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.

Raquel Moreno-Vicente^{a,b}, Zuriñe Fernández-Nieva^czurine.fernandez@tecnalia.com, Arantza Navarro^carantza.navarro@tecnalia.com, Irene Gascón-Crespí^digasconerespi@gmail.com, Magí Farré-Albadalejo^emfarre@imim.es, Manuela Igartua^{a,b}, Rosa María Hernández^{a,b}jose-luis.pedraz@ehu.es, José Luis Pedraz^{a,b*}
Raquel Moreno-Vicente^{a,b}

^a NanoBioCel Group, Laboratory of Pharmaceutics, University of the Basque Country, Faculty of Pharmacy, Vitoria-Gasteiz, Spain

^b Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Vitoria-Gasteiz, Spain

^c Pharmaceutical Development Department, Tecnalia Research & Innovation Foundation, Vitoria-Gasteiz, Spain

^c Pharmaceutical Development Department, Tecnalia Research & Innovation Foundation, Vitoria-Gasteiz, Spain

^d One-Dose Pharma, Barcelona, Spain

^e Human Pharmacology and Clinical Neurosciences Research Group, Hospital del Mar Medical Research Institute-IMIM, Parc de Salut Mar, Barcelona, Spain ()

24 <PA>NanoBioCel Group, Laboratory of Pharmaceuticals, University of the Basque
25 Country, Faculty of Pharmacy, Paseo de la Universidad 7, 01006 Vitoria-Gasteiz.
26 Biomedical Research Networking Center in Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN),
27 Paseo de la Universidad, 7, 01006, Vitoria-Gasteiz, Spain.Tel . : (+34)-945013091

28
29
30

31 Abstract:

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

52

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

54

55

56

57 ABBREVIATIONS

58 Heroin, diamorphine (DAM)

59 6-monoacetylmorphine (6OM)

60 Morphine (MOR)

61 Morphine-3-glucuronide (M3G)

62 Morphine-6-glucuronide (M6G)

63 Naloxone (NAL)

64 Naltrexone (NALT)

65 **1. Introduction**

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

81 In this work, all target compounds were determined and applied to a clinical trial in healthy
82 volunteers. NALT was administered by security 12 hours before oral administration of 50/2 mg of
83 DAM / NAL tablets. This formulation was developed as an Investigational Medicinal Product
84 (IMP) for drug substitution treatment in opioid dependents, and has a patent application
85 (Application number: PCT/ES2013/070272). This combination aims to prevent the abuse of
86 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

113 obtained from the Biobanco Vasco (Bilbao, Spain) who approved the analytical protocol of this
114 study (application number, CBVI371/1M).

115 2.2. Preparation of quality control samples and calibration standards

116 Stock solutions of all standards and IS were prepared at 1.0 mg/mL in DMSO. These solutions
117 were stored at 4°C. IS precipitation solution was prepared at 100 ng/mL in methanol from the IS
118 stock solution. Working solutions at the desired concentrations for calibration standard and quality
119 control samples were prepared by serial dilutions in combination from the stock solutions in
120 DMSO to make a single calibration curve for all compounds. Standard solutions were prepared by
121 spiking human plasma with working solutions at a concentration range of 10-2000 ng/mL for
122 M3G and 10-1000 ng/mL for DAM, 6OM, MOR, M6G and NAL. Linearity was studied within
123 these ranges by preparing seven concentrations of DAM, 6OM, M6G, MOR, NAL (10, 50, 100,
124 250, 500, 750, 1000 ng/mL) and eight concentrations of M3G (10, 50, 100, 250, 500, 750, 1000,
125 2000 ng/mL). Low, medium and high concentration quality control (QC) samples at
126 concentrations of 30, 400, 800 ng/mL for all compounds and at a higher concentration for M3G
127 (1500 ng/mL) were prepared in human plasma. All of these solutions were freshly prepared daily.

