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Validated assay for the determination of markers of illicit heroin in urine samples for the control of patients in a heroin prescription program

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Abstract

A fully validated procedure for the simultaneous determination of morphine (MOR), morphine-3-glucuronide (M3G), morphine-6-glucuronide (M6G), 6-acetylmorphine (6AM), codeine (COD), codeine-6-glucuronide (C6G), acetylcodeine (AC), noscapine (NOS) and papaverine (PAP) based on liquid chromatography followed by electrospray mass spectrometry applying multiple reaction monitoring (LC-ESI-MS/MS) in urine samples is described. The extraction was carried out on a Zymark Rapid Trace Workstation using C18 solid-phase extraction cartridges. The separation was performed in 19 min on an Agilent 1100 HPLC system, using a Phenomenex C18 AQUA column (4 μm, 150 mm × 2 mm), which is coupled with an Applied Biosystems API 2000 mass spectrometer. Deuterated analogues were used as internal standards. The limits of detection were in the range of 0.1 ng/ml (PAP) to 7.4 ng/ml (M6G), the coefficients of correlation were higher than 0.996, the precisions ranged from 3% to 12% and the absolute recoveries were between 45% (M3G) and 98% (MOR). Analyses of samples from patients of a heroin prescription program demonstrated the usefulness of the procedure for the analytical differentiation between prescribed synthetic heroin (diamorphine) use and non-prescription heroin abuse on the basis of urine analysis. After the ingestion of pharmaceutical heroin only general markers for heroin use were detected, which are MOR, M3G, M6G and 6AM, respectively. When illicit heroin was abused, additionally to further general markers (COD, C6G) specific markers for non-prescription heroin abuse (AC, NOS, PAP) were found. However, it must be kept in mind that only AC may be regarded as absolute specific marker of non-prescription heroin, because all other compounds may appear in urine after ingestion of opiate alkaloids containing medicines or foods (e.g. poppy seeds). Therefore, patients of a heroin prescription program should be advised not to ingest such products.

Keywords: Morphine; Morphine glucuronides; Codeine; Acetylcodeine; Noscapine; Papaverine; Heroin

1. Introduction

Heroin abuse is a widespread problem in society, present in diverse cultures and with extensive communal and economic consequences. In order to reduce illicit heroin abuse, crime, infectious disease and death, substitution programs were developed in several countries. A heroin prescription program for addicts, formerly known in Switzerland and Great Britain, was introduced in Germany 1.5 years ago [1]. A basic requirement is that patients participating in this program do not use any other illicit drugs, particularly non-prescription heroin. Part of the German heroin substitution program is the analytical differentiation between prescribed pharmaceutical heroin use and non-prescription heroin abuse on the basis of urine analysis.

Several reports have described the analysis of opiates in urine to confirm an illicit consumption of heroin and to distinguish heroin use from codeine (COD) or morphine (MOR) use [2–4]. The use of liquid chromatography mostly coupled with mass spectrometry (LC/MS) allowed the simultaneous detection of glucuronides [5–10]. However, interpretation of positive results can be difficult because of the presence of opiate alkaloids in medicines and foods (e.g. poppy seeds) [11–14], when ingestion of these products leads to excretion of COD and MOR in urine. Also other opium alkaloids like noscapine (NOS) or papaverine (PAP) were found in food [15].

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In urine 6-acetylmorphine (6AM) is a specific marker of heroin use, and several methods for its detection have been reported [16–19]; however, the detection window for 6AM is short (<8 h). COD and especially acetylcodeine (AC) have been suggested as markers of illicit heroin abuse [20–23]. COD is a natural constituent of illicit heroin preparations isolated from opium, which is subjected to acetylation and purification. AC content in non-prescription heroin ranged from 0.25% to 10.2% [20]. Other typical alkaloids in illicit heroin preparations are noscapine (NOS) (0–61%) and papaverine (PAP) (0.1–19.7%) [24,25]. As the pharmaceutical maintenance heroin is pure, the presence of COD and especially of AC or also of NOS or PAP in the urine of patients indicate that they may be supplementing their prescribed heroin doses with non-prescription heroin.

Therefore, a fully validated procedure was developed for the simultaneous determination of general markers of heroin use (6AM, MOR, M3G, M6G) together with specific markers of non-prescription heroin (AC, COD, C6G, NOS, PAP). Liquid chromatography followed by electrospray mass spectrometry applying multiple reaction monitoring was used (LC-ESI-MS/MS).

