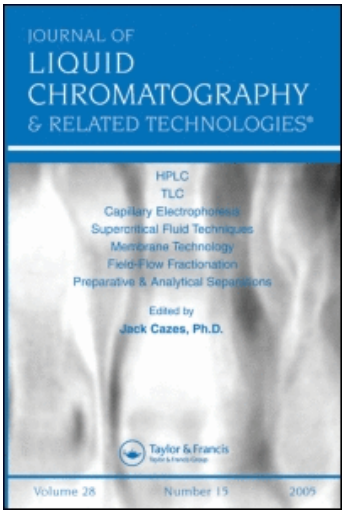

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ABSTRACT

A high-performance liquid chromatographic method with ultraviolet radiation absorbance detection has been developed for the determination of the principal opiates (heroin, 6-monoacetylmorphine, morphine, codeine) in human hair. An amount of 100 mg hair was incubated with 2 mL HCl 0.1 M at 56°C overnight and it was then extracted by solid-phase extraction using reversed-phase/ion exchange cartridges. Chromatography has been performed using a C_{18} reversed-phase column with a mobile phase consisting of water-acetonitrile (70 : 30 v/v) containing 0.01 M NaH_2PO_4 and 0.002 M sodium laurylsulphate at a final pH of 2.1. The linearity of the method was obtained in the concentration range of 0.5-50 ng/mg hair for 6-monoacetylmorphine, morphine and codeine, and of 5-200 ng/mg hair for heroin. The accuracy of the methodology was assessed using reference material from National Institute of Standards and Technology consisting of human hair segments and powdered human hair soaked with opiates.

Cocaine and benzoylecgonine did not interfere with the opiates in the chromatographic run, nor caffeine, nicotine and its metabolite cotinine, usually present in human hair. Using this methodology, hair from drug addicts were examined with a limit of quantitation of 0.5 ng morphine, codeine and 6-monoacetylmorphine/ mg hair and 5 ng heroin/mg hair.

INTRODUCTION

At present, hair analysis is being routinely used as a tool in forensic and clinical toxicology for detection of a previous exposure to drug.[1-5] In particular, hair analysis for opiates has been adopted in questions of drug related fatalities, revocation and restoration of driver's licenses, criminal responsibility, and prenatal drug exposure.[6-8] Furthermore, the analytical investigation of opiates in hair samples from drug addicts can be used to prove long-term abuse, to characterize abuse intensity, and addiction history.

Usually, hair analysis is performed by both chromatographic and immunological methods, with gas chromatography/mass spectrometry (GC/MS) being used as a method of choice because of its high specificity and sensitivity.[9-17] Nonetheless, we decided to investigate if high performance liquid chromatography (HPLC) with ultraviolet absorption detection could be applied as an alternative assay to analyze opiates in hair from heroin addicts with a reliability similar to that of GC/MS, to address those situations in which it is not possible and/or it is not necessary to perform GC/MS or LC/MS (analysis without legal impact, or executed also with immunochemical assay).

This paper describes an HPLC assay developed in our laboratory for the simultaneous determination of heroin and its metabolites 6-monoacetylmorphine and morphine together with codeine, a by-product in heroin street samples, in hair from heroin addicts and from cases of death due to acute narcotic intoxication.

EXPERIMENTAL

Chemicals

Heroin, morphine sulphate, codeine hydrochloride, cocaine hydrochloride, naltrexone hydrochloride, 6-monoacetylmorphine methanolic solution (1 mg/mL), and benzoylecgonine methanolic solution (1 mg/mL) were obtained from Salars (Milan, Italy). Caffeine, nicotine hydrogen tartrate, and cotinine were purchased from Sigma (Milan, Italy). Bond Elut Certify columns were

obtained from Varian S.p.A. (Torino, Italy). Extrelut-3 glass columns were purchased from Merck-Bracco (Milan, Italy). All reagents and solvents were analytical or HPLC grade. Reference materials consisting of drugs of abuse incorporated into human hair segments (Reference Material 8448) and in powdered human hair (Reference Material 8449) were provided by National Institute of Standards and Technology (Gaithersburg, MD, U.S.A.).

Chromatographic Instrumentation and Conditions

The HPLC system used in this study consisted of a Beckman 114 M solvent delivery system, a Beckman 160 fixed absorbance detector (Beckman Analytical, Milan, Italy) set at 254 nm, and a Varian 4290 integrator (Varian S.p.A., Torino, Italy).

