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Heroin, Morphine, and Hydromorphone Determination in Postmortem Material by High Performance Liquid Chromatography

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ABSTRACT: A procedure has been developed for the simultaneous determination of heroin, morphine, and hydromorphone from postmortem tissues by reversed phase high performance liquid chromatography (HPLC) using electrochemical detection. This method permits the direct determination of unmetabolized heroin from antemortem or postmortem urine as evidence of illegal drug use. Presumptive confirmation of heroin was based on the ability to hydrolyze the HPLC heroin fraction to morphine. Heroin was also confirmed in urine by gas chromatographic/mass spectroscopic (GC/MS) analysis of the HPLC fraction. Analysis of postmortem blood, gastric contents, urine, and injection site tissues have revealed the presence of morphine and hydromorphone, while heroin has only been identified in urine.

KEYWORDS: toxicology, heroin, morphine, hydromorphone, chromatographic analysis, diacetylmorphine, postmortem detection, high performance liquid chromatography

The analysis of postmortem tissues and body fluids for evidence of illegal heroin (diacetylmorphine) use is usually accomplished by measurement of morphine from postmortem samples. Heroin is known to be metabolized to 6-monoacetylmorphine and later to morphine in man [*1*]. Morphine determinations from postmortem samples are inconclusive as to the origin of the morphine. Morphine can originate from the metabolism of heroin, codeine, or directly from over-the-counter preparations of paregoric. Methods of morphine detection have included colorimetric [*2*], thin-layer chromatographic [*3*], spectrofluorometric [*4*], gas chromatographic [*5,6*], radioimmunoassay [*4,7,8*], enzyme immunoassay [*9*]. and high performance liquid chromatographic (HPLC) [*10–13*] techniques.

Direct assays for heroin have been limited by the instability of heroin during extraction and derivatization and by interference from structurally similar opiates. Assumptions that heroin is metabolized too rapidly by plasma esterase to be measured have also discouraged

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the development of analytical methods for postmortem heroin determinations. Urine provides a stable environment for heroin since heroin is known to have a relatively long half-life in slightly acidic aqueous solutions [*14*], and since urine is largely esterase free. Several methods have been reported for the analysis of heroin from illicit heroin exhibits [*15–22*], however, these methods were not designed for use with biological material or capable of detecting low concentrations of heroin. Heroin in antemortem body fluids and tissues has previously been studied using methods such as countercurrent partitioning followed by methyl orange spectrophotometric analysis [*2*] and gas chromatography [*1,2*]. Recently, a method for the detection of heroin from clinical blood specimens has been reported using HPLC with ultraviolet (UV) detection [*22*]. A method for the postmortem determination of 6-monoacetylmorphine has been published [*23*] using gas chromatography/mass spectroscopy (GC/MS), however no methods are currently available for the direct, unequivocal determination of heroin from postmortem samples. A direct assay for heroin would be useful for forensic science investigations since heroin is still a commonly encountered illicit drug and often implicated in sudden death.

The present work describes a method using liquid extraction, reverse phase HPLC separation, and electrochemical detection for the direct determination of these opiates. A confirmation procedure for heroin has also been developed involving hydrolysis of the heroin eluent fraction to morphine. Human postmortem material from cases of suspected heroin related death was examined to determine where and at what concentrations heroin and morphine could be detected.

Experimental Procedure

Equipment

HPLC analyses were accomplished using a Hewlett Packard 1084-B liquid chromatograph (Hewlett Packard Corp., Palo Alto, CA) with a Supelco Supelcosil LC-18-DB (5-μm) reversed phase column (Supelco, Inc., Bellefonte, PA) coupled to a BAS LC-3 Amperometric detector (Bioanalytical Systems Inc., West Lafayette, IN).

Reagents

Prosil-28 organosilane concentrate surface treating agent was obtained from SCM Specialty Chemicals, Gainesville, FL, and sodium fluoride, 99%, was purchased from Aldrich Chemical Co., Milwaukee, WI. Ammonium hydroxide and ammonium chloride were purchased from Fisher Scientific, Fairlawn, NJ, while citric acid monohydrate, sodium azide, sodium phosphate monobasic anhydrous, and sodium phosphate dibasic were purchased from Sigma Chemical Co., St. Louis, MO. Water was purified by reverse osmosis followed by distillation. Liquid chromatographic grade methanol was from EM Science, Cherry Hill, NJ.

