus be chosen, according to the practical and actual needs of patients/subjects and according to the purpose and scheduling of the analysis process.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest. Prof. Roberto Mandrioli received funding from Mundipharma Pharmaceuticals (Milan, Italy) to carry out previous analytical studies on oxycodone and metabolites in biological fluids but not to perform these experiments.

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Supplementary Material for

Determination of oxycodone and its major metabolites in haematic and urinary matrices: comparison of traditional and miniaturised sampling approaches

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Supplementary Material Legend

Table S1. Matrix effect and stability

Table S2. Sample analysis results on blood-based matrices

Table S3. Sample analysis results on urine-based matrices

Table S4. Accuracy assay results

Table S5. Result comparison linearity

Figure S1. Statistical correlation plots for the following result comparison: plasma vs DBS (a), plasma vs DPS (b), urine vs DUS (c), urine vs VAMS (d).

Table S1. Matrix effect and stability.

Analyte	Concentration level[a]	Matrix	Matrix effect, %RE[b]	Stability, % Recovery[b,c]	
				1 week	2 months
OXC	Low	Plasma	4.0	98.4	96.5
		DBS	4.2	98.6	94.3
		DPS	3.7	98.9	94.7
		Urine	5.1	100.4	98.1
		DUS	3.4	97.9	95.1
		VAMS	3.0	98.0	97.2
	Intermediate	Plasma	5.8	98.2	94.0
		DBS	6.0	98.3	93.3
		DPS	5.2	98.7	94.0
		Urine	6.6	101.1	97.3
		DUS	4.0	98.1	96.0
		VAMS	3.6	98.6	97.5
	High	Plasma	6.7	97.6	92.8
		DBS	6.7	98.0	95.6
		DPS	5.9	99.0	96.0
		Urine	7.4	102.6	97.0
		DUS	4.2	98.0	96.3
		VAMS	4.0	98.3	97.1
NOC	Low	Plasma	4.4	97.8	95.4
		DBS	4.3	97.0	94.1
		DPS	3.6	96.9	96.0
		Urine	5.2	101.4	97.1
		DUS	3.6	97.4	95.3
		VAMS	3.6	97.3	96.8
	Intermediate	Plasma	6.3	98.1	94.8
		DBS	6.0	97.5	95.3
		DPS	5.9	97.3	96.0
		Urine	7.4	102.1	96.8
		DUS	5.4	96.9	95.3
		VAMS	4.9	97.2	95.9
	High	Plasma	7.6	96.2	95.6
		DBS	6.9	96.9	94.4
		DPS	6.2	97.0	95.1
		Urine	7.8	102.3	96.1
		DUS	6.0	96.2	94.9
		VAMS	5.5	97.6	97.0

(continues on next page)

OMR	Low	Plasma	4.5	96.0	92.2
		DBS	4.0	96.3	94.5
		DPS	3.7	97.0	95.2
		Urine	5.7	100.6	97.6
		DUS	5.6	96.0	95.4
		VAMS	4.8	95.9	95.1
	Intermediate	Plasma	6.6	96.4	93.5
		DBS	6.1	96.5	94.2
		DPS	5.5	96.5	94.9
		Urine	7.1	101.0	97.2
		DUS	5.6	97.3	95.8
		VAMS	5.0	97.5	96.0
	High	Plasma	7.5	95.8	93.7
		DBS	6.9	97.1	93.9
		DPS	6.9	97.2	94.8
		Urine	8.0	102.0	95.9
		DUS	6.2	96.6	94.7
		VAMS	6.0	97.0	94.9

[a] For each matrix, “Low”, “Intermediate” and “High” correspond to the LOQ value, an intermediate point and the upper limit of the linearity curves, respectively.

[b] $n = 6$, mean value.

[c] Percentage recovery vs the same spiked samples analysed immediately.

Table S2. Sample analysis results on blood-based matrices.

