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Article

New HPLC Method to Detect Individual Opioids (Heroin and Tramadol) and their Metabolites in the Blood of Rats on Combination Treatment

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Drug abuse is both an age-old and a constantly evolving problem in society. Trends in illicit drug use are highly fluid, with new formulations increasing in popularity. For this reason, methods for illicit drug detection and analysis need to be continually updated so they remain useful and relevant. A recent trend in street heroin production has seen it diluted with large amounts of tramadol in addition to the classical diluents such as acetaminophen and caffeine. This study describes a sensitive, simple and accurate high-performance liquid chromatographic method with ultraviolet detection for the simultaneous detection of heroin, 6-acetylmorphine, morphine, tramadol and O-desmethyltramadol in the blood of rats using a liquid–liquid back-extraction method. The separation was performed on LichroCART RP-18e with particle size of 5 μm (250 × 4.6 mm) with mobile phase acetonitrile–50 mM KH_2PO_4 buffer, pH 7.1, using a gradient mode with a 1.0 mL/min flow rate. The calibration curves were linear in the concentration ranges 0.25–100 and 0.1–100 μg/mL for morphine and other analytes, respectively. Recovery values for the substances ranged between 59 and 83%. This technique was successfully used in pharmacokinetic studies measuring 6-acetylmorphine, morphine, tramadol and O-desmethyltramadol in the blood of rats intraperitoneally treated with a blend of 10 mg/kg heroin and 70 mg/kg tramadol. This technique shows promise for analysis of confiscated street heroin.

Introduction

Heroin is an illicit narcotic abused by millions of people worldwide. It is a semisynthetic analogue of morphine, prepared by the acetylation of morphine at elevated temperature. Most heroin seized by authorities is found to have been diluted or otherwise adulterated by dealers (1). In Egypt, a sample of every seized drug is sent to the Forensic Toxicology Lab of the Forensic Medicine Authority for chemical analysis. These drug analyses are increasingly detecting high concentrations of the adulterant tramadol (Tr). In most of street heroin seizures, samples contain high concentrations of Tr as an adulterant, as do many blood samples collected from abusers, which have contained the markers of heroin and Tr (T. Mahdy, personal data).

In humans, heroin crosses the blood–brain barrier more readily than morphine because of its acetyl-groups. Heroin is known to have an extremely short half-life (2–5 min) in human plasma because it is rapidly deacetylated to 6-monoacetylmorphine (6-AM), which is then deacetylated to morphine (M) by serum-esterases and liver carboxylesterases (2, 3) (Figure 1A). The pharmacological action of heroin is attributed to the formation of 6-AM and M (4). Because both 6-AM and M are pharmacologically active, the effects produced by a particular dose of heroin reflect the combined action of these metabolites (5). Thus, analysis to detect both 6-AM and M in biological fluids has been proposed to confirm heroin use.

Tr hydrochloride is a centrally acting analgesic, used in the treatment of moderate to severe and acute to chronic pain (6). Its mechanism of action is similar to that of an opiate agonist, in that it has selective activity at the opioid receptors (7). It inhibits reuptake of norepinephrine and serotonin, which appears to contribute to the drug's analgesic effect (8). Tr has been found to produce several positive responses in vertebrates (9). Tr is rapidly absorbed after oral administration and has a bioavailability of 65–70% due to first-pass metabolism (10). It is extensively metabolized in the liver by cytochrome P450 2D6 to O-desmethyltramadol (M1) and *N*-desmethyltramadol. The metabolite M1 is pharmacologically active and is largely responsible for the analgesic efficacy of Tr (11) (Figure 1B).

A variety of techniques have been described in the literature for the analysis of heroin and its metabolites by high-performance liquid chromatography (HPLC) in street samples (12, 13) and in biological specimens such as human plasma (14), human whole blood (15, 16) and urine (17). Simultaneous quantification of Tr and its metabolites in urine (18), saliva (19), plasma (20, 21) liver, bile, kidney (22) and brain tissue of rodents (23), using different analytical techniques, particularly HPLC coupled by ultraviolet (UV) (24, 25), fluorescence (22, 26, 27) or electrochemical detection (28), have also been reported. However, there is a lack of data for the simultaneous determination of heroin, M, 6-AM, Tr and M1 in biological specimens via these methods. To address this shortfall, the primary aim of this study was to develop and validate a new HPLC–UV method to simultaneously detect these substances in the blood of rats treated with a combination of the two opioids. Second, this method was also tested for street heroin seizure analyses.

