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DEVELOPMENT AND VALIDATION OF A HPLC-DAD/FLD METHOD FOR THE DETERMINATION OF MDMA, MDA, METHAMPHETAMINE, MORPHINE, MORPHINE-GLUCURONIDES AND 6-MONOACETYLMORPHINE IN HUMAN PLASMA

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Opiates and amphetamines are two of the most frequently used classes of illicit drugs. A liquid chromatographic method was developed for simultaneous quantification of heroin metabolites (6-acetylmorphine, morphine and morphine glucuronides) and some of the major amphetamine derivatives (methamphetamine, 3,4-methylenedioxymethamphetamine and 3,4-methylenedioxyamphetamine) in human plasma. Due to the very different polarities of the analytes, sample preparation involved two solid phase extraction methods using Oasis HLB cartridges. Chromatographic separation was performed on a C18 chromatographic column with a binary mixture of acetonitrile/10mM ammonium formate in water (pH = 4.0) as mobile phase, under gradient elution (flow rate = 0.5 mL/min). The total analysis run time was 27 min. The detection was performed in UV at 210nm, but also based on the strong native fluorescence of some of the analytes (λ_{exc}=210nm/ λ_{em}=340nm; λ_{exc}=288nm/ λ_{em}=324nm). The method was validated and proved to be sensitive enough to be used in simultaneous monitoring of heroin and amphetamines consumption.

INTRODUCTION

Identifying drug-addicts and including them in detoxification or substitution therapy programs is an important component of the war conducted by the authorities against illicit drug use and drug trafficking**.** Drug addicts' diagnosis, therapy monitoring and the estimation of substance use relapse during detoxification or substitution therapy require toxicological analysis in order to identify the parent drugs or their metabolites in different biological matrices.

Due to the fact that most of drug addicts are actually multidrug users, it is very important to elaborate analytical methods capable of detecting and quantifying simultaneously different types of illicit drugs and their metabolites in order to confirm the drug use.

Heroin (3,6-diacetylmorphine) is one of the oldest drugs of abuse known by man. In the human body, it is rapidly deacetylated to 6-acetylmorphine (6-AM), which is metabolized to morphine. In a final step morphine (MOR) is transformed in glucuronated metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G), respectively (Figure 1). Monitoring heroin consumption is based on the detection and quantification of these metabolites in biological samples (urine, plasma, hair, vitreous humor, cerebrospinal fluid**,** nails and teeth) by enzyme-linked immunosorbent assay (ELISA)[1], high performance liquid chromatography (HPLC) with ultraviolet (UV)[2], fluorescence (FL)[3-5] or mass spectrometric (MS or MS/MS) detection[6-10], gas chromatography (GC) with MS[1,11,12] detection or capillary electrophoresis (CE) with UV detection.[13,14]

Amphetamines represent another important class of illicit drugs used mainly as stimulants, but some of them are preferred also for their empathogenic and hallucinogenic effects. Methamphetamine (MA), 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) (Figure 1) are some of the major amphetamines used today.

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MDA represent a metabolite of MDMA, but it is also available on the illicit market. Various analytical methods such as enzyme-linked immunosorbent assay (ELISA)[15], HPLC-UV[16], HPLC-FL[17,18], HPLC-MS[19,20], GC-MS[21-23], GC with flame-ionization detection (FID)[24,25] and CE with UV[26] or laser induced fluorescence (CE-LIF)[27] detection have been used for the quantification of different amphetamines (MA, MDMA, MDA and other amphetamine derivatives) in biological matrices (plasma, urine, saliva).