The chromatographic separation was performed with a 250 X 4.6 mm I.D. LC-18-DB reversed-phase column (particle size 5mm) (Supelco, Milan, Italy). The isocratic mobile phase consisted of water-acetonitrile (70:30 v/v) containing 0.01 M NaH_2PO_4 and 0.002 M sodium laurylsulphate. The pH of the final solution was adjusted to 2.1 with phosphoric acid. The mobile phase was prepared daily and degassed by helium sparging.

The separation was performed at a flow rate of 1.7 mL/min for 15 minutes, then the flow was increased to 2.3 mL/min to elute more retained compounds (nicotine, cotinine, caffeine, and heroin) and components of the hair matrix. Run time was 35 minutes.

Standards and Controls

Solutions of stock reference standards (1mg/mL, 10μg/mL, 1μg/mL) were prepared in methanol and stored below 0°C. Dilutions were made fresh daily for each analysis. Aliquots of these standards were spiked into blank hair segments to create calibration curves and to determine solid-phase extraction recoveries, intraday and interday variabilities.

Hair Specimens

Hair samples were collected from drug-addicts attending a local addiction treatment center and from cases of death due to acute narcotic intoxication investigated by the Institute of Legal Medicine, University of Rome “Tor Vergata”.

From each subject, about 200 mg hair was collected from the back of the head, the hair was cut at the scalp and the full length was collected. None of hair samples collected exceeded 5 cm length, thus giving a maximum of 5-10 months consumption history.[18] Blank samples were collected as described above among laboratory personnel.

Hair Sample Preparation

Hair samples (100 mg) were washed two times with dichloromethane (3 mL) by vortex-mixing and dried under a stream of nitrogen. Then, they were cut into short segments and incubated in 2 mL of 0.1M HCl at 56°C overnight. Afterwards, the acid solution was mixed with 4 mL of 2 M Tris buffer (pH 8.1) and applied on a Bond Elut Certify solid-phase extraction column, which had been preconditioned with 2 mL methanol and 2 mL water. The column was washed with 2 mL water, 1 mL 0.1 M potassium acetate buffer (pH 4) and 2 mL methanol. The analytes were eluted with 2 mL dichloromethane:isopropyl alcohol (80:20 v/v) with 2 % ammonium hydroxide. The eluent was evaporated to dryness under a stream of nitrogen and redissolved in 200 μL of HPLC mobile phase. A 20 μL volume was injected into the HPLC column.

Study of Degradation of Acetylated Opiates During Incubation

In order to study the yield of hydrolysis of heroin and 6-monoacetyl-morphine during the overnight incubation in 0.1M HCl, six different portions of 100 mg of blank hair for each compound were spiked with heroin or 6-monoacetyl-morphine methanolic solutions at the level of 50 ng/mg hair and 10 ng/mg hair, respectively. After the evaporation of methanol at room temperature, three samples were redissolved with 2 mL 0.1M HCl and immediately extracted with Bond Elut Certify cartridges as previously described. The other three samples were incubated in 2 mL 0.1M HCl at 56°C overnight, then extracted with Bond Elut Certify cartridges. Finally, all the extracts were analysed by HPLC.

RESULTS AND DISCUSSION

The major problem in the analysis of hair samples is the quantitative extraction of analytes from the matrix. Complete dissolution of the hair is necessary to ensure that all the bound analytes are released from the hair. However, severe conditions used for the total digestion of the hair (eg. alkaline hydrolysis with NaOH) can completely degrade analytes of interest such as cocaine or 6-monoacetylmorphine. Therefore, in the context of method

development various digestion and extraction procedures reported in the literature[19-21] were evaluated in terms of analytical recovery and interfering peaks in the chromatographic profile that might lead to an incorrect identification and/or quantification of the investigated substances.

Digestion of hair samples with methanol, water, 0.1 N HCl and subsequent neutralization, followed by solvent evaporation and direct injection into HPLC column led to greatly interfered chromatograms. For this reason, different extraction procedures with organic solvents[20] or with solid phase extraction columns,[19] such as Extrelut and Bond Elut Certify were tested to be applied after the digestion process. From the results obtained (data not shown), acid digestion followed by solid-phase extraction with Bond Elut Certify columns appeared to be the best compromise providing the highest recovery of analytes and an absence of interfering substances.[22]

The separation of heroin, its metabolites and codeine, is shown in Figure 1. At a flow rate of 1.7 mL/min, the retention times were: 5.5 min for morphine, 9.3 min for codeine, 11.2 min for 6-monoacetylmorphine; then after the increase of the flow rate to 2.3 mL/min to shorten the duration of the chromatographic run, heroin was detected at 26.2 min.

