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The quantitative analysis of heroin, methadone and their metabolites and the simultaneous detection of cocaine, acetylcodeine and their metabolites in human plasma by high-performance liquid chromatography coupled with tandem mass spectrometry

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Abstract

For a pharmacokinetic–pharmacodynamic study in opioid tolerant patients, who were treated with heroin in combination with methadone, a liquid chromatographic assay with tandem mass spectrometry detection (LC–MS/MS) was developed for the simultaneous determination of heroin, methadone, heroin metabolites 6-monoacetylmorphine, morphine, and morphine-6 and 3-glucuronide and methadone metabolite EMDP. To detect any abuse of substances besides the prescribed opioids the assay was extended with the detection of cocaine, its metabolites benzoylecgonine and norcocaine and illicit heroin adulterants acetylcodeine and codeine. Heroin-d6, morphine-d3, morphine-3-glucuronide-d3 and methadone-d9 were used as internal standards. The sample pre-treatment consisted of solid phase extraction using mixed mode sorbent columns (MCX Oasis). Chromatographic separation was performed at 25 °C on a reversed phase Zorbax column with a gradient mobile phase consisting of ammonium formate (pH 4.0) and acetonitrile. The run time was 15 min. MS with relatively mild electrospray ionisation under atmospheric pressure was applied. The triple quadrupole MS was operating in the positive ion mode and multiple reaction monitoring (MRM) was used for drug quantification. The method was validated over a concentration range of 5–500 ng/mL for all analytes. The total recovery of heroin varied between 86 and 96% and of the heroin metabolites between 76 and 101%. Intra-assay and inter-assay accuracy and precision of all analytes were always within the designated limits (≤20% at lower limit of quantification (LLQ) and ≤15% for other samples). This specific and sensitive assay was successfully applied in pharmacokinetic studies with medically prescribed heroin and toxicological cases.

Keywords: Heroin; Acetylcodeine; Cocaine; Methadone; LC–MS–MS

1. Introduction

There is a growing international interest in the medical prescription of high doses of pharmaceutically pure heroin (diacetylmorphine) in otherwise treatment refractory heroin addicted patients. Heroin assisted treatment has resulted in significant improvements of the physical, mental and social well being of patients and their environment in Swiss and Dutch experiments [1,2]. In several other European countries and in Canada clinical trials with the administration of heroin to addicted patients are in progress [3].

Heroin is a semi-synthetic lipophilic morphine derivative. Two acetyl-groups are coupled to the 3- and 6-carbon site of the phenanthrene ring (Fig. 1). Heroin would pass the blood–brain barrier more easily than morphine because

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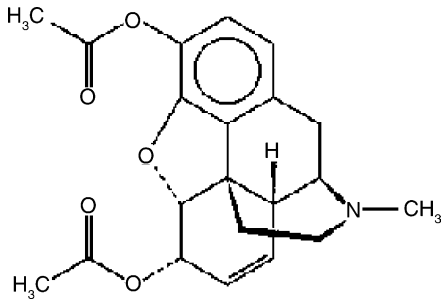

Fig. 1. Molecule structure of heroin.

of the acetyl-groups. The ester bonds, however, are very unstable in human plasma and heroin is rapidly hydrolysed to 6-monoacetylmorphine (6-MAM) and morphine (MOR) by serum-esterases and liver carboxylesterases [4]. Finally, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) conjugates are formed in liver, and probably in kidney and brain [5].

Heroin has a very short estimated half-life between 2 and 5 min but a prolonged pharmacodynamic action of several hours. It mainly acts by its more stable agonistic metabolites 6-monoacetylmorphine, morphine and morphine-6-glucuronide [6]. The major metabolite morphine-3-glucuronide has very low affinity to μ-opioid receptors, but is associated with adverse neuroexcitatory events in treatment with morphine and heroin [7]. Thus, for a pharmacokinetic and pharmacodynamic study it is pivotal to include the major metabolites 6-monoacetylmorphine, morphine, morphine-6-glucuronide and morphine-3-glucuronide.

Heroin treated patients who participated in our studies were all co-treated with the long acting opioid methadone to diminish craving and withdrawal symptoms. In order to study any interaction between both opioids, our bioanalytical assay was therefore extended with the quantification of methadone and its metabolite ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP) in plasma.