Experimental

Chemicals and reagents

Pure heroin, M, 6-AM, Tr, M1 and nalorphine (>99.0% purity) were supplied by LCG Promochem (Milan, Italy). Pure caffeine (CA) and acetaminophene (AC) analytical standards (>98.0 % purity) were purchased from Sigma-Aldrich (St. Louis, MO).

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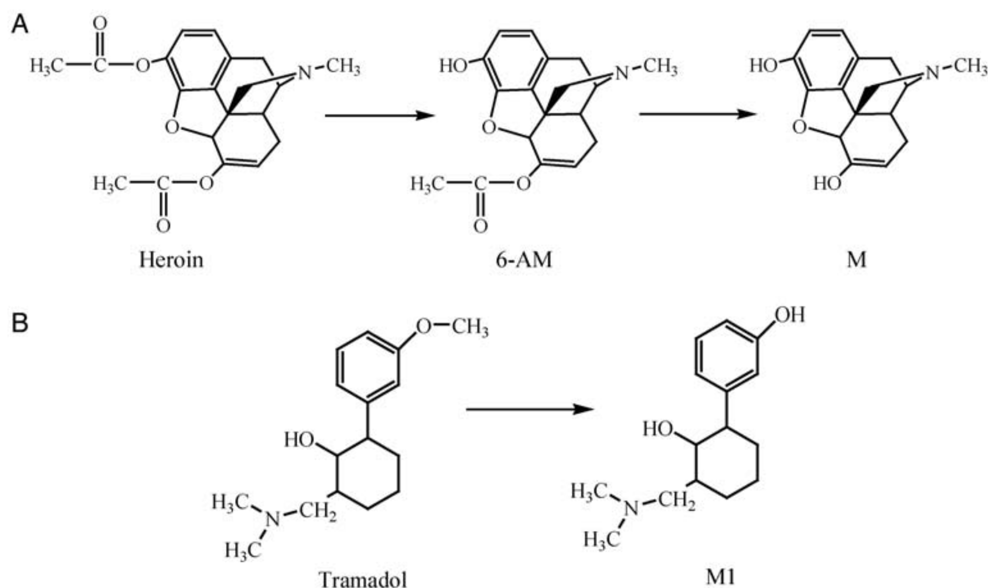

Figure 1. Molecular structures: heroin, 6-AM and M (A); Tr and M1 (B).

Nalorphine was used as internal standard (IS). HPLC-grade acetonitrile (ACN), methanol (MeOH), isopropanol (IP), chloroform ($CHCl_3$), ethyl acetate (AcOEt), methyl tertiary butyl ether (MTBE), and *n*-hexane (C_6H_{14}) were purchased from Merck (Darmstadt, Germany). Deionized water was produced by a Milli-Q Millipore Water System (Milford, MA). Potassium dihydrogen phosphate (KH_2PO_4) and sodium tetraborate decahydrate ($Na_2B_4O_7$ $10H_2O$) were from J.T. Baker (Deventer, Holland). All other reagents and materials were of analytical grade and supplied from commercial sources. The aqueous and organic components of the mobile phase, degassed under pressure, were mixed by the HPLC. The LC mobile phases were filtered through 0.2-μm cellulose acetate membrane filters (Sartorius Stedim Biotech S.A.; Aubagne Cedex, France) with a solvent filtration apparatus.

Standard solutions

Singular stock solutions of heroin, M, 6-AM, Tr, M1 and IS were prepared to a concentration of 1,000 μg/mL in MeOH using volumetric flasks. These were then stored at −20°C. To obtain a final concentration of 100 μg/mL, appropriate dilutions of stock standard solutions were prepared by diluting 1 mL of each solution to 10 mL. These solutions of heroin, M, 6-AM, Tr, M1 and IS were serially diluted in glass tubes (10 mL) to reach final concentrations of 10, 5, 1, 0.5 and 0.1 μg/mL. These were then stored at −20°C.

Instrumentation and chromatographic conditions

The ThermoFinnigan HPLC system consisted of a Spectra System P 2000 pump coupled with a Spectra System UV-Vis 2000 variable detector set at 285 nm, a Spectra System AS 3000 autosampler-processor with a variable loop (1–100 μL) and a SCM 1000 vacuum membrane degasser. Data were processed by ChromQuest 4.1 software (ThermoFinnigan, San Jose, CA). The analytical column was a LichroCART RP-18e with particle size of 5 μm (250 × 4.6 mm i.d.) maintained at room temperature (23–25°C). It was coupled to a Security Guard Cartridge with the same stationary phase (Phenomenex-USA) (5.0 μm: 4.0 × 3.0 mm i.d.).

