Determination of Opiates in Whole Blood and Vitreous Humor: A Study of the Matrix Effect and an Experimental Design to Optimize Conditions for the Enzymatic Hydrolysis of Glucuronides
Determination of Opiates in Whole Blood and Vitreous Humor: A Study of the Matrix Effect and an Experimental Design to Optimize Conditions for the Enzymatic Hydrolysis of Glucuronides

Livia Rentas Sanches1, Saskia Carolina Seulin2, Vilma Leyton2, Beatriz Aparecida Passos Bismara Paranhos1, Carlos Augusto Pasqualucci2,3, Daniel Romero Muñoz2, Michael David Osselton4 and Mauricio Yonamine1*

1Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil, 2Faculty of Medicine, University of São Paulo, Brazil, 3Death Verification Service, University of São Paulo, Brazil, and 4Department of Forensic & Biological Sciences, Bournemouth University, UK

Undoubtedly, whole blood and vitreous humor have been biological samples of great importance in forensic toxicology. The determination of opiates and their metabolites has been essential for better interpretation of toxicological findings. This report describes the application of experimental design and response surface methodology to optimize conditions for enzymatic hydrolysis of morphine-3-glucuronide and morphine-6-glucuronide. The analytes (free morphine, 6-acetylmorphine and codeine) were extracted from the samples using solid-phase extraction on mixed-mode cartridges, followed by derivatization to their trimethylsilyl derivatives. The extracts were analysed by gas chromatography–mass spectrometry with electron ionization and full scan mode. The method was validated for both specimens (whole blood and vitreous humor). A significant matrix effect was found by applying the F-test. Different recovery values were also found (82% on average for whole blood and 100% on average for vitreous humor). The calibration curves were linear for all analytes in the concentration range of 10–1,500 ng/mL. The limits of detection ranged from 2.0 to 5.0 ng/mL. The method was applied to a case in which a victim presented with a previous history of opiate use.

Introduction
The term “opiates” refers to substances derived from the opium poppy (Papaver somniferum), such as morphine and codeine, and also includes some semi-synthetic substances like heroin or oxycodone. Primarily prescribed to treat acute and chronic pain, these drugs are available in a varied number of pharmaceutical formulations. Heroin has also medical applications in many European countries, although it is classified as a Schedule I drug under United States law (1).

The opiates constitute a group of the most highly abused drugs in America, Europe and Asia. Fatalities involving opiate overdose have increased dramatically in the past few years. Heroin abuse is one of the major drug problems in society. In addition to the cases of overdose, incidental or negligent cases of death involving opiates, medical care and treatment have also been reported (2). Postmortem blood with an elevated morphine concentration was detected in a reported and curious case involving children who were withdrawn from life support, which emphasized the need for toxicological analysis in an attempt to solve these cases (3). The analysis and interpretation of opiates in postmortem specimens can present special challenges to the forensic toxicologist.

Morphine is primarily metabolised by glucuronidation to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), which may be found in large concentrations in the plasma due to the enterohepatic recycling. The pharmacological activity of M6G reaches levels that are even higher than morphine following oral administration, whereas M3G has little affinity for opioid receptors, but may also contribute to excitatory effects (4). If some time has passed after exposure, these metabolites can reach significant concentrations in biological specimens, and therefore, the concentrations of free and total morphine should be analyzed. Acid and enzymatic hydrolysis procedures for measuring total morphine have been reported, although inaccurate results or incomplete hydrolysis have been demonstrated (5).

Codeine analgesic activity is displayed by its metabolism to morphine and its glucuronides (6). Heroin is quickly metabolized to 6-acetylmorphine (6-AM), and the main metabolite detection allows the statement of heroin ingestion. However, 6-AM is also rapidly metabolized to morphine, which can make its detection difficult in many cases (7). This metabolite is unstable in blood samples due to conversion to morphine by the action of esterases. The stability of these substances is also influenced by pH. Therefore, the analysis of vitreous humor can be of great importance to better interpret heroin-related deaths. In the eye, there is a lack of enzymes, which preserves the integrity of 6-AM. This has been demonstrated in some cases in which 6-AM was present in the vitreous humor and absent in the blood (8). The addition of sodium fluoride preservative (2%) to the samples showed a significant increase in 6-AM stability owing to the inhibition of bacterial action (9). The primary biotransformation pathways of opiates are shown in Figure 1.

