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Article

Heroin-Related Compounds and Metabolic Ratios in Postmortem Samples Using LC–MS-MS

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Abstract

Analysis of postmortem samples with the presence of morphine can sometimes be challenging to interpret. Tolerance complicates interpretation of intoxications and causes of death due to overlap in therapeutic and fatal concentrations. Determination of metabolites and metabolic ratios can potentially differentiate between abstinence, continuous administration, and perhaps time of administration. The purpose of this study was to (a) develop and validate a method for quantitation of morphine-3β-D-glucuronide, morphine-6β-D-glucuronide, normorphine, codeine-6β-D-glucuronide, norcodeine, codeine, 6-acetylmorphine, and ethylmorphine in urine using liquid chromatography–tandem mass spectrometry; (b) apply the method to opiate related deaths; (c) compare metabolic ratios in urine in different causes of death (CoD) and after different drug intakes and (d) compare heroin intoxications in rapid and delayed deaths. Validation parameters such as precision, bias, matrix effects, stability, process efficiency, and dilution integrity were assessed and deemed acceptable. Lower limits of quantitation ranged from 0.01–0.2 μg/mL for all analytes. Autopsy cases (n=135) with paired blood and urine samples were analyzed. Cases were divided into three groups based on CoD; opiate intoxication, intoxication with other drugs than opiates, and other CoD. The cases were classified by intake: codeine (n=42), heroin (n=36), morphine (n=49), and ethylmorphine (n=3). Five cases were classified as mixed intakes and excluded. Heroin intoxications (n=35) were divided into rapid (n=15) or delayed (n=20) deaths. Parent drug groups were compared using metabolic ratio morphine-3β-D-glucuronide/morphine and significant differences were observed between codeine vs morphine ($p=0.005$) and codeine vs heroin ($p\leq0.0001$). Urine and blood concentrations, and metabolic ratios in rapid and delayed heroin intoxications were compared and determined a significant difference for morphine ($p=0.001$), codeine ($p=0.009$), 6-acetylmorphine ($p=0.02$) in urine, and morphine ($p=0.02$) in blood, but there was no significant difference ($p=0.9$) between metabolic ratios. Morphine-3β-D-glucuronide results suggested a period of abstinence prior to death in 25% of the heroin intoxications.

Introduction

According to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), the most common opioid abused in 2017 in the European Union (EU) was heroin (1). In the 2018 World Drug Report from the United Nations Office on Drugs and Crime (UNODC), there was more than a 58% increase in opioid related fatalities between 2012 and 2016 in England and Wales (2). In Sweden, heroin was one of the top five seized drugs as of 2017 (3). The abuse of other structurally related compounds such as codeine (COD) and morphine (MOR) is not uncommon. MOR was one of the 25 most frequently identified drugs in forensic laboratories in the USA in 2019 (4). New trends are developing among the illicit drug community, such as poppy seed tea, where individuals make tea using unwashed poppy seeds to achieve the intoxicant effects of MOR and/or COD (5). Trends like the one described increase the possibility of opiate intoxications rising; however, in the Nordic countries, the main intoxicants are illicit drugs such as heroin, methadone and fentanyl (6).

In the forensic community, interpretation issues can arise when analyzing MOR-related compounds. MOR shares metabolic pathways with other related substances. As such detected MOR can reflect the administration of heroin (diacetylmorphine), COD, ethylmorphine (EMOR) or MOR itself as shown in Figure 1. Heroin is quickly converted to 6-acetylmorphine (6-MAM) after intake, then metabolized to MOR. EMOR and COD also share the MOR metabolite and the conversion is mediated by the highly polymorphic cytochrome P-450 2D6 (CYP2D6) enzyme. This can influence the parent to metabolite ratio often used to interpret which drug was administered (7). MOR is then further metabolized into normorphine
(NMOR), morphine-3β-D-glucuronide (M3G) and morphine-6β-D-glucuronide (M6G) and COD is further metabolized to norcodeine (NCOD) and codeine-6β-D-glucuronide (C6G). Since several parent compounds are involved in the interpretation of metabolites, an understanding of the pharmacokinetics of the MOR-related compounds can help with interpretation. Previous studies have investigated the urinary pharmacokinetics of heroin in human subjects at various dosages and routes of administration (8–10). In a study performed by Mitchell et al., urinary excretion rate and metabolism of intramuscularly (IM) injected MOR in four male subjects were determined (9). The mean detection time for free MOR at 15-ng/mL cut-off was 51 hours and for total MOR at 40-ng/mL cut-off was 78 hours. In Cone et al., the urinary excretion profile of heroin metabolites was determined from six male subjects that snorted heroin (8). The subjects were given 6 and 12 mg of heroin HCl intranasally and 6 mg of heroin administered IM for comparison. In most subjects, metabolites were present in their first void, 1 to 8 hours after the administration. It was determined that peak concentrations and detection times for total MOR in urine from snorting and IM administration of heroin were comparable. One difference that was discovered was after intranasal administration, there were lower amounts of free MOR found. The concentrations of 6-MAM that were detected were highly variable and undetectable within 7 hours. In Smith et al., pharmacokinetics of total MOR, free MOR and 6-MAM in urine were evaluated on male subjects that either smoked heroin or had heroin intravenously (IV) administered (10). The doses were between 3 and 14 mg of heroin. The time to peak for lower dosages (3–7 mg) of smoked and IV administration for free and total MOR was 1.2 to 6.2 hours and 1.2 to 5.1 hours for 6-MAM. For the higher doses (10.5–13.9 mg), the time to peak free/total MOR and 6-MAM was 2.3 to 9.3 hours and 1.4 to 4.3 hours, respectively. It was determined that the route of administration did not have a significant impact on the parameters measured. In a study by Høiseth et al., a single dose of heroin was given to pigs to investigate the concentrations of heroin metabolites (6-MAM, MOR and M3G) in urine. This study was able to use a higher dose of heroin (20 mg) compared with other previous studies (8, 11, 12) due to avoiding the ethical concerns by using pigs rather than human subjects. Additionally, earlier time points were used for the collection of specimens compared with previous studies. It was reported that the T_{max} for MOR and 6-MAM in urine was achieved with 30 minutes (first collection) and M3G reached T_{max} with 90 minutes. Longer detection time (>6 hours) was also determined by this study for 6-MAM. Although this study used pigs, their physiology is similar to humans, and therefore, the reported detection times can be beneficial to forensic interpretations.

Using analyte ratios of MOR-related compounds could prove to be advantageous to interpretations. In Thaulow et al., ratios such as MOR/6-MAM, M3G/MOR and M6G/MOR in blood were proven to be helpful determining the influence of ethanol on the metabolism of heroin (13). The study concluded that ethanol inhibits the hydrolysis of 6-MAM to MOR and the glucuronidation of MOR to M3G and M6G. This finding further complicates interpretation of MOR-related compounds. Al-Asmari et al. validated a liquid chromatography–mass spectrometry (LC–MS) method for the quantification of various opioids and their metabolites in postmortem blood samples (14). Of the 32 cases analyzed, 11 were identified as heroin cases and were classified into three groups based on the blood concentrations of free MOR and its glucuronides (M3G and M6G). The three classifications were immediate death, subacute death and delayed death. Four cases were given the delayed death classification and these cases reported the highest M3G/MOR ratios compared with the other seven cases. This study showed the potential benefits of using metabolic ratios to help determine survival time in heroin-related deaths.

The aim of this study was to increase the diagnostic power in acute opiate overdose death investigations through the analysis of urine. We hypothesize that a short period of abstinence before administering the last dose can be identified by analyzing metabolites in urine. We also hypothesize that metabolic ratios change over time and that the time between administration and death is reflected by this change. The goals of this study were to (a) develop and validate a quantitative method for the determination of heroin metabolites and related substances in urine; (b) to apply the method to a series of opiate-related deaths; (c) to compare metabolic ratios in urine in different causes of death and after different drug intakes and (d) to compare toxicological findings in rapid and delayed deaths.

