Development and validation of a bioanalytical method for the simultaneous determination of heroin, its main metabolites, naloxone and naltrexone by LC-MS/MS in human plasma samples: Application to a clinical trial of oral administration of an heroin/naloxone formulation.

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Abstract:
A bioanalytical method using high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed and validated for simultaneous quantification of heroin, its main metabolites and naloxone. In addition, naltrexone was detected qualitatively. This method was used to analyse human plasma samples from a clinical trial after oral administration of an heroin/naloxone formulation in healthy volunteers. O-methylcodeine was used as an internal standard. Samples were kept in an ice-bath during their processing to minimize the degradation of heroin. A short methodology based on protein precipitation with methanol was used for sample preparation. After protein precipitation, only the addition of a formic acid solution was needed to elute heroin, 6-monoacetylmorphine, morphine, naloxone and naltrexone. Morphine metabolites were evaporated to dryness and reconstituted in a formic acid solution. Chromatographic separation was achieved at 35°C on an X-Bridge Phenyl column (150 x 4.6 mm, 5 μm) using a gradient elution with a mobile phase of ammonium formate buffer at pH 3.0 and formic acid in acetonitrile. The run time was 8 min. The analytes were monitored using a triple quadrupole mass spectrometer with positive electrospray ionization (ESI+) in multiple reaction monitoring (MRM) mode. The method was found to be linear in a concentration range of 10-2000 ng/mL for M3G and 10-1000 ng/mL for the rest of compounds. Quality controls showed accurate values between -3.6% and 4.0% and intra- and inter-day precisions were below 11.5% for all analytes. The overall recoveries were approximately 100% for all analytes including the internal standard. A rapid, specific, precise and simple method was developed for the determination of heroin, its metabolites, naloxone and naltrexone in human plasma. This method was successfully applied to a clinical trial in 12 healthy volunteers.

Keywords: Heroin; LC-MS/MS; Plasma; Validation; Protein Precipitation; Pharmacokinetic

ABBREVIATIONS
Heroin, diamorphine (DAM)
6-monoacetylmorphine (6OM)
Morphine (MOR)
Morphine-3-glucuronide (M3G)
1. Introduction

Heroin (diamorphine, DAM) is a semi-synthetic lipophilic morphine derivative and a powerful opioid analgesic. The half-life of DAM is short (approximately between 2 and 5 minutes), and DAM is rapidly hydrolyzed in human plasma by deacetylation to 6-monoacetylmorphine (6OM) and further to morphine (MOR) by serum-esterases and liver carboxylesterases. The primary site of morphine metabolism is the liver, where it undergoes rapid glucuronidation. MOR is metabolized by conjugation to morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). M3G is the main metabolite and has no pharmacological effect. These conjugates are hydrophilic compounds and are mainly excreted in urine [1-4]. It is known that the degradation of DAM and 6OM is dependent on pH and temperature. Therefore, it is necessary to take prevention measures in collecting samples to avoid the degradation [5]. Naloxone (NAL) is an opioid antagonist and is used to reduce constipation caused by orally administered opioid therapy. NAL concentrations in plasma are low after an oral administration because it is metabolized in the liver primarily by glucuronidation [6,7]. Naltrexone (NALT) is a competitive opioid antagonist that blocks the pharmacologic effect of heroin, morphine and other opioids. NALT also undergoes a significant first-pass metabolism [8,9].