Sample n°	Found concentration, ng/mL								
	OXC			NOC			OMR		
	Plasma	DBS	DPS	Plasma	DBS	DPS	Plasma	DBS	DPS
1	4.9 ± 0.6	4.5 ± 0.2	4.6 ± 0.2	2.4 ± 0.3	3.1 ± 0.2	2.3 ± 0.1	0.7 ± 0.2	1.0 ± 0.2	0.9 ± 0.1
2	5.6 ± 0.7	5.8 ± 0.3	5.6 ± 0.2	10.3 ± 1.3	10.0 ± 0.5	10.5 ± 0.5	0.5 ± 0.2	0.7 ± 0.1	0.5 ± 0.1
3	9.9 ± 1.2	10.1 ± 0.5	9.8 ± 0.4	7.3 ± 0.9	6.7 ± 0.3	7.2 ± 0.3	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
4	1.2 ± 0.2	1.8 ± 0.1	1.5 ± 0.1	4.6 ± 0.6	4.8 ± 0.2	4.5 ± 0.2	0.7 ± 0.2	0.9 ± 0.2	0.7 ± 0.2
5	15.9 ± 1.9	16.4 ± 0.8	15.5 ± 0.7	13.1 ± 1.6	12.5 ± 0.6	13.5 ± 0.5	2.3 ± 0.5	2.6 ± 0.3	2.2 ± 0.2
6	5.7 ± 0.7	6.0 ± 0.3	5.6 ± 0.2	16.2 ± 2.0	15.9 ± 0.8	16.0 ± 0.7	1.0 ± 0.3	0.8 ± 0.2	1.1 ± 0.2
7	14.8 ± 1.8	14.2 ± 0.7	15.5 ± 0.6	31.8 ± 3.9	30.9 ± 1.5	31.5 ± 1.3	2.6 ± 0.5	3.0 ± 0.4	2.5 ± 0.3
8	4.6 ± 0.6	4.0 ± 0.2	4.9 ± 0.2	6.1 ± 0.8	5.9 ± 0.3	6.0 ± 0.3	0.9 ± 0.3	1.2 ± 0.2	1.1 ± 0.1
9	33.7 ± 4.1	34.3 ± 1.7	33.8 ± 1.5	31.2 ± 3.9	30.8 ± 1.5	31.4 ± 1.3	1.9 ± 0.4	2.0 ± 0.4	1.9 ± 0.3
10	5.9 ± 0.7	6.3 ± 0.3	6.1 ± 0.2	14.4 ± 1.8	14.2 ± 0.7	14.4 ± 0.6	1.6 ± 0.4	2.1 ± 0.3	1.5 ± 0.2
11	3.4 ± 0.4	3.6 ± 0.2	3.4 ± 0.1	7.4 ± 0.9	7.2 ± 0.3	7.5 ± 0.3	0.7 ± 0.2	0.8 ± 0.2	0.7 ± 0.1
12	3.5 ± 0.4	4.0 ± 0.2	3.8 ±0.2	9.0 ± 1.1	8.7 ± 0.4	9.2 ± 0.4	0.8 ± 0.3	0.7 ± 0.2	1.1 ± 0.2
13	12.7 ± 1.5	12.3 ± 0.6	12.8 ± 0.5	9.5 ± 1.2	10.2 ± 0.5	10.0 ± 0.4	0.6 ± 0.2	1.0 ± 0.2	0.8 ± 0.1
14	18.3 ± 2.2	18.9 ± 1.0	18.3 ± 0.8	23.2 ± 2.9	22.5 ± 1.1	23.5 ± 0.9	2.2 ± 0.5	2.5 ± 0.4	2.4 ± 0.3
15	4.9 ± 0.6	5.6 ± 0.3	4.7 ± 0.2	7.0 ± 0.9	6.7 ± 0.3	7.2 ± 0.3	0.8 ± 0.3	1.1 ± 0.1	1.0 ± 0.1
16	1.4 ± 0.2	1.6 ± 0.1	1.4 ± 0.1	1.0 ± 0.2	1.2 ± 0.1	1.0 ± 0.1	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.1
17	32.5 ± 4.0	32.9 ± 1.7	32.4 ± 1.4	25.7 ± 3.2	26.4 ± 1.3	25.8 ± 1.1	2.8 ± 0.6	2.2 ± 0.4	2.5 ± 0.3
18	9.8 ± 1.2	10.3 ± 0.5	10.1 ± 0.4	8.7 ± 1.1	9.3 ± 0.5	9.0 ± 0.4	0.7 ± 0.3	1.1 ± 0.3	0.9 ± 0.2

n = 3, mean value.

Table S3. Sample analysis results on urine-based matrices.