128 2.3. Sample Preparation

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

133 Two-hundred microliters of IS precipitation solution (100 ng/mL in methanol) were added to 100
134 μ L of human plasma sample. The mixture was vortexed for 30 seconds and centrifuged at 14000
135 r.p.m. at 4°C for 5 minutes. Then, the supernatant was filtered into an Eppendorf tube. Finally, 50
136 μ L of supernatant were mixed with 50 μ L of 0.1% formic acid (method A) for the determination
137 of DAM, 6OM, MOR, NAL and NALT. For determination of M3G and M6G, evaporation was
138 necessary after protein precipitation to obtain higher signal intensity and better peak shapes for

these compounds (method B). To this end, 150 μ L of supernatant were evaporated to dryness at 25°C under a stream of nitrogen, and finally the dry residue was reconstituted with 50 μ L of 0.1% formic acid and transferred to a glass vial. A 50 μ L 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 10°C. Chromatographic separation was performed on an X-Bridge Phenyl column (150 x 4.6 mm, 5 μ m) from Waters and maintained at 35°C. 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 μ L 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, 120°C; desolvation temperature, 350°C, 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®).

161

2.5. Method validation

The present study was validated in compliance with GLP principles (Good Laboratory Practice)

164 and EMEA (European Medicines Agency) guidance for the validation of bioanalytical methods
165 [20]. Selectivity, carry over, matrix effects, linearity, precision, accuracy, lower limit of
166 quantification (LLOQ), recovery, stability and dilution integrity were evaluated during method
167 validation. Only selectivity was evaluated for NALT.

168 2.5.1. Selectivity and carry over

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

173 2.5.2. Linearity

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

179 2.5.3. LLOQ, accuracy and precision

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

187 2.5.4. Stability

188 To evaluate stability in solution, working solutions were prepared and kept at room temperature
189 (25 °C) for 6 hours and stored at 4-8°C. Stock solutions were stored at 4-8°C and quantified at
190 different times for more than 100 days to study long term stability.

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

197 2.5.5. Dilution integrity

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

201 2.5.6. Matrix effect and recovery

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

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

210

211 2.6. Pharmacokinetic study

212 The proposed LC-MS/MS method was used to evaluate the plasma profiles of DAM, 6OM, MOR,
 213 M3G, M6G and NAL after oral administration in humans. The study was performed on 12 healthy
 214 volunteers in the Clinical Trial Unit of the Hospital del Mar d'Investigacions Mèdiques (IMIM).
 215 All patients gave their written informed consent prior to participation in the study. The clinical
 216 trial was approved by the Clinical Research Ethical Committee of the Parc de Salut Mar. The
 217 volunteers received a single tablet of 50 mg heroin/2 mg naloxone by oral administration. NALT
 218 was administered in tablets of 50 mg naltrexone hydrochloride (Revia®, Bristol-Myers Squibb,
 219 S.A.) at 12 and 0.5 hours before and 12 hours after of the administration of DAM/NAL
 220 formulation. Blood samples were collected at different time points; -12, -0.5, 0 (pre-dose), 0.25,
 221 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24 hours after the administration. EDTA was used as an
 222 anticoagulant. The plasma was collected and stored at -80°C until its analysis by LC-MS/MS.
 223 The pharmacokinetics analysis of concentration-time data was performed according to non-
 224 compartmental analysis using the Phoenix WinNonlin Version 6.3 software package. The
 225 maximum plasma concentration ($C_{\max}$) and the time at which it is reached ($t_{\max}$) were obtained
 226 directly from the experimental data and confirmed by the same software. The area under the
 227 plasma concentration-time curve to the last experimental point (AUC_{all}) was obtained by the
 228 trapezoidal method and AUC extrapolated to infinity (AUC_{inf}) was calculated by adding the term
 229 $C_{\text{last}}/\lambda_z$ to AUC_{all} where C_{last} is the last measured concentration. The λ_z value (elimination rate
 230 constant) was calculated by linear regression of the logarithms of plasma concentrations in the
 231 terminal phase of the curve. The half-life ($t_{1/2}$) was calculated from $0.693 / \lambda_z$. The systemic
 232 clearance (CL) was calculated from $D \cdot F / (AUC_{\text{inf}})$, where D is the dose and F is the fraction of
 233 dose absorbed. The apparent volume of distribution (V_z) was obtained from $D \cdot F / (AUC_{\text{inf}} \cdot \lambda_z)$.
 234 In this case, F could not be calculated because the intravenous data are not available, so the CL
 235 and V_z parameters were normalized to F.