Heroin administration takes place in out-patient clinics under strict supervision of a nursing team [1]. However, many heroin dependent patients are multi-drug users and the use of cocaine and illicit heroin outside the clinic could not be fully prohibited in this setting. To get insight into any other use of drugs it was felt important to detect cocaine and its long-term metabolites benzoylecgonine and norcocaine in plasma.

Heroin is produced from opium poppies and the use of illicit heroin besides pharmaceutical grade heroin may be found by the detection of impurities of opium derivatives [8]. Opium content codeine, a by-product of illicit heroin, and its acetylated derivate acetylcodeine, were qualitatively measured as markers of illicit heroin in our study.

Gas chromatographic (GC) methods are suitable to analyse heroin, 6-monoacetylmorphine and morphine in human plasma [9]. The analysis of the large non-volatile morphine glucuronide metabolites however, cannot occur by GC without deconjugation of the glucuronide groups. However, the analysis of the morphine glucuronides was essential in our studies. For GC analysis, derivatisation of the analytes is obligatory, which is a time-consuming process that could induce further hydrolysis of heroin [10].

Successful HPLC methods with single MS, UV or diode array detection for the simultaneous measurement of heroin and four major metabolites are described in murine serum [11] and in human plasma [12]. Furthermore, several HPLC methods are described concerning the detection of long circulating heroin metabolites morphine and the morphine glucuronides, without actual measuring of the pro-drug heroin and its metabolite 6-acetylmorphine [13–15]. However, the recovery of heroin and metabolites only reached 44.8–66.8% in these studies in human plasma where automated sample preparation was applied on C18 columns at room temperature. When diode array detection was applied, endogenous compounds interfered with the quantification of heroin. In our study, MS/MS was applied to enhance specificity and sensitivity of the detection.

In summary, our aim was to develop a quantitative assay for heroin, methadone and their metabolites, which is also capable to detect any cocaine and illicit heroin use in heroin treated patients. We report a LC–MS/MS assay that fulfilled our requirements. Special attention was paid to the stability of the analytes during sample pre-treatment.

2. Experimental

2.1. Chemicals

Heroin hydrochloride and heroin-d6 originated from Akzo Nobel (Arnhem, The Netherlands). 6-Mono-acetylmorphine, morphine-3-glucuronide, morphine-6-glucuronide, morphine-d3 were obtained from Sigma (Poole, UK). Morphine-hydrochloride, cocaine hydrochloride and codeine phosphate were purchased from Bufa (Uitgeest, The Netherlands), methadone hydrochloride from Fagron (Nieuwerkerk aan de IJssel, The Netherlands) and methadone-d9, EMDP, acetylcodeine, morphine-3-glucuronide-d3 from Radian International LCC (Austin, Texas, USA). Diazepam and caffeine for specificity studies came from, respectively, Centrafarm Services (Etten-Leur, The Netherlands) and Bufa (Uitgeest, The Netherlands). All compounds showed purity between 98 and 100.6%. Drug free human plasma for quality control and calibration samples originated from Sanquin, Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). Distilled water was used throughout (Aqua B Braun, Emmenbrücke, Switzerland) and all other chemicals were of analytical or HPLC grade and used without further purification.

2.2. Preparation of stock solutions, quality control and calibration samples and internal standards

Stock solutions of heroin, 6-monoacetylmorphine, morphine-3-glucuronide, morphine-6-glucuronide, were

prepared in a 10 mM ammonium acetate (pH 4.5) in methanol solution 1:1 (v/v). Stock solutions of morphine, methadone and EMDP were prepared in methanol. Stock solutions for calibration samples and QC samples were prepared independently. All stock solutions were stored at −20 °C for a period of maximal 25 months. Fifty microlitres of working solutions of 0.1, 0.2, 1, 2, 5 and 10 μg/mL were diluted with 950 μL drug-free plasma, resulting in calibration standards with analyte concentrations of, respectively, 5, 10, 50, 100, 250 and 500 ng/mL. Quality control samples of 50, 250, and 400 ng/mL were prepared by adding 50 μL working solution of, respectively, 1, 5 and 8 μg/mL to 950 μL drug-free plasma. Quality control and calibration samples were freshly prepared for every analysis.

For the preparation of internal standards stock solutions amounts of morphine-d3, morphine-3-glucuronide-d3 and methadone-d9 were diluted in methanol. Heroin-d6 was dissolved in 10 mM ammonium acetate (pH 4.5) in methanol (1:1, v/v). A working solution was prepared from these stock solutions containing 1.25 μg/mL of each internal standard in methanol.