Vitreous humor is composed of 99% water, with the remaining 1% made up of sugar, salts and proteins. It is also subjected to less contamination and bacterial degradation due to the protected environment inside the eye, which makes it available for analysis in cases in which blood samples have already been degraded (10).

The quantitative methods for the determination of opiates in postmortem specimens have been described in the scientific literature. The use of gas chromatography–mass spectrometry (GC–MS) (11–14) and liquid chromatography–mass spectrometry (LC–MS) (15–17) has been reported by several authors. The most commonly used sample preparation techniques have included liquid–liquid extraction (13, 14) and solid-phase extraction (SPE) procedures (11, 12, 15–17).
Despite all the available methods for the determination of opiates in biological samples, the conditions for glucuronide hydrolysis (which can be enzymatic or by means of addition of strong acids) have varied widely among these methods, and there is still a lack of information concerning the efficiency of these procedures (12, 13, 18, 19). Another point to be considered is the evaluation of the matrix effect for the quantification of substances in different biological specimens, a common situation in forensic toxicology. Therefore, the objective of this work was to optimize enzymatic hydrolysis conditions by means of experimental design and provide validation data to quantify opiates (total and free morphine, 6-AM and codeine) in whole blood and vitreous humor, also considering the possible matrix effect. The validated method was applied to a case in which the victim presented with a previous history of opiate use.

**Experimental**

*Chemicals and reagents*

Morphine, codeine, 6-AM, morphine-3βd-glucuronide, morphine-6βd-glucuronide and the deuterated internal standard morphine-d3 were purchased from Cerilliant (Round Rock, TX) as solutions in methanol or acetonitrile at a concentration of 1 mg/mL or 100 μg/mL. β-Glucuronidase from Helix Pomatia (type H-1) 500 KU and N,O-bis-(N,O-trimethylsilyl) trifluoroacetamide (BSTFA) with 1% of TCMS were obtained from Sigma Aldrich (St. Louis, MO). Bond Elut Certify SPE cartridges (130 mg) were obtained from Varian Inc. (Palo Alto, CA). All other solvents and reagents of analytical grade were supplied by Merck (Darmstadt, Germany).

*Specimens*

The drug-free blood samples were obtained from non-user volunteers (laboratory staff) and were collected in gray-top tubes containing 2% of sodium fluoride and stored at −20°C. Due to similarities in chemical composition, a saline solution (1% NaCl) was used as drug-free vitreous humor. The drug-free samples were used for the validation process of the analytical methods. The case samples (blood and humor vitreous) were obtained from a body attended by the Death Verification Service at the University of São Paulo, Brazil. These samples were also collected in gray-top tubes containing 2% of sodium fluoride. This study was approved by the Research Ethics Committee of the Clinics Hospital at the Faculty of Medicine of University of São Paulo – CAPesq HC-FMUSP (Ethics Protocol Approval number 0352/09).

*Extraction and derivatization*

One milliliter of each sample (whole blood or vitreous humor) was mixed with 10 μL of a 10 μg/mL internal standard solution (morphine-d3) and 5.0 mL of a 0.1M phosphate buffer solution (pH 6.0) in a 15-mL cap tube. The mixture was vortexed and centrifuged for 12 min at 300 g, and the supernatant was placed on SPE columns. The SPE cartridges were conditioned with 2 mL of methanol, followed by 2 mL of 0.1M phosphate buffer (pH 6.0). Following conditioning, the samples were added to the cartridges and allowed to flow under gravity. Then the columns were washed by the sequential addition of 2.0 mL of deionized water, 1.0 mL of 0.1M acetate buffer (pH 4.0) and 2.0 mL of methanol; they were then dried under maximum vacuum for 2 min. The analytes were eluted with...
2.0 mL of a fresh dichloromethane–isopropanol–ammonium hydroxide solution (20:5:0.5 v/v/v) and collected in 3.0-mL conical-shaped vials. Each sample eluent was evaporated under a stream of nitrogen at 40 °C. The residue was reconstituted in 50 μL of BSTFA + 1% of TCMS, capped, vortexed and heated for 20 min at 70 °C. After cooling, the solution was transferred to autosampler vials, and 1 μL of the solution was injected into the GC–MS system.