236

237 3. Results and discussion

238 3.1. LC-MS/MS analysis

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

263 3.2. Sample preparation

264 During sample preparation, it was necessary to avoid exposure of samples to ambient conditions
265 for a long time to preserve the stability of heroin, an easily degradable compound [5,21]. Plasma
266 samples were kept in an ice-bath during processing. Centrifugation was conducted at 4°C, and
267 prepared samples were immediately transferred to an autosampler at 10°C or a refrigerator at 4°C.
268 Most reported procedures used SPE to extract heroin and metabolites from human plasma
269 [2,18,19]. Based on that, different pre-treatment methods were tested. However, the aim of this
270 study was to develop a simple and a rapid method, so protein precipitation (PP) was chosen as the
271 extraction procedure. Only one LC-MS/MS assay using a pretreatment with protein precipitation
272 has been published for the determination of heroin and its metabolites [22]. Nevertheless, this PP
273 method was not tested in plasma, showed a long chromatographic run time (approximately 16
274 minutes) and required time-consuming evaporation and reconstitution steps. Moreover, there are
275 no published studies that evaluate NAL and NALT in combination with heroin and its metabolites.
276 Methanol and acetonitrile were tested as extraction solvents for PP. Methanol was found to give
277 better peak shapes for all analytes compared to acetonitrile. Then, the addition of a formic acid
278 solution enhanced the signal responses and improved the peak shapes for DAM, 6OM, MOR,
279 NAL and NALT compounds without needing evaporation (method A) and saving time in sample
280 preparation. However, it was necessary to do evaporation and reconstitution in formic acid
281 solution after PP for M3G and M6G compounds in order to concentrate the samples and to
282 improve their chromatographic profiles (method B). This procedure was accepted after checking
283 the matrix effect and recovery data (section 3.3.6).

284 3.3. Validation

285 3.3.1. Selectivity and carry over

286 The method was found to have high selectivity for the analytes. No interfering peaks from
287 endogenous compounds were observed at the retention times for each compound and the IS (Fig.
288 2A). Furthermore, no quantifiable carryover effect was obtained in this study after injecting the
289 highest calibration standard.

290 3.3.2. Linearity

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

297 3.3.3. LLOQ, Precision and accuracy

298 The method was found to be highly accurate and precise. The mean accuracies of the quality
299 controls ranged between -3.6% and 4.0%, and the intra- and inter-assay precisions (CV) were $<$
300 11.5% for all the analytes. For all compounds, the LLOQ was 10 ng/mL in human plasma
301 showing acceptable accuracies within the range of -4.5% to 6.9% and precisions below 12.3%.

302 3.3.4. Stability

303 Stability tests were evaluated in human plasma and in solution under different conditions as
304 described in section 2.5.4. The results are shown in Table 2.

305 Working solutions were found to be stable at least for 6 hours at room temperature, and stock
306 solutions of each compound were stable for more than 100 days when stored at 4°C in the fridge.
307 However, the internal standard solution was stable for only 14 days after storage in the refrigerator
308 at 4°C and for 6 hours at room temperature. In human plasma, the results of long-term stability
309 were found to be within $\pm 15\%$ accuracy, indicating that all compounds are stable for more than
310 three months at -80°C. Additionally, no detectable degradation was observed after three freeze-
311 thaw cycles for all compounds. Similar results were observed on-instrument stability when
312 samples were stored in the autosampler for at least 20 hours at 10°C. The stability during sample
313 processing was evaluated for 6 hours using an ice-bath (0 °C) and all compounds were stable
314 under these conditions.

315 3.3.5. Dilution Integrity

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

319 3.3.6. Matrix Effect and Recovery

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

325 3.3.7. In-study validation

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

335 3.3.8. Incurred sample reanalysis

336 The total number of incurred samples analysed in different batches was 24 samples (12% of the
337 total samples in the study) including two samples per volunteer with concentrations close to the
338 maximal concentration ($C_{\max}$) and in the elimination phase. The percentage difference between the
339 concentrations of incurred samples reanalysis and the initial values was determined for each

340 analyte. 95.8%, 87.5%, 91.7% and 100% of the repeats showed less than 20% differences for
341 M3G, M6G, MOR and the rest of the analytes, respectively. These values complied with the
342 acceptance criteria, where at least 67% of the repeats should not be greater than 20% differences.