Heroin-d6 was used as internal standard for quantification of heroin and morphine-d3 for the quantification of 6-monoacetylmorphine and morphine. Morphine-3-glucuronide-d3 was applied as internal standard for both analysed glucuronides (morphine-3-glucuronide and morphine-6-glucuronide) and the methadone metabolite EMDP. Methadone-d9 was used as internal standard for quantification of methadone.

2.3. Patients' samples

In this study, plasma concentrations of two heroin addicted patients, one heroin injector and one heroin inhaler, who were on steady state treatment with pharmaceutical prepared heroin in combination with methadone are shown. For injection, 300 mg heroin hydrochloride was formulated in 3 mL Water for Injection and for inhalation, 300 mg heroin base was mixed with 100 mg caffeine. Caffeine was added because it lowers the melting point of heroin and therefore diminishes the disintegration of heroin during the smoking procedure [16].

Blood samples were collected in 7 mL glass tubes containing 15 mg potassium oxalate and 12 mg sodium fluoride (Benson Dickinson Vacutainer™, Plymouth, UK). Sodium fluoride containing tubes were applied for blood sampling, in order to inhibit the plasma esterase activity and thereby stabilising the amount of heroin [17].

Samples were taken at baseline level ($t = 0$), during inhalation period and at 2, 5, 10, 15, 30, 45, 60, 115, 180, 240 and 480 min after finishing heroin administration. Blood samples were immediately put on ice after sampling. Within a period of 15 min after blood sampling, samples were centrifuged at 2000 × *g* and at 4 °C. Plasma was shock frozen in a dry ice/methanol bath and kept in polypropylene tubes at −20 °C until analysis.

2.4. Sample pre-treatment

All patients' plasma samples were analysed both undiluted as well as diluted with blank plasma containing sodium fluoride in a 1:20 ratio of the heroin dosage. Plasma samples were thawed in an ice/water bath and to 250 μL of plasma a volume of 50 μL internal standard solution was added. Samples were then diluted with 300 μL 0.15 N hydrochloric acid in water. After vortex mixing, the diluted samples were kept in ice-water.

Mixed mode sorbent Oasis MCX 1 mL extraction columns were used for solid phase extraction (Waters Corporation, Milford, MA, USA). The MCX columns were conditioned with, respectively, 2 mL of methanol, 1 mL of water and 2 mL 10 mM citrate buffer (pH 3.0). The SPE columns were not allowed to run dry during the conditioning phase. The acidified plasma samples were loaded onto the columns and the flow was kept at approximately 1 mL/min. After sample load the cartridges were washed with 500 μL water pH 3.0 (acidified with acetic acid) and the columns were dried at maximal vacuum for 1 min. Elution occurred next within 10 s with 500 μL 0.5% ammonium acetate in methanol solution 1:20 (v/v), which was kept at −20 °C to promote stability of heroin. The eluate was collected in a well containing 250 μL 50 mM ammonium acetate buffer (pH 3.0) and immediately vortexed. Since heroin is not stable in the basic elution solution, the eluate was collected in an acidic buffer to improve stability of heroin compound of the eluate. The solvent was evaporated under a nitrogen stream at 20 °C till on average 250 μL was left. The extract was kept at 4 °C until analysis.

2.5. HPLC

The HPLC system consisted of an Agilent 1100 series binary pump and autosampler (Agilent, Palo Alto, CA, USA). The 96 wells auto-sampler temperature was remained at 4 °C. Six microlitres of the extract was injected to a reversed phase Zorbax Bonus column (150 mm × 4.6 mm i.d., particle size 5 μm; Rockland Technologies Inc., Newport, Delaware, USA). A reversed phase 10 mm × 3 mm column was used as guard column (Varian).

Gradient elution was performed using a mixture of 5 mM ammonium formate in water (pH 4.0) and acetonitrile, as described in Table 1. Flow rate of the mobile phase was kept constant at 1 mL/min and the total run time was 15 min. The column oven temperature was kept at 25 °C. The autosampler needle was washed with 100% methanol for 10 s after each injection.