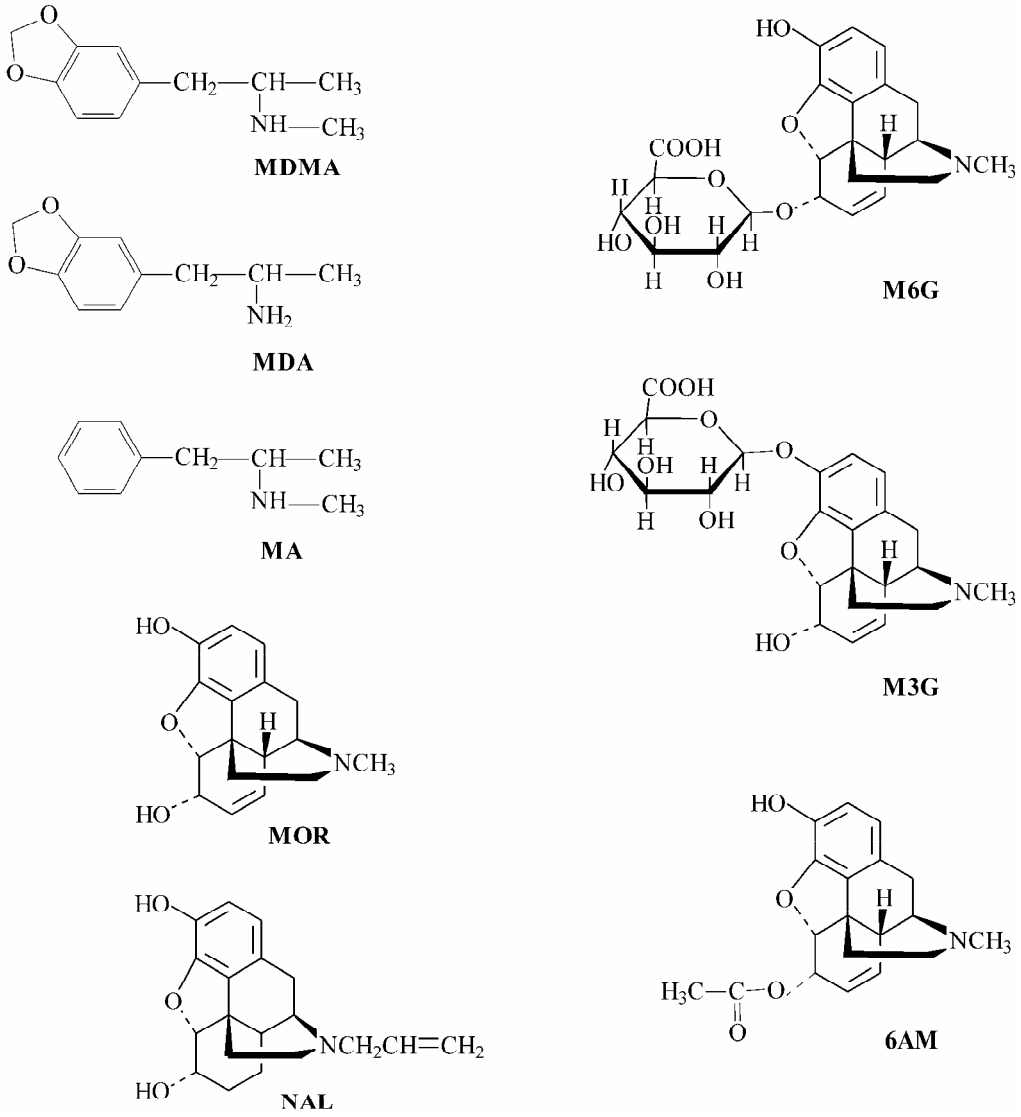

Fig. 1 – Chemical structure of the analyzed heroin metabolites, amphetamine derivatives and internal standard.

To date there are only few methods described in the literature for the simultaneous quantification of heroin metabolites and amphetamines from human biological samples. The majority of these assays were elaborated for hair analysis based on GC-MS[28], GC-MS/MS[29], capillary zone electrophoresis (CZE) with MS detection[30,31] or sweeping micellar electrokinetic chromatography (MECK) with UV detection[32]. Chen et al. elaborated a HPLC-MS/MS method for screening of abused drugs and their metabolites in urine (a total of 19 analytes, including MDA, MDMA and MOR).[33]

Regarding the simultaneous quantification of heroin metabolites and amphetamines from human blood/plasma samples three methods were found in the literature, all based on GC with MS or MS/MS detection.[34-36]

The present work was aimed at developing and validating a sensitive HPLC method with UV and FL detection as an alternative to the existing GC-MS assays for the simultaneous quantification of heroin metabolites (6-AM, MOR, M3G and M6G) and some of the major amphetamine derivatives (MA, MDMA, MDA) from human plasma.