The mobile phase consisted of acetonitrile (A)–50 μM KH_2PO_4 buffer (B) adjusted to pH 7.1 with NaOH at a flow rate of 1 mL/min. The analyses were carried out in gradient mode (Table I).

Table I
Composition of Mobile Phase*

Time (min)	A%	B%
0	5	95
5	10	90
10	20	80
12	25	75
25	25	75
30	5	95

*Note: A, acetonitrile; B, phosphate buffer, pH 7.1.

Animal treatment and sampling

Animal experiments were conducted at the animal experimental facility of the Faculty of Veterinary Medicine (University of Pisa). Animal care and handling was performed according to the provision of the EC council Directive 86/609 EEC. The study protocol was approved by the University of Pisa's ethics committee for animal welfare (CEASA) [authorization number 8630 (issued on July 1, 2011)] and transmitted to the Italian Ministry of Health.

Sixteen Wistar breed rats weighing 170 to 215 g were used for the experiment. The rats were randomly divided into eight groups consisting of two animals. These rats were housed two per cage, under conventional ventilation, temperature (18–22°C) and lighting (16 h light/day) conditions. During the study, they were given free access to water and food. The health of the rats was monitored daily by qualified personnel supervised by a veterinarian for the duration of the study. The

animals were given one week to adjust to their new environment before commencement of the experiment. During this adjustment period, all animals were kept on drug-free feed. After the adjustment period, Group I received a single injection of saline while the other groups received a combination of Tr at 70 mg/kg and heroin at 10 mg/kg; all injections were given intraperitoneally. The animal groups were sacrificed after 15 min (II), 30 min (III), 1 (IV), 2 (V), 6 (VI), 10 (VII) and 24 (VIII) h, respectively, following administration. The animals were decapitated and the blood samples collected immediately. All samples were immediately frozen at −20°C until analysis (within five days).

Sample extraction

The procedure was performed in a 15-mL screw-capped polypropylene vial. A 500-μL aliquot of whole blood was added to 50 μL of IS (5 μg/mL). After vortexing for 30 s, 250 μL of $Na_2B_4O_7$ $10H_2O$ buffer (0.2M, pH 9.0) was added and the sample was vortexed again. Five mL of MTBE was then added, then the sample was vortexed (30 s), shaken (60 osc/min, 10 min) and centrifuged at 2,191 g (rotor radius 10 cm) for 10 min. Four mL of the supernatant was collected in a clean screw-capped polypropylene vial containing 1 mL of 0.01M HCl. This latter blend was vortexed (30 s), shaken (60 osc/min, 5 min) and centrifuged at 2,191 g (rotor radius 10 cm) for 5 min. The organic layer was discarded. The aqueous layer was alkalinized with 0.5 mL of 0.2M borate buffer and the mixture was extracted with 5 mL of MTBE by shaking for 5 min followed by centrifugation at 2,191 g (rotor radius 10 cm) for 3 min. Four mL of organic layer was transferred to a 5-mL glass tube for complete evaporation under nitrogen flow in a 40°C water bath. The residue was reconstituted with 500 μL of a mobile phase of acetonitrile–KH_2PO_4 buffer (10:90) and 100 μL was injected onto the HPLC.

Bioanalytical method validation

The described method was validated in terms of linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, specificity, stability, precision and accuracy according to international guidelines on the bioanalytical method validation (29).

Calibration curves were obtained by spiking the blank matrix with a known concentration of each analyte and IS to provide concentrations of 0.1, 0.25, 0.50, 1, 5, 10, 25, 50 and 100 μg/mL. The calibration curves of peak area versus concentration (μg/mL) of the analytes were plotted. Least squares regression parameters for the calibration curves were calculated, and the concentrations of the test samples were interpolated from the regression parameters. Sample concentrations were determined by linear regression, using the formula $Y = mX + b$, where $Y =$ peak area, $X =$ concentration of the standard in μg/mL, m = the slope of the curve and b = the intercept with Y axis. Correlation coefficients for each of the calibration curves were > 0.998.