2.6. MS/MS

The LC flow was split 1:20 by an Accurate Flow Post Column Splitter (LC Packings, Sunnyvale, CA, USA) before entering the electrospray interface (Turbo Ionspray®) of an API 3000 triple quadrupole mass spectrometry system (PE

Table 1
HPLC eluent gradient Eluent A consisted of 5 mM ammonium formate (pH 4.0) and Eluent B of 100% acetonitrile

Time (min)	Eluent A (%)	Eluent B (%)
0.0	97.0	3.0
2.0	97.0	3.0
2.6	87.0	13.0
8.0	84.5	15.5
8.1	20.0	80.0
11.0	20.0	80.0
11.1	97.0	3.0
15.0	97.0	3.0

Sciex, Ont., Canada). Ions were created in the positive ion mode under atmospheric pressure at 5.5 kV and at a source temperature of 350 °C. Nebulisation took place using zero air at 1.8 L/min. Curtain flow (nitrogen gas grade 5.0) was set at 1.0 L/min and turbo flow at 6.5 L/min zero air. Ions were induced to fragmentation in the collision cell (Q2) by nitrogen gas grade 5.0 with a collision gas pressure of 261 molecules/cm^2. The collision energy for the protonated analytes varied from 23 to 81 V. Dwell times for each transition were set at 80 ms with a 5 ms pause between scans. Multiple reaction monitoring (MRM) was used for drug quantification. Selected ion masses of the protonated precursors and fragment ions are shown in Table 2. Chromatographic peaks were integrated using Analyst^{TM} software (version1.2, Sciex).

3. Validation

3.1. Linearity

Calibration standards of 5, 10, 50, 100, 250, and 500 ng/mL were analysed in duplicate in three different runs. Calibration curves were calculated by least-squares linear regression using a weighing factor of $1/x$ (x = concentration) for morphine and methadone and a weighing factor of $1/x^2$ for heroin, 6-mono-acetylmorphine, morphine-3-glucuronide, morphine-6-glucuronide and EMDP.

Deviations of calculated concentrations from the nominal concentrations exceeding ±15% were excluded from linear regression. At the lower limit of quantification (LLQ) deviations within ±20% from the nominal value were permitted.

3.2. Accuracy and precision

To assess accuracy and precision, three replicates of the spiked quality control samples at four concentration levels (LLQ: 5 ng/mL; low: 50 ng/mL; mid: 250 ng/mL; high: 400 ng/mL) were measured for all analytes in five separate runs within 1 week. Accuracy was calculated as: ((mean concentration quality control − nominal concentration)/nominal concentration) × 100%. Precision was calculated as: (standard deviation of the mean/mean) × 100%. The intra-assay accuracy and precision were calculated for each of the five runs separately. The calculation of inter-assay accuracy and precision was based on the mean and standard deviation of five runs together. The acceptance criteria of precision and accuracy were less than 20% deviation at LLQ level and less than 15% deviation at higher concentration levels.

3.3. Recovery

To determine any ion-suppression, the mean response of processed blank plasma samples that were reconstituted with working solutions of the analytes, were compared to the mean results of the analysis of working solutions, representing 100% recovery.

The SPE recovery was calculated by comparing the results of processed quality control samples to the results of processed blank samples reconstituted with working solutions. To assess the total recovery, the mean outcomes of three processed QC samples were compared to working solutions. All experiments were performed in triplicate at three concentra-

Table 2
Retention times and selected ions of each analyte and internal standard (IS)

Analyte/IS	Retention time (min)	Precursor/product ions (m/z)	IS applied	Type of determination
M3G	2.4	462.2/286.0	M3G-d3	Quantitative
M3G-d3	2.4	465.2/289.0		
Morphine-d3	2.9	289.2/151.9		
MOR	2.9	286.3/151.9	Morphine-d3	Quantitative
M6G	4.5	462.2/286.0	M3G-d3	Quantitative
Codeine	5.3	300.1/215.2		Qualitative
6-MAM	6.1	328.1/165.0	Morphine-d3	Quantitative
Acetylcodeine	7.8	342.1/225.1		Qualitative
Cocaine	7.9	304.1/182.0		Qualitative
Heroin-d6	8.0	376.1/268.1		
Heroin	8.0	370.1/268.1	Heroin-d6	Quantitative
Norcocaine	8.8	290.1/136.0		Qualitative
Benzoylecgonine	9.3	290.2/82.1		Qualitative
Methadone	10.8	310.2/265.0	Methadone-d9	Quantitative
Methadone-d9	10.8	319.3/268.2		
EMDP	12.2	264.1/220.1	M3G-d3	Quantitative

tion levels for all analytes (low: 50 ng/mL; mid: 250 ng/mL; high: 400 ng/mL).