Materials and Methods

Chemicals and reagents

M3G and morphine-3β-D-glucuronide-d_3 (M3G-d_3) standards were obtained from Lipomed (Arlesheim, Switzerland). M6G, NMOR, MOR, C6G, NCOD, COD, 6-MAM, EMOR, morphine-6β-D-glucuronide-d_3 (M6G-d_3), morphine-d_3 (MOR-d_3), codeine-6β-D-glucuronide-d_3 (C6G-d_3), norcodeine-d_3 (NCOD-d_3), codeine-d_3 (COD-d_3) and 6-acetylmorphine-d_3 (6-MAM-d_3) standards were purchased from Cerilliant (Round Rock, TX, USA). Concentrated formic acid (LC–MS grade) for mobile phase was purchased from Fisher Scientific (Gothenburg, Sweden). Purified water was supplied by MilliQ® system (Millipore, Billerica, MA, USA). Ammonium

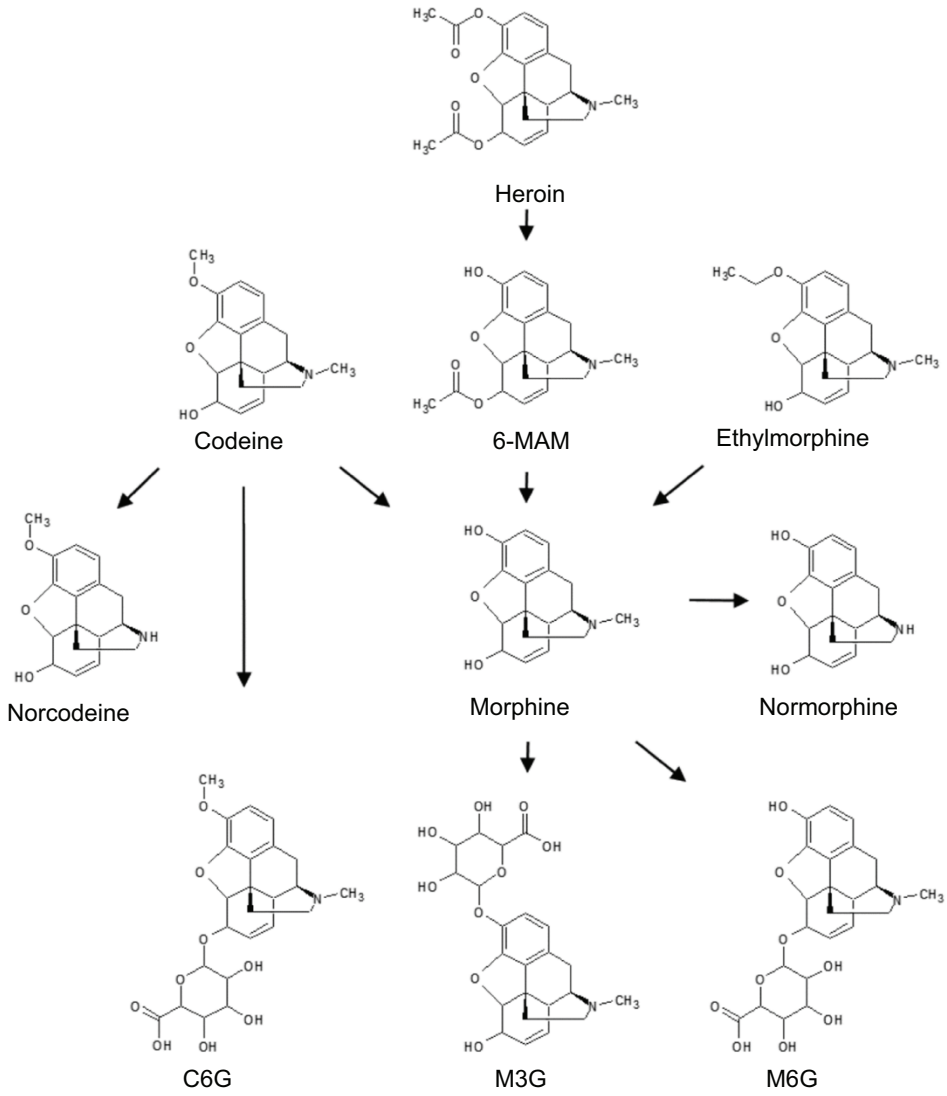

Figure 1. Metabolism of heroin-related compounds.

formate (BioUltra ≥99.0%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (gradient grade) was purchased from Merck (Darmstadt, Germany).

Liquid chromatography

An Acquity UPLC® I-class (Waters Corp., Milford, MA, USA) coupled to a Waters Xevo TQD was used to perform analysis. Chromatographic separation was achieved using an ACE Excel C18-PFP column (2 μm, 2.1 × 100 mm) at 60°C. Gradient elution was based on a mobile phase consisting of 0.001% formic acid in 10-mM ammonium formate (pH 5.2) and 0.001% formic acid in methanol at a constant flow rate of 0.5 mL/min. The gradient conditions were initially 1% organic mobile phase during 1.5 min, which were then increased to 41% during the next 7.5 min, followed by a 95% organic mobile phase wash for 1 min before re-equilibration at 1% for 1 min. Total run time was 10.1 min and injection volume 3 μL.

Mass spectrometry

Data were acquired and analyzed using Waters MassLynx™ software (Waters, version 4.1 SCN 940). Multiple reaction monitoring (MRM) was utilized in positive electrospray ionization mode with two transitions for each analyte and one for each internal standard as seen in Table I. Source parameters were as follows: capillary voltage at 0.40 kV, source temperature at 150°C, desolvation temperature at 550°C, source gas flow at 1,000 L/h (desolvation) and 50 L/h (cone).

Sample preparation

Urine samples were prepared by diluting 100 μL urine sample with 900 μL internal standard solution in MilliQ-water before direct injection on the liquid chromatograph–tandem mass spectrometer (LC–MS-MS) instrument. The final concentration of internal standard in samples was 1.125 μg/mL for M6G-d_3, C6G-d_3, NCOD-d_3, COD-d_3 and 6-MAM-d_3; 2.25 μg/mL for M3G-d_3 and 5.625 μg/mL for MOR-d_3. Calibration samples and control samples were prepared in batches by spiking drug-free human urine. The calibration samples and control samples were then prepared as the urine samples before analysis.

Validation

Method validation was performed according to international guidelines (15, 16), which comprised of selectivity, matrix interferences,

Table I. Optimized Parameters for Morphine-3β-D-Glucuronide, Morphine-6β-D-Glucuronide, Normorphine, Morphine, Codeine-6β-D-Glucuronide, Norcodeine, Codeine, 6-Acetylmorphine, Ethylmorphine and Deuterated Internal Standards

Compound name	Parent (m/z)	Product (m/z)	Cone (V)	Collision (V)	Retention time (min)	Transition ratio	Internal standard
M3G	*462.11*	*286.15*	50	34	1.13		M3G-d_3
	462.11	165.10	50	68		0.06	
M6G	*462.10*	*286.15*	60	36	2.33		M6G-d_3
	462.10	165.10	60	64		0.09	
NMOR	*272.05*	*165.00*	50	40	2.40		MOR-d_3
	272.05	121.00	50	28		1.95	
MOR	*286.05*	*165.00*	54	36	3.16		MOR-d_3
	286.05	201.05	54	26		1.03	
C6G	*476.15*	*300.10*	62	34	3.61		C6G-d_3
	476.15	165.00	62	64		0.11	
NCOD	*286.10*	*165.05*	46	44	4.37		NCOD-d_3
	286.10	121.00	46	28		1.18	
COD	*300.10*	*165.00*	40	40	4.93		COD-d_3
	300.10	215.05	40	26		0.90	
6-MAM	*328.10*	*165.05*	50	42	5.31		6-MAM-d_3
	328.10	211.05	50	28		0.66	
EMOR	*314.10*	*165.05*	52	46	6.07		COD-d_3
	314.10	229.10	52	26		0.61	
M3G-d_3	465.10	289.15	50	32	1.13		
M6G-d_3	465.11	289.15	60	34	2.32		
MOR-d_3	289.10	165.05	50	42	3.13		
C6G-d_3	479.15	303.15	60	34	3.59		
NCOD-d_3	289.11	165.05	50	40	4.34		
COD-d_3	303.15	165.05	50	44	4.89		
6-MAM-d_3	331.10	165.05	50	40	5.29		