Sample n°	Found concentration, ng/mL								
	OXC			NOC			OMR		
	Urine	DUS	VAMS	Urine	DUS	VAMS	Urine	DUS	VAMS
1	240.6 ± 9.6	244.6 ± 8.6	238.5 ± 4.3	312.9 ± 21.9	315.9 ± 8.8	310.3 ± 7.1	68.2 ± 5.4	65.3 ± 3.4	66.6 ± 2.5
2	321.4 ± 12.9	315.2 ± 11.0	319.7 ± 6.4	223.9 ± 15.3	225.3 ± 6.3	222.7 ± 4.5	84.8 ± 6.7	86.1 ± 4.4	85.0 ± 3.3
3	908.2 ± 36.3	916.4 ± 28.4	905.3 ± 15.8	612.4 ± 41.6	618.3 ± 17.3	615.0 ± 13.5	317.6 ± 25.1	312.1 ± 17.2	320.2 ± 12.8
4	112.9 ± 4.5	107.4 ± 3.9	110.9 ± 2.2	130.2 ± 9.1	133.9 ± 3.7	130.5 ± 2.6	59.2 ± 4.7	55.7 ± 3.0	60.3 ± 2.3
5	216.8 ± 8.7	220.2 ± 7.5	215.4 ± 5.0	112.0 ± 7.7	111.1 ± 3.1	116.9 ± 2.2	30.2 ± 2.4	32.3 ± 1.6	30.5 ± 1.2
6	385.4 ± 16.2	374.9 ± 13.1	389.7 ± 9.4	237.1 ± 16.4	240.9 ± 7.2	235.6 ± 4.9	134.5 ± 10.6	130.1 ± 6.8	135.7 ± 5.4
7	184.9 ± 7.6	190.3 ± 6.1	185.5 ± 4.3	160.9 ± 11.3	163.9 ± 4.8	160.5 ± 3.0	66.2 ± 5.2	67.9 ± 3.7	65.3 ± 2.5
8	478.6 ± 20.6	468.3 ± 15.0	475.0 ± 9.5	456.7 ± 31.0	467.9 ± 14.0	452.5 ± 8.6	200.7 ± 15.8	197.9 ± 10.5	198.5 ± 7.7
9	98.7 ± 4.0	102.3 ± 3.4	100.2 ± 2.1	202.6 ± 14.0	196.7 ± 5.1	200.2 ± 4.2	74.6 ± 5.9	76.8 ± 4.1	75.9 ± 3.0
10	106.3 ± 4.5	108.9 ± 3.5	105.6 ±	190.3 ± 12.9	201.0 ± 6.0	195.4 ± 3.5	36.3 ± 2.9	32.9 ± 1.6	35.9 ± 1.3
11	160.8 ± 6.6	163.9 ± 5.6	162.5 ±	108.4 ± 7.4	112.3 ± 3.3	105.6 ± 2.1	53.6 ± 4.2	56.2 ± 3.0	55.0 ± 2.2
12	212.3 ± 8.7	205.4 ± 6.6	207.5 ±	185.9 ± 13.0	187.3 ± 5.2	185.5 ± 3.9	60.8 ± 4.8	59.3 ± 3.1	60.9 ± 2.4

n = 3, mean value.

Table S4. Accuracy assay results.

Analyte	Concentration level[a]	Accuracy (Mean % Recovery)[b]					
		Plasma	DBS	DPS	Urine	DUS	VAMS
OXC	Low	83	80	82	84	84	86
	Intermediate	87	84	88	86	87	87
	High	93	85	90	90	92	94
NOC	Low	79	80	82	77	80	83
	Intermediate	85	87	84	82	84	86
	High	88	89	89	86	88	90
OMR	Low	80	74	79	74	75	79
	Intermediate	84	77	81	80	83	84
	High	87	83	86	83	85	87

[a] For each matrix, “Low”, “Intermediate” and “High” correspond to the LOQ value, an intermediate point and the upper limit of the linearity curves, respectively.

[b] $n = 3$.

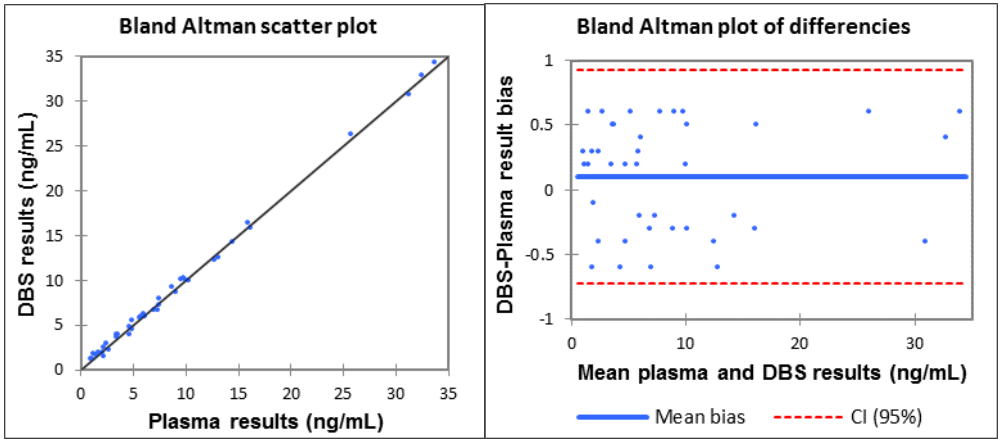
Table S5. Result comparison linearity.