343 3.4. Application to pharmacokinetic study

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

349 As can be observed, plasma concentration levels were only obtained for MOR, M3G and M6G
350 compounds where M3G was found to be the major metabolite. As mentioned in the literature
351 review, DAM and 6OM were not detected in plasma after oral administration just like NAL due to
352 the first-pass metabolism [23-25]. The method was sensitive to quantify M3G and M6G in human
353 plasma for 24 hours after a single oral dose and until 180 min for MOR. The mean $AUC_{0-\infty}$ was
354 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
355 compound. These results are consistent with those previously reported in the literature obtained
356 after the oral administration of heroin at several doses [26,27]. Regarding NALT determination,
357 plasma levels of NALT were found in some of volunteers' samples although the signals obtained
358 for all of them were not very intense, so that these concentrations may be near the limit of
359 quantification for NALT [8].

360 4. Conclusions

361 In conclusion, a rapid, specific and precise LC-MS/MS method was developed and validated for
362 the simultaneous quantification of DAM, its main metabolites (6OM, MOR, M3G and M6G) and
363 NAL in human plasma using a small volume of sample (100 µL). In addition, this method was
364 used for the qualitative detection of NALT. A simple protein precipitation were used for pre-
365 treatment without the need to evaporate DAM, 6OM, MOR, NAL and NALT compounds, which

366 saves time during samples preparation. Although evaporation and reconstitution were necessary
367 for the M3G and M6G extraction in plasma samples, good recoveries and negligible matrix effects
368 were observed by using protein precipitation for all compounds.
369 Therefore, this method was successfully applied to human plasma samples from a clinical trial
370 after the oral administration of a DAM/NAL formulation in healthy volunteers. The short
371 methodology for sample preparation, the selectivity, precision, accuracy and the short LC-MS/MS
372 run time make this method reliable and suited to the analysis of pharmacokinetics for large
373 numbers of samples.

374

375 **REFERENCES**

- 376 [1]. P.Zuccaro, R. Ricciarello, S. Pichini, Simultaneous determination of heroin, 6-
 377 monoacetylmorphine, morphine, and its glucuronides by liquid chromatography-
 378 atmospheric pressure ionspray-mass spectrometry, *J. Anal. Toxicol.* 21 (1997) 268-277.
- 379 [2]. Elisabeth J. Rook, Michel J.X. Hillebrand, Hilde Rosing, The quantitative analysis of
 380 heroin, methadone and their metabolites and the simultaneous detection of cocaine,
 381 acetylcodeine and their metabolites in human plasma by high-performance liquid
 382 chromatography coupled with tandem mass spectrometry, *J. Chromatogr. B.* 824 (2005)
 383 213-221.
- 384 [3]. S.Y. Yeh, R.L. McQuinn, C.W. Gorodetzky, Urinary excretion of heroin and its
 385 metabolites in man, *J. Pharmacol. Exp. Ther.* 196 (1976) 249-256.
- 386 [4]. Asher Y Salmon, Zafir Goren, Yaniv Avissar, Hermona Soreq, Human erythrocyte but not
 387 brain acetylcholinesterase hydrolyses heroin to morphine, *Clin. Exp. Pharmacol. Physiol.*
 388 26 (1999) 596-600.
- 389 [5]. D.A. Barrett, A.L. Dyssegaard. P.N. Shaw, The effect of temperature and pH on the
 390 deacetylation of diamorphine in aqueous solution and in human plasma, *J. Pharm.*
 391 *Pharmacol.* 44 (1992) 606-608.
- 392 [6]. M. Liu, E. Wittbrodt, Low-dose naloxone reverses opioid-induced constipation and
 393 analgesia, *J. Pain symptom Manag.* 23 (2002) 48-53.
- 394 [7]. Ji Dong, Shuaibing Liu, Hua Zhang, Determination of naloxone-3-glucuronide in human
 395 plasma and urine by HILIC-MS/MS, *J. Chromatogr. B* 942-943 (2013) 83-87.