Cocaine and benzoylecgonine could also be present in hair samples and could be extracted by the procedure used for the opiates; these analytes did not interfere with the opiates in the chromatographic run, as cocaine co-eluted with the front of the solvent while benzoylecgonine had a retention time around 20 min. Furthermore, caffeine, nicotine, and cotinine, usually present in human hair, had retention times of ca. 22, 17, and 19 min, respectively.

The linearity of the method was evaluated with calibration curves of the peak height (y, mm) versus the amount of analytes (x, ng/mg hair). These were prepared daily from blank hair spiked with seven different concentrations of the analytes and carried through the entire extraction procedure. The calibration curves were linear over the ranges 0.5-50 ng/mg hair for 6-monoacetylmorphine ($y = 3.0x + 0.2$, $r = 0.997$), morphine ($y = 5.2x + 0.9$, $r = 0.998$), and codeine ($y = 4.2x + 0.4$, $r = 0.998$), and 5-200 ng/mg hair for heroin ($y = 0.3x + 0.2$, $r = 0.997$).

The quantification limits, defined as the lowest quantitatively measurable concentration of the different compounds at a signal-to-noise ratio > 3, were calculated as ng of compound per mg of hair and were: 0.5 ng/mg hair in case of morphine, codeine, and 6-monoacetylmorphine, 5 ng/mg hair in case of heroin. The analytical recoveries of the solid-phase extraction procedure for these compounds and the intra-day and inter-day variabilities were evaluated at three different concentrations on six days and are summarized in Table 1.