In this work, all target compounds were determined and applied to a clinical trial in healthy volunteers. NALT was administered by security 12 hours before oral administration of 50/2 mg of DAM / NAL tablets. This formulation was developed as an Investigational Medicinal Product (IMP) for drug substitution treatment in opioid dependents, and has a patent application (Application number: PCT/ES2013/070272). This combination aims to prevent the abuse of heroin and to avoid medical risks. Several analytical methods based on HPLC with ultraviolet
(UV), diode-array (DAD), fluorescence [10-12], gas chromatography with mass spectrometry (MS) detection [13,14] and HPLC tandem MS [1,15-17] were published for the simultaneous quantification of some of these compounds in biological matrices. Recently LC-MS/MS methods were applied to the determination of heroin and its metabolites because of their high selectivity and sensitivity [16-18]. Most of these methods were reported using solid phase extraction (SPE) for sample preparation, which is a time-consuming process and requires large volumes [2,18,19]. Furthermore, the difficulty of developing short HPLC methods in reverse phase for the simultaneous determination of extremely polar compounds, such as glucuronides, and nonpolar compounds in the same analysis has led to long chromatographic run times [1,2,18]. So far, no published papers are available regarding the simultaneous determination of all these compounds. The aim of this work is developing a rapid and simple methodology for sample preparation and its analysis by LC-MS/MS to apply it in clinical pharmacokinetic studies where large numbers of samples are determined. In that respect, the present study describes the development of a fast LC-MS/MS method for determination of all these compounds (DAM, 6OM, MOR, M3G, M6G, NAL and NALT) in human plasma using a small sample volume and sample preparation based on protein precipitation, saving time in the analysis and minimizing the degradation of DAM and 6OM in plasma. This work was performed under GLP’s standards, fulfilling EMEA guidance requirements [20], and was applied to a clinical trial of healthy volunteers after oral administration of a DAM/NAL formulation.

2. Experimental

2.1. Reagents and standards

All compounds (DAM, 6OM, MOR, M3G, M6G, NAL, NALT) were supplied by OneDose Pharma (Barcelona, Spain), and O-methylcodeine (internal standard, IS) was obtained from EDQM (Council of Europe, France). Dimethylsulphoxide (DMSO), methanol and acetonitrile (HPLC-grade) were purchased from Scharlab (Barcelona, Spain). Ammonium formate and formic acid were obtained from Panreac (Spain). The method was developed using blank human plasma.
obtained from the Biobanco Vasco (Bilbao, Spain) who approved the analytical protocol of this
study (application number, CBVI371/1M).

2.2. Preparation of quality control samples and calibration standards

Stock solutions of all standards and IS were prepared at 1.0 mg/mL in DMSO. These solutions
were stored at 4ºC. IS precipitation solution was prepared at 100 ng/mL in methanol from the IS
stock solution. Working solutions at the desired concentrations for calibration standard and quality
control samples were prepared by serial dilutions in combination from the stock solutions in
DMSO to make a single calibration curve for all compounds. Standard solutions were prepared by
spiking human plasma with working solutions at a concentration range of 10-2000 ng/mL for
M3G and 10-1000 ng/mL for DAM, 6OM, MOR, M6G and NAL. Linearity was studied within
these ranges by preparing seven concentrations of DAM, 6OM, M6G, MOR, NAL (10, 50, 100,
250, 500, 750, 1000 ng/mL) and eight concentrations of M3G (10, 50, 100, 250, 500, 750, 1000,
2000 ng/mL). Low, medium and high concentration quality control (QC) samples at
concentrations of 30, 400, 800 ng/mL for all compounds and at a higher concentration for M3G
(1500 ng/mL) were prepared in human plasma. All of these solutions were freshly prepared daily.

2.3. Sample Preparation

In this study, human plasma samples from clinical trials were collected at several time points
(section 2.6) after oral administration of an heroin/naloxone formulation and stored at -80ºC until
their analysis by LC-MS/MS. Plasma aliquots were kept in an ice bath during the sample
processing to avoid the degradation of DAM and 6OM.

Two-hundred microliters of IS precipitation solution (100 ng/mL in methanol) were added to 100
µL of human plasma sample. The mixture was vortexed for 30 seconds and centrifuged at 14000
r.p.m. at 4ºC for 5 minutes. Then, the supernatant was filtered into an Eppendorf tube. Finally, 50
µL of supernatant were mixed with 50 µL of 0.1% formic acid (method A) for the determination
of DAM, 6OM, MOR, NAL and NALT. For determination of M3G and M6G, evaporation was
necessary after protein precipitation to obtain higher signal intensity and better peak shapes for
these compounds (method B). To this end, 150 µL of supernatant were evaporated to dryness at 25ºC under a stream of nitrogen, and finally the dry residue was reconstituted with 50 µL of 0.1% formic acid and transferred to a glass vial. A 50 µL aliquot of sample obtained by method A was added to the same vial for simultaneous determination by LC-MS/MS.