Quantifying transitions are indicated with italics.

linearity, precision, bias, carryover and processed sample stability. Selectivity was evaluated in reference to matrix interferences, purity of standard/internal standard, and relevant substances consisting of benzodiazepines, opioids and stimulants at concentrations ranging between 0.2 and 10 μg/mL in urine ($n = 72$). Matrix interferences were assessed by analyzing 10 authentic drug-free postmortem human urine specimens. To determine purity of standards, drug-free human urine was spiked with only internal standard mix solution. Drug-free human urine was also spiked with single standards of analytes of interest at the upper calibration range. Qualitative matrix effects were determined using postcolumn infusion and 10 drug-free human postmortem urine samples that were diluted 10 times with MilliQ-water and analyzed. Linearity was evaluated using 6 replicates of at least 8 nonzero calibrators. Precision and bias were determined over 8 separate occasions spread over 2 weeks in triplicate at low, medium and high concentration levels. Carryover was determined by the injection of a blank matrix sample after the highest calibrator. The lower limit of quantitation (LLOQ) was determined as the lowest level concentration that met criteria for accuracy and percent coefficient of variance (CV%). Processed stability was assessed by analyzing extracts stored at ±10°C at medium level concentrations at 6, 24 and 72 hours. Process efficiency was evaluated using 10 drug-free urine samples (100 μL) that were prepared at low (0.2 μg/mL) and high (4 μg/mL) concentrations of analytes and internal standards then diluted to 1 mL with MilliQ water. References of the standards and internal standards were prepared in mobile phase (100 μL) and diluted with MilliQ water (900 μL). The references were injected 6 times at each concentration to determine an average area. Process efficiency was calculated by dividing the area of sample by the reference area and multiplying by 100. Samples ($n = 3$) were fortified at 3.4 μg/mL (NMOR and NCOD), 7.4 μg/mL (COD, 6-MAM and EMOR) and 34 μg/mL (M3G, M6G, MOR and C6G) then diluted 10x with drug-free urine to evaluate dilution integrity.

Authentic cases

The study was approved by the regional ethics committee in Linköping (Dnr: 2017-387-32). Postmortem urine samples were collected from autopsy cases screened positive (17) for MOR, COD and/or 6-MAM and confirmed in blood in routine work at National Board of Forensic Medicine in Linköping, Sweden (18). The previously published quantitative method had LOQ of 0.005 μg/g in blood for MOR, COD and 6-MAM (18). From each case, an aliquot of 1 to 2 mL of urine was collected and stored at −20°C up to 12 months prior to analysis.

Data processing and statistical methods

Demographic data, cause of death (CoD), and mode of death were obtained from the National Board of Forensic Medicine's in-house database. Cases were divided into three groups based on the CoD; opiate intoxication, intoxication with other drugs than opiates and other causes of death. In addition, cases were assigned to a group accordingly to the opiate administered. Based on the toxicological findings these were heroin (6-MAM detected in blood or urine), MOR (only MOR detected in blood), COD (COD detected in blood in concentrations significantly higher than MOR) or EMOR (EMOR detected in blood). Heroin intoxications ($n = 35$) were classified as rapid if 6-MAM was present in blood or as delayed if 6-MAM was negative in blood. Graph Pad Prism V 8.3.0 (San Diego, CA, USA) was used for statistical analyses. Normality of the concentrations

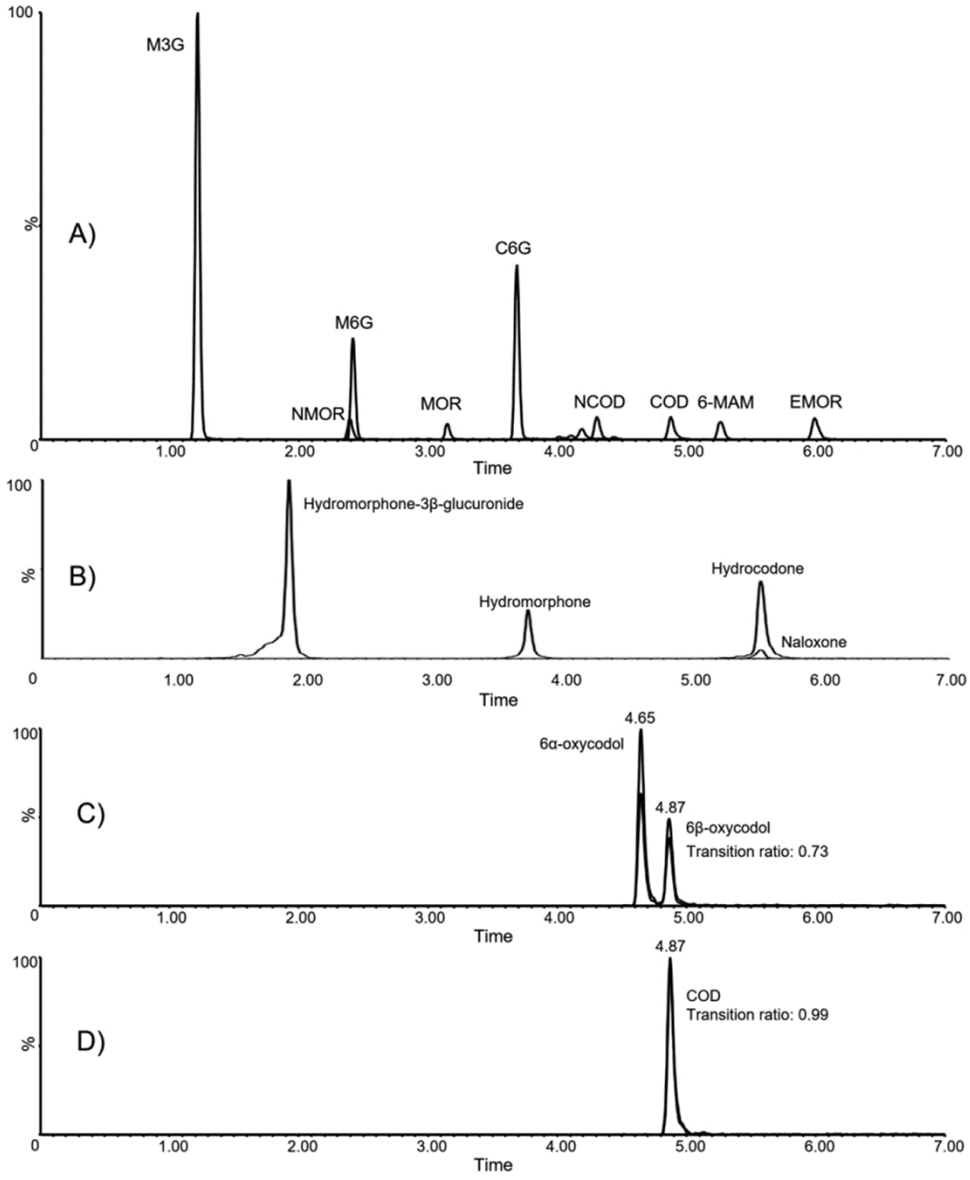

Figure 2. Chromatogram of analytes and possible interfering compounds. (a) Morphine-3β-D-glucuronide, morphine-6β-D-glucuronide, normorphine, codeine-6β-D-glucuronide, norcodeine, codeine, 6-acetylmorphine and ethylmorphine from a quality control sample; (b) the isobaric compounds hydromorphone-3β-glucuronide, hydromorphone, hydrocodone, and naloxone; (c) α/β oxycodol shown in transitions for codeine (m/z 300.10/165.00 and 300.10/121.00) and (d) codeine shown in transition m/z 300.10/165.00 and 300.10/121.00.

was tested using the Kolmogorov–Smirnov test. The concentrations were not all normally distributed, and thus, median and range concentrations are shown. The Kruskal–Wallis one-way analysis of variance test was used to determine differences between metabolic ratios in the parent drug groups. Mann–Whitney *U* tests were performed to compare results between rapid and delayed deaths from heroin. Differences were considered significant if P-value < 0.05.