For the determination of total morphine, the enzymatic hydrolysis was performed using β-glucuronidase from Helix Pomatia (type H-1). The lyophilized enzyme was reconstituted with 1.0M acetate buffer (pH 5.0) to yield 100.000 units/mL β-glucuronidase activity. Then, a second aliquot of 1.0 mL of each sample (whole blood or vitreous humor) received 10 μL of a 10 μg/mL internal standard solution (morphine-d3), 1.0 mL of 1.0 M acetate buffer (pH 5.0) and 200 μL of the H. Pomatia β-glucuronidase solution. The tubes were vortexed and incubated at 37 °C for 4 h. After hydrolysis and cooling to room temperature, 50 mL of 0.1M phosphate buffer (pH 6) was added to each tube and samples were submitted to the same SPE procedure described previously.

**Instrumentation**

The quantitative analysis was performed on a Focus GC coupled to a Polaris Q ion trap MS from Thermo Scientific (Waltham, MA), which was operated in the electron ionization mode. The chromatographic separation was achieved using an HP-5MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 µm film thickness). Helium was the carrier gas, which was set at a flow rate of 0.7 mL/min. The injector was operated in the splitless mode at 270 °C, and the transfer line temperature was set at 280 °C. The GC oven was initially held at 150 °C for 1 min, with a ramp of 10 °C/min to 250 °C (held 8 min). Full scan analyses were performed in the mass range of 200–500 m/z. The acceptance criteria of qualification were: retention time within 2% compared with standards analyzed in the same batch, and the mass spectrum should have a good visual match to that of the standards. The monitored ions for quantification of analytes were as follows: morphine, m/z (429); morphine-d3, m/z (432); codeine, m/z (371); 6-acetylmorphine, m/z (399).

The experimental design for the optimization of the enzymatic hydrolysis conditions for morphine glucuronides

The optimization study was conducted using a multivariate statistical technique called response surface methodology (RSM), which made statistical previsions based on the fit of a polynomial equation to the experimental data (20). The application of this methodology made it possible to simultaneously optimize the levels of the variables, which allowed us to observe the interactive effects among them.

An experimental design called Box-Behnken was chosen to define which experiments should be performed before the application of the RSM methodology. Box-Behnken is a three-level factorial design that requires only 17 experiments, in which five of them are replicates of the center point (21).

The enzymatic hydrolysis was performed using β-glucuronidase from Helix Pomatia (type H-1). The three factors that were analyzed with significant relevance and chosen levels (low and high) were as follows: incubation temperature (37–60 °C), incubation time (4–24 h) and enzyme concentration (2,500–20,000 units/mL). The experiments were conducted in random order to provide an accurate estimation of the experimental error. The fortified blood samples (1.0 mL) with a total concentration of 1,000 ng/mL of the glucuronides were subjected to the selected conditions (temperature, time and enzyme concentration) and analyzed according to the procedure described previously. Analysis of variance was applied to the regression results, which verified whether a lack of fit was evident.

The ratio between the morphine and morphine-d3 peak areas was used as the response. Design-Expert (version 8), a statistical software, was used to select the experimental design and to perform the analysis through the application of response surface methodology.

**Method validation**

The method was validated by determining sensitivity, selectivity, linearity, extraction efficiency, precision (intra-assay and inter-assay) and stability. To obtain the validation data, quality control (QC) samples were prepared by spiking drug-free matrices, which resulted in the following concentrations of morphine, 6-AM and codeine: 30, 550 and 1,100 ng/mL (QC1, QC2 and QC-3). All parameters were analyzed in both the blood and saline solutions.

The limit of detection and the limit of quantitation

The limit of detection (LOD) was defined as the concentration giving a signal to noise ratio of >3. The limit of quantitation (LOQ) was defined as the concentration giving a signal to noise ratio of >10. LOD and LOQ should still satisfy the predetermined acceptance criteria of qualification (retention time within 2% compared with standards analyzed in the same batch and a good visual match of mass spectrum to that of the standards).