2.4. LC-MS/MS conditions

The LC-MS/MS system consisted of the Waters Alliance 2795 HPLC system (Waters Corp., Milford, MA, USA) coupled to the Waters Quattro Premier mass spectrometer (Waters Corp., Milford, MA, USA). The HPLC autosampler temperature was remained at 10ºC. Chromatographic separation was performed on an X-Bridge Phenyl column (150 x 4.6 mm, 5 µm) from Waters and maintained at 35ºC. Gradient elution was carried out with a mobile phase consisting of ammonium formate buffer (pH 3.0; 5 mM) (mobile phase A) and formic acid 0.1% in acetonitrile (mobile phase B). The gradient started at 5% B for 1 min, increased to 90% B for 3 min and was held at 90% B for 2 min before returning to its initial conditions. The total run time was 8 min. The injection volume was 30 µL and the flow rate through the column was 1.0 mL/min.

MS/MS detection was performed using electrospray positive ionization (ESI+) in multiple reaction monitoring (MRM) mode. The following MS/MS conditions were applied to the ESI source: capillary voltage, 3.0 kV; source temperature, 120ºC; desolvation temperature, 350ºC, cone gas flow, 50 L/h and desolvation gas (nitrogen) flow, 800 L/h. A dwell time of 0.1 sec was used for all transitions. The m/z transitions, cone voltage, collision energy and retention times for all compounds are shown in Table 1. Data acquisition and peak integration were evaluated using MassLynx 4.0 software with QuanLynx v4.1 (Waters®).

2.5. Method validation

The present study was validated in compliance with GLP principles (Good Laboratory Practice).
and EMEA (European Medicines Agency) guidance for the validation of bioanalytical methods
[20]. Selectivity, carry over, matrix effects, linearity, precision, accuracy, lower limit of
quantification (LLOQ), recovery, stability and dilution integrity were evaluated during method
validation. Only selectivity was evaluated for NALT.

2.5.1. Selectivity and carry over
Selectivity was demonstrated by comparing six different batches of blank human plasma from
healthy volunteers and drug-containing plasma. These samples were individually assessed for the
presence of any interference across the retention window of each analyte and IS. Carry-over was
evaluated by injecting six blank samples after an upper limit of quantification (ULOQ) sample.

2.5.2. Linearity
Linearity was assessed by analysing the calibration standard samples in a range of 10-2000 ng/mL
for M3G and 10-1000 ng/mL for the rest of analytes (DAM, 6OM, MOR, M3G, M6G and NAL).
A calibration (standard) curve was generated for each analyte and analysed on three consecutive
analytical days. The best fit was achieved by a linear regression of the peak area ratios of the
analytes to the IS versus nominal standard concentrations and a weighing factor of 1/x.

2.5.3. LLOQ, accuracy and precision
The LLOQ is the lowest concentration of analyte in a sample which can be quantified with an
acceptable accuracy (±20%) and precision (<20%). The LLOQ determined the lowest calibration
standard for each analyte. To evaluate accuracy and precision, quality control samples in plasma
were prepared at 30, 400, 800 ng/mL for all compounds and 30, 800, 1500 ng/mL for M3G,
covering the linearity range. Intra-day and inter-day accuracy and precision were measured using
five determinations per concentration (LLOQ and QC concentration levels) on the same day and
on different days, respectively.

2.5.4. Stability
To evaluate stability in solution, working solutions were prepared and kept at room temperature (25 ºC) for 6 hours and stored at 4-8ºC. Stock solutions were stored at 4-8ºC and quantified at different times for more than 100 days to study long term stability.

Stability in plasma was evaluated at low and high QC concentrations (n=3) under different temperatures and storage conditions. Stability during sample processing was studied for 6 hours at room temperature (25ºC). Freeze and thaw stability of analytes was evaluated after thawing at room temperature and refreezing at -80ºC for at least 12 hours in three cycles. QC samples were stored at -80ºC for more than 90 days to evaluate long-term stability. On-instrument stability was evaluated by analysing QC samples kept in the autosampler at 10ºC for more than 20 hours.