Results

Validation

The initial goal for this study was to develop and validate a method to quantify M3G, M6G, NMOR, MOR, C6G, NCOD, COD, 6-MAM and EMOR in urine. In Figure 2a a chromatogram from a low quality control sample is shown. No qualitative matrix effects were detected using postcolumn infusion. After analyzing 10 postmortem drug-free urine samples, there were no detectable interferences for all analytes except for C6G. Although there was an interference detected for C6G, it was able to be discriminated by transition ratio criteria and relative retention time. There were no analyte peaks identified in internal standard only mix and vice-versa, therefore the purity of standards and internal standards were deemed acceptable. There were peaks identified from the relevant substances that were evaluated. In Figure 2b, hydromorphone-3β-glucuronide, hydromorphone, hydrocodone and naloxone are shown. These compounds were evaluated as potential interferences for M3G, M6G, MOR, COD, NCOD or 6-MAM, but were differentiated by retention time. The presence of 6β-oxycodol was detected as an interference for COD when analyzing authentic cases (Figure 2c, d), with identical retention time and a similar transition ratio (6β-oxycodol 0.73,

COD 0.99). There was no carryover observed in the blank injected after the highest calibrator.

In Table II, validation parameters such as, LLOQ, upper limit of quantitation (ULOQ), precision, bias, dilution integrity and process efficiency are shown. After evaluating residual plots for all analytes, a quadratic regression was determined to be the best fit with a 1/x (NMOR, NCOD and 6-MAM) or $1/x^2$ (M3G, M6G, MOR, C6G, COD and EMOR) weighting. Eleven calibration levels were used for M3G, M6G and C6G: 0.10, 0.20, 0.40, 0.50, 1.0, 2.5, 5.0, 10, 25, 50 and 100 µg/mL. Ten calibration levels were used for MOR: 0.025, 0.050, 0.10, 0.50, 1.0, 2.5, 5.0, 10, 25 and 50 µg/mL. The same calibration levels were used for COD as for MOR, except the level 50 µg/mL was left out. Nine calibration levels were also used for EMOR: 0.010, 0.020, 0.040, 0.50, 1.0, 2.5, 5.0, 10 and 25 µg/mL. The same calibration levels were used for 6-MAM as for EMOR, except the level 25 µg/mL was left out. For NMOR and NCOD, seven calibration levels were used: 0.025, 0.050, 0.10, 0.50, 1.0, 2.5 and 5.0. The LLOQ was determined to be 0.01 µg/mL for 6-MAM, 0.02 µg/mL for EMOR, 0.025 µg/mL for MOR and COD, 0.05 µg/mL for NMOR and NCOD and 0.2 µg/mL for M3G, M6G, and C6G. These concentrations met the validation criteria for signal to noise > 10, accuracy between 75% and 125%, and CV% within ±25%. Intraday and interday precision was calculated as %CV and deemed acceptable if it was ±20%CV. Interday precision ranged from 1.6 to 7.5%CV and intraday precision ranged from 1.7 to 8.1%CV for all analytes, both well within the acceptable limits. Bias ranged −15.8 to 6.4% for all analytes, which is within the acceptable limits of ±20%. At the three time intervals (6, 24 and 72 hours) at ±10°C, all analytes were within ±20% of target concentration and stability deemed acceptable. To determine dilution integrity, samples were diluted 10x with drug-free urine and analyzed. The quantitated results were between 98% and 113% from the target concentration for all analytes, and 10x dilution were therefore deemed accepted.

Process efficiency was deemed acceptable if it was within ±25% of the reference (Table II). There was ion enhancement observed for both M6G (111% and 150%) and C6G (119% and 123%) at both concentrations. Each of these analytes has a match deuterated internal standard that also exhibited the ion enhancement, therefore able to mitigate this effect. The process efficiency of NMOR at the low concentration was 61%, which does not meet criteria. There is no commercially available deuterated internal standard for NMOR. NCOD-d_3 was used as the internal standard for NMOR. Although this does not completely mitigate the suppression observed, it was considered acceptable for this study because it did not compromise other validation parameters such as LLOQ, precision or bias.

Authentic cases

There were a total of 135 autopsy cases with paired blood and urine samples analyzed in this study. Of the 135 cases, 5 cases were excluded due to being classified as mixed intakes and outside the scope of this study. The demographics of the remaining 130 cases such as CoD, manner of death, and place of death are shown in Supplemental Table I. Most of the cases were from male decedents (72%). The overall age ranged between 17 and 96 with a median age of 56 years old. The leading CoD was nonintoxication (53%) followed by opiate-intoxication (34%) and other intoxication (13%). Accidental (46%) was the most common manner of death from these cases followed by natural (28%). Home (53%) and hospital (25%) were the most frequent place of death. An objective was to compare metabolic ratios between the different causes of death but since the

Table II. Validation Parameters for Morphine-3β-D-Glucuronide, Morphine-6β-D-Glucuronide, Normorphine, Morphine, Codeine-6β-D-Glucuronide, Norcodeine, Codeine, 6-Acetylmorphine, and Ethylmorphine

			Interday Precision (%CV)			Intraday Precision (%CV)			Bias (%)				Process Efficiency (%)Analyte (d_3)	
Analyte	LLOQ (µg/mL)	ULOQ (µg/mL)	Low[a]	Mid[b]	High[c]	Low[a]	Mid[b]	High[c]	Low[a]	Mid[b]	High[c]	DilutionIntegrity (%)	Low[d]	High[e]
M3G	0.2	100	2.2	1.6	3.3	2.2	1.7	2.9	−1.2	−2.3	−4.9	98	84 (97)	100 (104)
M6G	0.2	100	4.2	2.9	3.2	3.4	2.5	3.5	−0.8	−1.2	−3.7	110	111 (117)	150 (133)
NMOR	0.05	5.0	6.3	3.2	3.8	5.3	3.1	3.1	−2.7	0.7	1.7	111	61	88
MOR	0.025	50	7.5	2.7	3.7	8.1	2.0	2.5	5.9	−0.4	3.0	113	77 (76)	92 (89)
C6G	0.2	100	4.3	2.3	3.6	4.0	1.9	3.6	−3.9	−5.5	−8.3	107	119 (138)	123 (128)
NCOD	0.05	5.0	4.7	2.5	3.2	5.3	1.8	3.2	−0.2	1.5	0.8	102	86 (93)	103 (106)
COD	0.025	25	6.1	2.4	2.4	5.6	2.3	2.5	−1.0	4.8	2.6	111	95 (100)	108 (111)
6-MAM	0.01	10	6.2	2.0	3.2	4.5	1.7	3.0	1.5	3.1	2.4	103	92 (101)	104 (107)
EMOR	0.02	25	4.6	2.4	2.8	4.2	2.6	2.7	−15.8	6.4	3.0	112	95	107

[a]Low concentration: 0.02 µg/mL for 6-MAM, 0.05 µg/mL for MOR, COD, and EMOR, 0.1 µg/mL for NMOR and NCOD, and 0.4 µg/mL for M3G, M6G, and C6G
[b]Middle concentration: 1.7 µg/mL for NMOR and NCOD, 3.7 µg/mL for COD, 6-MAM, and EMOR, and 17 µg/mL for M3G, M6G, MOR, and C6G
[c]High concentration: 3.4 µg/mL for NMOR and NCOD, 7.4 µg/mL for COD, 6-MAM, and EMOR, and 34 µg/mL for M3G, M6G, MOR, and C6G
[d]Low concentration was 0.2 µg/mL for all analytes
[e]High concentration was 4 µg/mL for all analytes