Mobile Phase

Mobile phase was prepared by dissolving 48.0 g of sodium phosphate, monobasic (NaH_2PO_4) into 2700 mL of glass distilled water followed by the addition of 1300 mL of methanol (32.5%) and adjusting the pH with 10 to 15 mL of 60% sodium hydroxide (NaOH) to pH 7.30 using a calibrated pH meter. Mobile phase was filtered through a 0.45-μm millipore filter and degassed with heat under vacuum before use.

Preparation of Standards, Controls, and Buffers

Pure standards of heroin hydrochloride (HCl) were obtained from Bayer Pharmaceutical, Germany; morphine sulfate was from Eli Lilly and Co., Indianapolis, IN; hydromorphone

HCl from Wyeth Pharmaceuticals, Philadelphia, PA; and nalorphine HCl, from Sigma Chemical Co., St. Louis, MO. Stock standards of morphine (0.100 mg/mL), hydromorphone (0.100 mg/mL), and nalorphine (0.004 ng/mL) were prepared in water and found to be stable, while aqueous heroin stock standards (0.100 mg/mL) were prepared fresh before each assay or kept frozen between use. Heroin assay standards were prepared by spiking drug free human urine with heroin stock standard to achieve final concentrations from 250 to 2000 ng/mL. Morphine standards were prepared from pooled human blank plasma to final concentrations of morphine ranging from 10 to 500 ng/mL. Hydromorphone standards were prepared from the stock standard in the same manner to final concentrations ranging from 62.5 to 1000 ng/mL.

Ammonium chloride/ammonium hydroxide extraction buffer was prepared fresh every two weeks by adding 53.5 g of ammonium chloride (5*M*) to 182 mL of water and adjusting the pH to 8.50 with 2 to 7 mL of fresh concentrated ammonium hydroxide. The buffer was sealed and stored cold when not in use.

Acidic buffer was prepared by adding 30.0 g of sodium phosphate monobasic (1*M*) and 13.1 g of citric acid (0.25*M*) to 250-mL glass distilled water for a final pH of 3.0. The acidic buffer was stored cold and renewed every three weeks.

A buffered preservative was prepared by adding 2.13 g of sodium phosphate dibasic (0.05*M*), 0.75 g of sodium phosphate monobasic (0.018*M*), 1.2 g of sodium azide (0.4%), and 6.0 g of sodium fluoride (2.0%) to 300 mL of glass distilled water, final pH 7.0.

Preparation of Samples

Postmortem samples of whole blood, urine, or vitreous humor were assayed without pretreatment, while body tissues were buffered and preserved with an equal weight of buffered preservative. Before assay, tissues were thoroughly homogenized and assayed on a twofold dilution of buffered preservative within 2 h or stored at −30°C until assay. The buffered preservative was found to prevent any change in heroin or morphine levels in spiked samples incubated at 25°C up to 2 h. The sodium fluoride/sodium azide (NaF/NaN_3) preservative may best be incorporated during sample acquisition to prevent degradation.

Assay Procedure

One-hundred microlitre aliquots of plasma or urine standard, blank, controls, and specimen (blood, urine, vitreous, or tissue homogenates) were pipetted into silanized 125- by 15-mm glass extraction tubes. One hundred microlitres of nalorphine internal standard was then added to each tube followed by one hundred microlitres of the 5*M* ammonium chloride/ammonium hydroxide buffer. Five millilitres of extraction solvent (dichloromethane : isopropanol (96 : 4, v/v) was added to each tube followed by a vigorous 1-min vortex and 40-min high-speed shake (280 shakes/minute). Following centrifugation (10 min at 2500 rpm), the extraction solvent was transferred to a second 125- by 15-mm silanized extraction tube. Acidic extraction was accomplished by the addition of 200 μL of phosphate-citrate buffer (pH 3.0), vigorously vortexing for 10 s, and shaking at high speed for 30 min. The extraction solvent (organic layer) was then removed and discarded. Final extraction was accomplished by the addition of 1.5 mL of the basic buffer (pH 8.50) and 10 mL of the extraction solvent, followed by a 10-s vortex and 30-min high-speed shake. The extraction solvent was then transferred to a silanized conical tube and evaporated to dryness under a stream of dry nitrogen. Immediately following solvent evaporation, samples were reconstituted with 100 μL of mobile phase and were injected onto the column. A single-step extraction procedure was also developed using a 99.5 : 05 dichloromethane isopropanol solution. We do not recommend the use of a single-step extraction procedure, however, as a result of increased risk of interference from endogenous metabolites in urine or other commonly encountered drugs.