2.5.5. Dilution integrity

The effect of dilution was assessed by a 2-fold, 5-fold and 10-fold dilution of the ULOQ plasma sample with blank plasma (five replicates per dilution factor). The acceptable precision and accuracy were required to be within ±15%.

2.5.6. Matrix effect and recovery

The matrix effect of each compound and IS was evaluated by comparing peak area ratio in the presence of plasma, with the peak area ratio in the absence of plasma. Six different batches of blank human plasma spiked with QCs at low and high concentration (30 and 800 ng/mL, respectively) were analysed. The matrix effect was also evaluated on haemolyzed and hyperlipemic plasma.

Recoveries of each compound and IS were evaluated by comparing the peak areas of extracted QC samples (n=5) with the peak areas of post-spiked QC samples (n=5) at the QC concentrations levels (30, 400,800 ng/mL for the analytes and 100 ng/mL for IS).

2.6. Pharmacokinetic study
The proposed LC-MS/MS method was used to evaluate the plasma profiles of DAM, 6OM, MOR, M3G, M6G and NAL after oral administration in humans. The study was performed on 12 healthy volunteers in the Clinical Trial Unit of the Hospital del Mar d’Investigacions Mèdiques (IMIM). All patients gave their written informed consent prior to participation in the study. The clinical trial was approved by the Clinical Research Ethical Committee of the Parc de Salut Mar. The volunteers received a single tablet of 50 mg heroin/2 mg naloxone by oral administration. NALT was administered in tablets of 50 mg naltrexone hydrochloride (Revia®, Bristol-Myers Squibb, S.A.) at 12 and 0.5 hours before and 12 hours after of the administration of DAM/NAL formulation. Blood samples were collected at different time points: -12, -0.5, 0 (pre-dose), 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24 hours after the administration. EDTA was used as an anticoagulant. The plasma was collected and stored at -80°C until its analysis by LC-MS/MS.

The pharmacokinetics analysis of concentration-time data was performed according to non-compartmental analysis using the Phoenix WinNonlin Version 6.3 software package. The maximum plasma concentration (C$_{\text{max}}$) and the time at which it is reached (t$_{\text{max}}$) were obtained directly from the experimental data and confirmed by the same software. The area under the plasma concentration-time curve to the last experimental point (AUC$_{\text{all}}$) was obtained by the trapezoidal method and AUC extrapolated to infinity (AUC$_{\text{inf}}$) was calculated by adding the term C$_{\text{last}}$/λ$_{\text{z}}$ to AUC$_{\text{all}}$ where C$_{\text{last}}$ is the last measured concentration. The λ$_{\text{z}}$ value (elimination rate constant) was calculated by linear regression of the logarithms of plasma concentrations in the terminal phase of the curve. The half-life (t$_{1/2}$) was calculated from 0.693 / λ$_{\text{z}}$. The systemic clearance (CL) was calculated from D*F / (AUC$_{\text{inf}}$), where D is the dose and F is the fraction of dose absorbed. The apparent volume of distribution (V$_{\text{z}}$) was obtained from D*F / (AUC$_{\text{inf}}$* λ$_{\text{z}}$). In this case, F could not be calculated because the intravenous data are not available, so the CL and V$_{\text{z}}$ parameters were normalized to F.

