Research Article

Quantification of Morphine and Tramadol in Urine after Oral Co-administration

Ahmed I. Sabbah[1*], Sameh M. Hafeez[1], Safwat A. Ahmed[2], Shehab A. Sallam[3], Ghada M. Hadad[4]
[1]Chemical Laboratory, Forensic Medicine Authority, Ministry of Justice, Ismailia, Egypt.
[2]Department of Pharmacognosy, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt.
[3]Chemistry Department, Faculty of Science, Suez Canal University, Ismailia, Egypt.
[4]Analytical Chemistry Department, Faculty of Pharmacy, Suez Canal University, Ismailia, Egypt.
***Corresponding author's E-mail:** sabah987@hotmail.com

Accepted on: 27-11-2014; **Finalized on:** 31-01-2015.

ABSTRACT

Morphine and Tramadol remain the primary targets in clinical and forensic urine drug testing. UPLC–MS triple stage quadrupole is used for the simultaneous analysis of morphine and tramadol in urine of ten healthy volunteers after a single exposure are developed and validated. Urine samples were collected with 6 h intervals for three days after drug administration using solid phase extraction. Stability study is performed on urine samples after 24 h single dose co-administration. The detection of endogenous morphine and tramadol in human urine has an average value of 3.4 and 155.7 ng/ml after 72 h. Recovery values were not less than 98.12 % for all drugs. The potential degradation of both drugs under various stress conditions over one month is shown. The proposed method was found to be a suitable technique for simultaneous determination of both drugs in human urine. Possible toxicity could happen when both drugs are co-administered in higher as concluded from both drug excretions.

Keywords: Morphine; Tramadol; single dose; SPE; LC-MS.

INTRODUCTION

Opiates like MOR (MOR) and its synthetic analogues like TRM (TRM) are among the most widely abused drugs in every social and economic strata of society. Although they are highly addictive, they are commonly prescribed after surgery, trauma, or for chronic pain relief.[1] Because of the high consumption of these substances worldwide, they are involved in many forensic cases.[2] The influence of these drugs on driving and in jobs in which the presence of high serum concentrations in employees could be dangerous has been documented.[3] These drugs also play a role in drug facilitated sexual assault (DFSA).[4]

Measurement of [free]/[total] MOR ratio have been routinely carried out by various methods including radio-immunoassay and GC–MS.[5] This approach employs measuring total morphine directly using glucuronic acid resulting by acid hydrolysis or glucuronidase enzyme of the molecule.[6] Acid hydrolysis appeared to be more efficient at both positions of the morphine glucuronide molecule and is more suitable for urine.[7] In order to negate the need for deconjugation, a number of techniques have been published that measure M3G and M6G directly, such as HPLC with fluorescence, dual electrochemical and spectrophotometric detection.[8,9] Recently, various papers have described the quantification of morphine and glucuronides by HPLC–MS, which offers significantly more sensitivity and specificity.[10-12]

Several methods for determination of TRM concentration in human urine have been reported. HPLC-based methods with UV detection have been employed for determination of relatively higher concentrations of TRM and its metabolites.[13,14] An electrochemical method has been also reported.[15] LC/MS has been used in the identification, structure characterization and quantitative analysis of TRM and its metabolites.[16-20]

Recently, the co-administration of both MOR and TMR is relevantly observed in several forensic cases without any analytical or toxicity studies. So, this paper describes an accurate, rapid, low sample volume, specific and validated LC–MS method using SPE for the quantification of MOR and TRM showing excretion of both drugs when taken together as a single dose. Also, the degradation effect at different conditions, e.g. sunlight, room temperature and refrigeration on urine samples after 24 h single oral dose co-administration over one month is also discussed.

MATERIALS AND METHODS

Apparatus

Mass spectra is performed using a TSQ Quantum Access MAX triple stage quadrupole mass spectrometer equipped with an electrospray (ESI) ionization source. An Accela U-HPLC equipped with Accela 1250 quaternary pumps and Xcalibur software (version 2.2) was used. The optimized parameters were as follows: turbo ion spray temperature 400 °C, capillary temperature (270 °C), sheath gas (20 psi) and auxiliary gas (2 psi).

Chemicals

All solvents were HPLC grade. Diazepam (DZ), morphine and tramadol (99.9%) were kindly gifted from EIPICO and ADWIA (10th of Ramadan, Egypt) and Misr Co. (Cairo, Egypt), respectively. Solid phase extraction SPE (Strata™-

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.