2. Experimental

2.1. Chemicals

COD, MOR, 6AM, M3G, M6G and their deuterated analogues were purchased from LGC Promochem^{TM} (Wesel, Germany), AC, NOS, PAP and C6G from Lipomed AG GmbH (Bad Saeckingen, Germany); AC-d_3 was synthesised by acetylation of COD-d_3 (using acetic anhydride/pyridine (3:2, v/v; reagents from Sigma, Deisenhofen, Germany) and incubation for 30 min at 80 °C). The concentrations in the text given as ng/ml refer to concentration of the compounds as base.

CertiPur® pH 9 boric acid buffer solution was provided by Merck KgaA (Darmstadt, Germany). All chemicals were analytical reagent grade and were used without further purification.

2.2. Standard solutions

A methanolic stock solution containing COD, C6G, MOR, M3G, M6G, NOS and PAP and a second solution containing 6AM and AC in acetonitrile were prepared. Four concentration ranges, adapted to the expected concentrations in the authentic samples, with nine different standard concentrations were created: 1–100 ng/ml (PAP), 5–500 ng/ml (AC, NOS), 10–1000 ng/ml (COD) and 25–2500 ng/ml (C6G, 6AM, MOR, M3G, M6G).

A methanolic stock solution containing COD-d_3, MOR-d_3 and M3G-d_3 and a solution of 6AM-d_3 with AC-d_3 were prepared in concentrations of 1 μg/ml, respectively. All solutions were stored at −18 °C.

2.3. Solid-phase extraction system

Solid-phase extraction was performed on a Rapid Trace Workstation from Zymark GmbH (Ruesselsheim, Germany) using Chromabond® C18ec-SPE-columns from Macherey-Nagel (Dueren, Germany).

2.4. Chromatographic and mass spectrometric equipment and conditions

The LC-MS/MS system consisted of an Agilent (Waldbronn, Germany) 1100 HPLC system (binary pump, degasser and autosampler) coupled with an Applied Biosystems (Darmstadt, Germany) API 2000 triple quadrupole mass spectrometer.

LC separation was performed on a Phenomenex C18 AQUA column (4 μm, 150 mm × 2 mm) using mobile phase A (water/acetonitrile 98:2, 5 mM ammonium acetate) and mobile phase B (water/acetonitrile 10:90, 5 mM ammonium acetate) in a gradient program with a flow of 200 μl/min: 0–6 min: 95% A → 0% A; 6–10 min: 0% A → 0% A; 10–13 min: 0% A → 95% A; 13–19 min: 95% A → 95% A.

TurboIon Spray for ESI-MS/MS in positive ion mode used the temperature of 380 °C and ionspray voltage of 5000 V. In multiple reaction monitoring (MRM) mode following transitions were monitored: AC (*m/z* 342.1 → 225.2), COD (*m/z* 300.0 → 165.0), 6AM (*m/z* 328.1 → 164.9), MOR (*m/z* 286.1 → 151.8), NOS (*m/z* 414.2 → 220.0), PAP (*m/z* 340.1 → 202.0), C6G (*m/z* 476.3 → 300.0), M3G (*m/z* 462.3 → 286.0), M6G (*m/z* 462.2 → 286.1), AC-d_3 (*m/z* 344.9 → 265.5), COD-d_3 (*m/z* 303.1 → 199.1), 6AM-d_3 (*m/z* 331.1 → 164.9), MOR-d_3 (*m/z* 289.1 → 151.9) M3G-d_3 (*m/z* 465.3 → 289.0). For identity confirmation besides the specific precursor ion and product ion listed above a second structural-specific product ion was detected. For quantification, peak area ratios of the analytes to the corresponding deuterated standards were calculated as a function of the concentration of the substances. For some analytes no deuterated analogues were available, so that C6G and NOS were calculated using COD-d_3, AC-d_3 was used for PAP and M3G-d_3 for M6G.

2.5. Sample preparation

Urine samples (stored at −18 °C) were thawed, a volume of 1000 μl was combined with 1920 μl pH 9 buffer solution and spiked with 40 μl of both internal standard solutions. After mixing on a rotary shaker (vortex), the 3 ml samples were extracted automatically in a Zymark Rapid Trace Workstation using the following protocol.