Table III. Urine and Blood Concentrations From Authentic Samples in This Study

		Urine results			Blood results		
Intake classification	Analytes	*N*	Concentration range (μg/mL)	Median (μg/mL)	*N*	Concentration range (μg/g)	Median (μg/g)
Codeine intake	M3G	37	0.25–77.8	1.95	–	–	–
***N* = 42**	M6G	24	0.27–12.8	0.45	–	–	–
	NMOR	27	0.06–4.88	0.16	–	–	–
	MOR	25	0.03–3.54	0.15	14	0.005–0.18	0.01
	C6G	42	1.51–398	46.4	–	–	–
	NCOD	37	0.06–34.2	0.39	–	–	–
	COD	42	0.10–36.0	2.59	42	0.006–2.4	0.07
	Metabolite Ratios		**Ratio Range**	**Ratio Median**			
	M3G/MOR	24	1.6–83.2	21.9	–	–	–
	C6G/COD	42	0.5–262	22.0	–	–	–
Morphine intake	M3G	44	0.29–318	10.1	–	–	–
***N* = 49**	M6G	37	0.29–63.5	3.35	–	–	–
	NMOR	14	0.06–2.10	0.13	–	–	–
	MOR	46	0.05–47.8	1.05	49	0.01–6.9	0.07
	Metabolite Ratios		**Ratio Range**	**Ratio Median**			
	M3G/MOR	43	2.3–70.9	9.0	–	–	–
Heroin intake	M3G	31	0.21–823	26.4	–	–	–
***N* = 36**	M6G	29	0.22–174	4.88	–	–	–
	NMOR	14	0.05–1.59	0.13	–	–	–
	MOR	36	0.04–97.0	3.07	36	0.02–0.89	0.22
	C6G	27	0.21–77.8	2.60	–	–	–
	NCOD	10	0.05–1.19	0.10	–	–	–
	COD	31	0.04–7.98	0.38	32	0.005–0.12	0.02
	6-MAM	36	0.02–25.6	0.47	16	0.005–0.03	0.02
	Metabolite Ratios		**Ratio Range**	**Ratio Median**			
	M3G/MOR	32	1.3–33.5	6.4	–	–	–
	C6G/COD	27	1.2–32.7	6.0	–	–	–

distribution of opiate intoxication was dominated by heroin intakes and the nonintoxications by COD and MOR a comparison was not deemed appropriate.

The results for the urine and blood concentrations from the cases in this study are shown in Table III. The 130 cases were classified by intake: COD ($n = 42$), heroin ($n = 36$), MOR ($n = 49$) and EMOR ($n = 3$). Due to the small sample size, the EMOR concentrations were not included in the table. For these three cases, the blood MOR concentrations were 0.005, 0.06 and 0.06 μg/g and for the same cases, the blood EMOR concentrations were 0.29, 0.08 and 0.28 μg/g. The urine concentrations were 3.44, 27.8 and 31 μg/mL for M3G, 0.77, 4.52 and 4.69 μg/mL for M6G, 0.22 μg/mL, not present, and not present for NMOR, not present, 4.28, and 2.54 μg/mL for MOR, and 0.72, 10.1 and 7.68 μg/mL for EMOR. Due to absence of MOR, there were only two M3G/MOR ratios (6.5 and 12.2).

In this study population, two cases were completely negative in urine and another 12 cases were negative for the phase II metabolite M3G. Eight of those were opiate intoxications, one from MOR, two from COD, and five from heroin with four rapid deaths and one delayed.

In Figure 3a, a comparison between the parent drug groups (excluding EMOR) regardless of CoD using M3G/MOR ratios is shown. The number of cases for each group that had M3G/MOR ratios reported were COD ($n = 25$), MOR ($n = 46$) and heroin ($n = 35$). The spread and median of the three groups are visually different, but in order to properly assess significance, a Kruskal–Wallis test was performed. There was a significant difference observed between COD versus MOR ($P = 0.005$), COD versus heroin ($P < 0.0001$), but not between MOR versus heroin ($P = 0.10$).

In Table IV, results for cases classified as heroin overdoses ($n = 35$) are shown with the cases divided into rapid ($n = 15$) or delayed ($n = 20$) deaths. In these cases, there was only one female (case 123) reported. In most of the cases (77%) the mode of death was determined to be accidental. The median age for rapid and delayed deaths was 35 and 34, respectively. Mann–Whitney *U* tests were performed between all the urine concentrations and MOR blood concentrations for rapid and delayed death groups. There was a significant difference determined for MOR ($P = 0.001$), COD ($P = 0.009$), 6-MAM ($P = 0.02$) in urine and MOR ($P = 0.02$) in blood. The median values from the delayed death urine concentrations were all higher compared with the rapid death medians. Additionally, a Mann–Whitney *U* test was used to determine if there was a significant difference in the M3G/MOR ratios between the two groups (Figure 3b). It was determined that there was not a significant difference ($P = 0.9$).

Discussion

In this study, the distribution of opiate metabolites in urine in a postmortem population positive for MOR or COD in blood has been described. Initially, an LC–MS-MS method was developed and validated for the quantitative determination of several metabolites including conjugated ones. The method proved precise and accurate