X-Drug B33 μm, Cation Mixed-Mode Polymeric Sorbent, 60 mg/6ml) was obtained from Phenomenex Inc. Hydrochloric acid, potassium hydroxide, ammonium hydroxide; formic acid and sodium acetate were purchased from Sigma–Aldrich. Isopropanol, ethyl acetate, acetonitrile and PTFE filters (0.2μm×25mm) were supplied by Merck. Deionised water (18.1 MΩ) was obtained from a Millipore-Q water system.

Standard Solutions

Due to the adsorption of MOR and its glucuronide metabolites to the glassware, all stock and working solutions were prepared in plastic tubes or silanized glassware.[21] Stock solutions of DZ, MOR and TRM were prepared in methanol (1 mg/ml). They were stored frozen at -20 °C for no more than two months. Aliquots of the working solutions were evaporated under nitrogen and then reconstituted in urine so that the calibration range of MOR 10 ng/ml-15μg/ml and 10 ng/ml-45 μg/ml for TRM were achieved.

Treatment and Sampling

This investigation conforms to the Egyptian Community guidelines for the use of humans in experiments. The Human Ethics Committee of Faculty of pharmacy, Suez Canal University, approved the study (license no. is 20148H3). Ten healthy male (normal liver, kidney functions and electrocardiogram), informed, adult volunteers were instructed to abstain from all medications for 2 weeks before single oral administration. MOR immediate release syrup (morphine hydrochloride® 2 mg/ml, ORDINE®, GSK, Australia) and TRM immediate release tablet (Amadol® 50 mg ADWIA, 10th of Ramadan, Egypt) were used. Urine was collected in 100 ml amber glass containers before intake and every 6 h for three days after administration. The spiked and real samples were then frozen quickly and stored at -20 °C immediately before the analysis; the samples were thawed at room temperature.

Solid-Phase Extraction Method

For each 2 mL mixture of centrifuged urine sample (spiked and real) and the internal standard (100ng/mL), add 500 μl of conc. hydrochloric acid and heat at 90 °C for 2 h. Add 2 mL of 200 mM sodium acetate buffer (pH 4.0) followed by 1 ml 6 N KOH, centrifuge for 5 minutes at 5000 rpm and verify that pH is 4.0-6.0.

In this method, Strata™-X-Drug B 60 mg/6 ml (SPE) cartridges was used. After initial sample preparation, 2 ml urine of the real sample or spiked standard was loaded onto the column and allowed to elute at approximately 1 ml/min. The column was washed with 2 ml of 100 mM sodium acetate buffer (pH 5.0), followed by 2 ml methanol and then flow dried under full vacuum for 10 min. Elution was achieved with 2 ml of ethyl acetate/isopropanol/ammonium hydroxide (70:20:10). The eluent was collected and evaporated to dryness under air at 50 °C and reconstituted with 1 ml acetonitrile. This was transferred to HPLC vial insert for injection. The injection volume was (10 μl).[22]

LC–MS Conditions

Chromatographic separation was performed on Hypersil-Gold C18 column, 20 mm x 2.0 mm (1.9 μm) (Thermo scientific, New York, USA). Gradient elution was performed at room temperature with0.1% formic acid (mobile phase A) and acetonitrile (mobile phase B) at flow rate 250 μl/m, injection volume 10 μl, total run time for each sample 5 min, as follows: 0–0.5 min 10-20% mobile phase B, 0.5–0.75 min 20–30% mobile phase B, 0.75–1.00 min 30-40% mobile phase B, 1.00–1.25 min 40-50% mobile phase B, 1.25–1.50 min 50-60% mobile phase B, 1.50–2.50 min 60-70% mobile phase B, 2.50-3.00 min 70-80% mobile phase B, 3.00-3.50 min 80-90% mobile phase B, 3.5-4.00 min 90-10% mobile phase B, 4-4.5 min 10-20% mobile phase B, 4.5-5.00 min 20-30% mobile phase B.

The following MRM transitions are recorded: m/z 286.3/286.3, 286.3/165.2 and 286.3/201.1for MOR; 264.2/58.2, 264.2/264.2 and 264.2/246.2 for TRM and 284.6/284.6, 284.6/193.1 and 284.6/153.9 for DZ. Ion spray voltage (3600 V), Capillary offset (35) and three different collision energy (CE) 38, 24; 16, 11 and 31, 26eV., and cone voltage 43, 20 and 43 V, respectively, were investigated for all analytes. The mass spectrometric conditions were optimized for each compound by continuously infusing a standard solution (1 μg/ml in mobile phase) at 20μl/min.

