LC/MS/MS method of 6-MAM, morphine, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) for quantitative analysis in serum

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Abstract

Aim: Heroin is mainly metabolized via 6-MAM to morphine and its glucuronides. Predominantly, the inactive morphine-3-glucuronide is formed, apart from a smaller amount of morphine-6-glucuronide, an active metabolite. Therefore, it is of forensic interest to quantify the glucuronides separately. An analytical LC/MS/MS method for the quantification of 6-MAM, morphine, morphine-3-glucuronide and morphine-6-glucuronide in serum was developed and validated according to the guidelines of GTFCh.

Methods: Solid phase extraction was used for sample preparation. The LC/MS/MS analysis was performed using a HPLC from Shimadzu coupled to a triple quadrupole mass spectrometer (AB Sciex 4000). Due to the high polarity of the compounds of interest, a HILIC-column was used for chromatographic separation. For each analyte, two MRMs were selected for measurements.

Results: The calibration curve was found to be linear in a range of 10-1000 ng/ml for all four compounds. The limits of detection (LOD) for the analytes ranged from 0,7 ng/ml to 5,8 ng/ml. Accordingly, the limits of quantification (LOQ) were in a range of 9,0-18,5 ng/ml. Intraday und interday precision were tested for low (35 ng/ml) and high (350 ng/ml) concentration levels, fulfilling the criteria to be less than 15 %. Good recovery rates for all analytes were achieved (79,8-98,9%).

Conclusion: The LC/MS/MS-method presented was successfully developed and validated and can therefore be applied to forensic cases.

1. Introduction

Heroin is rapidly metabolized (figure 1) via 6-monoacetylmorphine (6-MAM) into morphine. Morphine itself is mainly metabolized into morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G). The pharmacokinetics of the metabolism depends on the application route [1]. In terms of abuse intravenous administration is the most common application route of heroin apart from inhalation. Basically, glucuronidation occurs in liver, catalyzed by UGT2B7 [2].

For the determination of morphine concentrations, GC/MS is a common analytical method. After enzymatic hydrolysis, it can also be used to measure indirectly the total amount of glucuronides.

From a forensic point of view, it can be of interest to measure M3G and M6G concentrations separately, since M6G is an active metabolite of morphine, but normally formed in smaller amounts (8-10%) than M3G (45-55%), an inactive metabolite. Hence, the concentration of M6G besides morphine is valuable for the interpretation of an intoxication or impairment of an individual [3, 4]. Therefore, a LC/MS/MS method allows determining morphine level and those of its glucuronides directly and parallel.

2. Material and Methods

2.1. Chemicals

Standard solutions of 6-MAM, morphine, M3G and M6G as well as the deuterium labeled substances of all analytes were purchased from Cerilliant (Texas, USA). All chemicals and solutions used were of analytical grades.

2.2. Solid-Phase Extraction

The extraction procedure is mainly based on the earlier described method [5]. For sample preparation 1 ml of serum was used. At first the internal standard (100 ng/ml of 6-MAM-d3 and morphine-d3 and 250 ng/ml of M3G-d3 and M6G-d3) and 2 ml of buffer solution (0.1 M ammonium acetate buffer; pH 9) were added. Afterwards, the samples were centrifuged for 8 minutes at 4000 rpm.

For solid-phase extraction, Chromabond C18ec-SPE-columns from Macherey-Nagel GmbH & Co. KG (Dueren, Germany) were used. The columns were conditioned by 2 ml methanol, 2 ml of bidestilled water and 2 ml of a buffer solution (0.1 M ammonium acetate buffer; pH 9). Subsequently, the samples were loaded onto the columns, followed by washing with 2 ml of buffer solution. Ahead of elution, the cannula was cleaned by 5 ml of bidistilled water and 1 ml of methanol. Analytes were eluted using 0.7 ml methanol followed by 0.7 ml methanol/acetic acid (9:1). The eluate was evaporated under a stream of nitrogen at 60 °C, reconstituted in 100 µl of HPLC mobile phase A and centrifuged for 10 minutes at 13,000 rpm. Samples were transferred into microvials and stored at -20 °C until analysis.

2.3. LC/MS/MS Conditions

The LC/MS/MS system consisted of a HPLC from Shimadzu coupled to a triple quadrupole mass spectrometer (AB Sciex 4000) using the positive ion mode.