3. Results and discussion
3.1. LC-MS/MS analysis

The MRM transitions, cone voltage and collision energy were optimized by direct infusion of each analyte including the IS. Positive ion mode was used for MS detection of these compounds. Fig. 1 shows the mass spectra of the protonated [M+H]+ ion of heroin, its metabolites, NAL, NALT and the IS. Only the major ion transitions were monitored (Table 1). Although the same ion transition was used for monitoring both M3G and M6G, their separation was possible by HPLC. The main challenge during method development was to find the best conditions for the simultaneous separation of all the analytes in a single chromatogram, due to the large difference in their chromatographic behaviour. The major difficulty was to optimize the reverse phase conditions to achieve the separation of M3G and M6G glucuronides, very polar compounds, both having the same MRM transition. Several columns were evaluated for HPLC method development. An X-Bridge Phenyl 5 µm, 150 x 4.6 mm column showed the best separation, retention and good peak shapes, so it was chosen to develop the analytical method. Ammonium formate buffer (pH 3.0; 5 mM) as mobile phase A and 0.1% formic acid in ACN as mobile phase B gave the best retention and high response for all compounds. Gradient elution ensured the separation of the compounds with good resolution in a short period of time (5 minutes) and allowed for programming the MS detection of ion transitions by time segments, thus increasing the sensitivity. The flow was diverted to waste both before and after elution of peaks of interest to prevent entrance of any matrix components into the MS source. Different time programs used in the analysis and the retention times for each compound are shown in Table 1. Fig. 2A shows the chromatogram of a blank human plasma sample, Fig. 2B shows a human plasma sample spiked with all the quantifiable standards and IS at 500 ng/mL and 100 ng/mL, respectively, and a volunteer’s sample at 30 min after oral administration of the DAM/NAL formulation is pictured in Fig. 2C. In the last one, only four compounds (M3G, M6G, MOR, NALT) and the IS were detected in human plasma.

3.2. Sample preparation
During sample preparation, it was necessary to avoid exposure of samples to ambient conditions for a long time to preserve the stability of heroin, an easily degradable compound [5,21]. Plasma samples were kept in an ice-bath during processing. Centrifugation was conducted at 4ºC, and prepared samples were immediately transferred to an autosampler at 10ºC or a refrigerator at 4ºC. Most reported procedures used SPE to extract heroin and metabolites from human plasma [2,18,19]. Based on that, different pre-treatment methods were tested. However, the aim of this study was to develop a simple and a rapid method, so protein precipitation (PP) was chosen as the extraction procedure. Only one LC-MS/MS assay using a pretreatment with protein precipitation has been published for the determination of heroin and its metabolites [22]. Nevertheless, this PP method was not tested in plasma, showed a long chromatographic run time (approximately 16 minutes) and required time-consuming evaporation and reconstitution steps. Moreover, there are no published studies that evaluate NAL and NALT in combination with heroin and its metabolites. Methanol and acetonitrile were tested as extraction solvents for PP. Methanol was found to give better peak shapes for all analytes compared to acetonitrile. Then, the addition of a formic acid solution enhanced the signal responses and improved the peak shapes for DAM, 6OM, MOR, NAL and NALT compounds without needing evaporation (method A) and saving time in sample preparation. However, it was necessary to do evaporation and reconstitution in formic acid solution after PP for M3G and M6G compounds in order to concentrate the samples and to improve their chromatographic profiles (method B). This procedure was accepted after checking the matrix effect and recovery data (section 3.3.6).

3.3. Validation

3.3.1. Selectivity and carry over

The method was found to have high selectivity for the analytes. No interfering peaks from endogenous compounds were observed at the retention times for each compound and the IS (Fig. 2A). Furthermore, no quantifiable carryover effect was obtained in this study after injecting the highest calibration standard.
3.3.2. Linearity

Linearity was assessed based on the average of three calibration curves analysed on three separate days. For all analytes, calibration curves were fitted according to a linear regression (1/x weighing), with correlation coefficients ($r^2$) >0.99, indicating a good linearity in the established range during the validation and quantification. This modelling with a weight of 1/x leads to better predictions than an unweighted model for all compounds (bias from the calibration curves is less than 15% for all calibration points and the precision of three replicates is less than 15%).

3.3.3. LLOQ, Precision and accuracy

The method was found to be highly accurate and precise. The mean accuracies of the quality controls ranged between -3.6% and 4.0%, and the intra- and inter-assay precisions (CV) were <11.5% for all the analytes. For all compounds, the LLOQ was 10 ng/mL in human plasma showing acceptable accuracies within the range of -4.5% to 6.9% and precisions below 12.3%.