Validation

Preparation of Urine Quality Controls

Quality control urine samples were screened for drug free using the assay procedure, 62.5, 250 and 500ng/ml were used during clinical analysis. All QCs, working standards, and stock solutions were stored frozen at-20 °C prior to use. DZ as an internal standard (I.S.) was used to prepare 100 ng/ml during solid-phase extraction.

Accuracy and Precision

Accuracy, intra- and inter-day precisions for all analytes were evaluated according to the requirements of FDA guideline on bioanalytical method validation.[23] Urine standards of two concentrations 45 μg/ml rations over the tested range (10 ng/ml, LQC) for MOR and TRM, (15μg/ml and 45μg/ml, HQC) for MOR and TRM were analyzed daily over 7 days to determine the intra- and inter-day accuracy and precision values. Accuracy was determined by the percentage deviation of the mean calculated concentration compared to the spiked concentration. Precision was determined by calculating the coefficient of variation (CV %) at each concentration level based on the mean concentration and the standard deviation.

Stability

Determination of the degradation percent of these drugs were calculated on urine samples occurring after 24 h

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.

intake under various stress conditions: (A) sunlight, (B) room temperature,(C) refrigeration at 5 °C during time intervals 1, 7, 14, 21 and 30 days. 100 ml urine samples were exposed in transparent glass containers to the mentioned conditions.

Matrix Effects

The extraction efficiency was determined by injecting five replicates at 10ng/ml and 15 µg/ml for MOR and 10 ng/ml and 45 µg/ml for TRM. Blank urine was fortified with analyte solution and internal standard before and after SPE. Matrix effect was calculated by dividing peak area of each analyte and the internal standard in samples from set 2 (five extracts of each different drug-free urine spiked with analytes after extraction) by those in samples from set 1 (five neat standards) matrix effects were evaluated according to.[24] Peak area ratios (analyte/I.S.) were used for determination of concentration from extracted matrix.

Carryover

Carryover was evaluated by injecting blank urine containing I.S. immediately after a sample spiked with 100 ng/ml of all target analytes. The measured concentration of the blank sample was used to calculate the carryover rate. Carryover was considered negligible if the measured concentration was below the LOQ.

Recoveries

The relative recoveries at all QCs concentrations and limit of quantifications were measured by comparing the response obtained for samples that were subjected to the extraction procedure with those obtained from blank urine extracts that were spiked post extraction to the same nominal concentrations. Recoveries were calculated using the peak ratio (peak area of analyte divided by peak area of I.S.).

RESULTS AND DISCUSSION

The method was validated for the quantification of MOR and TRM as shown in Table 1. Calibration curve for MOR and TRM were linear over the range 10 ng/ml - 15 µg/ml and 10 ng/ml – 45 µg/ml with a correlation coefficient (r^2 value) of 0.9999 with no interferences with any of the standards.

Accuracy and precision were calculated for all real samples and RSD% was found to be less than 1%. The detection limit was calculated for each analyte based on the blank urine noise (below 2ng/ml for the MRM). Identification was based on three MRM transitions for each analyte. Transition ion ratios were also calculated for all analytes. In order to satisfy this identification criterion, it was found that an analyte concentration of 10 ng/ml (LQC) and 15 µg/mL (HQC) was required for MOR. However, for TRM the satisfactory of identification criterion was 10 ng/ml (LQC) and 45 µg/ml (HQC), there was often sufficient ion intensity for successful EPI formation to enable library matching. Production of a specific EPI provides a significant advantage over existing methods which incorporate tandem mass only. Recovery of MOR and TRM in all spiked analytes was found to be greater than 98.12%.

The limit of detection (LOD, signal-to-noise, higher than 3:1) was found to be 0.3 ng/ml and 1 ng/ml for MOR and TRM. The limit of quantification (LOQ, signal-to-noise, higher than10:1) was found to be 10 ng/ml for both MOR and TRM. The LOQ was to be measured with a relative standard deviation percent (RSD, %) less than 10% for accuracy and precision.