HPLC Determination

The HPLC and electrochemical detector parameters are presented in Table 1. Analyses from clinical or coroner's specimens were accomplished without interference from endogenous material using these parameters. Hydrodynamic voltammograms were obtained by comparing peak heights from chromatograms of 15-μL test injections of a 100-ng/mL morphine standard while successively increasing the electrochemical cell voltage from 0 to 1 V. A voltage just below the plateau of maximal electrolysis was selected as this working voltage provided both the best signal to noise ratio and selectivity.

Standard curves were constructed by plotting heroin, morphine, or hydromorphone/nalorphine peak height response ratios against morphine concentration. Drug recovery was measured by comparison of peak heights of extracted standards to those of aqueous standards injected directly onto the column without prior extraction. Intrarun and interrun precision studies were accomplished for morphine using two levels of pooled plasma controls which lie near the upper and lower limits of the standard curve.

Confirmation of heroin from coroner's urine specimens was accomplished by collecting the HPLC heroin fraction on a second injection and hydrolyzing it in vitro to morphine. The recovered HPLC eluent fraction was buffered to pH 9.1 and heated under pressure to 100°C for 30 min to hydrolyze the heroin to morphine. Hydrolyzed eluent was then carried through the first step of the extraction procedure using proportionally larger quantities of buffer, internal standard, and extraction solvent to accommodate the larger sample size. Appearance of a single morphine peak was used as confirmation for heroin. Unhydrolyzed HPLC eluent fraction was also extracted and analyzed by GC/MS for heroin (courtesy of Dr. John C. Baenziger, Indiana University School of Medicine, Department of Pathology). The GC/MS analyses were performed using a Hewlett Packard 5955 GC/MS. A HP Ultra-Z (SE-54) phenyl-methyl-Si capillary column (0.2-mm inside diameter (ID) by 25 m) was used for separation. The oven was temperature programmed from 200 to 280°C over 15 min. The select ion spectrum mode was used for the detection of heroin, 6-monoacetyl morphine, and morphine.

Results and Discussion

Chromatograms obtained from the analysis of coroners' specimens of blood containing morphine (Fig. 1*b*) and urine containing morphine and heroin (Fig. 1*d*) are shown. Chromatogram A (Fig. 1) shows chromatographic separation of an opiate mixture following direct injection onto the HPLC column. Chromatographic conditions were adjusted to minimize possible interference from endogenous compounds and other drugs that have similar extraction and chromatographic properties. Chromatogram C (Fig. 1) was obtained by extracting drug-free urine as described. To achieve complete separation from interfering endogenous compounds and acetaminophen, it was necessary to use a mobile phase pH of 7.30 which increased the lipophilicity of the opiate analytes, enhancing the interaction of the opi-

TABLE 1—*Summary of HPLC parameters.*

Instrument	HP 1084-B liquid chromatograph
Column	Supelcosil LC-18-DB reverse-phase
Detector	BAS LC-3 amperometric detector
Detector voltage	+0.4 to 0.6 V, 100 nA/V
Mobile phase	0.1*M* NaH_2PO_4: 32.5% methanol, pH 7.30
Temperature	28°C, isothermal
Flow rate	1.0 mL/min, isocratic
Injection volume	15 μL
Attenuation	4