Selectivity (matrix effects)

The interfering compounds present in each matrix may strengthen or reduce the detection of the analytes of interest, which can lead to different results from the analysis of fortified samples with the same analyte concentration, but in different matrices (22). This assay was performed to identify the differences in analyte detection caused by different matrix effects of the blood and saline solutions. The test was performed by preparing two sets of samples (n = 6): one contained blood, and the other contained a saline solution, and both samples had the same concentration of analytes at each target concentration level (30, 550 and 1,100 ng/mL). Data were analyzed by the application of the F-test to test for differences among sample variance, followed by the application of the t-test to assess whether the means of the two groups were statistically different from each other (Eq. 1).

\[ F_{cal} = \frac{s_1^2}{s_2^2} \]  

(1)

In this equation, \( s_1 \) and \( s_2 \) represent the standard deviations of the analyte response for each set of samples, and the larger
variance should be used as the nominator. \( F_{cal} \) represents the calculated value of \( F \); while \( F_{tab} \) represents the tabulated value.

If \( F_{cal} < F_{tab} \), the \( F \)-test is not significant (5% significance level), and it can be considered that the variances are similar. Then, the difference of the means of the two groups can be tested with Student’s \( t \)-test (Eqs. 2 and 3).

\[
\begin{align*}
\text{tcal} &= \frac{|\bar{x}_1 - \bar{x}_2|}{\sqrt{s^2\left(\frac{1}{n_1} + \frac{1}{n_2}\right)}} \\
\text{s^2} &= \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 + n_2 - 2)}
\end{align*}
\]

In these equations, \( x_1 \) and \( x_2 \) represent the means of the analyte response for each set of samples; \( n_1 \) and \( n_2 \) represent the number of replicates in each set of samples; \( t_{cal} \) represents the calculated value of \( t \), while \( t_{tab} \) represents the tabulated value from the Student’s \( t \)-distribution.

On the other hand, if \( F_{cal} > F_{tab} \), the \( F \)-test is significant, which means that the variances between the groups are different; then, the \( t \)-test of unequal variances can be used to test the means (23) (Eqs. 4 and 5).

\[
\begin{align*}
\text{tcal} &= \frac{\sqrt{n}}{s_d} \\
\text{s_d} &= \sqrt{\frac{\sum_{i=1}^{n} (d_{i1} - d_{i2})^2}{n}}
\end{align*}
\]

In these equations, \( d_{i1} \) and \( d_{i2} \) are the responses for a sample pair in each group, \( x_d \) represents the mean of differences between analyte response pairs, \( s_d \) represents the standard deviation of differences between them and \( n \) is the number of pairs.

If the calculated \( t \) value (for equal or unequal variances) is below the value that is expected in the \( t \)-test tables, it is possible that the analytes do not suffer a different interference from the matrices and vice versa.

**Extraction efficiency—Recovery**

The extraction efficiency was determined using the QC samples at the target concentrations of 30, 550 and 1,100 ng/mL for all analytes. The recovery study was performed by preparing one set of samples that were fortified with a control solution at the initial time of sample preparation before SPE and by preparing another set of samples that were only fortified after extraction but before evaporation and derivatization. The relative recovery was calculated by comparing the mean response in the first group (extracted) to the mean response of the second group (non-extracted) and then multiplying by 100.

**Precision (intra- and inter-assay)**

The precision assay was performed by the analysis of replicate blood and saline samples (\( n = 6 \)) at the target analyte concentrations of 30, 550 and 1,100 ng/mL. The intra-assay precision for the samples was determined on the same day and performed within one analytical run, while the inter-assay precision for the samples was determined on three different days. Data were evaluated through the analysis of variance in each group (intra-assay and inter-assay), which was established by the relative standard deviation (RSD%).

**Stability**

The handling of the samples and different storage conditions can influence analyte stability. The stability assay was assessed using blood and saline solutions fortified with analytes of interest at low and high concentrations (30 and 1,100 ng/mL). The storage conditions studied were as follows: room temperature for 24 h, 4°C for 7 days, –20°C for 30 days followed by thawing at room temperature (three freeze-thaw cycles) and –20°C for three months. Analyses were performed in triplicate for each condition and level of concentration. In this assay, the stability of morphine, 6-AM and codeine, along with M3G and M6G was determined to verify the occurrence of the natural hydrolysis of these metabolites to morphine. The concentrations obtained in the QC samples after each storage condition were compared to those from freshly prepared QC samples. Acceptable results included a maximum concentration difference of 20% when comparing QC samples under storage with fresh QC samples.