2.5.1. Column conditioning

The columns were conditioned with 2 ml methanol, followed by 2 ml bidestilled water and 2 ml pH 9 buffer solution at a flow rate of 1 ml/min.

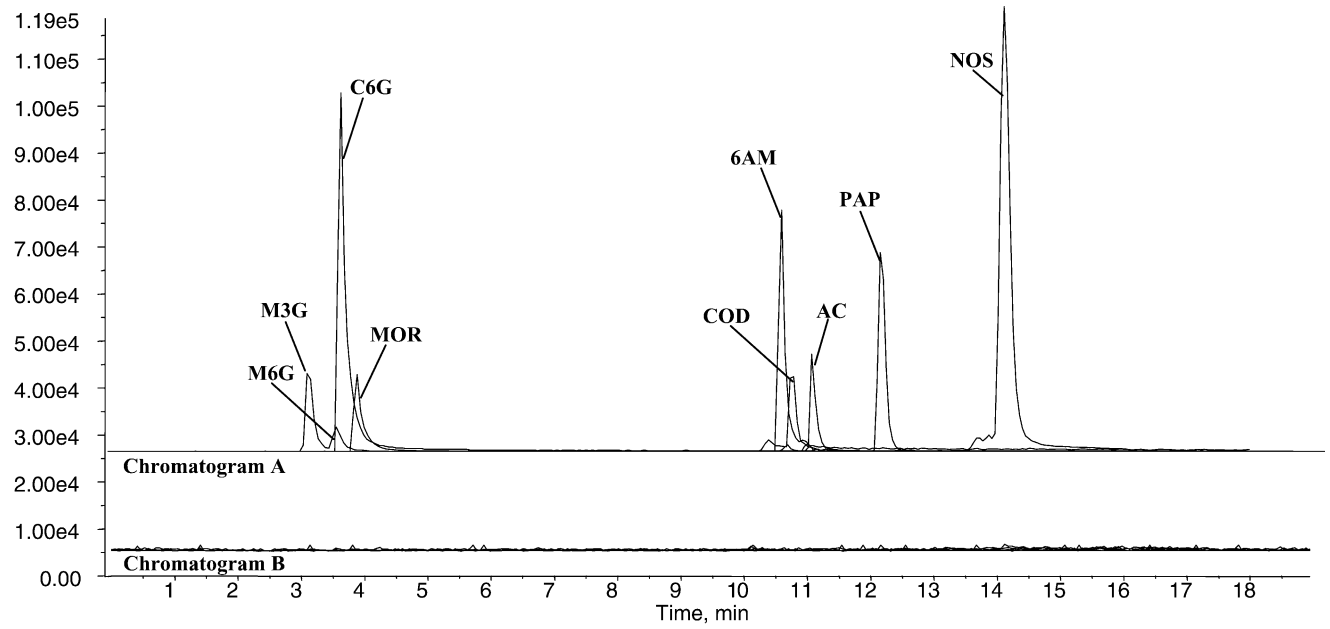

Fig. 1. (A) Chromatogram of a spiked urine sample assayed to contain following concentrations: AC 50 ng/ml, COD 500 ng/ml, 6AM 1250 ng/ml, MOR 1250 ng/ml, M3G 1250 ng/ml, M6G 1250 ng/ml, NOS 250 ng/ml, PAP 50 ng/ml, AC 50 ng/ml. (B) Chromatogram of a blank urine sample.

2.5.2. Sample extraction

The samples were loaded onto the columns at a flow rate of 1.2 ml/min and washed with 2 ml pH 9 buffer solution (flow rate 6 ml/min). Afterwards the cannula was cleaned by a purge of 5 ml bidistilled water at a flow rate of 30 ml/min, and the columns were dried with air for 3 min.

2.5.3. Elution

Two fractions were collected in one vial. The first fraction was collected with 0.7 ml of methanol, the second fraction with 0.7 ml methanol/acetic acid (9:1), both at a flow rate of 1.2 ml/min.

2.5.4. Preparation for the HPLC-MS/MS analysis

The eluates were evaporated under a stream of nitrogen at 60 °C, reconstituted in 100 μl of HPLC mobile phase A and put into the ultrasonic bath for 5 min in order to improve dissolution.