MOR, TRM and DZ were shown to be stable over 2 months period, urine standards are always stored in the freezer (-20 °C), this was not considered a significant issue for casework. With regards stability of extracts, no observable decrease in concentration was seen in the urine standards stored at room temperature for 2 h. However, if extracts are stored overnight at room temperature, significant decreases in all analytes are seen. Therefore, samples are extracted and analyzed on the same day and are not stored prior to analysis.

Analysis of isostructural chemical compounds by MS is complicated because some often have a molecular weight as well as a basic structure in common, which can produce same major MRM transitions and retention times. Among the analytes in the present study MOR have the molecular formula ($C_{17}H_{19}NO_3$) and TRM has a structure formula ($C_{16}H_{25}NO_2$), a structure based on morphinan. However, different MRM transitions with good sensitivity, in spite of not being major ones, were chosen for MOR and TRM. In addition to this, gradient analysis made it possible to separate both compounds on chromatography. LC–MS method was explored to save time and to avoid using the toxic derivatization reagents used by the former GC–MS methods. Efficient chromatographic separation with narrow and symmetrical peaks is important to obtain a selective LC–MS method and to reduce the possibility of ion suppression and/or ion enhancement in the MS source.[24,25]

Focusing of the analytes on the column inlet at the gradient start minimizes the effect of pre-column peak broadening and gives a better control of the chromatographic separation. Hence, conditions providing increased retention of the most polar compounds are favorable. To minimize the LC–MS instrument operation time and to ease the operator job, short analysis time is desirable as well. The mean urine concentration–time curves of MOR and TRM are depicted in (Fig. 1).

The mean maximum excretion concentration of both drugs in the urine was 23.7 ng/ml and 22 µg/ml for MOR and TRM. It obtained after 24h of single dose co-administration of 2 mg/ml MOR and 50 mg/ml TRM. The mean minimum excretion concentration of both drugs in the urine was 3.4 ng/ml for MOR and 155.7 ng/ml for TRM which is obtained after 72 h (Fig. 2).

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.

The mean urine excretion concentrations of both drugs with 6 h intervals for 3 days are shown in (Table 2).

The severe decrease in the excretion of MOR in urine may be due to fitting and greater affinity of MOR to μ receptors compared to TRM (approximately 6000 fold) resulting in the high excretion rate of unchanged TRM.[26] Thus, our study can explain properly why possible toxicity could happen when both drugs are co-administered together leading to possible death in different forensic cases.

After 24 h administration, proper sample collection and storage under various stress conditions (sunlight, room temperature and refrigeration at 5 °C) during time intervals 24 h, 7, 14, 21 and 30 days.

The mean concentration–time stability curve of urine samples of MOR and TRM are depicted in (Fig. 3).

There are significant degradation effects of sunlight than when stored at room temperature on both drugs. Refrigeration showed up a non-significant effect on TRM however, there was a significant degradation effect on MOR. This is may be due to adsorption of MOR glassware as shown before (Table 3).

Table 1: Validation Data for the quantification of MOR and TRM (n=15).

TRM			MOR			Parameters
264/246	264/58	264/264	286/201	286/165	286/286	MRMs
1.22	1	1	0.9	0.3	0.4	LOD (ng/mL)
10.15	10.27	10.04	10.36	10.12	10.42	LOQ (ng/mL)
 9.52 44.96	 9.83 44.98	 9.94 44.97	 9.61 14.95	 9.72 14.96	 9.93 14.92	Inter-day (mean: n=15) LQC (ng/mL) HQC (μg/mL)
 5.33 3.84	 2.12 1.14	 2.63 2.11	 3.42 2.93	 4.22 3.21	 2.91 2.72	Inter-day accuracy LQC (%deviation) HQC (%deviation)
 4.11 5.87	 2.74 5.93	 6.42 5.64	 5.11 6.13	 2.93 4.21	 4.22 3.81	Inter-day precision LQC (%CV) HQC (%CV)
 10.43 44.97	 9.91 44.99	 10.15 44.98	 9.92 14.97	 9.81 14.97	 9.13 14.98	Intra-day (mean: n=15) LQC (ng/mL) HQC (μg/mL)
 3.22 0.63	 1.64 0.92	 1.91 2.34	 2.73 3.80	 3.64 1.72	 2.42 0.94	Intra-day accuracy LQC (%deviation) HQC (%deviation)
 3.62 5.34	 3.13 4.90	 5.23 3.92	 4.72 3.11	 3.10 2.84	 1.43 1.92	Intra-day precision LQC (%CV) HQC (%CV)
 98.72 99.53	 99.47 98.68	 99.81 99.63	 98.12 99.31	 99.64 99.26	 99.53 99.28	Recovery (%) LQC HQC