Within-run and between-run accuracy and precision were assessed on quality control samples (QC samples) and determined by replicate analysis using seven determinations of different concentration levels: LOQ (0.1 μg/mL; 0.25 μg/mL for M), low QC (0.5 and 1 μg/mL), medium QC (5, 10 and 25 μg/mL) and high QC (100 μg/mL).

Quantification

When unknown samples were assayed, a control and a fortified blank sample were processed simultaneously for quality control. LODs and LOQs were determined as analyte concentrations giving signal-to-noise ratios of 3 and 10, respectively.

Statistical analysis and pharmacokinetic analysis

The statistical analyses were evaluated using an analysis of variance (ANOVA) test. The results were presented as mean ± standard deviation (SD). All the analyses were conducted using GraphPad InStat (GraphPad Software; La Jolla, CA). For all the experiments, differences were considered significant if the associated probability level (P) was lower than 0.05. The pharmacokinetic calculations were carried out using WinNonLin v 5.2.1 (Pharsight Corp.; Sunnyvale, CA). Maximum concentration (C_{max}) for all the analytes in blood and the time required to reach C_{max} (T_{max}) were predicted from the data. The area under the concentration versus time curve ($AUC_{0-\infty}$) was calculated using the linear trapezoidal rule. Changes in blood concentrations for all the analytes were evaluated using the standard non-compartmental analysis and the relative pharmacokinetic parameters were determined using standard non-compartmental equations.

Results and Discussion

Detection method development

The mobile phase was chosen on the basis of a previously published method (30). The NaH_2PO_4 buffer was tested at different concentrations (0.01, 0.025, 0.05 and 0.1M). At the lower concentration, Tr and IS resulted in the same retention time. At 0.025M, the pure substances were well resolved; however, interfering peaks that overlapped with 6-AM, Tr and IS were produced when the substances were spiked into matrix. The analytes were well separated from blood impurities at buffer concentrations of 0.05 and 0.1M, and 0.05M was chosen as optimal because higher concentrations can cause salt precipitation in the HLPC.

A range of buffer pH (3.0 to 7.2) was assayed to optimize the chromatographic separation. In the pH range 3.0 to 5.5, the peaks of M1, 6-AM and IS overlapped. Optimal peak separation for pure analytes was produced using a pH ranging between 6 and 7.2. However, when the spiked matrix samples were tested, the peaks from some of the blood impurities obscured those of 6-AM and IS. A pH value of 7.1 was found to be optimal for the complete separation of analytes and impurities. However, heroin was the most sensitive to pH changes and this pH altered its retention time to 36 min. Fortunately, because of the rapid *in vivo* metabolism of heroin, this factor did not affect *in vivo* results. Tr and M1 were affected by pH to a lesser degree, while M, 6-AM and IS were relatively insensitive to pH change.

The final mobile phase resulted in acetonitrile–NaH_2PO_4 (0.05M), pH 7.1 with a 1 mL/min flow rate. This was found to be an excellent compromise in terms of sensitivity and peak separation.

The wavelengths tested in the present study were: 210 nm (31, 32), 275 nm (22) and 285 nm (30).

The maximum wave lengths for the investigated compounds were: heroin (279 and 299 nm), 6-AM (210 and 287 nm), M (210 and 285 nm), Tr and M1 (272 and 279 nm) and IS (285 nm). The wavelength value of 285 nm was found to be optimal in terms of sensitivity for all the analytes and avoiding several matrix impurities that became problematic at lower wavelengths.

The IS was chosen based on previous studies on M and 6-AM (14, 33, 34). It was also found to be appropriate for comparison with Tr and M1.

Optimization of the extraction method

The influence of the kind of solvents (an important tool for the selectivity of the method) was studied to find the optimal extraction protocol for the analytes. The solvents ethylacetate–*n*-hexane (4:1) (25), ethylacetate (21, 19), chloroform–isopropanol (9:1) (35), MTBE (20) and chloroform (commonly used in the Forensic Toxicology Lab; Cairo, Egypt) were compared. MTBE was selected as the most suitable organic solvent in terms of analyte extraction and minimization of matrix interference (Table II).

Optimal pH value for extraction was 9; further increases in alkalinity cause hydrolysis of 6-AM to M due to 6-AM's instability under alkaline conditions (14).

Method validation

It was necessary to validate each step of the analytical method because there are no published methodologies for the simultaneous determination of M, M1, 6-AM, Tr and IS from rat blood samples using HPLC–UV.