Table IV. Blood and Urine Results from Rapid and Delayed Death Heroin Cases

Case	MoD	Age	Hx	Blood concentration µg/g			Urine Concentration µg/mL								Metabolite ratio	Case details	Other drugs in femoral blood
				6-MAM	MOR	COD	6-MAM	MOR	M3G	M6G	NMOR	COD	C6G	NCOD	M3G/MOR		
R.1	A	41	–	0.02	0.25	0.02	0.06	0.77	25.7	5.15		0.12	3.80		33.5	Injection	ALP, AMP, BE, BUP, DZP, GBP, OXY, TZP
R.2	U	42	–	0.01	0.35	0.02	0.03	2.09	25.7	4.75	0.05	0.10	0.86		12.3	Injection	ALP, BUP
R.3	A	29	Y	0.02	0.36	0.02	0.61	11.2	89.9	18.9	0.16	0.52	5.12		8.03	Injection, RR-rehab	–
R.4	U	30	–	0.02	0.89	0.06	0.25	0.58	1.29	0.22		0.04			2.24	Injection, Syringe in right thigh, drugs	ALP, EtOH, THC
R.5	U	42	Y	0.03	0.24	0.01	0.90	1.22	11.00	2.55		0.08	0.47		9.03	Injection, Syringe in left arm, RR-prison	ALP
R.6	A	35	–	0.006	0.13	0.01	0.32	1.38	12.6	2.02		0.14	0.71		9.13	Injection	ALP, AMZ
R.7	A	30	Y	0.02	0.51	0.03	0.17	0.60	0.81			0.04			1.35	–	ALP, BE, DZP, MZP
R.8	U	58	Y	0.03	0.36	0.03	0.44	1.58	23.8	4.88	0.03	0.18	2.51	0.06	15.1	–	AMZ
R.9	A	60	Y	0.02	0.35	0.06	0.02	0.13							0.00	Injection, crushed tablets beside body	AMZ, BUP, ZOP
R.10	A	19	–	0.02	0.08	0.006	0.15	0.15							0.00	FU, CPR, died on scene	DHProp, EtOH
R.11	A	46	Y	0.006	0.11	0.01	0.31	0.52	2.57	0.31		0.05			4.93	–	EtOH
R.12	A	31	–	0.02	0.62	0.03	0.05	0.09							0.00	Injection, crushed medications	ALP, DZP, PRO
R.13	A	24	Y	0.005	0.15	0.007	0.07	0.04							0.00	–	ALP, EtOH
R.14	U	26	Y	0.03	0.21	0.01	0.19	1.23	9.48	2.45		0.14	1.23		7.72	–	AMZ, BPN, PGB, SER
R.15	A	60	-	0.006	0.76	0.12	1.73	13.0	127	20.1		2.32	22.2	0.19	9.77	FU, injection, expires at ER (1h)	ALP, BUP, PGB, ZOP
D.1	A	43	Y		0.09	0.007	0.08	0.66	4.76	1.12		0.05	0.21		7.18	Injection, relapsed after long sobriety	ALP, EtOH, THC
D.2	A	40	–		0.47	0.07	2.11	28.7	134	28.2	0.13	2.02	7.23	0.16	4.67	FU, injection, CPR, dies at hospital	ALP, AMZ, BUP, DZP, PGB
D.3	A	28	Y		0.34	0.03	0.87	67.3			0.67	3.81		0.07	0.00	Injection	DZP, TRA
D.4	A	36	–		0.12	0.01	0.19	0.11	0.21						2.02	Heroin powder and a rolled bill	EtOH
D.5	U	29	Y		0.02		0.50	2.54	28.0	4.11	0.13	0.47	4.68	0.48	11.0	Powders/tablets, & paraphernalia	AMP, BE, DXN, DZP, TRA
D.6	A	33	–		0.25	0.02	1.39	4.65	22.6	4.20	0.38	0.25	1.46		4.86	FU, CPR, dies at hospital	ALP, EtOH, PGB, THC
D.7	U	27	Y		0.02		1.16	7.15	45.6	11.4	0.19	1.13	7.04	0.13	6.38	–	COC, BE, PZN, ZPX
D.8	A	34	–		0.03		0.56	5.97	37.8	7.38	0.17	0.44	2.75	0.05	6.33	–	ALP, BUP, PGB, THC
D.9	U	33	Y		0.25	0.02	0.55	5.08	26.4	4.69	0.04	0.38	1.20		5.19	Injection	ALP, DZP, OZP
D.10	A	32	Y		0.38	0.03	0.43	1.92	9.93	1.91		0.13	0.63		5.17	Injection, RR-treatment home	ALP, EtOH, THC, QZP
D.11	A	51	–		0.03	0.005	0.03	6.10	60.0	11.6		1.18	2.37	0.05	9.83	FU, injection, CPR, expires at ER (1d)	ALP, AMP
D.12	A	35	–		0.1	0.01	0.70	3.98	50.9	11.5		0.40	5.54		12.8	FU, injection, CPR, dies at scene	AMZ, BUP, LPZ, MZP
D.13	A	24	–		0.09	0.01	1.24	6.54	60.0	8.84	0.06	0.60	2.94		9.18	Injection, RR-prison	ALP, BE, MZP
D.14	A	25	–		0.16	0.02	0.84	6.12	28.1	2.82		0.43	0.50		4.59	Injection	ALP, DZP, MDA, MDMA, MGN, PGB
D.15	A	26	–		0.02		0.12	3.84	16.3	2.69	0.06	0.13	0.85		4.25	Injection	ALP, AMP, THC
D.16	A	65	Y		0.37	0.04	1.46	17.9	77.5	11.2	0.09	1.20	2.80		4.33	–	ALP
D.17	A	42	Y		0.23	0.02	0.69	6.03	46.6	7.12	0.10	0.52	3.05		7.73	Paraphernalia for smoking heroin	EtOH, SER
D.18	A	39	Y		0.16	0.01	0.08	1.16	11.4	1.96	0.10	0.14	1.24		9.86	–	ALP, BUP
D.19	A	59	Y		0.21	0.02	0.68	3.59	27.0	5.65		0.27	2.60		7.53	Injection, other paraphernalia	AMP
D.20	A	45	Y		0.14	0.01	0.58	4.91	31.5	6.31		0.48	2.77	0.06	6.41	Was trying heroin	COC, BE, SER, THC

Summary of rapid versus delayed deaths

		Blood Concentrations µg/g			Urine Concentrations µg/mL								Metabolite Ratio
		6-MAM	MOR	COD	6-MAM	MOR	M3G	M6G	NMOR	COD	C6G	NCOD	M3G/MOR
R.1-R.15	**Median**	0.02	0.35	0.02	0.19	0.77	12.6	3.65	0.05	0.12	1.87	N/A	7.72
	Range	0.005–0.03	0.08–0.89	0.006–0.12	0.02–1.73	0.04–13.0	0.81–127	0.22–20.1	0.03–0.16	0.04–2.32	0.47–22.2	0.06–0.19	0.00–33.5
D.1-D.20	**Median**		0.13	0.02	0.63	5.00	28.1	5.98	0.12	0.44	2.68	0.07	6.36
	Range		0.02–0.47	0.005–0.07	0.03–2.11	0.11–67.3	0.21–134	1.12–28.2	0.04–0.67	0.05–3.81	0.21–7.23	0.05–0.48	0.00–12.8

D.# denotes delayed death case#; R.# denotes rapid death case#.
MoD denotes mode of death; Hx, known drug history; A, accidental; U, uncertain; 1d, one day; RR, recently released from; Injection, syringe was found at scene; FU, found unconscious.
ALP, alprazolam, AMP, amphetamine; AMZ, alimemazine; BE, benzoylecgonine; BPN, bupropion; BUP, buprenorphine; COC, cocaine; DXN, duloxetine; DZP, diazepam; EtOH, ethanol; GBP, gabapentin; LPZ, levomepromazine; MDA, methylenedioxyamphetamine; MDMA, 3,4-methylenedioxy-methamphetamine; MGN, mitragynine; MZP, mirtazapine; OXY, oxycodone; OZP, oxazepam; PGB, pregabalin; PRO, propranolol; PZN, perphenazine; SER, sertraline; THC, tetrahydrocannabinol; TRA, tramadol; TZP, temazepam; QTP, quetiapine; ZOP, zopiclone; ZPX, zuclopentixol.

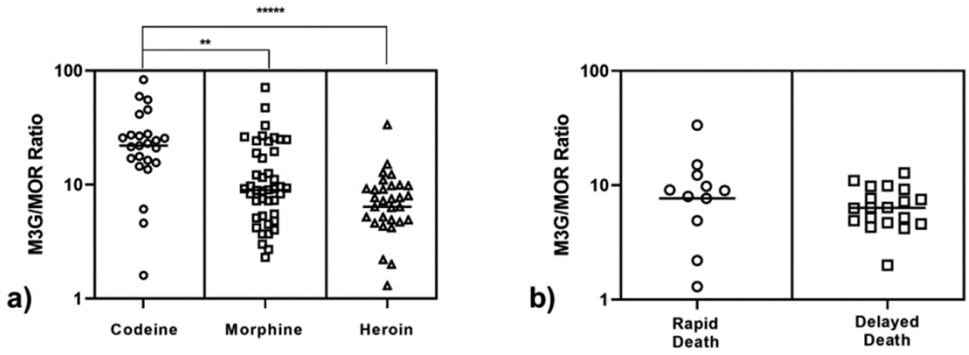

Figure 3. (a) Comparison of the M3G/MOR ratio between parent drug groups regardless of cause of death and (b) Comparison of the M3G/MOR ratio between rapid and delayed heroin intoxications.

with calibration ranges covering 94% of the findings. The co-elution of a minor oxycodone metabolite, 6β-oxycodol with COD, posed a potential issue with analysis. It has been shown earlier that in-source fragmentation of 6β-oxycodol produces a precursor isobaric to COD (19). To identify a possible problem with quantification of COD, 6α-oxycodol can be monitored. In this method, the cone voltage was set to minimize in-source fragmentation of 6β-oxycodol, without losing signal intensity for COD. The chromatographic separation of COD and 6β-oxycodol could not be achieved when keeping the retention of the earliest eluting compound, M3G, to a k' above 2.

Concentration data for heroin metabolites in urine are scarce. Smith et al. (10) reported ranges of peak concentrations between 0.15 and 2.6 μg/mL for free MOR in subjects administering doses of 10 to 12 mg. In another study by Cone et al., intranasal administration of 6 or 12 mg heroin resulted in peak concentrations of free MOR between 0.15 and 0.61 μg/mL and 0.36 and 2.1 μg/mL, respectively (8). Bogusz et al. described urinary concentrations of heroin metabolites in six heroin-related deaths and reported concentrations of MOR, M3G, M6G between 0.05 and 1.2, 1.3 and 13.6 and 0.9 and 3.7 (μg/mL) respectively (20). Al-Asmari reported urine free MOR concentrations between 0.045 and 42.264 (μg/mL) in 19 heroin-related deaths (21). Compared with the published data from controlled administration studies as well as case reports, the urinary concentrations in heroin cases in this study were considerably higher suggesting substantially higher doses.