3.3.4. Stability

Stability tests were evaluated in human plasma and in solution under different conditions as described in section 2.5.4. The results are shown in Table 2. Working solutions were found to be stable at least for 6 hours at room temperature, and stock solutions of each compound were stable for more than 100 days when stored at 4ºC in the fridge. However, the internal standard solution was stable for only 14 days after storage in the refrigerator at 4ºC and for 6 hours at room temperature. In human plasma, the results of long-term stability were found to be within ±15% accuracy, indicating that all compounds are stable for more than three months at -80ºC. Additionally, no detectable degradation was observed after three freeze-thaw cycles for all compounds. Similar results were observed on-instrument stability when samples were stored in the autosampler for at least 20 hours at 10ºC. The stability during sample processing was evaluated for 6 hours using an ice-bath (0 ºC) and all compounds were stable under these conditions.
3.3.5. Dilution Integrity

In the dilution integrity study, the precision was less than 6.5% and the accuracy was within the range of -11.6% to 7.7% for all compounds. These results proved that the samples with higher concentrations than the ULOQ could be diluted and re-analysed.

3.3.6. Matrix Effect and Recovery

The overall CV of the IS normalized matrix effect was within the acceptable limits (2.4-12.5%). These data proved that the matrix effect in human plasma for all compounds was negligible in this study. The mean recoveries obtained for all compounds and the internal standard were approximately 100% (above 93%). These results demonstrated that PP was a good choice for a sample preparation method.

3.3.7. In-study validation

To assure that the assay was performing under the pre-defined specifications, a calibration curve was created for each analytical run to calculate the concentration of each analyte in the unknown samples. The number of quality control samples to be analysed was between 5% and 20% of the total unknown samples number for each analytical run. At least 77% of the quality controls analysed every day were within ±15% of their respective nominal values, and the coefficients of variation (CV) were less than 15%. The overall precision (CV %) obtained in the total analytical runs during sample quantification was 8.6% for DAM, 7.9% for 6OM, 6.7% for MOR, 8.6% for M3G, 9.1% for M6G and 5.3% for NAL. The total accuracy was in the range of -7.4 to 2.01% for all compounds.

3.3.8. Incurred sample reanalysis

The total number of incurred samples analysed in different batches was 24 samples (12% of the total samples in the study) including two samples per volunteer with concentrations close to the maximal concentration ($C_{\text{max}}$) and in the elimination phase. The percentage difference between the concentrations of incurred samples reanalysis and the initial values was determined for each
analyte. 95.8%, 87.5%, 91.7% and 100% of the repeats showed less than 20% differences for M3G, M6G, MOR and the rest of the analytes, respectively. These values complied with the acceptance criteria, where at least 67% of the repeats should not be greater than 20% differences.

3.4. Application to pharmacokinetic study

The validated method enabled the simultaneous quantification of DAM, its metabolites and NAL and the qualitative determination of NALT in human plasma samples from a clinical trial after the oral administration of 50/2 mg of DAM/NAL formulation in healthy volunteers. The mean plasma concentration-time profiles are in Fig. 3, and the respective pharmacokinetic parameters are listed in Table 3.

As can be observed, plasma concentration levels were only obtained for MOR, M3G and M6G compounds where M3G was found to be the major metabolite. As mentioned in the literature review, DAM and 6OM were not detected in plasma after oral administration just like NAL due to the first-pass metabolism [23-25]. The method was sensitive to quantify M3G and M6G in human plasma for 24 hours after a single oral dose and until 180 min for MOR. The mean AUC$_{0-\infty}$ was 83.1 ± 22.6 ng/mL*h for MOR, 5.6 ± 0.6 µg/mL*h for M3G and 853.4 ± 113.8 ng/mL*h for M6G compound. These results are consistent with those previously reported in the literature obtained after the oral administration of heroin at several doses [26,27]. Regarding NALT determination, plasma levels of NALT were found in some of volunteers’ samples although the signals obtained for all of them were not very intense, so that these concentrations may be near the limit of quantification for NALT [8].

4. Conclusions

In conclusion, a rapid, specific and precise LC-MS/MS method was developed and validated for the simultaneous quantification of DAM, its main metabolites (6OM, MOR, M3G and M6G) and NAL in human plasma using a small volume of sample (100 µL). In addition, this method was used for the qualitative detection of NALT. A simple protein precipitation were used for pre-treatment without the need to evaporate DAM, 6OM, MOR, NAL and NALT compounds, which
saves time during samples preparation. Although evaporation and reconstitution were necessary for the M3G and M6G extraction in plasma samples, good recoveries and negligible matrix effects were observed by using protein precipitation for all compounds.