Table II
Single Extraction Recovery Percent (± SD) of M, M1, 6-AM, Tr and IS Spiked at 10 μg/mL with Different Organic Solvents ($n = 3$)

Organic solvents	Recovery				
	M	M1	6-AM	Tr	IS
Ethylacetate	40.2 ± 0.9	38.4 ± 0.6	41.9 ± 0.7	85.1 ± 5.4	44.47 ± 0.6
Ethylacetate–n-hexane (4:1)	45.4 ± 1.1	37.1 ± 0.3	43.1 ± 1.4	81.7 ± 6.9	32.40 ± 0.5
Chloroform	62.2 ± 2.4	59.7 ± 6.4	81.7 ± 3.4	76.0 ± 6.4	71.1 ± 4.3
Chloroform–isopropanol (9:1)	76.5 ± 3.4	42.1 ± 2.4	44.6 ± 5.2	40.1 ± 2.3	44.8 ± 3.4
MTBE	59.4 ± 5.3	64.5 ± 4.4	79.6 ± 3.2	83.7 ± 6.1	78.8 ± 3.5

The calibration curves were constructed by plotting the ratio of the peak areas versus concentrations in the working range. Good linearity was achieved for M, M1, 6-AM, Tr and IS in the range studied. The linear regression equations are reported in Table III.

According to Europea Medicine Evaluation Agency (EMEA) guidelines (29), LODs and LOQs were calculated based on a signal-to-noise approach. These calculations were performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples. In this way, the minimum concentration at which the analyte can be reliably quantified (LOQ) or detected (LOD) was determined. The typical signal-to-noise ratios were 10:1 and 3:1 for LOQ and LOD, respectively (Table III). Both the accuracy and the precision of these values lay within the proposed criteria [relative standard deviation (RSD) <20%].

Specificity and interference by co-eluting components were determined by comparing the chromatograms of different batches of blank matrices to those from spiked whole blood and test samples. It was found that under optimized chromatographic conditions, peaks due to the matrix did not interfere with M, M1, 6-AM, Tr and IS. Typical retention times for M, M1, 6-AM, Tr and IS were 13.25 ± 0.02, 14.96 ± 0.02, 18.34 ± 0.03, 19.93 ± 0.03 and 24.07 ± 0.05 min, respectively (Figures 2A, 2B, 2C, and 2D).

Recoveries were 59.4 ± 5.3 % for M, 64.5 ± 4.4% for M1, 79.6 ± 3.2% for 6-AM, 83.7 ± 6.1% for T and 78.8 ± 3.5% for IS. The respective coefficient of variation (CV) (%) values varied from 0.41 to 11.01, 0.19 to 5.31, 0.82 to 4.99, 0.75 to 4.6 and 1.06 to 4.41 for M, M1, 6-AM, Tr and IS, respectively. Intra-day value consistency (repeatability) was evaluated for five replicates of each QC sample during the same day. Inter-day value consistency (intermediate precision) was evaluated by quantization of M, M1, 6-AM, Tr and IS in QC samples on five different days. Relative errors for both the intra-day and inter-day accuracy were <7%.

Stability studies were performed to ensure good reproducibility of the method. Stock solutions of the analytes and IS (10 μg/mL) and high and low QC samples were tested for stability under short-term room temperature conditions, long-term storage conditions (−20°C) and freeze-thaw treatment. M, M1, Tr and IS were very stable at both +20°C for 24 h and −20°C for 30 days. 6-AM proved relatively unstable after 6 h at +20°C; a loss of 10–12% was observed and after 10 days the

Table III
Summary of Validation Data for M, M1, 6-AM, Tr and IS

Property	M	M1	6-AM	Tr	IS
Linear range (μg/mL)	0.1–100	0.1–100	0.1–100	0.1–100	0.1–100
Calibration equation	$y = 7778x - 1357$	$y = 24042x + 3580$	$y = 7907x + 880$	$y = 18890x + 18491$	$y = 7794x + 1624$
Correlation coefficient (r^2)	0.9997	0.9992	0.998	0.998	0.9993
Recovery (%)	59.4 ± 5.3	64.5 ± 4.4	79.6 ± 3.2	83.7 ± 6.1	78.8 ± 3.5
LOQ (μg/mL)	0.25	0.1	0.1	0.1	0.1
LOD (μg/mL)	0.08	0.03	0.03	0.03	0.03
Accuracy	85.8 ± 4.3	89.4 ± 3.5	97.8 ± 5.9	110 ± 4.6	—
Precision (%)					
Intra-day	0.41–11.01	0.19–5.31	0.82–4.99	0.75–4.6	1.06–4.41
Inter-day	1.15–4.34	0.6–6.01	0.56–3.74	1.42–5.73	1.20–5.32
Specificity	Specific	Specific	Specific	Specific	Specific