However, as seen in Table III, the variation in urinary concentrations was wide for all analytes. For heroin intakes, M3G had the largest range (0.21–823 μg/mL) of an almost 4,000-fold difference. On the other hand, the M3G/MOR ratio ranged only between 1.3 and 33.5 (25-fold) with a median of 6.4 suggesting that the ratio is less dependent on dose. Similar to heroin intakes, the largest range was observed for M3G (0.29–318 μg/mL) for MOR intakes with M3G/MOR ratios showing much less variation ranging from 2.3 to 70.9 (30-fold) with a median of 9.0. The glucuronidation of MOR is mediated by UDP-glucuronosyltransferase-2B7 (UGT2B7) that exhibits genetic variations believed to impact enzyme activity but studies have shown that these variations were not associated with changes in pharmacokinetics of MOR (22).

Additionally, M3G/MOR ratios between the three intake groups (COD, MOR and heroin) were compared. Significant differences between COD and both MOR and heroin intakes were found, suggesting a change in glucuronidation rate of MOR when COD was the drug administered. M3G/MOR ratios after heroin and MOR intake were not significantly different with medians of 6.4, 9.0. This suggests that metabolism of heroin and MOR is similar and that studies on MOR may be helpful when interpreting heroin intake.

The rationale for analyzing the conjugates directly rather than performing hydrolysis is primarily to avoid the uncertainty of hydrolysis efficiency for the different conjugates when comparing concentrations or metabolic ratios (23–26). Another is to improve the interpretation of results comparing phase I and phase II metabolites in possible overdose cases. The concentrations of phase II metabolites and the conjugated/free analyte concentration ratios are expected to be higher in delayed compared with rapid deaths because conjugation has proceeded for a longer time in the delayed deaths (27). In addition, when conjugates are not present in urine, it suggests a time of abstinence before the last dose. Abstinence and the consequent loss of tolerance against opiates have been suggested as a risk factor in heroin overdose deaths (28–30). However, the time course of loss of tolerance to opiates is not very well studied, but it has been shown that a period of abstinence will affect the sensitivity to heroin in an animal model (31). The longer the time of abstinence, the more likely that some tolerance has been lost. Studies with controlled administration of heroin have generally had short detection times for free or total MOR owing to low doses or limited urine collection times (8, 10). However, Wang et al. (2020) investigated the detection times of conjugated and unconjugated heroin metabolites in 20 users and found that M3G was present up to 11 days in 8 of the subjects (32). In this study population, only 2 cases were completely negative in urine and another 12 cases were negative for M3G suggesting first use or a period of abstinence before administration. Eight of those were opiate intoxications, one from MOR, two from COD, and five from heroin with four rapid deaths and one delayed.

The presence of 6-MAM in blood has been used as a marker of rapid deaths because of its very short half-life (27, 33). Accordingly, the 35 acute heroin deaths were grouped into rapid and delayed, and then compared the toxicological findings in blood and urine. The blood MOR concentrations were significantly different between the groups with a median concentration of 0.35 μg/g in the rapid deaths compared with 0.13 μg/g in the delayed deaths confirming the rapid onset or suggesting higher doses in the rapid deaths. This is in accordance with the results from Darke et al. (33) who found twice as high MOR concentrations in rapid deaths (0.26 mg/L) compared with delayed (0.12 mg/L).

In addition to a high dose, co-administration of other central nervous system (CNS) depressants can shorten the time course.

However, as seen in Table IV, CNS depressants were equally common in both groups with all but two decedents (R.3 and D.19) having other drugs on board that could have contributed. The most common drug was alprazolam ($N = 21$), confirming conclusions from previous studies on opiate-related deaths (34–36) that have identified alprazolam as a contributing drug.

Another risk factor that can contribute to a rapid death is low tolerance to opiates originating from first use, abstinence, or sporadic use (33). A negative result in urine suggests a rapid death, where the excretion of heroin metabolites has not occurred and that the subject was abstinent prior to administering heroin. In the current study, no heroin intoxication had completely negative results, but four (27%) of the rapid cases presented with negative results for M3G and M6G as did one (5%) delayed death. This confirms a period of abstinence before death that could indicate loss of tolerance. Additional four decedents had very low M3G concentrations (<3 μg/mL) together with low M3G/MOR ratios (<5.0) that could have arisen from the final administration, which also indicates prior abstinence (R.4, R.7, R.11 and D.4). However, most decedents had both phase I and phase II metabolites present indicating previous use. In general, delayed deaths had higher concentrations of metabolites in their urine and significant differences were seen for MOR, 6-MAM and COD explained by the increased time for excretion before death.

Lower M3G/MOR ratios would be expected with short survival times. However, there was no difference in ratios between rapid and delayed deaths (Figure 3b), which may be explained by residual conjugated metabolites from previous use.

In abstinent subjects, the ratio might be used to estimate the time of administration or time between administration and death. Previous studies that have investigated the excretion of heroin metabolites after controlled administration are minimal. In a paper by Cone et al. (8) and one by Smith et al. (10), intranasal, smoking and intravenous routes were used to describe the excretion of free and total MOR over several days. Unfortunately, neither study determined the conjugated metabolites. Their designs included collection of all urine samples without any specific sampling scheme. After intravenous injection of 12-mg heroin total MOR concentration always exceeded that of free MOR even at the earliest collection time, but increased ratios between total and free MOR were seen during the first 12 hours after injection (10). In this study, the acute heroin intoxications with detectable M3G presented with higher M3G concentrations than MOR even though ratios were low in several cases. Unfortunately, reliable information about the time between administration and death was missing and no relationship between ratios and time could be investigated. Further investigations into the excretion of free and conjugated metabolites are needed to enable a more detailed interpretation of M3G/MOR ratios shortly after administration. At present, the major rational for analyzing phase II metabolites in urine is to confirm a period of abstinence prior to death.

Conclusions

In conclusion, the developed and validated method was suitable to quantitatively determine phase I and phase II metabolites of heroin and related compounds. The direct determination of conjugates such as M3G and M6G can objectively identify a period of abstinence before death. In this study population of 35 acute heroin overdoses, 14% were confirmed abstinent and another 11% presented with results pointing toward abstinence suggesting other factors than a short period of abstinence may be equally or more important for a fatal outcome. Blood MOR concentrations were higher in rapid than in delayed deaths; however, the significance of this finding is unclear since it can be explained by metabolism and elimination.

Supplementary data

Supplementary data is available at *Journal of Analytical Toxicology* online.