RESULTS AND DISCUSSION

The analysis of heroin metabolites and amphetamines from human plasma included a sample preparation step through solid phase extraction, followed by a HPLC analysis with UV detection and FL detection based on the native fluorescence of some of the analytes. Due to the very different polarities of the analytes and in order to eliminate the endogenous interferences due to the complex biological matrix, two extraction methods were needed. Neither one of the two extraction methods which were elaborated could not guarantee high extraction yields and selectivity for all analytes.

Regarding the selection of the detection method, fluorescence is of first choice due to its major advantage over the UV detection, the superior sensitivity. This detection was chosen for most of the analytes, with the exception of 6AM and MA, two compounds with very weak native fluorescence. In case of these two analytes UV detection was used at 210nm. Usually this wavelength has the disadvantage of poor selectivity, but guarantees a better sensitivity than higher wavelengths from the UV spectra.

The fluorescence excitation and emission spectra of the analytes were recorded in order to facilitate the selection of optimum excitation and emission wavelengths. Based on the spectral analysis in case of MDMA and MDA a λ_{exc} = 288nm and λ_{em} = 324nm were chosen. In case of heroin metabolites a maximum was observed in the emission spectra at 325nm, but the analysis of blank and spiked plasma samples showed significant interferences at λ_{em} = 325nm (with λ_{exc} = 288nm) at their retention times. Finally, based on the selectivity studies and on the S/N ratios, the heroin metabolites were monitored at λ_{em} = 340nm (with λ_{exc} = 210nm).

The optimum sample preparation method and UV or FL detector settings for all analytes are summarized in Table 1. In order to eliminate the errors due to the variability of the extraction yield an internal standard was used (NAL).

Table 1

Sample preparation, detection and retention parameters

Analyte	Sample preparation	Detection	Retention time (min)
M6G	EFS 1	FLD – λ_{exc}=210nm/ λ_{em}=340nm	7.36
MOR		FLD – λ_{exc}=210nm/ λ_{em}=340nm	7.91
MA	EFS 2	UV – 210nm	12.92
6AM		UV – 210nm	12.08
MDMA		FLD – λ_{exc}=288nm/ λ_{em}=324nm	13.77
MDA		FLD – λ_{exc}=288nm/ λ_{em}=324nm	12.54
M3G		FLD – λ_{exc}=210nm/ λ_{em}=340nm	3.65

Using the described HPLC conditions all analytes were well resolved and eluted in symmetrical peaks with a total analysis run time of 27 minutes (including washing of the column and reequilibration to the initial composition of the mobile phase). The retention times corresponding to the analytes are presented in Table 1.

The method was validated according to the bioanalytical method validation guide with regard to the selectivity, linearity, limit of quantitation, accuracy, recovery and precision.

The assay was found to be selective for all seven analytes and I.S. as no interfering peaks were observed in the extracts (obtained by SPE1 or SPE2) of the blank plasma samples, with the effluent being monitored with the photodiode array detector (210nm) and with the fluorescence detector (λ_{exc}=210nm/ λ_{em}=340nm; λ_{exc}=288nm/ λ_{em}=324nm) (Figures 2-5).

The method proved to be linear over the studied concentration range in case of each drug or metabolite (Table 2). Calibration curves were constructed for each analyte, based on the relation between peak area ratios and concentration ratios of the analyte and internal standard (weighted (1/*y*) linear regression).

The accuracy values were lower than ± 15% for all samples (± 20% for LLOQ), in agreement to international regulations regarding bioanalytical methods' validation (Table 2). The recoveries of all seven analytes and of IS from plasma were determined by comparing the analytical results for extracted samples at all ten calibration levels with unextracted standards that represents 100% recovery. Despite of the significant differences regarding the polarity of the analyzed molecules, the two extraction methods using the Oasis HLB cartridges guaranteed very good recoveries for all of them (Table 2). The mean recovery % (± SD) of internal standard was 99.98% ± 4.41.