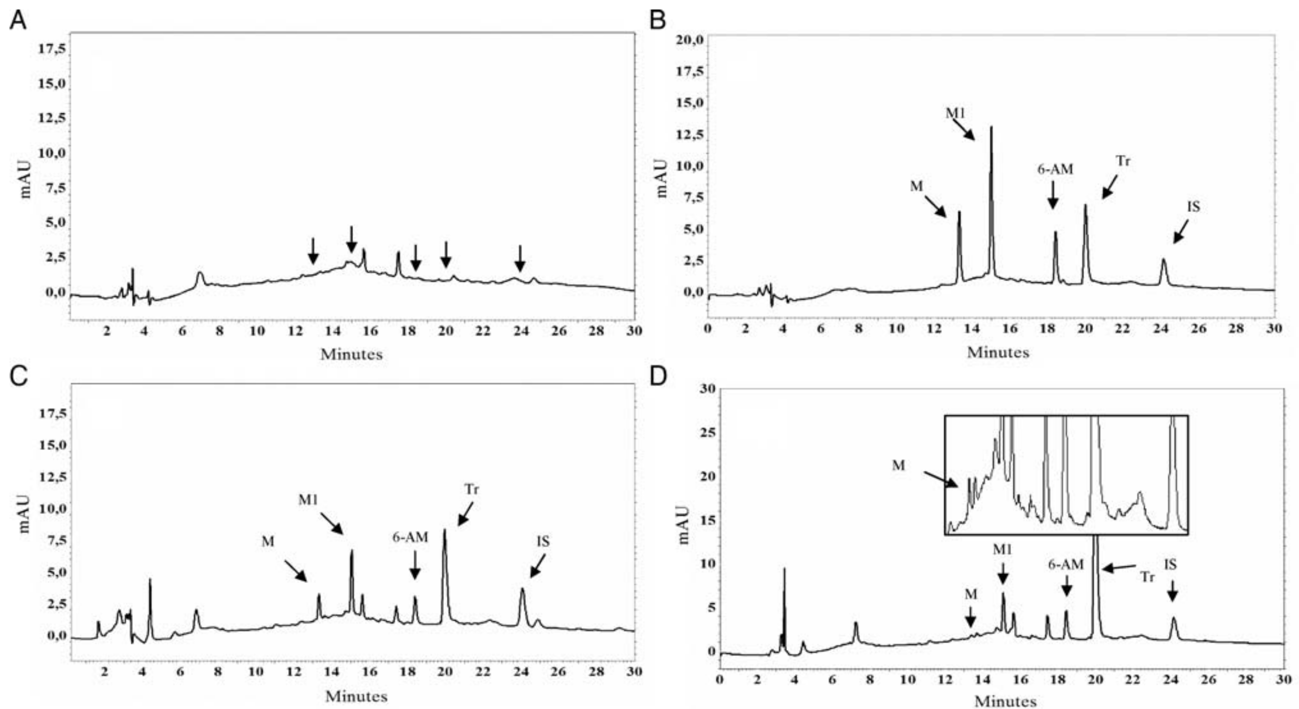

Figure 2. Chromatographic curve from blanks of Wistar rat blood (A); chromatographic curve from pure substances and IS (5 μg/mL) (B); chromatographic curve from fortified sample (5 μg/mL) of Wistar rat blood (C); chromatographic curve from blood sample collected from Wistar rat intraperitoneally injected with a combination of heroin (10 mg/kg) and Tr (70 mg/kg) (collection at 30 min): window reports a magnification of the chromatographic run showing M peak (D).