2.6. Calibration curves

In order to improve accuracy of measurement the concentration ranges of each analyte were divided into two segments for the analysis of high and low concentrations (e.g. AC: 10–100 ng/ml urine (high) and 1–10 ng/ml urine (low)). This procedure seemed to be superior to weighted calibration curves.

2.7. Method validation

The method was fully validated according to international guidelines using the program VALISTAT® for statistics [26].

In order to evaluate method selectivity blank urine samples from different sources were prepared as described, but without adding any analyte or internal standard mix (n = 6). Furthermore blank samples were analysed to check the absence of analyte ions in the respective peaks of the

Table 1
Recoveries of the whole procedure with automated off-line solid-phase extraction

	High concentration		Low concentration	
	Concentration (ng/ml)	Absolute recovery (%)	Concentration (ng/ml)	Absolute recovery (%)
AC	50	52.28 ± 3.60	2.5	55.09 ± 17.3
COD	500	90.96 ± 9.61	25	92.43 ± 11.34
C6G	1250	96.45 ± 9.21	62.5	80.11 ± 10.27
6AM	1250	59.44 ± 6.09	62.5	59.44 ± 7.08
MOR	1250	98.28 ± 15.70	62.5	97.41 ± 11.37
M3G	1250	39.75 ± 7.58	62.5	45.5 ± 5.81
M6G	1250	46.76 ± 6.83	62.5	52.78 ± 5.48
NOS	250	84.61 ± 6.64	12.5	70.06 ± 3.14
PAP	50	59.75 ± 3.31	2.5	50.28 ± 1.88