	OXC				NOC				OMR			
	Slope	Intercept	r^2	S[a]	Slope	Intercept	r^2	S[a]	Slope	Intercept	r^2	S[a]
Plasma-DBS	1.0067	0.2013	0.9986	0.3724	0.9989	0.1454	0.9966	0.4768	0.8916	0.0245	0.8456	0.2441
Plasma-DPS	1.0033	-0.0518	0.9995	0.2227	0.9950	-0.0657	0.9993	0.2148	0.8124	0.3786	0.8584	0.2338
Urine-DUS	1.0014	-1.1682	0.9992	6.8999	1.0128	0.3040	0.9992	4.3385	0.9798	0.8243	0.9991	2.5986
Urine-VAMS	1.0028	0.1227	0.9999	2.5956	1.0016	-01758	0.9996	3.1252	0.9955	0.1890	0.9997	1.3859

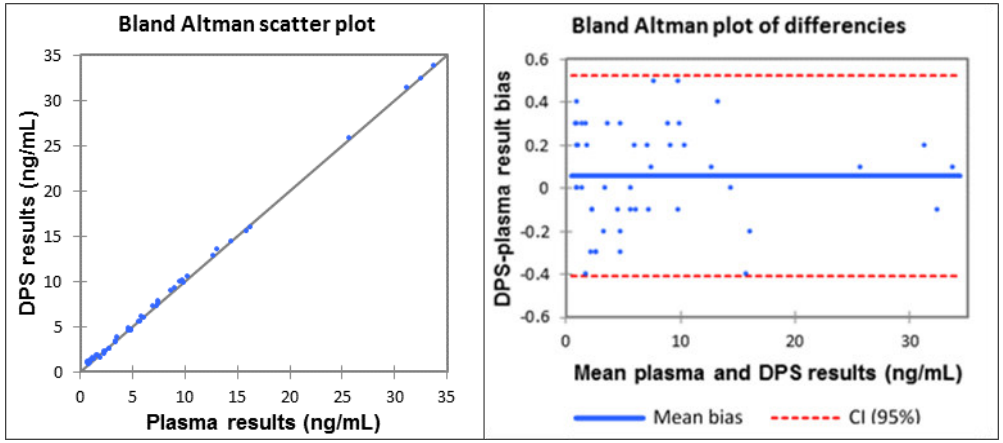
[a] Standard error of the regression.

Figure S1. Statistical correlation plots for the following result comparison: plasma vs DBS (a), plasma vs DPS (b), urine vs DUS (c), urine vs VAMS (d).

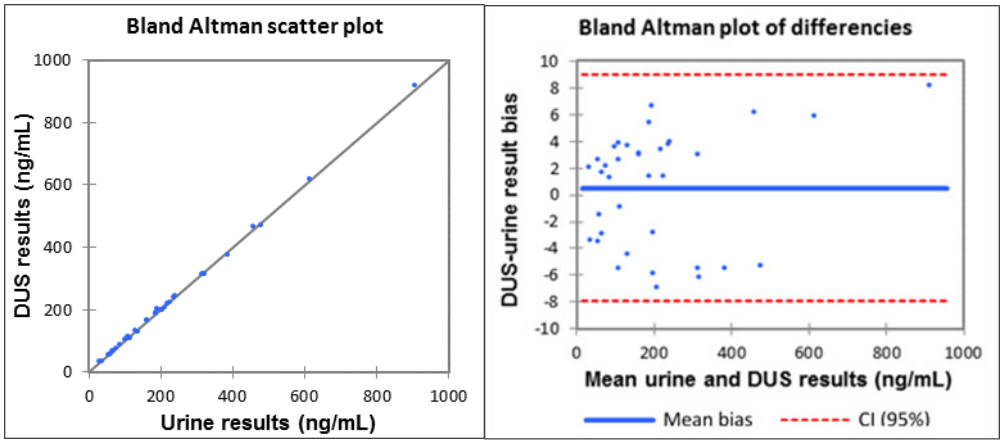
a - Plasma vs DBS results



b - Plasma vs DPS results



c - Urine vs DUS results



d - Urine vs VAMS results