References

1. (2019) European Drug Report: Trends and Developments 2019. European Monitoring Centre for Drugs and Drug Addiction. http://www.emcdda.europa.eu/system/files/publications/11364/20191724_TDAT19001ENN_PDF.pdf.
2. (2018) UNODC-World Drug Report 2018. United Nations Office on Drugs and Crime. https://www.unodc.org/pdf/opioids-crisis/WDR18_Booklet_3_DRUG_MARKETS.PDF.
3. (2019) Sweden Country Drug Report 2019. European Monitoring Centre for Drugs and Drug Addiction. https://www.emcdda.europa.eu/system/files/publications/11354/sweden-cdr-2019_0.pdf.
4. (2019) NFLIS-DRUG 2019 Midyear Report. National Forensic. Laboratory Information System. https://www.nflis.deadiversion.usdoj.gov/DesktopModules/ReportDownloads/Reports/13408NFLISDrugMidYear2019.pdf.
5. Powers, D., Erickson, S., Swortwood, M.J. (2018) Quantification of morphine, codeine, and thebaine in home-brewed poppy seed tea by LC-MS/MS. *Journal of Forensic Sciences*, **63**, 1229–1235.
6. Simonsen, K.W., Kriikku, P., Thelander, G., Edvardsen, H.M.E., Thordardottir, S., Andersen, C.U., et al. others (2020) Fatal poisoning in drug addicts in the Nordic countries in 2017. *Forensic Science International*, **313**, 110343.
7. He, Y.J., Brockmöller, J., Schmidt, H., Roots, I., Kirchheiner, J. (2008) CYP2D6 ultrarapid metabolism and morphine/codeine ratios in blood: was it codeine or heroin?. *Journal of Analytical Toxicology*, **32**, 178–182.
8. Cone, E.J., Jufer, R., Darwin, W.D., Needleman, S.B. (1996) Forensic drug testing for opiates. VII. Urinary excretion profile of intranasal (snorted) heroin. *Journal of Analytical Toxicology*, **20**, 379–392.
9. Mitchell, J.M., Paul, B.D., Welch, P., Cone, E.J. (1991) Forensic drug testing for opiates. II. Metabolism and excretion rate of morphine in humans after morphine administration. *Journal of Analytical Toxicology*, **15**, 49–53.
10. Smith, M.L., Shimomura, E.T., Summers, J., Paul, B.D., Jenkins, A.J., Darwin, W.D., et al. (2001) Urinary excretion profiles for total morphine, free morphine, and 6-acetylmorphine following smoked and intravenous heroin. *Journal of Analytical Toxicology*, **25**, 504–514.
11. Cone, E.J., Welch, P., Mitchell, J.M., Paul, B.D. (1991) Forensic drug testing for opiates: i. detection of 6-acetylmorphine in urine as an indicator of recent heroin exposure; drug and assay considerations and detection times*. *Journal of Analytical Toxicology*, **15**, 1–7.
12. Cone, E.J., Dickerson, S., Paul, B.D., Mitchell, J.M. (1993) Forensic drug testing for opiates. v. Urine testing for heroin, morphine, and codeine with commercial opiate immunoassays. *Journal of Analytical Toxicology*, **17**, 156–164.
13. Thaulow, C.H., Høiseth, G., Andersen, J.M., Handal, M., Mørland, J. (2014) Pharmacokinetic interactions between ethanol and heroin: A study on post-mortem cases. *Forensic Science International*, **242**, 127–134.
14. Al-Asmari, A.I., Anderson, R.A. (2007) Method for quantification of opioids and their metabolites in autopsy blood by liquid chromatography-tandem mass spectrometry. *Journal of Analytical Toxicology*, **31**, 394–408.
15. (2013) Scientific working group for forensic toxicology (SWGTOX) standard practices for method validation in forensic toxicology. *Journal of Analytical Toxicology*, **37**, 452–474.
16. Peters, F.T., Drummer, O.H., Musshoff, F. (2007) Validation of new methods. *Forensic Science International*, **165**, 216–224.

17. Roman, M., Ström, L., Tell, H., Josefsson, M. (2013) Liquid chromatography/time-of-flight mass spectrometry analysis of postmortem blood samples for targeted toxicological screening. *Analytical and Bioanalytical Chemistry*, **405**, 4107–4125.
18. Jones, A.W., Holmgren, A., Ahlner, J. (2012) Concentrations of free-morphine in peripheral blood after recent use of heroin in overdose deaths and in apprehended drivers. *Forensic Science International*, **215**, 18–24.
19. Muñoz-Muñoz, A.C., Pekol, T., Schubring, D., Johnson, C., Andrade, L. (2018) Identification of novel opioid interferences using high-resolution mass spectrometry. *Journal of Analytical Toxicology*, **42**, 6–16.
20. Bogusz, M.J., Maier R-D, D.S. (1997) Morphine, morphine-3-glucuronide, morphine-6-glucuronide, and 6-monoacetylmorphine determined by means of atmospheric pressure chemical ionization-mass spectrometry-liquid chromatography in body fluids of heroin victims. *Journal of Analytical Toxicology*, **21**, 346–355.
21. Al-Asmari, A.I. (2020) Postmortem fluid concentrations of heroin biomarkers and their metabolites. *Journal of Forensic Sciences*, **65**, 570–579.
22. Nielsen, L.M., Sverrisdóttir, E., Stage, T.B., Feddersen, S., Brøsen, K., Christrup, L.L., et al. (2017) Lack of genetic association between OCT1, ABCB1, and UGT2B7 variants and morphine pharmacokinetics. *European Journal of Pharmaceutical Sciences*, **99**, 337–342.
23. Sitasuwan, P., Melendez, C., Marinova, M., Mastrianni, K.R., Darragh, A., Ryan, E., et al. (2016) Degradation of opioids and opiates during acid hydrolysis leads to reduced recovery compared to enzymatic hydrolysis. *Journal of Analytical Toxicology*, **40**, 601–607.
24. Malik-Wolf, B., Vorce, S., Holler, J., Bosy, T. (2014) Evaluation of abalone β-glucuronidase substitution in current urine hydrolysis procedures. *Journal of Analytical Toxicology*, **38**, 171–176.
25. Costa, S., Barroso, M., Castañera, A., Dias, M. (2010) Design of experiments, a powerful tool for method development in forensic toxicology: application to the optimization of urinary morphine 3-glucuronide acid hydrolysis. *Analytical and Bioanalytical Chemistry*, **396**, 2533–2542.
26. Wang, P., Stone, J.A., Chen, K.H., Gross, S.F., Haller, C.A., Wu, A.H.B. (2006) Incomplete recovery of prescription opioids in urine using enzymatic hydrolysis of glucuronide metabolites. *Journal of Analytical Toxicology*, **30**, 570–575.
27. Thaulow, C.H., Å Ml, Ø., Rogde, S., Andersen, J.M., Høiseth, G., Handal, M., et al. (2018) Can measurements of heroin metabolites in post-mortem matrices other than peripheral blood indicate if death was rapid or delayed?. *Forensic Science International*, **290**, 121–128.
28. Warner-Smith, M., Darke, S., Lynskey, M., Hall, W. (2001) Heroin overdose: causes and consequences. *Addiction*, **96**, 1113–1125.
29. Fugelstad, A., Ahlner, J., Brandt, L., Ceder, G., Eksborg, S., Rajs, J., et al. (2003) Use of morphine and 6-monoacetylmorphine in blood for the evaluation of possible risk factors for sudden death in 192 heroin users. *Addiction*, **98**, 463–470.
30. Thiblin, I., Eksborg, S., Petersson, A., Fugelstad, A., Rajs, J. (2004) Fatal intoxication as a consequence of intranasal administration (snorting) or pulmonary inhalation (smoking) of heroin. *Forensic Science International*, **139**, 241–247.
31. Strandberg, J.J., Kugelberg, F.C., Alkass, K., Gustavsson, A., Zahlsen, K., Spigset, O., et al. (2006) Toxicological analysis in rats subjected to heroin and morphine overdose. *Toxicology Letters*, **166**, 11–18.
32. Wang, L., Ni, C., Shen, H., Sheng, Z., Liang, C., Wang, R., et al. (2019) Comparison of the detection windows of heroin metabolites in human urine using online SPE and LC–MS/MS: importance of morphine-3-glucuronide. *Journal of Analytical Toxicology*, **44**, 22–28.
33. Darke, S., Duflou, J. (2016) The toxicology of heroin-related death: Estimating survival times. *Addiction*, **111**, 1607–1613.
34. Seldén, T., Ahlner, J., Druid, H., Kronstrand, R. (2012) Toxicological and pathological findings in a series of buprenorphine related deaths. possible risk factors for fatal outcome. *Forensic Science International*, **220**, 284–290.
35. Rintoul, A.C., Dobbin, M.D.H., Nielsen, S., Degenhardt, L., Drummer, O.H. (2013) Recent increase in detection of alprazolam in Victorian heroin-related deaths. *Medical Journal of Australia*, **198**, 206–209.
36. Fagiola, M., Hahn, T., Avella, J. (2019) Evaluation of acetylfentanyl following suspected heroin overdose when complicated by the presence of toxic fentanyl and alprazolam concentrations. *Academic Forensic Pathology*, **9**, 191–199.