Due to the high polarity of the compounds of interest, a HILIC column (Nucleodur) from Macherey-Nagel GmbH & Co. KG, Dueren, Germany) was used for chromatographic separation. Mobile phase A (water, 15 mM ammonium acetate, pH = 4.3) and mobile phase B (pure acetonitrile) in a gradient program with a flow of 400 µl/min: 0-1 min: 95% B; 1-8 min 95% → 50% B; 8-10 min: 50% B → 10% B; 10-11 min: 10% B; 11-12 min: 10% B → 95% B; 12-15 min: 95% B.

The transitions in multiple reaction monitoring (MRM) mode are listed in table 1 for all compounds. Since M3G and M6G are showing the same MRM transitions, good chromatographic separation is necessary.

3. Results and Discussion

The method was successfully validated according to the guidelines of GTFCh [6]. All validation parameters were fulfilling the given criteria and listed in table 2. Contrary to earlier published methods, very good recoveries were achieved due to reducing matrices effects by use of 0.1 M ammonium acetate buffer pH 9 instead of other buffer solutions.

Figure 2 shows a chromatogram of the quality control sample for a low concentration level containing 35 ng/ml of each analyte.

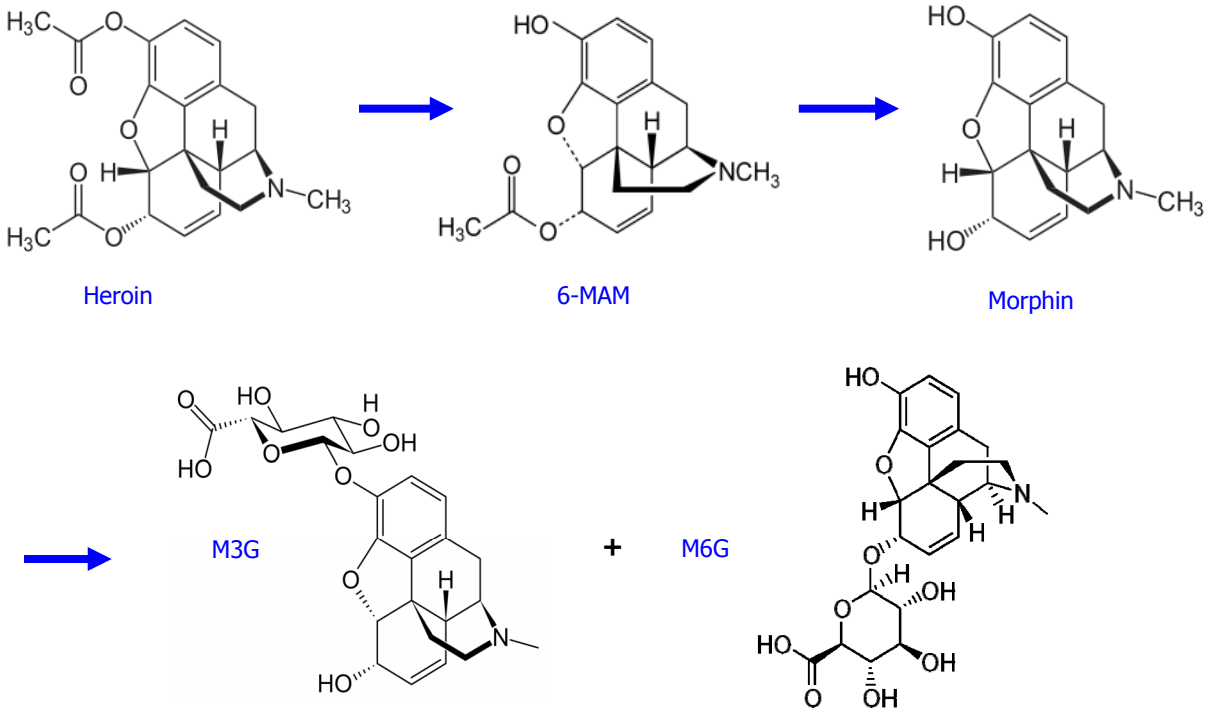

Fig. 1. Metabolism of heroin.