Based on the statistical evaluation (% CV) of the analytical data obtained during the intra- and inter-

day precision study, the method proved to be precise at all three concentration levels corresponding to the in-house QC samples (Table 3).

Table 2

Linearity, accuracy and recovery data (n=10)

Analyte	Linearity range (ng/mL)	Correlation coefficient (r^2)	Accuracy %	Mean absolute recovery % (±S.D.)
MDMA	4.22 – 843.00	0.9984 – 0.9995	92.07 – 108.27	93.60 (16.93)
MDA	4.30 – 861.00	0.9972 – 0.9993	89.35 – 110.14%	90.08 (13.68)
MA	24.21 – 807.00	0.9898 – 0.9949	87.10 – 112.13%	81.49 (7.75)
MOR	17.80 – 890.00	0.9943 – 0.9992	85.13 – 111.04%	76.53 (13.64)
M3G	9.95 – 995.00	0.9888 – 0.9988	85.38 – 112.89% (115.96 at LLOQ)	71.10 (10.34)
M6G	40.32 – 1008.00	0.9925 – 0.9988	87.93 – 114.26	75.74 (12.54)
6AM	10.74 – 1074.00	0.9897 – 0.9944	87.53 – 113.27	98.91 (8.24)

Table 3

Intra- and inter-day precision (n=5)

Analyte	Nominal concentration (ng/mL)	Intra-day precision CV%	Inter-day precision CV%
MDMA	8.43	10.30	10.70
	84.3	12.01	8.86
	421.5	9.82	9.51
MDA	8.61	10.15	9.74
	86.1	13.12	10.88
	430.5	11.3	10.39
MA	24.21	7.52	10.32
	80.7	8.50	9.27
	403.5	8.37	6.87
MOR	17.80	7.22	7.63
	89.00	8.53	7.83
	445.00	13.44	14.24
M3G	19.90	7.41	8.31
	99.50	5.96	9.81
	497.50	6.36	8.40
M6G	60.48	8.46	8.58
	120.96	11.54	10.15
	504.00	8.46	7.86
6AM	21.48	7.96	7.29
	107.40	8.32	7.63
	537.00	9.23	7.10