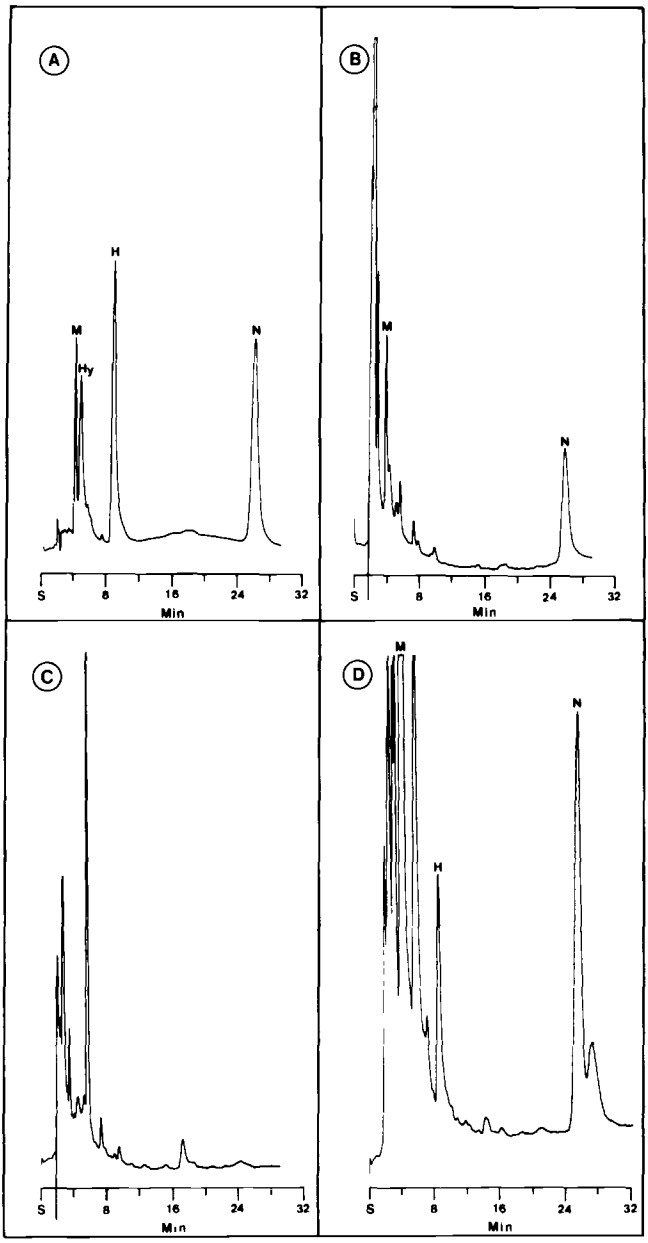

FIG. 1—*Separation of mixed opiates, 100 ng/mL of morphine* (M), *500 ng/mL of hydromorphone* (Hy), *1000 ng/mL of heroin* (H), *and 350 ng/mL of nalorphine* (N) (Chromatogram A). *Coroner's specimen (blood) with morphine* (Chromatogram B). *Drug-free urine blank* (Chromatogram C). *Coroner's specimen (urine) with morphine and heroin* (Chromatogram D).

ates to the reversed phase C18 beyond that of the interfering substances. Possible interference by opiate derivatives, other drugs, and metabolites was investigated by direct injection of 1 to 5-μg/mL aqueous or methanolic standard solution of drug onto the column and reporting the retention time, if any (Table 2). Few compounds were amenable to electrolysis at the low electrochemical cell voltage chosen or retained on the C18 column at pH 7.30.

TABLE 2—*Retention times of drugs tested for interference.*[a]

Drugs and Metabolites	t_R, minutes	Drugs and Metabolites	t_R, minutes
Acetaminophen	2.76	Imipramine	ND
Aminopyrine	13.63	Ketamine	14.98
Amitriptyline	ND	Lidocaine	ND
Barbiturates	ND	Meperidine	ND
Benzoyl ecgonine	ND	Methadone	ND
Caffeine	ND	Methaqualone	ND
Chlorpheniramine	ND	Morphine	3.76
Chlordiazepoxide	ND	*N*-Acetyl procainamide	ND
Cocaine	ND	Nalorphine	24.73
Codeine	ND	Naloxone	ND
Cyanide	2.11	*N*-Propionyl procainamide	ND
Disopyramide	11.00	*N*-Propylamphetamine	ND
D-Methylamphetamine	ND	Norpropoxyphene	ND
Desipramine	ND	Nortriptyline	ND
Diazepam	ND	Oxycodone	ND
Diphenhydramine	ND	Pentazocine	ND
Doxepin	ND	Phencyclidine	ND
Ethchlorvynol	ND	Phenothiazines	ND
Fluoxetine	ND	Phenylpropanolamine	ND
Fluphenazine	ND	Phenytoin	ND
Flurazepam	ND	Procainamide	ND
Glutethimide	ND	Propoxyphene	ND
Heroin	8.22	Strychnine	ND
Hydromorphone	4.40	Trihexyphenidyl	ND

[a]ND = not detected.
t_R = retention time (minutes).