- 396 [8]. Hwi-yeol Yun, Seong-chul Ban, Simultaneous analysis of naltrexone and its major
397 metabolite, 6- β -naltrexol, in human plasma using liquid chromatography-tandem mass
398 spectrometry: Application to a parent-metabolite kinetic model in humans, *Talanta* 71
399 (2007) 1553-1559.
- 400 [9]. Claudia Clavijo, Jamie Bendrick-Peart, An automated, highly sensitive LC-MS/MS assay
401 for the quantification of the opiate antagonist naltrexone and its major metabolite 6 β -
402 naltrexol in dog and human plasma, *J. Chromatogr. B.* 874 (2008) 33-41.
- 403 [10]. D. Bourquin, T. Lehmann, High-performance liquid chromatographic monitoring of
404 intravenously administered diacetylmorphine and morphine and their metabolites in human
405 plasma, *J. Chromatogr. B.* 694 (1997) 233-238.
- 406 [11]. G. Chari, A. Gulati, High-performance liquid chromatographic determination of morphine,
407 morphine-3-glucuronide, morphine-6-glucuronide and codeine in biological samples using
408 multi-wavelength forward optical detection, *J. Chromatogr. B.* 571 (1991) 263-270.
- 409 [12]. P.A. Glare, T.D. Walsh, C.E. Pippenger, A simple, rapid method for the simultaneous
410 determination of morphine and its principal metabolites in plasma using high-performance
411 liquid chromatography and fluorometric detection, *Ther. Drug. Monit.* 13 (1991) 226-232.
- 412 [13]. J.G. Guillot, M. Lefebvre, J.P. Weber, Determination of heroin, 6-acetylmorphine, and
413 morphine in biological fluids using their propionyl derivatives with ion trap GC-MS, *J.*
414 *Anal. Toxicol.* 21 (1997) 127-133.
- 415 [14]. D.G. Watson, Q. Su, J.M. Midgley, Analysis of unconjugated morphine, codeine,
416 normorphine and morphine as glucuronides in small volumes of plasma from children, *J.*
417 *Pharm. Biomed. Anal.* 13 (1995) 27-32.

- 418 [15]. D. Projean, T.M. Tu, J. Ducharme, Rapid and simple method to determine morphine and
419 its metabolites in rat plasma by liquid chromatography-mass spectrometry, *J. Chromatogr.*
420 *B.* 787 (2003) 243-253.
- 421 [16]. Hongliang Jiang, YuRong Wang, Development and validation of a sensitive LC/MS/MS
422 method for the simultaneous determination of naloxone and its metabolites in mouse
423 plasma, *J. Chromatogr. B.* 879 (2011) 2663-2668.
- 424 [17]. Xiujun Wu, Weiping Zhang, Simultaneous analysis of codeine and its active metabolites in
425 human plasma using liquid chromatography-tandem mass spectrometry: Application to a
426 pharmacokinetic study after oral administration of codeine, *J. Pharm. Biomed. Anal.* 78-79
427 (2013) 261-268.
- 428 [18]. Ahmed Al-Asmari, Robert A. Anderson, Susan Kidd and Alison H. Thomson, Method for
429 the quantification of diamorphine and its metabolites in pediatric plasma samples by liquid
430 chromatography-tandem mass spectrometry, *J. Anal. Toxicol.* 34 (2010) 177-195.
- 431 [19]. Jessica M. Jones, Michael D. Raleigh, Stability of heroin, 6-monoacetylmorphine, and
432 morphine in biological samples and validation of an LC-MS assay for delayed analyses of
433 pharmacokinetic samples in rats, *J. Pharm. Biomed. Anal.* 74 (2013) 291-297.
- 434 [20]. European Medicines Agency (EMA), Guideline on bioanalytical method validation,
435 Committee for Medicinal Products for Human Use (CHMP), 2011.
- 436 [21]. G.K. Poochikian, J. C. Cradock, Simple high-performance liquid chromatographic method
437 for the separation of 3, 6- diacetylmorphine hydrochloride (heroin) and hydrolysis
438 products, *J. Chromatogr.* 171 (1979) 371-376.
- 439 [22]. Ritva Karinen, Jannike Mørch Andersen, Determination of heroin and its main metabolites
440 in small sample volumes of whole blood and brain tissue by reversed-phase liquid
441 chromatography- tandem mass spectrometry, *J. Anal. Toxicol.* 33 (2009) 345-350.