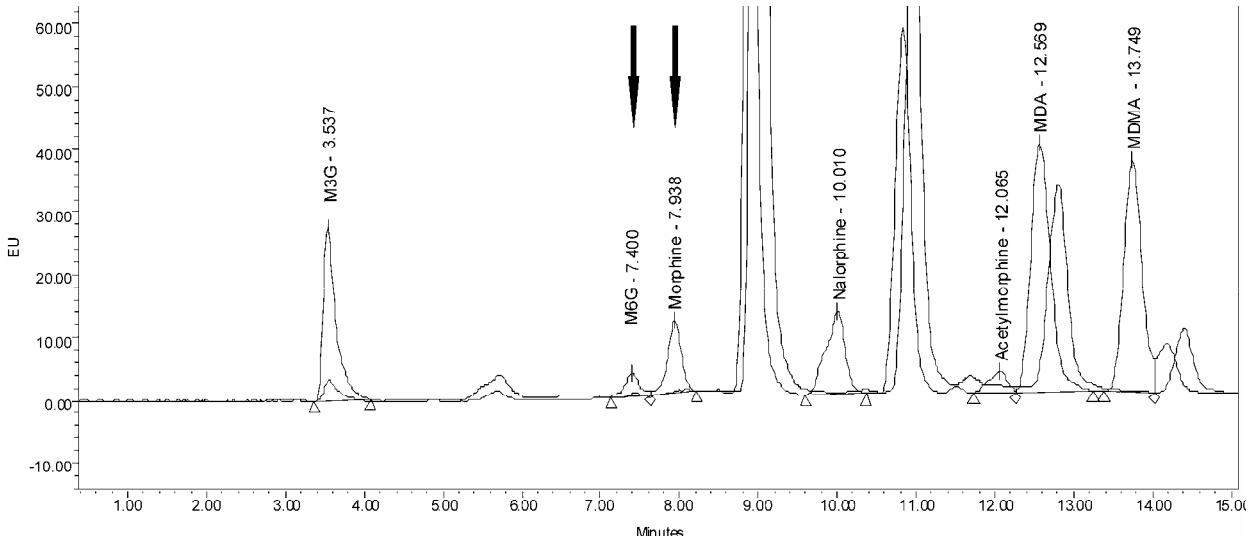

Fig. 2 – Chromatograms of plasma blank and of plasma spiked with M3G (549.00 ng/mL), M6G (507.00 ng/mL), MOR (502.00 ng/mL), 6AM (516.00 ng/mL), MDA (234.00 ng/mL), MDMA (237.50 ng/mL), MA (403.00 ng/mL) and NAL (547.00 ng/mL). FL detection (λ_{ex} = 210 nm, λ_{em} = 340 nm); sample preparation – SPE method nr. 1.

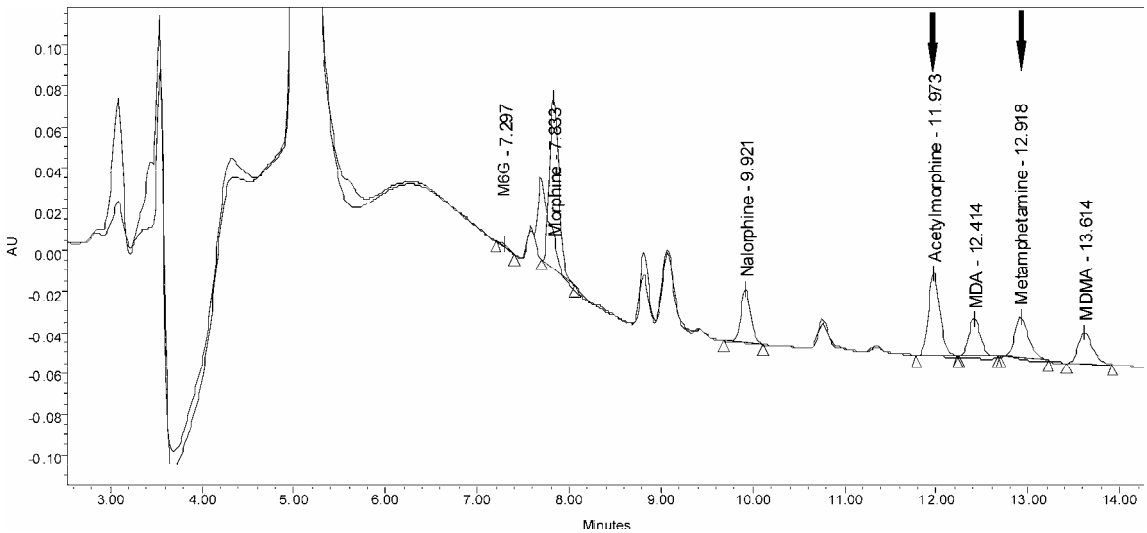

Fig. 3 – Chromatograms of plasma blank and of plasma spiked with M3G (995.00 ng/mL), M6G (1008.00 ng/mL), MOR (890.00 ng/mL), 6AM (1074.00 ng/mL), MDA (861.00 ng/mL), MDMA (843.00 ng/mL), MA (807.00 ng/mL) and NAL (547.00 ng/mL). UV detection (λ = 210 nm); sample preparation – SPE method nr. 2