**Case study**

To prove the applicability of this analytical method, a case that involved opiate consumption was analyzed. The quantification was based on the ratios of the ion peak areas of the compounds to the internal standard ion peak areas. The calibration curves were used to determine the opiate concentrations, which used a weighting factor of \( 1/x^2 \) for blood and \( 1/y^2 \) for vitreous humor.

A woman who was approximately 80 years old and had traces of cancer in the liver was examined at the Death Verification Service, University of São Paulo, Brazil. A rapid immunoassay test for drugs (Triage, Biosite) in the urine was used at the autopsy and was positive for opiates. No other details about the history of the case were accessed.
Results and Discussion

**GC–MS analyses**

Opiates have often been found in postmortem biological fluids in overdose cases or even in cases of treatment with legitimate prescriptions. Gas chromatographic analysis of these substances has been difficult due to their high polarity and low volatility. It has been necessary to apply derivatization techniques to produce thermal and volatile stable derivatives (25). In this study, the silylation derivatizing technique was chosen because it is one of the most commonly applied techniques. It is easy to use and does not require additional solvent or clean-up steps. Good reproducibility was obtained using this technique.

All analytes were successfully extracted and separated using this method. All ion peaks were Gaussian-shaped and demonstrated baseline resolution. The molecular ion was usually the most abundant in the mass spectra of opiate-TMS derivatives; therefore, it was selected as the quantification ion.

**Optimisation of the enzymatic hydrolysis conditions of morphine glucuronides**

The morphine glucuronides are very important analytes to be detected in toxicological analysis. Nevertheless, because of its extreme polarity, it is necessary to include a hydrolysis step, which enables the determination of total morphine by gas chromatography.

Good glucuronide hydrolysis rates in urine have been reported in the literature with acid hydrolysis (26, 27), which is even faster than the enzymatic process. However, this drastic condition can convert some opiates such as 6-AM or acetylcodine into morphine or codeine, which overestimates the concentrations. Therefore, we opted for the enzymatic hydrolysis, which used β-glucuronidase.

To obtain optimal conditions for the hydrolysis of glucuronides, some parameters such as the incubation time, incubation temperature and enzyme concentration of β-glucuronidase from Helix Pomatia were evaluated. The response surface methodology was used to perform the optimisation. This technique simultaneously optimized the levels of variables, which enabled us to observe the interactive effects between the variables, to reduce the number of experiments necessary to conduct the research, and to decrease the time and expenses consumed when compared to the traditional one-variable-at-a-time optimization technique. Box-Behnken was chosen as the experimental design because it seemed to be the most efficient and economical three-level factorial design, requiring only 17 experiments (21). Costa et al. (28) have also applied the design of experiments technique to perform the optimization of urinary morphine glucuronide hydrolysis; however, the procedure was performed with strong acids instead of enzymes, and only M3G hydrolysis was focused on in the study. M6G does not seem to be as readily hydrolyzed as M3G. In fact, M6G has glucuronide acid bound to an alcoholic position, whereas in M3G, glucuronide acid is bound to a phenolic position, which is electron-rich. This position facilitates the hydrolysis due to the formation of a pheno-late anion that can be stabilized through resonance (29).

No reports have related the optimization methods of morphine glucuronide hydrolysis in blood samples. The vast majority of the studies have compared the difference between the acid and enzymatic hydrolysis conditions only in urine samples (27, 29, 30), almost always reporting better hydrolysis rates with the acid condition. Hackett et al. (27) reported that more than 90% of M3G is hydrolyzed in urine samples, with an incubation time of 4 or 24 h at 60°C with β-glucuronidase from H. Pomatia or at 45°C with β-glucuronidase from E. coli, whereas the use of acid hydrolysis resulted in the conversion of 95% of morphine glucuronide to morphine. The conditions for the hydrolysis of morphine glucuronides in blood, such as 1 h of incubation at 60°C with 2 mL of β-glucuronidase (31) or 16 h of incubation at 37°C with 5,000 U/mL of β-glucuronidase (13), were also reported in the scientific literature; however, the efficiency of these procedures was not mentioned.