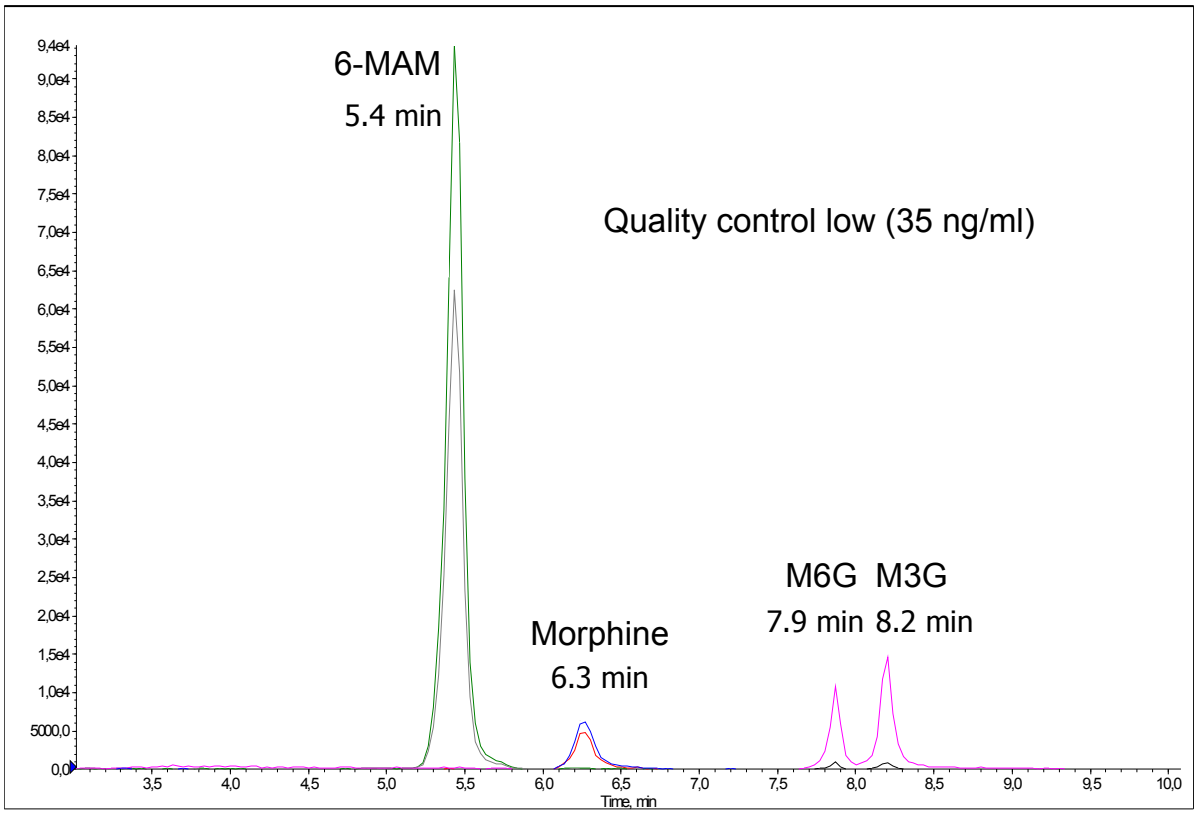

Fig. 2. Chromatogram of the quality control sample (35 ng/ml of each analyte).

Tab. 1. MRM transitions of the analytes.

Substances	**6-MAM**	**Morphine**	**M3G**	**M6G**
Target-MRM (m/z)	328 → 165	286 → 152	462 → 286	462 → 286
Qualifier-MRM (m/z)	328 → 193	286 → 165	462 → 165	462 → 165

Tab. 2. Validation parameters of the presented method.

Validation parameters/substances	**6-MAM**	**Morphine**	**M3G**	**M6G**
Linearity in ng/ml	10-1000	10-1000	10-1000	10-1000
Intraday precision quality control low	2.7 %	4.0 %	2.6 %	2.7 %
Interday precision quality control low	8.2 %	12.7 %	5.3 %	8.2 %
Intraday precision quality control high	2.3 %	3.3 %	2.1 %	2.3 %
Interday precision quality control high	11.2 %	8.1 %	8.0 %	4.8 %
Limit of detection (LOD) in ng/ml	5.8	1.7	0.7	4.1
Limit of quantification (LOQ) in ng/ml	18.5	10.2	9.0	8.0
Recovery of the solid phase extraction low	89.0 %	83.3 %	92.8 %	98.9 %
Recovery of the solid phase extraction high	79.8 %	81.5 %	86.3 %	82.4 %
Matrix effects (low: 35 ng/ml)	71.0 %	75.5 %	59.5 %	43.8 %
Matrix effects (high: 350 ng/ml)	79.7 %	81.8 %	62.8 %	50.7 %

4. Conclusions

The described method was applied to cases of driving under the influence of drugs and also intoxication cases. For the latter ones concentration ratios of M3G/morphine and M6G/morphine were used for estimation of the time interval between the last consumption of heroin and death.

5. References

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