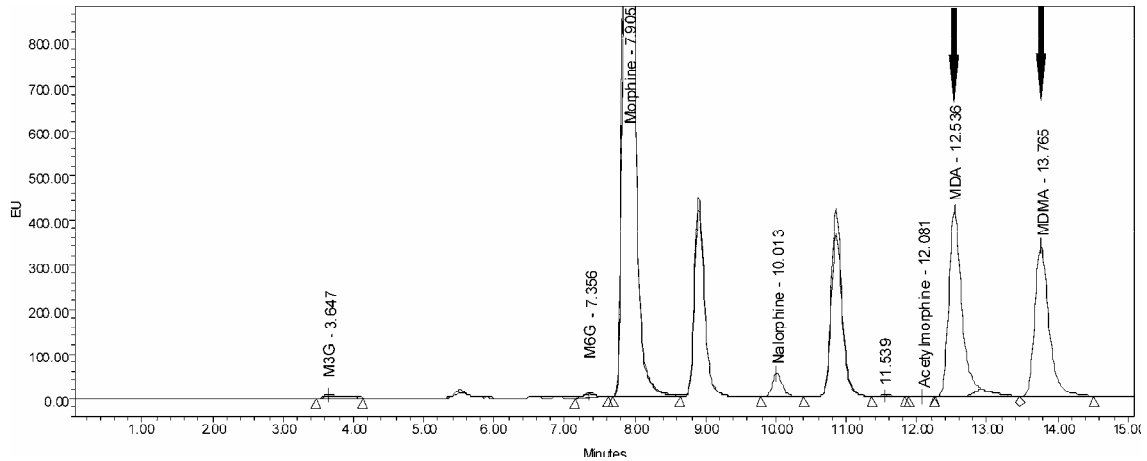

Fig.4. Chromatograms of plasma blank and of plasma spiked with M3G (87.84 ng/mL), M6G (81.12 ng/mL), MOR (80.32 ng/mL), 6AM (82.56 ng/mL), MDA (56.16 ng/mL), MDMA (57.00 ng/mL), MA (64.48 ng/mL) and NAL (547 ng/mL). FL detection (λ_{ex} = 288 nm, λ_{em} = 324 nm); sample preparation – SPE method nr. 2

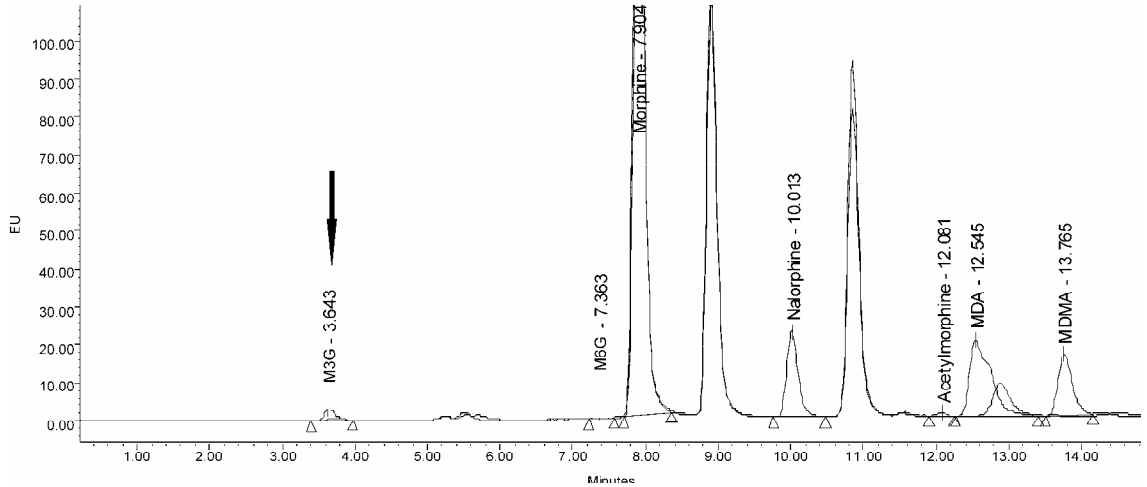

Fig.5. Chromatograms of plasma blank and of plasma spiked with M3G (87.84 ng/mL), M6G (81.12 ng/mL), MOR (80.32 ng/mL), 6AM (82.56 ng/mL), MDA (56.16 ng/mL), MDMA (57.00 ng/mL), MA (64.48 ng/mL) and NAL (547 ng/mL). FL detection (λ_{ex} = 210 nm, λ_{em} = 340 nm); sample preparation – SPE method nr. 2

Since the accuracy and precision parameters corresponding to the lowest calibrators were in the limits of the 20% (admitted by the international validation guidelines for bioanalytical methods), the lower limit of quantification (LLOQ) was set at these concentration levels for each analyte.