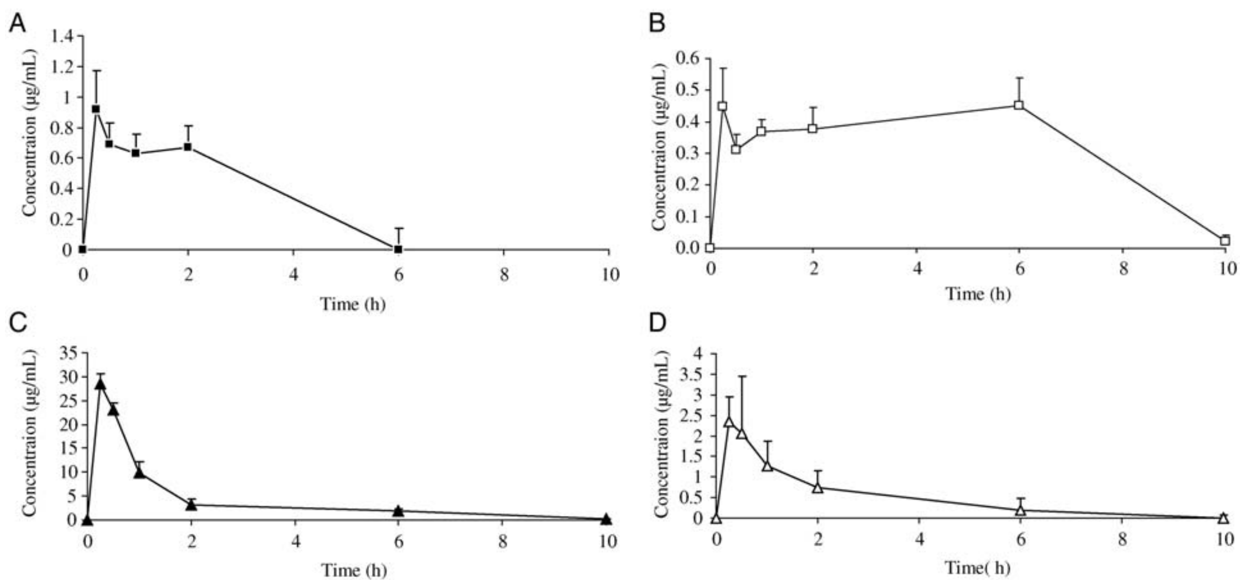

Figure 3. Observed values of blood concentrations versus time: 6-AM (A); M (B); Tr (C); M1 (D), following a single intraperitoneal dose [heroin (10 mg/kg) plus Tr (70 mg/kg)] in adult Wistar rats ($n = 16$).

compound was almost completely hydrolyzed. At −20°C, 6-AM was stable for up to 10 days, and then its concentration decreased slowly with a significant loss of 7−10% after fourteen days, which concurs with previous findings (15). Data obtained after three freeze-thaw cycles showed that the analytes were stable in rat blood (CV < 7%). These findings indicated that the storage of analytes in blood samples at −20°C is adequate, and no stability-related problems would be expected during routine analyses for analytical studies within 10 days.

Robustness of the methodology was determined by the reproducibility of results using the (analytical) method in different laboratories or under different circumstances. The present

study evaluated three blood aliquots from the treated rats in two different labs (Veterinary Pharmacology and Forensic Toxicology, both at the University of Pisa) and obtained variations of less than 7.2%.

These results demonstrate that the method enables accurate quantification of M, M1, 6-AM and T. The validation parameters were in agreement with the EMEA guidelines (29).

Although to the best of our knowledge, no simultaneous detection of heroin, Tr and related metabolites in rat blood is present in the literature, the present findings are in line with previous GC–MS studies (critical in forensic medicine), taking into consideration the singular analytes (23, 36).

Application of the method

The applicability of this method was verified by determining M, M1, 6-AM and Tr in rat blood samples after intraperitoneal administration of a combination of heroin and Tr. HPLC analysis of the blood confirmed the presence of M, M1, 6-AM and Tr in time-related amounts (Figure 3). The amount of M, M1, 6-AM and Tr in blood ranged between 0.312 and 0.450 μg/mL, 0.179 and 2.343 μg/mL, 0.631 and 0.925 μg/mL and 0.226 and 28.445 μg/mL, respectively. The described method allowed the pharmacokinetics of the four analytes to be followed. Heroin was not detectable in any sample due to its fast metabolism to 6-AM and M (3). M, M1, 6-AM and Tr had a C_{max} after 15 min. 6-AM T_{max} found in the present study is in agreement with previous heroin metabolic studies showing that heroin is immediately converted in 6-AM (4, 5). The 6-AM T_{max} might also be expected to come earlier, but no collection points before 15 min were scheduled in the present study. T_{max} of Tr and M1 are also in line with a recent pharmacokinetic study on rats (37). In that study, after oral administration, T_{max} of both analytes was 30 min. In the present study, the intraperitoneal administration could have evoked a faster absorption reducing the T_{max}. M, M1, and Tr concentration subsequently dropped to close to LOD after 10 h, while 6-AM dropped after 6 h. M had the lowest blood concentration among the analytes, showing a plateau between 0.5 and 6 h; this peculiar trend prevented determination of all the pharmacokinetic parameters measured for the other molecules (Table IV). For this reason and others, because the present data have been derived using only two rats for each collection time, a large-scale study is indicated to fully determine the significance of the results.