3.4. Stability

Short-term stability was investigated of plasma quality control samples of different concentrations (low: 50 ng/mL; mid: 250 ng/mL; high: 400 ng/mL) that were kept in ice-water for 60 min. Long-term stability in plasma was investigated in 10 patients' plasma samples that were kept at −20 °C for a period of 14 months. Freeze-thaw stability of the analytes in plasma was determined in quality control samples containing 250 ng/mL of the analytes, after thawing in ice-water and refreezing at −20 °C in three cycles within a period of 3 weeks.

Stability of the SPE extracts of quality control samples was assessed at three concentration levels (low: 50 ng/mL; mid: 250 ng/mL; high: 400 ng/mL) after a 17 days period at 4 °C. Stability of the stock solutions was measured after a storage period of 25 months at −20 °C.

3.5. Selectivity and specificity

Interference of endogenous compounds with the analytes was investigated by analysis of blank plasma samples of six different volunteers (unspiked, spiked with internal standard and with the analytes at LLQ level). Specificity was tested with diazepam (500 ng/mL in drug free plasma) and caffeine (500 ng/mL in drug free plasma).

Table 3
Recovery means of three determinations are presented

Analyte	Concentration (ng/mL)	SPE recovery (%)	Total recovery (%)
Heroin	50	104	96
	250	88	91
	400	87	86
6-MAM	50	95	94
	250	92	86
	400	94	88
MOR	50	99	101
	250	88	86
	400	88	83
M3G	50	83	79
	250	78	80
	400	79	79
M6G	50	89	81
	250	84	79
	400	82	76
Methadone	50	80	86
	250	76	79
	400	77	77
EMDP	50	84	84
	250	83	82
	400	90	88

4. Results and discussion

4.1. Sample pre-treatment

Plasma samples were kept in an ice/water bath and were acidified immediately after thawing and before solid phase extraction to promote stability of heroin. Heroin in plasma is most stable at low temperatures [18] and at low pH (<5.2) [18,19]. Furthermore, the analytes were protonated under acidic conditions, which is essential to perform solid phase extraction with cation exchange columns.

The application of solid phase extraction with mixed mode strong cation exchange columns (MCX OASIS) resulted in good recovery of both apolar substances (like heroin and methadone) and of polar compounds (morphine-glucuronides) (Table 3).