The aim of the stability studies was to identify any possible errors due to analyte loss by degradation during sample manipulation and short term storage. Regarding the post-preparative stability (PPS), the inaccuracy values (%) indicated no major changes in the analyte content for MOR, M6G, 6AM, MDA and MDMA after 48h of storage at 4°C in the autosampler, protected from light. M3G and MA proved to be stable in the same conditions in the final extracts for 24h, but at 48h some stability problems were observed (inaccuracy of – 40.53% and 16.96%, respectively). Based on these results it would be recommended to extract simultaneously only sample sets which could be analyzed by the elaborated HPLC method in maximum 24h.

All analytes proved to be stable in human plasma for at least 48h when stored at room temperature, protected from light (maximum inaccuracy obtained for M3G (QC3) = -13.34%). Freeze-thaw stability studies showed only minor losses of analyte for all seven substances in the biological matrix, with the maximum inaccuracy obtained in case of QC1 for M3G (-12.43%).

In general, GC-MS is considered to be the standard method recommended to be used in toxicology laboratories for confirmation of drug consumption. As it was mentioned earlier, only GC-MS methods were found in the literature for the simultaneous quantification of heroin metabolites, MDMA, MDA and MA in human plasma.[34-36] Beside the indisputable advantages of high sensitivity and selectivity, these methods present some major drawbacks: the need for a supplementary derivatization step to increase the volatility of polar analytes (a time consuming and costly operation which represents an additional source of errors) and that it is not suitable for quantification of morphine glucuronides (only the quantification of total morphine is possible, after a previous hydrolysis step). Other disadvantages of these assays could be the high costs and that they are not accessible for all laboratories.

In contrast to GC-MS analysis, the elaborated LC method presented a series of advantages: more accessible detection with lower costs (UV and FL detection versus MS or MS/MS), but with very good sensitivity, no derivatization step, no limitations due to different polarities of analytes and the possibility to analyze simultaneously free and conjugated morphine. The elaborated method proved to be sensitive enough to allow the quantification of all seven analytes at the concentration levels which could be found in real plasma samples from drug users.

EXPERIMENTAL

Chemicals

MOR, M3G, M6G, 6-AM, MDMA, MDA, MA and NAL standards were obtained from Lipomed (Lipomed AG, Arlesheim, Switzerland). Acetonitrile and methanol (HPLC gradient grade) were purchased from Sigma (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). 25% Ammonium hydroxide, ammonium formate, formic acid and acetic acid (analytical grade) were obtained from Fluka (Fluka Chemie GmbH, Buchs, Switzerland). Distilled deionised water was produced by a Direct Q-5 Millipore (Millipore SA, Molsheim, France) water purification system. Drug free human plasma was supplied by the Local Bleeding Centre Cluj-Napoca, Romania.

Standard solutions

Primary stock standard solutions were prepared by dissolving each analyte in an adequate solvent to obtain a concentration of 1mg/mL and they were stored in the refrigerator at 4°C. The used solvents were: methanol (in case of MOR, M6G, 6AM, MDA and NAL), a 4/1 (v/v) mixture of methanol/water (in case of MA and MDMA) and a 1/4 (v/v) mixture of methanol/water (in case of M3G). Working standard solutions, containing all the analytes, were prepared daily by dilution of stock solutions with deionised water.

Calibration standards and quality control (QC) samples were prepared by addition of the determined quantity of working stock solution to drug free plasma. Three different levels of plasma QC samples were prepared, corresponding to low (QC1), medium (QC2) and high (QC3) concentration.

Instrumentation and chromatographic conditions

The analysis were performed using a 2695 Waters Alliance HPLC (Waters, Milford, MA, USA) system composed of a ternary pump, autosampler, column heater and solvent degasser. The HPLC unit was linked to a 996 PDA (photodiode array) detector and a 2475 FLD (fluorescence detector) (Waters, Milford, MA, USA).