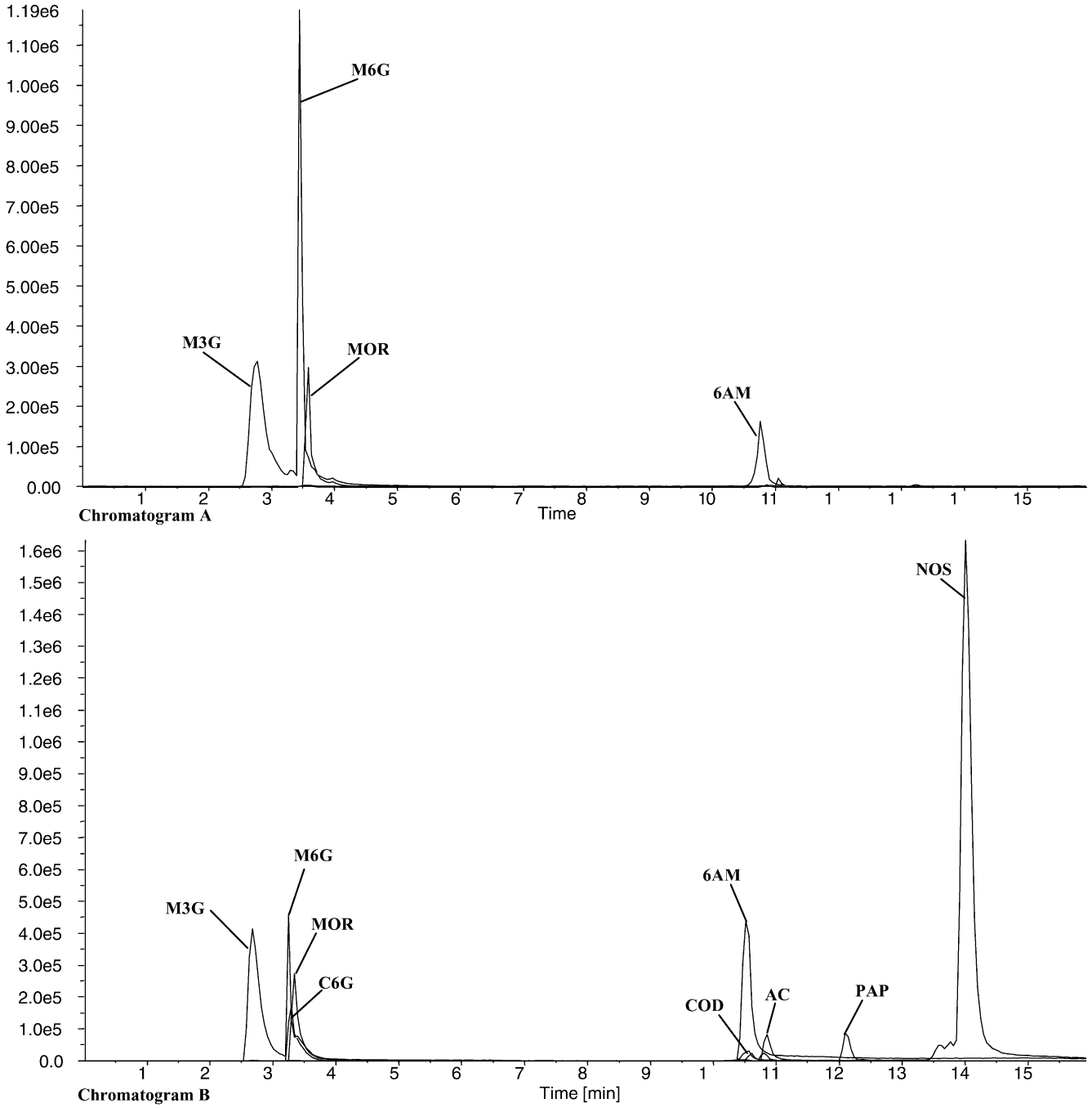

Fig. 2. Comparison of two chromatograms. (A) Urine sample extract of a patient from the heroin prescription program, who ingested synthetic heroin. Only general markers for heroin use were detected (MOR, M3G, M6G and 6AM). (B) In contrast in an authentic urine sample extract of a forensic case additionally to further general markers (COD, C6G) specific markers for non-prescription heroin abuse (AC, NOS, PAP) were detected.

internal standards (n = 2). No matrix effect was found (Fig. 1B).

For calibration blank urine samples were spiked with analytes in equidistant concentrations. Replicates of all standard samples were processed as described above, checked for outliers (Grubbs-test) and averaged (n = 6). Using the mean values, calibration curves were checked for variance homogeneity (F-test) and for linearity (Mandel-test).

Additionally, two quality control (QC) samples (high and low, Table 1) were prepared and analysed in duplicate over 8 days.

For determination of analytical limits (limit of detection, LOD; limit of quantitation, LOQ) blank urine samples were spiked with 10 calibration standard mixes near the expected LOD. These samples were processed and analyzed in duplicate as described above.

Table 2
Linear regression data at low and high concentration ranges

	High concentration				Low concentration			
	Linear range (ng/ml)	Coeffiecient of correlation	Slope	Intercept	Linear range (ng/ml)	Coeffiecient of correlation.	Slope	Intercept
AC	10–100	0.999	26.90	0.89	1–10	0.999	27.5	−7.4
COD	100–1000	0.999	29.7	72.2	10–100	0.999	36.6	9.88
C6G	250–2500	0.998	0.17	−0.01	25–250	0.999	0.192	0.017
6AM	250–2500	0.998	1.2	1.4	25–250	0.999	1.17	0.12
MOR	250–2500	0.996	0.6	0.057	25–250	0.998	0.72	0.088
M3G	250–2500	0.998	0.17	0.017	25–250	0.998	0.19	0.0027
M6G	250–2500	0.998	0.528	0.01	25–250	0.998	0.61	0.016
NOS	50–500	0.999	3.41	0.98	5–50	0.999	2.31	1.147
PAP	10–100	0.998	1.51	0.6	1–10	0.998	1.78	0.02

Analytical recoveries were calculated on each analyte by comparing the absolute peak-area ratios of the two different QC standard solution and the corresponding QC sample replicates ($n = 6$).

To evaluate freeze/thaw stability a standard of medium concentration ($n = 6$) was analyzed before and after two freeze/thaw cycles. For each freeze/thaw cycle, the samples were frozen at −18 °C for 21 h, thawed, and kept at ambient temperature for 3 h. Freeze/thaw cycles were repeated after 4, 7, 14, 28, 56 and 84 days.

3. Results

3.1. Solid-phase extraction

The application of an automated off-line solid-phase extraction system revealed satisfactory results. Absolute extraction recoveries of the whole method ranged from 45% (M3G) to 98% (MOR), only M3G and M6G had recoveries lower than 50% (Table 1). Using an automated system human, errors concerning the performance could be minimized.