Our results showed that the optimal condition for hydrolysis is 4 h of incubation at 37°C with the addition of 20,000 U/mL of β-glucuronidase, which results in hydrolysis efficiency rate of 75% (Figure 2). A progressive increase in efficiency was observed when the amount of enzyme was increased. A decrease in the hydrolysis rate occurred when the incubation temperature was increased, which was probably because of enzymatic degradation at high temperatures. The best efficiencies of hydrolysis were obtained using incubation times of 4 and
24 h. From this, it was found that 4 h are enough to complete the hydrolysis, and this time was selected to perform the enzyme incubation. The blood samples with and without sodium fluoride were tested to assess the enzymatic hydrolysis efficiency; no significant differences were found.

The analysis of variance showed that the regression model is significant (p = 0.016), and no significant lack of fit was found (p > 0.05).

**Extraction procedure**

The amphoteric nature of morphine has made it difficult to use some extraction procedures, such as liquid–liquid extraction, because it forms charged species at all pHs other than its isoelectric point, which makes it water soluble and difficult to extract, resulting in poor recovery. The use of SPE with cation exchange columns has improved the efficiency of extracting drugs such as morphine because it has allowed the drug to be extracted in the charged form.

Additionally, concerning SPE extraction, the difficulty encountered, also reported in literature (32), is related to the passage of postmortem blood through the SPE columns; this specimen is often clotted and not homogenous, easily plugging the column. Hence, the samples were diluted five-fold in buffer and centrifuged before extraction, which enabled a smooth passage through the cartridges and did not compromise the quantitative determination.

The employment of vitreous humor to analyze drugs can be of great importance because it is a clean matrix; it is composed of 99% water and contains few proteins and enzymes (10).

**Method validation**

The results obtained in the method validation for the determination of opiates in blood and saline solution samples are shown in Table I.

<table>
<thead>
<tr>
<th>LOD (ng/mL)</th>
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The LODs were 5.0 ng/mL in the blood samples and 2.0 ng/mL in the saline samples for all the analytes. The LOQ was 10.0 ng/mL in both the blood and saline samples for all the opiates. These limits were considered to be good values that compared well with previously published work.

The linear dynamic range was determined based on the opiates’ therapeutic and fatal concentrations and in addition to previous works; these values ranged from 10 to 1,500 ng/mL. The method showed that the data were heteroscedastic, which was probably attributable to the large concentration range (two orders of magnitude). In this case, the larger deviations that were present at higher concentrations tended to influence the linear regression more than the smaller deviations that were present at smaller concentrations, which can impair the accuracy in the lower part of the range (23). Weighted least square linear regression was applied in this case, which decreased the sum of the relative error percentages over the entire range. The correlation coefficients for the calibration curves were all greater than 0.995 for the blood samples when a weighting factor of 1/x² was used (morphine: y = 0.0062x + 0.0019; 6-AM: y = 0.0039x + 0.0004; codeine: y = 0.0025x – 0.0011), and were greater than 0.999 for the saline samples when a weighting factor of 1/y² was used (morphine: y = 0.003459x – 0.00134; 6-AM: y = 0.00415x – 0.00654; codeine: y = 0.002558x – 0.00364).

The average extraction efficiencies ranged from 76 to 88% in the blood samples and from 98 to 103% in the saline samples. The recovery rates above 75% were considered good values when considering the analysis of biological fluids.

**Table I**

Confidence Parameters for the Opiates’ Method Validation

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The means obtained between the blood and saline solution sets of samples showed a significant difference in analyte responses at all concentration levels (30, 550 and 1,100 ng/mL). Hence, all the confidence parameters were obtained in both matrices, and we recommend that the calibration curves for the analysis of case samples should be performed in each specific matrix, which minimizes errors.

The LODs were 5.0 ng/mL in the blood samples and 2.0 ng/mL in the saline samples for all the analytes. The LOQ was 10.0 ng/mL in both the blood and saline samples for all the opiates. These limits were considered to be good values that compared well with previously published work.

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The average extraction efficiencies ranged from 76 to 88% in the blood samples and from 98 to 103% in the saline samples. The recovery rates above 75% were considered good values when considering the analysis of biological fluids.