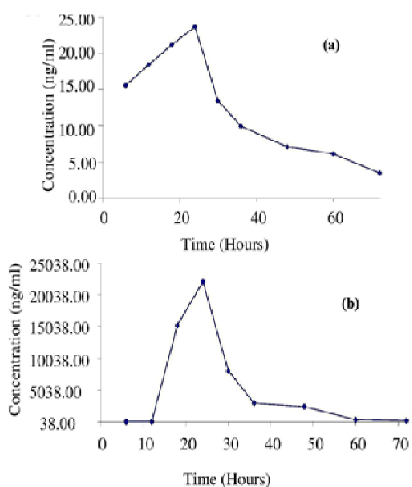

Figure 1: The mean concentration curves (ng/ml) of (a) MOR and (b) TRM excretion for 3 days.

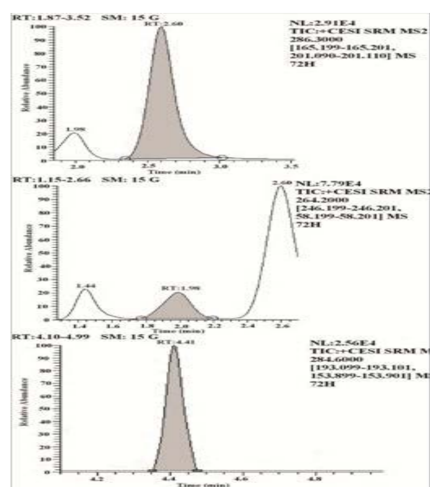

Figure 2: The excretion chromatogram of both MOR and TRM after 72 h of oral dose co-adminstration.

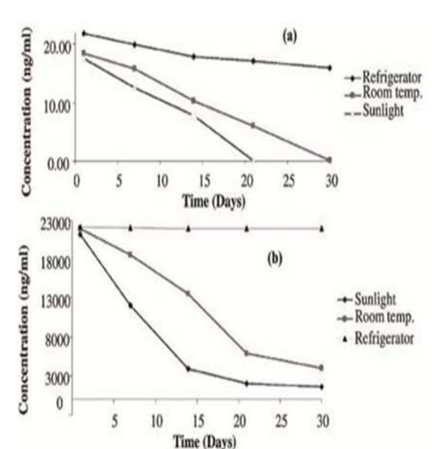

Figure 3: Mean concentration of (a) MOR and (b) TRM stability (room temperature, sunlight and refrigerator).

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.

Table 2: Mean Urine Excretion of MOR and TRM concentrations after Co-administration at 6 h interval for 3 days.

	MOR				TRM			
Time (h)	Conc. (ng/ml)	RT[a]	RSD[b] %	RE[c] %	Conc. (µg/ml)	RT[a]	RSD[b] %	RE[c] %
6 h	15.52	2.64	0.1	0.02	0.04	2.01	0.02	0.05
12 h	18.37	2.64	0.01	0.02	0.05	2.05	0.03	0.08
18 h	21.21	2.60	0.01	0.01	15.23	2.01	0.01	0.03
24 h	23.65	2.60	0.004	0.01	22.19	2.01	0.01	0.04
30 h	13.48	2.60	0.01	0.02	8.03	2.01	0.02	0.04
36 h	9.91	2.60	0.01	0.02	2.93	2.01	0.02	0.05
48 h	7.04	2.60	0.01	0.01	2.33	2.01	0.01	0.02
60 h	6.14	2.60	0.01	0.03	0.35	2.01	0.02	0.04
72 h	3.42	2.60	0.002	0.005	0.16	1.98	0.02	0.06

a retention time; b relative standard deviation of concentration; c relative error

Table 3: The mean concentrations of MOR and TRM in urine samples stored under various stress conditions (room temperature, sunlight and refrigeration) at different time intervals.