Electrochemical cell voltage (E = 0.4 to 0.6 V) was maintained at a level which provided the largest peak height for morphine before reaching a plateau with additional voltage. White [*11*] has demonstrated two waves of oxidation for morphine by electrochemical detection, the first wave with a $E1/2$ = +0.44 V and a second wave $E1/2$ = +0.70 V. White found a cell voltage of +0.60 to be most suitable in his early electrochemical detection methods for morphine analysis. Our experience has shown that voltages above +0.6 V increased peak heights for morphine but also increased detector noise and baseline instability to an unacceptable degree and caused the appearance of numerous interfering peaks. Using these detector parameters, it was not possible to include codeine in the procedure. It may be feasible to run a UV detector in series for the detection of codeine. Posey and Kimble [*24*] have described a UV method for codeine. A more elaborate, dual cell detector might permit the use of a higher cell voltage for increased sensitivity and detection of codeine which has been shown to be electroactive at +0.90 V [*13*], however, +0.5 V was the optimal voltage for use with our BAS LC-3 single cell detector and extraction procedure for these select opiates.

The extraction procedure used with standards prepared from postmortem blood or urine produced quantitative results which were reproducible and linear from 10 to 500 ng/mL (morphine), 62 to 1000 ng/mL (hydromorphone), and 250 to 2000 ng/mL (heroin). Figure 2 shows a typical standard curve for morphine from plasma standards ranging in morphine concentration from 10 to 500 ng/mL. Standard curves were analyzed by regression analysis with the correlation coefficient, slope, and y-intercept for each run compiled. Quality control was established by including controls prepared from pooled drug-free plasma at two levels within the limits of the standard curve. From these controls, the within assay mean (intrarun $\bar{x}$), between assay mean (interrun $\bar{x}$), and standard error of the mean (SEM) at each control level were established. As would be expected, the interrun assay variability was found to be greater than the within run variation (Fig. 2). The limit of sensitivity for a 100-μL

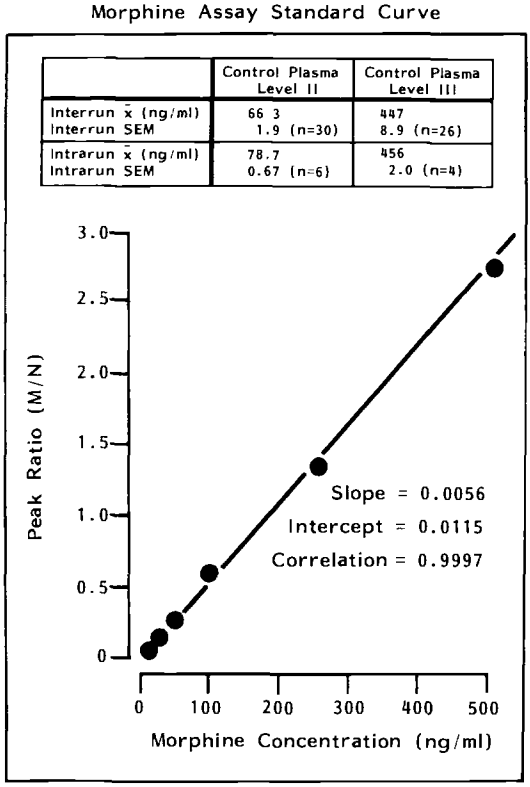

FIG. 2—*A representative standard curve for morphine from plasma standards. Actual amounts of morphine injected onto the column were 0.150, 0.375, 0.750, 1.50, 3.75, and 7.50 ng. Interrun and intrarun quality control data was established over a six-month period from two levels of plasma control.*

extracted sample was 0.5 ng/mL (morphine), 3.1 ng/mL (hydromorphone), and 12.5 ng/mL (heroin).