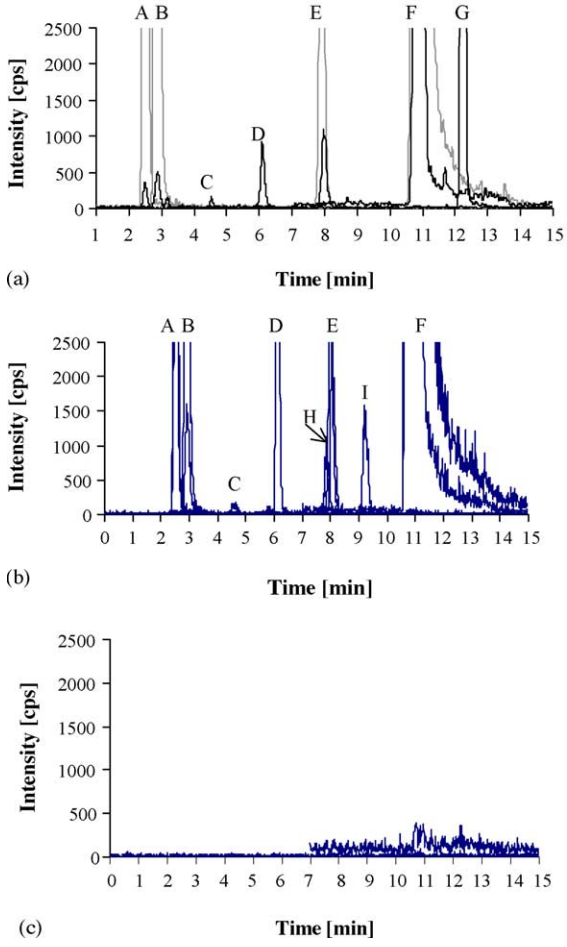

Fig. 2. (a) Representative total ion chromatograms of all quantifiable analytes spiked at LLQ level in human plasma (5 ng/mL). The intensity of the deuterated analytes was above 2500 [cps]. (b) Representative total ion chromatograms of random chosen patient' plasma sample. (c) Total ion chromatogram of a plasma sample of a non-drug using volunteer. (A) M3G and M3G-d3; (B) morphine and morphine-d3; (C) M6G; (D) 6-MAM; (E) heroin and heroin-d6; (F) = methadone and methadone-d9; (G) EMDP; (H) cocaine; (I) benzoylecgonine.

Table 4
Inter-assay accuracy and precision inter-assay accuracy and precision of five analytical runs

Analyte	Nominal concentration (ng/mL)	Measured concentration (ng/mL)	Inter-assay accuracy (%)	Inter-assay precision (%)
Heroin	5.1	5.1	1.7	11.1
	49.7	50.9	2.4	7.9
	250	252	0.8	4.9
	398	403	1.3	2.4
6-MAM	5.0	5.0	0.0	9.5
	49.7	51.4	3.4	5.1
	251	251	0.2	4.6
	398	405	1.7	4.3
MOR	4.9	4.7	−4.7	10.9
	50.0	49.3	−1.4	5.5
	252	246	−2.3	5.7
	400	396	−1.0	2.9
M3G	5.0	5.0	−0.7	7.4
	49.9	50.2	0.6	2.8
	251	248	−1.2	4.5
	400	401	0.2	3.2
M6G	5.0	4.8	−4.6	10.1
	49.8	50.2	0.8	6.8
	251	256	2.2	6.7
	399	410	2.6	6.4
Methadone	5.0	4.6	−8.6	10.3
	50.5	52.8	5.6	3.7
	252	265	5.3	4.9
	400	429	7.2	2.5
EMDP	5.0	4.8	−5.0	7.4
	53.2	54.0	1.4	9.1
	268	265	−1.0	9.9
	426	434	1.8	11.2

In each run quality control samples were measured at three concentration levels.

A basic methanol buffer had to be applied to elute the cations from the column. By lowering the temperature of the eluent to −20 °C, shortening the elution time to maximal 10 s, and the collection of the elution solvent in an acidic buffer, the recovery of heroin significantly improved from 40 to 93%.

Although a large variety of compounds with different polarities could be analysed, the analysis of one of the methadone metabolites 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) did not succeed. The cyclic and apolar character of the EDDP molecule probably prohibited an acceptable recovery.

4.2. HPLC

The 16 analytes of interest were analysed using a reversed phase Zorbax Bonus column (Table 2). This column was chosen because up to 100% aqueous eluents of a broad pH range can be applied. Morphine-3-glucuronide and morphine-6-glucuronide, which share the same molecule masses and tandem MS fragmentation characteristics, could chromatographically be separated from each other on this column, with retention times of 2.4 and 4.5 min, respectively (Fig. 2a). The run time was shortened to 15 min by switching from a polar aqueous mobile phase (ammonium formate buffer pH 4.0) to an apolar mobile phase (acetonitrile) after 8 min. The retention times are listed in Table 2.

4.3. MS/MS

All analytes were selected for mass spectrometry based on ion masses of their protonated parent masses. The *m*/*z* ratio of the most common product formed in the collision cell was chosen to refine mass spectrometry (Table 2).

4.4. Validation

The assay for all quantified compounds was linear between 5 and 500 ng/mL with correlation coefficients exceeding 0.9987. Deviations from the nominal concentrations of the calibration samples of all analytes varied from 0.1 to 10%. The inter-assay accuracy and precision data were all within acceptance criteria of ±20% at LLQ level and ±15% for other concentrations (Table 4). Intra-assay accuracy was below 10.5% for heroin and below 14% for the other compounds. The intra-assay precision did not exceed 8.6%.

The total recovery and SPE recovery of all quantified analytes are summarised in Table 4. Ion suppression was below acceptance criteria throughout the validation procedure.