Stress condition		MOR						TRM					
	Time (day)	Initial Conc. (ng/ml)	Conc. (ng/ml)	Degraded %	RE[a] %	RT[b]	RSD[c] %	Initial Conc. (µg/ml)	Conc. (µg/ml)	Degraded %	RE[a] %	RT[b]	RSD[c] %
Sunlight	1	23.65	17.57	25.65	0.01	2.64	0.21	22.19	21.23	4.31	0.02	1.98	0.01
	7	23.65	12.57	46.81	0.02	2.64	0.42	22.19	12.13	45.34	0.03	2.01	0.02
	14	23.65	7.83	66.88	0.01	2.53	0.31	22.19	3.91	82.39	0.04	1.98	0.02
	21	23.65	NF[d]	NF[d]	NF[d]	NF[d]	NF[d]	22.19	2.02	90.90	0.02	1.97	0.01
	30	23.65	NF[d]	NF[d]	NF[d]	NF[d]	NF[d]	22.19	1.56	92.95	0.02	1.91	0.01
Room Temp.	1	23.65	18.58	21.41	0.02	2.56	0.77	22.19	21.99	0.86	0.03	1.89	0.02
	7	23.65	15.68	33.67	0.03	2.52	0.84	22.19	18.56	16.32	0.03	1.86	0.01
	14	23.65	10.32	56.33	0.02	2.60	0.32	22.19	13.58	38.80	0.02	1.98	0.01
	21	23.65	5.87	75.17	0.01	2.54	0.64	22.19	5.84	73.66	0.03	2.01	0.02
	30	23.65	NF[d]	NF[d]	NF[d]	NF[d]	NF[d]	22.19	4.02	81.87	0.04	1.92	0.02
Refrigerator	1	23.65	21.88	7.43	0.02	2.58	0.36	22.19	22.17	0.07	0.03	1.95	0.01
	7	23.65	19.88	15.90	0.04	2.62	0.68	22.19	22.06	0.56	0.04	1.90	0.02
	14	23.65	17.88	24.35	0.02	2.60	0.26	22.19	21.98	0.92	0.03	1.89	0.01
	21	23.65	17.07	27.77	0.01	2.63	0.73	22.19	21.93	1.15	0.03	1.93	0.01
	30	23.65	15.84	32.99	0.03	2.60	0.89	22.19	21.81	1.66	0.03	1.89	0.01

[a] relative error; [b] retention time; [c] relative standard deviation of concentration[d] not found

CONCLUSION

LC–MS is a powerful analytical technique for the determination of low levels of MOR and TRM in urine after a single oral dose co-administration. Our proposed method is a suitable technique for simultaneous determination of both drugs in human urine. It is simple, rapid, accurate, sensitive, cost-effective and utilizes SPE as a sensitive extraction method. LC-MS gradient chromatography makes the method suitable for analysis of a large number of samples to give a response to the increasing demand of justice. Our study can explain that possible toxicity could happen when both drugs administered orally together in higher doses in different forensic cases and the degradation effects when urine samples exposed to different stress conditions.

REFERENCES

1. Savage SR, Joranson DE, Covington EC, Definitions related to the medical use of opioids: evolution towards universal agreement, Journal of pain and symptom management, 26, 2003, 655-667.
2. Ojaniemi KK, Lintonen TP, Impinen AO, Trends in driving under the influence of drugs: a register-based study of DUID suspects during 1977–2007, Accident Analysis & Prevention, 41, 2009, 191-196.
3. Drummer OH, Gerostamoulos J, Batziris H, The involvement of drugs in drivers of motor vehicles killed in Australian road traffic crashes, Accident Analysis & Prevention, 36, 2004, 239-248.

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.

4. Akers RL, Rational choice, deterrence, and social learning theory in criminology: The path not taken, J Crim L & Criminology, 81, 1990, 653.

5. Meadway C, George S, Braithwaite R, A rapid GC–MS method for the determination of dihydrocodeine, codeine, norcodeine, morphine, normorphine and 6-MAM in urine, Forensic science international, 127, 2002, 136-141.

6. Wang P, Stone JA, Chen KH, Incomplete recovery of prescription opioids in urine using enzymatic hydrolysis of glucuronide metabolites, Journal of analytical toxicology, 30, 2006, 570-575.

7. Romberg RW, Lee L, Comparison of the hydrolysis rates of morphine-3-glucuronide and morphine-6-glucuronide with acid and beta-glucuronidase, J Anal Toxicol, 19, 1995, 157-162.

8. Aderjan R, Hofmann S, Schmitt G, Morphine and morphine glucuronides in serum of heroin consumers and in heroin-related deaths determined by HPLC with native fluorescence detection, Journal of analytical toxicology, 19, 1995, 163-168.