3.2. Method validation

3.2.1. Selectivity

A chromatographic run was performed in 19 min, including 6 min of conditioning the LC column. In Fig. 1 chromatogram A shows the separation of all nine analytes. M6G and C6G as well as 6AM and COD could not be separated chromatographically but could easily be differentiated by different MRM-fragmentations. Chromatograms of blank urine samples gave no interference (Fig. 1B). Additionally, blank urine samples combined with internal standard solutions showed the absence of analyte ions in the respective peaks. Cross interference between different analytes (e.g. MOR and 6AM because of detoriation of 6AM) have not been detected.

3.2.2. Calibration

The resulting calibration curves were divided into two concentration ranges (high and low). All coefficients of correlation were higher than 0.996 (Table 2).

3.2.3. Precisions and accuracy

Respectively, two QC samples were analysed in duplicate over 8 days to determine precision and accuracy (Table 3). Intraday precision ranged from 4% (COD) to 12% (6AM), interday precision from 5% (AC) to 12% (6AM) and accuracy from 1% (6AM) to 11% (M6G). With a significance of 95% and a results' uncertainty of 33% the LODs and LOQs were calculated and LODs ranged from 0.1 ng/ml (PAP) to 7.4 ng/ml (M6G), LOQs from 0.33 ng/ml (PAP) to 26.1 ng/ml (M6G).

3.2.4. Stability

Concentration variations after freeze/thaw cycles were 8% for AC and 9% for 6AM, for the other compounds variations were all smaller than 10%.

Table 3
Precision, accuracy and analytical limits

	Concentration (ng/ml)		Intraday precision (%)		Interday precision (%)		Accuracy (%)		Limits (ng/ml)	
	High	Low	High	Low	High	Low	High	Low	LOD	LOQ
AC	50	2.5	4.67	7.38	4.67	8.23	−8.95	5.9	0.35	1.16
COD	500	25	3.9	7.01	5.54	7.01	−3.27	6.23	1.74	5.5
C6G	1250	62.5	8.77	6.65	10.15	7.36	−8.23	2.05	3.04	10
6AM	1250	62.5	11.65	6.57	11.65	10.57	1.42	−10.71	2.83	9.86
MOR	1250	62.5	6.27	8.43	6.49	8.43	−3.05	−2.5	3.4	12.38
M3G	1250	62.5	7.52	8.07	7.52	8.07	4.37	−8.46	5	17.82
M6G	1250	62.5	5.57	6.35	10.19	5.63	11.46	8.09	7.4	26.1
NOS	250	12.5	6.57	7.41	7.56	10.1	10.7	5.15	0.48	1.56
PAP	50	2.5	6.96	6.47	8.33	6.36	3.56	4.37	0.1	0.33

3.2.5. Applicability

The applicability of the method was proven by analyses of urine samples from patients of a heroin prescription program as well as from users of illicit non-prescription heroin. In Fig. 2 a comparison of two chromatograms is given. Chromatogram A was achieved from a urine sample extract of a patient from the heroin substitution program, who ingested synthetic heroin. Only general markers for heroin use were detected, which are MOR, M3G, M6G and 6AM, respectively. No COD, C6G, AC, PAP or NOS were found in this sample. In contrast in chromatogram B—an authentic urine sample extract of a forensic case—additionally to further general markers (COD, C6G) specific markers for non-prescription heroin abuse (AC, NOS, PAP) were detected. These findings demonstrate the usefulness of the procedure for the analytical differentiation between prescribed pharmaceutical heroin use and non-prescription heroin abuse on the basis of urine examination.

4. Discussion

The described procedure permit the simultaneous determination of 9 substances, which are considered as general markers of heroin use or rather as specific markers of non-prescription heroin use [20–23]. Satisfactory validation data were achieved for selectivity, linearity, recovery and reproducibility. The procedure is useful for the analytical differentiation between prescribed pharmaceutical heroin use and non-prescription heroin abuse on the basis of urine examination. However, it must be kept in mind that only AC may be regarded as absolute specific marker of non-prescription heroin. All other compounds may appear in urine after ingestion of morphine or codeine containing medicines or other sources of opium alkaloids (e.g. poppy seeds). Therefore, patients of a heroin substitution program should be advised not to ingest any MOR/COD containing medicines and not to consume foods containing poppy seeds.

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