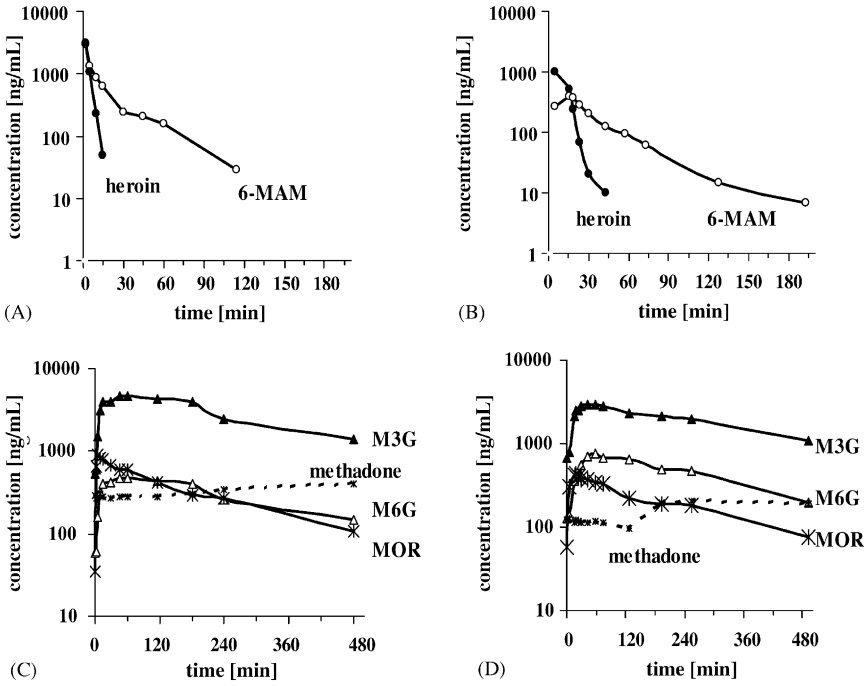

Fig. 3. Plasma concentrations of heroin, its metabolites and methadone after injection (A and B) or inhalation (C and D) of 300 mg heroin. The heroin injector used 100 mg methadone and the heroin inhaler 60 mg methadone orally. Both patients were in steady state treatment for heroin in combination with methadone.

Stability results are summarised in Table 5. Heroin and the metabolites were stable in plasma at 0–4 °C for a period of 1 h (recovery above 94.5%). A period of 1 h was chosen, because in practice samples were kept in ice-water for a period of 10–60 min before SPE columns were ready for sample loading. Stability of all analytes was also established after three freeze-thaw-cycles.

The chromatograms of six batches of control human plasma contained no endogenous peaks co-eluting with any of the analytes (Fig. 2c). All LLQ samples prepared in these six batches of human plasma could be quantified within the required 20% deviation from the nominal concentration, except for one heroin LLQ sample with a deviation of 35% from the nominal value (data not shown).

For specificity studies, diazepam was chosen because its use is very common in this special patient population. Caffeine was selected because the formulation for inhalation of heroin base contained caffeine. These agents did not interfere the chromatography and detection of the analytes (data not shown).

4.5. Plasma concentrations in patients

Plasma concentration–time curves of heroin and its major metabolites after 300 mg heroin in two patients (one heroin inhaler and one heroin injector) are pictured in Fig. 3. Methadone doses of 60 mg in the heroin inhaler and 100 mg in the heroin injector were administered orally 120 min after the heroin administration. Both patients were in steady state treatment of heroin and methadone. Methadone and the more stable heroin metabolites morphine, morphine-3- and morphine-6-glucuronide were above LLQ at baseline. The plasma concentrations of the methadone metabolite EMDP did not reach above the LLQ in both patients.

During and after heroin inhalation, lower plasma concentrations of heroin and its metabolites were seen compared to intravenous injection of a bolus. Bioavailability is limited in heroin inhalation because a part of the heroin dose is lost due to degradation during the vaporisation procedure of heroin [20]. After heroin inhalation, relatively large morphine-6-glucuronide concentrations were seen compared to intravenous use. The ratio between both glucuronides in plasma after heroin inhalation resembles the glucuronides ratio after oral morphine administration [21]. Possibly a part of the heroin dose is swallowed during the inhalation procedure and absorbed through the digestive tract.

In these two patients, who participated in a study that took place under controlled circumstances in a research clinic, no traces of cocaine or traces of illicit heroin use were found. However, in samples of patients who were treated with heroin on medical prescription in an outpatient clinic, cocaine and its metabolite benzoylecgonine were commonly found, and in rare cases acetylcodeine or codeine (Fig. 2b).