9. Gerostamoulos J, Drummer OH, Solid phase extraction of morphine and its metabolites from postmortem blood, Forensic science international, 77, 1996, 53-63.

10. Murphy CM, Huestis MA, LC-ESI-MS/MS analysis for the quantification of morphine, codeine, morphine-3-beta-D-glucuronide, morphine-6-beta-D-glucuronide, and codeine-6-beta-D-glucuronide in human urine, Journal of mass spectrometry, 40, 2005, 1412-1416.

11. Musshoff F, Trafkowski J, Kuepper U, An automated and fully validated LC-MS/MS procedure for the simultaneous determination of 11 opioids used in palliative care, with 5 of their metabolites, Journal of mass spectrometry, 41, 2006, 633-640.

12. Gustavsson E, Andersson M, Stephanson N, Validation of direct injection electrospray LC-MS/MS for confirmation of opiates in urine drug testing, Journal of mass spectrometry, 42, 2007, 881-889.

13. Ebrahimzadeh H, Yamini Y, Sedighi A, Determination of tramadol in human plasma and urine samples using liquid phase microextraction with back extraction combined with high performance liquid chromatography, Journal of Chromatography B, 863, 2008, 229-234.

14. Javanbakht M, Moein MM, Akbari-adergani B, On-line clean-up and determination of tramadol in human plasma and urine samples using molecularly imprinted monolithic column coupling with HPLC, Journal of Chromatography B, 911, 2012, 49-54.

15. Valle M, Pavon J, Calvo R, Simultaneous determination of tramadol and its major active metabolite- O-demethyltramadol by high-performance liquid chromatography with electrochemical detection, Journal of Chromatography B: Biomedical Sciences and Applications, 724, 1999, 83-89.

16. Malonne H, Sonet B, Streel B, Pharmacokinetic evaluation of a new oral sustained release dosage form of tramadol, British journal of clinical pharmacology, 57, 2004, 270-278.

17. Ardakani YH, Rouini MR, Improved liquid chromatographic method for the simultaneous determination of tramadol and its three main metabolites in human plasma, urine and saliva, Journal of pharmaceutical and biomedical analysis, 44, 2007, 1168-1173.

18. Verplaetse R, Tytgat J, Development and validation of a sensitive UPLC–MS/MS method for the analysis of narcotic analgesics in urine and whole blood in forensic context, Forensic science international, 215, 2012, 136-145.

19. Al-Saffar Y, Stephanson NN, Beck O, Multi component LC–MS/MS screening method for detection of new psychoactive drugs in urine—Experience from the Swedish population, Journal of Chromatography B, 930, 2013, 112-120.

20. Saccomanni G, Del Carlo S, Giorgi M, Determination of tramadol and metabolites by HPLC-FL and HPLC–MS/MS in urine of dogs, Journal of pharmaceutical and biomedical analysis, 53, 2010, 194-199.

21. Heinig K, Wirz T, Bucheli F, Sensitive determination of a pharmaceutical compound and its metabolites in human plasma by ultra-high performance liquid chromatography–tandem mass spectrometry with on-line solid-phase extraction, Journal of pharmaceutical and biomedical analysis, 54, 2011, 742-749.

22. Michael Rummel MT, Seyed Sadjadi, and Erica Pike, A Simplified and Streamlined Approach to Solid Phase Extraction using Strata™-X-Drug B for SAMHSA's Low Cutoff Drugs of Abuse Panel. Available from https://phenomenexblobcorewindowsnet/documents/f87ba1cb-4f9e-4312-ac61-8dbdd64c0e97pdf: Phenomenex, Inc., 411 Madrid Ave., Torrance, CA, 90501, USA.

23. FDA C. Guidance for industry: bioanalytical method validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CV). 2001.

24. Matuszewski B, Constanzer M, Chavez-Eng C, Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS, Analytical Chemistry, 75, 2003, 3019-3030.

25. Chambers E, Wagrowski-Diehl DM, Lu Z, Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses, Journal of Chromatography B, 852, 2007, 22-34.

26. Raffa RB, Friderichs E, Reimann W, Opioid and nonopioid components independently contribute to the mechanism of action of tramadol, an 'atypical' opioid analgesic, Journal of Pharmacology and Experimental Therapeutics, 260, 1992, 275-285.

Source of Support: Nil, **Conflict of Interest:** None.

© Copyright protected. Unauthorised republication, reproduction, distribution, dissemination and copying of this document in whole or in part is strictly prohibited.