Therefore, this method was successfully applied to human plasma samples from a clinical trial after the oral administration of a DAM/NAL formulation in healthy volunteers. The short methodology for sample preparation, the selectivity, precision, accuracy and the short LC-MS/MS run time make this method reliable and suited to the analysis of pharmacokinetics for large numbers of samples.
REFERENCES


[20]. European Medicines Agency (EMEA), Guideline on bioanalytical method validation, Committee for Medicinal Products for Human Use (CHMP), 2011.


TABLE CAPTIONS

Table 1. MS/MS conditions

Table 2. Results of stability study in solution and in human plasma.

Table 3. Pharmacokinetic parameters in human plasma after an oral administration of DAM/NAL formulation in healthy volunteers (n = 12, data are means ± standard deviations).

FIGURE CAPTIONS

Fig. 1. MS/MS spectra of [M+H]+ ion for DAM (a), 6OM (b), MOR (c), M3G (d), M6G (e), NAL (f), NALT (g) and IS (h).

Fig. 2. Representative MRM chromatograms of extracted from: (A) a blank human plasma; (B) a human plasma sample spiked with DAM, 6OM, MOR, M3G, M6G, NAL (500 ng/mL) and IS; (C) and a volunteer’s sample taken 30 min after oral administration of DAM/NAL formulation (50/2 mg).

Fig. 3. Human plasma concentrations after an oral administration of DAM/NAL formulation in healthy volunteers (n = 12, data are means ± standard deviations).
HIGHLIGHTS

- Simultaneous determination of heroin, its metabolites, naloxone and naltrexone.
- A rapid, specific and precise LC-MS/MS method was developed and validated.
- The short pretreatment methodology allowed saving time during samples preparation.
- Suitable to apply for the analysis of clinical trials.
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<th>Collision Energy (eV)</th>
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<td>Long-term (4°C)</td>
<td>Nominal Conc. (ng/mL)</td>
<td>Long term (-80°C)</td>
<td>Freeze-thaw (3 cycles)</td>
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<td>DAM</td>
<td>100.2</td>
<td>99.8</td>
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<td>112.0</td>
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<td>91.1 87.0 95.2 96.7</td>
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</tr>
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<td>M6G</td>
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</tr>
<tr>
<td>$t_{1/2}$ (hr)</td>
<td>0.7 ± 0.2</td>
<td>12.2 ± 2.7</td>
<td>4.2 ± 2.4</td>
<td></td>
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</tr>
<tr>
<td>$T_{\text{max}}$ (hr)</td>
<td>0.4 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$C_{\text{max}}$ (ng/mL)</td>
<td>85.1 ± 23.7</td>
<td>1165.0 ± 227.0</td>
<td>251.0 ± 38.2</td>
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</tr>
<tr>
<td>$AUC_{\text{all}}$ (hr*ng/mL)</td>
<td>70.3 ± 19.7</td>
<td>4677.7 ± 544.0</td>
<td>771.6 ± 98.0</td>
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<tr>
<td>$AUC_{\text{inf}}$ (hr*ng/mL)</td>
<td>83.1 ± 22.6</td>
<td>5564.5 ± 604.3</td>
<td>853.4 ± 113.8</td>
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<tr>
<td>$AUC_{\text{ext}}$ (%)</td>
<td>15.5 ± 3.2</td>
<td>15.8 ± 5.7</td>
<td>9.5 ± 3.3</td>
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</tr>
<tr>
<td>$V_z/F_{\text{obs}}$ (L)</td>
<td>637.3 ± 103.1</td>
<td>159.4 ± 37.9</td>
<td>352.2 ± 169.3</td>
<td></td>
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<tr>
<td>$Cl/F_{\text{obs}}$ (L/min)</td>
<td>10.7 ± 2.8</td>
<td>0.2 ± 0.0</td>
<td>1.0 ± 0.1</td>
<td></td>
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</table>
Graphical Abstract