Extraction recovery was established by dividing each extracted standard peak height value by the corresponding peak height value obtained from direct injection of aqueous standards onto the column without prior extraction. Average extraction recovery expressed as percent of aqueous standard was 70% (morphine), 57% (hydromorphone), 55% (heroin), and 78% (nalorphine). Although nalorphine was retained longer, it served as an excellent internal standard as it maintained similar ratios to the opiates of interest regardless of the individual tube recovery efficiency. Other basic drugs with shorter retention times were attempted for use as the internal standard, but did not extract similarly to morphine. With the use of the buffers described, less than 2% of the heroin from prepared standards were found to hydrolyze to morphine during the extraction procedure. Since 6-monoacetylmorphine could not be detected by these methods, the extent of conversion of heroin to 6-monoacetylmorphine was not evaluated; however, with the use of freshly silanized tubes, recovery of heroin from aqueous standards was as high as 95%. For optimal recovery and selectivity, the extraction procedure requires the concentrated (5*M*) buffer to be used in the prescribed proportion to sample volume and extraction solvent. Substituting a larger volume of a less potent buffer or increasing sample size will result in altered drug recovery. In developing these methods, the amphoteric compounds were found to distribute into the aqueous phase, even at a basic pH. This was reduced by the use of the concentrated buffers. The buffers described in these procedures must be renewed and checked on a regular basis as they are only stable for two to

three weeks when kept well sealed. For optimal drug recovery, it was essential that the buffer maintained a pH greater than 8.2 when challenged with an equal volume of 0.18*N* sulfuric acid. Also of special consideration in replicating these procedures is the value of the 1-min vortex and 40-min shake required for consistent recovery. Furthermore, excessive drying of the extraction solvent or allowing the dry conical tubes to stand for time intervals greater than 30 min before reconstituting was also found to reduce recovery, possibly as a result of irreversible binding of drug to glass.

Confirmation of heroin by off-column in vitro hydrolysis of the HPLC heroin fraction to morphine offered a convenient and straightforward approach for verification of the heroin HPLC peak. Although numerous drugs were checked for interference, this does not eliminate the possibility of interference from another drug source or endogenous compounds. The procedure of in vitro hydrolysis of heroin from the pure HPLC fraction to produce a pure single peak of morphine upon injection into the HPLC may be used for confirmation.

Using these techniques, urine specimens from six coroners' cases were found to contain significant levels of heroin. The presence of heroin in postmortem urine was expected since the pH and low esterase activity of urine would minimize hydrolysis. Heroin was never identified in blood, liver, kidney, lung, or vitreous humor. All of the tissue sites from these cadavers tested positive for morphine. The possibility of heroin remaining stable in other body tissues or fluids is unlikely due to the extensive distribution of esterase enzymes in man. Table 3 summarizes the levels of heroin and morphine from six coroners' cases that were believed to be heroin related deaths. Morphine radioimmunoassay (RIA) (Coat-A-Count®, Diagnostic Products Corp.) was used for screening of blood and urine specimens for unconjugated morphine. RIA levels of morphine are presented along with the HPLC data for comparison in Table 3. GC/MS analysis of the heroin containing HPLC fraction from coroners' urine specimens further confirmed the heroin; also present, however, were very low concentrations of 6-monoacetylmorphine and morphine. Although these procedures include an off-column hydrolysis technique for heroin confirmation, this method should be used with other confirming techniques, as is standard practice in forensic toxicology.

Conclusion

The separation and identification of heroin, morphine, and hydromorphone from postmortem material was accomplished using an organic extraction cleanup followed by reverse-phase HPLC and electrochemical detection. The identification of heroin was based on three parameters: retention time matching with standard, off-column hydrolysis of the heroin HPLC fraction to morphine and GC/MS confirmation of the HPLC fraction. Conversion of heroin to morphine using the extraction procedure was less than 2%, while the conversion to

TABLE 3—*Heroin and morphine concentrations from coroners' specimens.*[a]

	Heroin Concentration, μg/mL		Morphine Concentration, μg/mL			
Autopsy Case	Blood (HPLC)	Urine (HPLC)	Blood (HPLC)	Blood (RIA)	Urine (HPLC)	Urine (RIA)
TC9585	ND	0.40	0.045	0.080	1.20	>0.45
667486	ND	0.25	...	...	3.60	...
TC686	ND	0.25	0.051	0.030	1.10	>0.28
TC34686	ND	7.0	0.044	0.031	1.30	1.3
TC45586	ND	2.0	0.305	0.360	3.00	4.3
TC56986	ND	5.0	0.060	>0.100	3.60	>0.10

[a]ND = not detected.

6-monoacetylmorphine was not evaluated. Assay parameters allowed simultaneous determination of heroin, morphine, and hydromorphone from biological fluids and tissues without interference from endogenous compounds or the common drugs tested. This method is unique as it permits the direct determination of heroin from postmortem urine as evidence of illegal drug use.

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