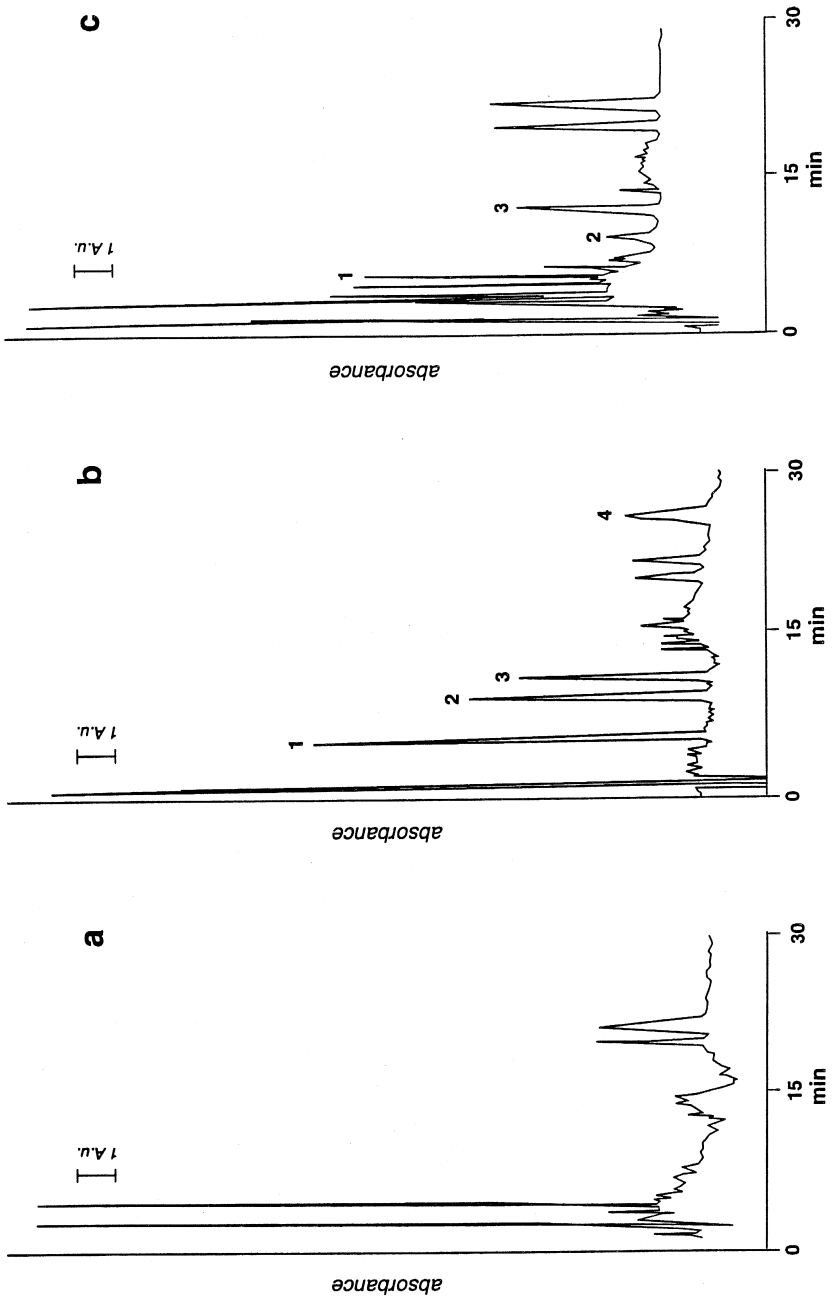

Figure 1. a) Chromatogram of an extract of blank hair sample; b) chromatogram of an extract of blank hair spiked with 10 ng/mg hair of morphine (1), codeine (2), 6-monoacetylmorphine (3), and 40 ng/mg hair of heroin (4); c) chromatogram of an extract from a heroin-user's hair sample containing 6.6 ng/mg hair morphine (1), 1 ng/mg hair codeine (2), 1.4 ng/mg hair 6-monoacetylmorphine (3).

Table 1

Recovery and Variability*

Concentration (ng/mg Hair)	Recovery (Mean + S.D.) (%)	Variability (%) Intra-Day	Inter-Day
Heroin			
10	91.0 ± 3.5	3.9	5.1
50	94.2 ± 2.7	3.0	4.0
100	96.5 ± 2.3	2.4	2.6
6-Monoacetylmorphine			
1	94.3 ± 4.3	4.5	5.2
2	96.5 ± 4.0	4.1	4.8
10	96.8 ± 4.3	4.4	5.0
Morphine			
1	95.0 ± 3.7	3.8	4.2
2	95.2 ± 3.0	3.1	4.0
10	96.5 ± 3.7	3.9	4.8
Codeine			
1	90.3 ± 3.5	3.7	4.0
2	90.6 ± 2.8	3.0	3.2
10	91.0 ± 2.5	2.6	3.3

* n = 6.

The accuracy of the entire methodology was verified with reference material consisting of drugs of abuse in human hair segments (Reference Material 8448) and drugs of abuse in powdered human hair (Reference Material 8449) provided by National Institute of Standards and Technology (NIST). Three samples were analyzed for both materials. Our results were in good agreement with the reported concentrations (Table 2).

The method described here was developed without the use of an internal standard. In the case of hair analysis by HPLC, the internal standard is useful to verify, and eventually correct the analytical recovery of the extraction procedure, and the relative retention times of the various compounds. It cannot give information about the percentage of extraction of the analytes from hair matrix, nor about the presence of interfering peaks which can create false

Table 2

Accuracy Determined with Standard Material from US National Institute of Standard and Technology*

Material	Certified Value (ng/mg Hair)	Concentration Found (ng/mg Hair)
Ref. Mat. 8448		
Morphine	11.9 ± 0.9	11.2 ± 0.2
Codeine	6.7 ± 0.5	5.3 ± 0.1
Ref. Mat. 8449		
Morphine	4.3 ± 0.2	4.5 ± 0.1
Codeine	2.9 ± 0.2	2.2 ± 0.1

* n = 3.

positives. Furthermore, using this methodology, very high recoveries (Table 1) and good accuracy were obtained (Table 2). Nonetheless, an accidental error can occur and for this reason the possibility of using naltrexone or thebaine as internal standards was investigated. Using the chromatographic conditions described above, both naltrexone and thebaine elute after 6-monoacetylmorphine with a retention time of 13 and 15 min, respectively. Furthermore, preliminary results obtained with blank hair spiked with the two compounds show that acid digestion does not affect the substances and solid-phase extraction with Bond Elut Certify gives analytical recoveries in the range of 88-93 % for naltrexone and of 90-95% for thebaine.