As far as we know, this is the first time that liquid chromatography with tandem mass spectrometry was applied for the simultaneous quantitative analysis of heroin and methadone and their metabolites in human plasma.

In our clinic, this bioanalytical method was not only applied for pharmacokinetic studies on heroin and methadone, but also in toxicological cases when drug-abuse was suspected. For bio-analysis only 250 μL plasma was

Table 5
Stability data short-term stability was measured in spiked plasma samples that were kept in ice-water for 1 h ($n = 3$)

Analyte	Short-term			Long-term		Freeze-thaw			Final extract		
	Concentration $t = 0$ (ng/mL)	CV (%)	Recovery (%)	Mean concentration (ng/mL)	Recovery (%)	Concentration $t = 0$ (ng/mL)	CV (%)	Recovery (%)	Concentration $t = 0$ (ng/mL)	CV (%)	Recovery (%)
Heroin	48	8.4	113.9	261	102.2	255	2.8	100.0	48.3	1.4	100.9
	248	7.3	99.6						248	4.8	102.6
	412	2.3	98.0						412	6.0	97.7
6-MAM	53	4.5	94.5	343	95.6	241	1.1	101.9	53	5.0	97.4
	253	4.0	96.3						253	3.3	104.1
	403	2.0	96.7						403	4.3	104.1
MOR	51	7.0	96.5	306	93.5	238	3.1	100.1	51.2	2.1	97.3
	257	2.4	97.8						257	0.6	97.8
	404	3.5	101.6						404	3.5	99.1
M3G	51	2.5	100.1	2110	96.7	254	0.6	97.8	50.9	6.1	96.1
	255	5.0	101.8						255	1.8	98.7
	413	1.7	101.5						413	2.6	99.6
M6G	50	7.0	102.2	240	102.1	268	3.4	93.1	49.8	2.7	99.0
	261	1.6	98.1						261	1.9	93.7
	418	3.0	95.2						418	2.3	90.0
Methadone	56	4.1	96.7	264	101.9	261	1.6	98.7	55.5	3.4	90.7
	275	2.3	97.8						275	2.6	98.5
	435	0.7	100.0						435	1.3	99.8
EMDP	58	2.8	96.6	<LLQ	NA	241	2.1	89.8	57.5	7.3	91.8
	270	3.7	99.6						270	5.0	95.9
	420	3.0	104.8						420	5.4	98.5

Long-term stability was assessed in patients' plasma samples after various heroin doses and sampling times that were kept at −20 °C for 14 months ($n = 9$). Stability of spiked samples after three freeze-thaw cycles (−20 °C to +4 °C) was measured ($n = 3$). Extracts were kept 17 days at 4 °C ($n = 3$).

required, which is an advantage in pharmacokinetic studies in malnourished heroin addicted patients.

5. Conclusion

To study pharmacokinetic and pharmacodynamic relations in patients on heroin-assisted methadone therapy, an accurate and reproducible LC–MS/MS method with SPE as sample pre-treatment was developed, for the simultaneous quantitative analysis of heroin, its metabolites 6-monoacetylmorphine, morphine, morphine-3-glucuronide and morphine-6-glucuronide, methadone and EMDP. Apart from the quantitative analysis of the prescribed opioids, the analysis was extended with the qualitative detection of unprescribed substances, like cocaine, cocaine metabolites norcocaine and benzoylecgonine and illicit heroin impurities acetylcodeine and codeine.

To measure such a large amount of analytes with different polarities that could be transformed into their metabolites during sample pre-treatment and ionisation process, good separation on the HPLC column was essential. This could be achieved within 15 min on a Zorbax Bonus reversed phase column with gradient elution, with an aqueous ammonium formate mobile phase followed up by acetonitrile. Morphine-3-glucuronide and morphine-6-glucuronide, which share the same molecule mass and could therefore not be distinguished by MS/MS, could be separated on the HPLC column.

Heroin is most stable at 0–4 °C and at pH 3.5–5.2 [18,19]. Several measurements to prevent hydrolysis of the unstable heroin were taken throughout the process of sample preparation and analysis, like adding serum esterase blocking fluoride in the plasma tubes, the low temperatures and the low pH (3–4) conditions during SPE and HPLC. The combination of these measures resulted in a high recovery rate and low LLQ not only for heroin, but also for its major metabolites.

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