This method could also be applied to the detection of heroin in street samples, given its capability to resolve peaks produced by heroine metabolites from those of other additives/diluents such as caffeine and paracetamol, as shown in Figure 4.

Table IV
Predicted Pharmacokinetic Parameters (mean ± SD) of M, 6-AM, M1 and Tr Following a Single Intraperitoneal Dose [Heroin (10 mg/kg) Plus Tr (70 mg/kg)] in Adult Wistar Rats ($n = 16$)*

Parameters	M	6-AM	M1	Tr
λz	—	0.27 ± 0.01	0.51 ± 0.01	0.38 ± 0.07
HLλz	—	2.55 ± 0.05	1.34 ± 0.34	1.87 ± 0.35
T_{max} (h)	6.0 ± 0	0.25 ± 0	0.25 ± 0	0.25 ± 0
C_{max} (μg/mL)	0.45 ± 0.07	7.57 ± 0.85	2.34 ± 0.16	28.44 ± 2.89
$AUC_{0-\infty}$ (h μg/mL)	3.22 ± 0.38	20.96 ± 0.59	4.92 ± 0.46	38.78 ± 4.63
MRT (h)	4.18 ± 0.11	2.71 ± 0.15	1.35 ± 0.04	1.98 ± 0.58

*Note: λz, terminal phase rate constant; HLλz, terminal half-life; MRT, mean resident time.

Conclusion

The analytical method described in this work provides selective and accurate analyses of M, M1, 6-AM and Tr without the need for expensive cleanup steps, solvent-consuming flows or expensive devices. The LOQs are within acceptable limits and show that the method could be useful for forensic toxicological analysis on abusers. These features also make the described method suitable for pharmacokinetic investigations, including drug–drug interaction. In summary, this is the first time that the HPLC–UV technique has been reported to simultaneously detect M, M1, 6-AM and Tr. This method (extraction, separation and applied techniques) is simple and efficacious for the determination of analytes in rat blood and raw street heroin.

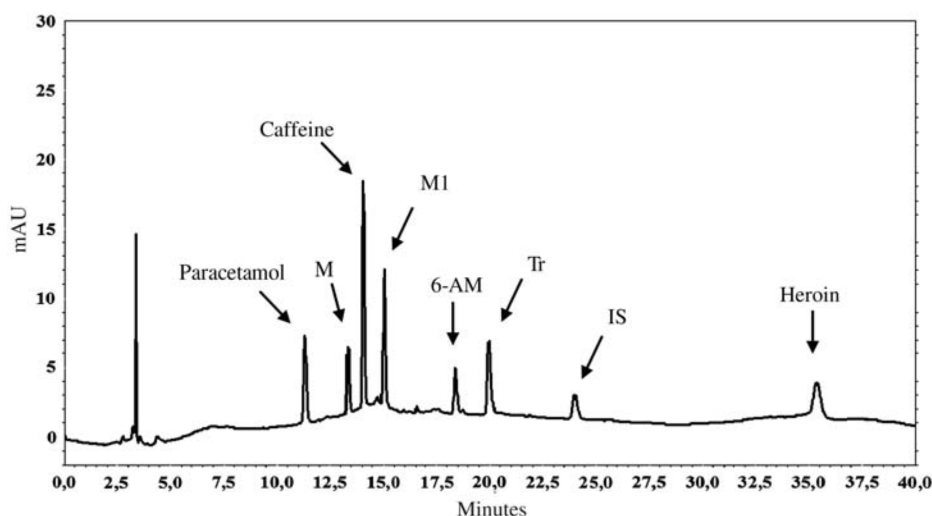

Figure 4. Chromatographic curve of substances commonly detected in street heroin.

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