In order to evaluate the extent of hydrolysis of acetylated opiates during sample digestion, blank hair samples spiked with known amounts of heroin and 6-monoacetylmorphine were submitted to the digestion and extraction procedure applied to the real samples (Table 3). On the basis of HPLC results, it can be said that a hydrolysis process occurs for both compounds, in agreement with what is reported in the literature.[23]

This is a critical point considering the acid digestion used to extract analytes from our hair samples, particularly with the poor quantitation limit obtained in case of heroin and when low 6-monoacetylmorphine concentrations are present in hair samples.

Table 3

Concentration of Heroin, 6-Monoacetylmorphine and Morphine in Hair Spiked with 50 ng Heroin/mg Hair and 10 ng 6-Monoacetylmorphine/mg Hair Before and After Incubation with HCl 0.1 M at 56°C Overnight

Sample	Conc. Found (ng/mg Hair) T = 0 hr	T = 18 hr	Yield of Hydrolysis (%)
Heroin	47.9 ± 0.9	8.0 ± 3.8	83.3 ± 7.6
6-Monoacetylmorphine	1.5 ± 0.7	2.8 ± 1.7	
Morphine	0.7 ± 0.2	38.9 ± 3.5	
6-Monoacetylmorphine	7.8 ± 0.8	0.9 ± 0.1	88.4 ± 1.2
Morphine	2.2 ± 0.3	9.1 ± 0.5	

Regarding the choice of acid digestion to extract analytes from hair, it has to be added that the real situation of opiates in terms of presence and concentration in hair is difficult to be described. On one hand, methanolic extraction is not quantitative and so incorrect in terms of real concentration of all analytes in hair specimens. On the other, acid, basic, or enzymatic digestion are incorrect in term of real concentration of each analyte and presence of analytes which can undergo hydrolysis processes. Nonetheless, acid digestion as described above, provided the highest analytes recovery and was used in agreement with other authors.[15 20]

Heroin was reported to be present in human hair and in hair washes of heroin users.[17] Hence, it was included in the method to validate a simultaneous separation of opiates starting from the parent drug. Furthermore, at the beginning more extractions procedures were evaluated, e.g.. methanolic extraction, which does not hydrolyze heroin.

However, with the acid digestion at high temperature performed on hair samples and the limit of quantitation reported for heroin with this assay, heroin can not be considered a predictor of abuse due to the low levels found in hair of abusers, but only of external contamination. Indeed, in cases of external contamination, some could be found in hair extracts, but above all in hair washes, as reported by the literature.[17]

On the other hand, 6-monoacetylmorphine has been reported to be the major indicator of heroin abuse in hair when using methanolic digestion of hair samples, but it cannot be considered when using acid or alkaline digestion which hydrolyze this acetylated opiate.

Table 4

Concentrations of Opiates in Heroin Users' Hair

Subject	6-Monoacetylmorphine (ng/mg Hair)	Morphine (ng/mg Hair)	Codeine (ng/mg Hair)
Heroin Users			
A	1.4	6.6	1.0
B	4.2	5.1	2.1
C	2.9	3.8	N.D.
D	3.1	3.0	0.8
E	0.7	2.8	1.6
F	0.5	0.8	0.5
G	2.6	2.6	0.9
Heroin-Related Deaths			
H	0.8	2.5	0.9
I	7.7	24.3	3.4
L	2.4	2.0	N.D.
M	7.0	7.0	N.D.
N	4.0	2.8	N.D.
O	9.0	15.0	1.7
P	0.4	0.8	0.5

N.D.: Not detected.

Hence, when low 6-monoacetylmorphine concentrations are present in hair samples, the analyte can be totally converted to morphine. However, in our experience, we never obtained an hair extract with morphine alone (Table 4), but even in this case, morphine alone can be accepted as a reliable marker of heroin abuse when analyzing samples coming from heroin addicts and heroin-related deaths.[23]

In conclusion, a new method is proposed for the quantitative determination of heroin, 6-monoacetylmorphine, morphine, and codeine from human hair extracts or washes. The use of HPLC-UV detection for analysis of opiates in hair enlarges the possibility of routine hair analysis to those laboratories, which do not usually perform GC/MS. Indeed, HPLC/UV does not claim to be as reliable as GC/MS, but as an alternative to GC/MS in those cases in which GC/MS can not be used or legal or administrative information is not requested.

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