- 442 [23]. Inturrisi CE, Max MB, Foley KM, The pharmacokinetics of heroin in patients with chronic
443 pain, *N. Engl. J. Med.* 310 (1984) 1213-1217.
- 444 [24]. Elisabeth J. Rook, Alwin D. R. Huitema, Wim van den Brink, Pharmacokinetics and
445 pharmacokinetic variability of heroin and its metabolites: Review of the literature, *Curr.*
446 *Clin. Pharmacol.* 1 (2006) 109-118.
- 447 [25]. Joan A. Culpepper-Morgan, Charles E. Inturrisi, Russell K. Portenoy, Treatment of opioid-
448 induced constipation with oral naloxone: a pilot study, *Clin. Pharmacol. Ther.* 52 (1992)
449 90-95.
- 450 [26]. Girardin F, Rentsch KM, Schwab MA, Pharmacokinetics of high doses of intramuscular
451 and oral heroin in narcotic addicts, *Clin. Pharmacol. Ther.* 74 (2003) 341-352.
- 452 [27]. Ludwig Perger, Katharina M. Rentsch, Oral heroin in opioid-dependent patients:
453 Pharmacokinetic comparison of immediate and extended release tablets, *Eur. J. Pharm.*
454 *Sci.* 36 (2009) 421-432.
- 455

455 **TABLE CAPTIONS**

456 **Table 1. MS/MS conditions**

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

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

460 **FIGURE CAPTIONS**

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

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

467 **Fig. 3. Human plasma concentrations after an oral administration of DAM/NAL formulation**
468 **in healthy volunteers (n = 12, data are means $\pm$ standard deviations).**

469

469 HIGHLIGHTS

470

- 471 • Simultaneous determination of heroin, its metabolites, naloxone and naltrexone.
- 472 • A rapid, specific and precise LC-MS/MS method was developed and validated.
- 473 • The short pretreatment methodology allowed saving time during samples
- 474 preparation.
- 475 • Suitable to apply for the analysis of clinical trials.

476

477

477

Compound	Retention time (min)	MRM	Cone Voltage (V)	Collision Energy (eV)	MS program (time period, min)	Type of determination
DAM	4.75	370.19 / 165.02	50	40	4.5-5.0	Quantitative
6OM	4.26	328.33 / 165.11	35	35	4.0-4.5	Quantitative
MOR	2.97	286.22 / 164.99	45	30	2.5-4.0	Quantitative
M3G	2.16	462.30 / 286.27	55	25	1.2-3.0	Quantitative
M6G	2.46	462.30 / 286.30	50	30	1.5-3.2	Quantitative
NAL	4.09	328.27 / 212.14	25	35	3.8-4.4	Quantitative
NALT	4.27	342.30 / 212.22	30	40	4.0-4.5	Qualitative
IS	4.53	314.24 / 165.06	40	40	4.3-4.8	Quantitative

478

479

479

Compound	Mean Recovery (%)						
	<i>In solution</i>		Nominal Conc. (ng/mL)	<i>In human plasma</i>			
	Room Temp. (6h)	Long-term (4°C)		Long term (-80°C)	Freeze-thaw (3 cycles)	Autosampler (10°C)	During Processing
DAM	100.2	99.8	30	108.0	85.6	104.3	107.3
			800	88.4	89.8	91.5	103.5
6OM	99.9	100.4	30	91.8	114.3	108.6	112.0
			800	86.3	114.6	105.6	114.3
MOR	100.3	99.7	30	91.1	95.2	96.7	110.0
			800	87.0	100.1	94.6	111.9
M3G	100.7	99.9	30	94.4	112.0	91.0	93.7
			800	98.1	113.0	89.1	109.7
M6G	100.4	100.1	30	104.6	98.2	89.0	95.3
			800	93.3	92.5	104.2	107.1
NAL	99.8	100.1	30	85.9	97.2	103.7	111.7
			800	89.1	100.7	99.0	107.6
IS	101.2	97.9					

480

481

481

		MOR			M3G			M6G		
t_{1/2}	(hr)	0.7	±	0.2	12.2	±	2.7	4.2	±	2.4
T_{max}	(hr)	0.4	±	0.2	0.7	±	0.3	0.9	±	0.2
C_{max}	(ng/mL)	85.1	±	23.7	1165.0	±	227.0	251.0	±	38.2
AUC_{all}	(hr*ng/mL)	70.3	±	19.7	4677.7	±	544.0	771.6	±	98.0
AUC_{inf}	(hr*ng/mL)	83.1	±	22.6	5564.5	±	604.3	853.4	±	113.8
AUC_{ext}	(%)	15.5	±	3.2	15.8	±	5.7	9.5	±	3.3
Vz/F_{obs}	(L)	637.3	±	103.1	159.4	±	37.9	352.2	±	169.3
Cl/F_{obs}	(L/min)	10.7	±	2.8	0.2	±	0.0	1.0	±	0.1

482

483

Figure 2