**Table II**

Free and Total Codeine and Morphine Concentrations Found in the Real Case Samples

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Morphine (ng/mL)</th>
<th>Codeine (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Femoral blood</td>
<td>269.9</td>
<td>1279.4</td>
</tr>
<tr>
<td>Cardiac blood</td>
<td>397.3</td>
<td>1256.9</td>
</tr>
<tr>
<td>Vitreous humor</td>
<td>162.1</td>
<td>798.6</td>
</tr>
</tbody>
</table>

*n.d.: not detected*
Figure 3. Chromatographic profile obtained with a SPE–GC–MS analysis of a fortified blood sample containing codeine (1), morphine and morphine-d3 (2, 3) and 6-acetylmorphine (4) at a middle concentration (550 ng/mL) (A). Drug-free blood sample (B). Postmortem cardiac blood hydrolyzed sample of the related case (C). Postmortem femoral blood hydrolyzed sample of the related case (D). Postmortem vitreous humor hydrolyzed sample of the related case (E). The ions acquired to generate the chromatograms were as follows: 429 + 432 + 399 + 371.

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The inter-assay precision ranged from 4.3 to 8.5% (RSD) in the blood samples and from 2.8 to 6.0% in the saline samples. The intra-assay precision yielded coefficients of variance that ranged from 5.6 to 9.3% in the blood samples and from 4.1 to 5.4% in the saline samples. The use of morphine-d3 as an internal standard did not affect the reproducibility of the analysis, with the RSDs not exceeding the predefined limit of 10% for the intra-assay precision and 20% for the inter-assay precision for all analytes.

The investigation of opiate stability in the blood samples did not indicate any degradation under all the conditions studied, with the exception of 6-AM, which showed instability at room temperature and after being stored for a long period (3 months in freezer). With respect to analyte stability in the saline samples, only 6-AM and codeine showed instability after being stored for a long period. The results indicated that case samples must be analyzed as soon as possible because the analytes can degrade after being stored for a long period. In all storage conditions studied, neither M3G and M6G showed any degradation to morphine, even in the blood and saline samples; this could be a result of the sodium fluoride effect, which eliminates bacterial contamination and inhibits enzymatic activity.

**Case study**

Codeine and morphine were found in the case analysis. The drug concentrations found in samples are shown in Table II.

A high total codeine concentration was observed in all analyzed samples, which was a result of the hydrolysis of a codeine metabolite, codeine-6-glucuronide. The deceased had suffered from cancer and had probably received codeine treatment for pain control. The free codeine concentration in the blood was in the therapeutic range, and the higher amount that was detected in the total form indicated chronic use.

The presence of morphine in the samples was a result of codeine metabolism, which was explained by the lower amounts of this drug in relation to codeine. The larger amount was in the total form, while the free concentration was detected only in trace amounts.

A higher concentration of free codeine was observed in the cardiac blood than in the femoral blood. This yielded a C/F ratio of 1.8 and indicated a possible postmortem redistribution of this drug, which was not observed in the total concentration, possibly because the conjugate form did not suffer this phenomenon.

With respect to the concentrations detected in the vitreous humor samples, the concentrations of the total form of both analytes, morphine and codeine, were lower than the blood samples. The metabolites might have had difficulty crossing the lipid barriers due to their polar character. Otherwise, the free codeine concentration was higher in this fluid, which could be a result of the apparent delay in the drug excretion process of the vitreous humor. The chromatographic profiles of a fortified blood sample, a drug-free blood sample and the case samples are shown in Figure 3.

**Conclusions**

The use of dilution followed by centrifugation and SPE enabled us to obtain clean extracts even when working with whole blood samples, which did not interfere with the drug quantitation and eliminated the deproteinization procedure steps.

The optimization of the enzymatic hydrolysis of glucuronides facilitated more accurate and efficient results for the application of total analytes, which is of great importance to better interpret opiate cases and can help establish the time of death using the proportion of free and total analytes.

The method was validated for both specimens (whole blood and vitreous humor) and a significant matrix effect was found by applying the F-test. Therefore, it is recommendable that calibration curves should be performed in each specific matrix to minimize errors.

This method fit well for the purpose for which it was intended, which was obtaining precise and accurate results for laboratory routines.

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