Chromatographic separation was achieved on a Atlantis (Waters, Milford, MA, USA) dC18 chromatography column (150mm x 3.9mm i.d., 3µm particle size) and a Atlantis dC18 guard column (20mm x 3.9mm i.d., 3µm particle size), maintained at 35°C. Gradient elution was performed using a mixture of acetonitrile and 10mM ammonium formate in water (pH = 4.0, with formic acid), as described in Table 4. Each solvent was degassed in an ultrasonic bath before use for HPLC. The mobile phase was delivered at a flow rate of 0.5mL/min. The autosampler injection volume was set at 25 µL. The sample compartment was maintained at 4°C.

Table 4

Mobile phase (elution gradient)

Time (min)	Acetonitrile % (v/v)	10mM $HCOONH_4$ (pH = 4.0) % (v/v)
0	3	97
3	15	85
15	20	80
20	20	80
20,05	3	97
27	3	97

The PDA detector was set at 210nm, while fluorescence detection was performed using $\lambda_{exc}/\lambda_{em}$ = 210nm/340nm and 288nm/324nm, respectively. Chromatographic data acquisition and processing were performed using Empower 2 software (Waters, Milford, MA, USA).

Sample preparation

In order to extract all analytes from human plasma samples, two solid phase extraction (SPE) methods were elaborated. Both extractions were performed using Oasis HLB, 1cc, 30mg extraction cartridges (Waters Corp., Dublin, Ireland).

SPE Method 1: 1mL plasma sample was spiked with 50 µL internal standard working solution (10.94 µg/mL), corresponding to a final concentration in plasma of 547 ng/mL nalorphine. An Oasis HLB cartridge was conditioned with 1mL methanol, and equilibrated with 1mL water, followed by loading of the sample and a washing step with 1mL, 5/95 (v/v) mixture of methanol/water. After drying the cartridge, analytes were eluted with methanol (0.5mL). The extract was transferred to an autosampler vial and a 25µL aliquot were injected into the HPLC system.

SPE Method 2: 1mL plasma sample was spiked with 50 µL internal standard working solution (10.94 µg/mL) and alkalized with 50µL concentrated ammonium hydroxide. An Oasis HLB cartridge was conditioned with 1mL methanol, and equilibrated with 1mL water, followed by loading of the sample and a washing step with 1mL, 5/95 (v/v) mixture of methanol/2% ammonium hydroxide in water. After drying the cartridge, analytes were eluted with a 50/50 (v/v) mixture of methanol/2% acetic acid in water (0.5mL). The extract was transferred to an autosampler vial and a 25µL aliquot were injected into the HPLC system.

Validation of the analytical method

The method was validated according to the FDA Guidance for Bioanalytical Method Validation. [18] The selectivity of the method was tested by extracting and analyzing six plasma samples from different sources. The linearity was evaluated by injecting a total of 10 calibration standards in plasma and the calibration curves were constructed by plotting peak area ratios of analytes and I.S. against corresponding concentrations. The lower limit of quantification (LLOQ) was defined as the lowest concentration with a precision of less than 20% CV and an accuracy of 80 to 120%. Accuracy was determined from the back-calculated results of the linearity study. Intra- and inter-day precision, defined as coefficient of variation (CV%), was determined using QC samples corresponding to low, medium and high concentrations. Five replicates were analyzed at each concentration on three consecutive days.

Stability studies were carried out at three concentration levels (QC1, QC2 and QC3). The stability study of the analytes in human plasma included the evaluation of room-temperature stability (RTS), post-preparative stability (PPS) in the autosampler and freeze-thaw stability. For all storage conditions plasma samples were analyzed in triplicate for each concentration and at each time point.

CONCLUSIONS

A LC-UV/FLD method was developed and validated as a more accessible alternative to the existing GC-MS assays for the simultaneous quantification of heroin metabolites and some of the most frequently used amphetamine derivatives in human plasma. Some of the heroin metabolites (M3G, M6G) were impossible to analyze by GC-MS, a major limitation which was solved in case of the HPLC method described in this work. The detection parameters together with the solid phase extraction and chromatographic separation guaranteed the absence of any interference from the endogenous compounds. The assay showed good linearity, accuracy, recovery and precision for all analytes, over the studied concentration range and proved to be adequate for the quantification of these molecules in the monitoring process of drug addicts.

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