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Hair analysis for opiates: evaluation of washing and incubation procedures

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Abstract

Hair analysis of drugs of abuse has been a subject of interest from a clinical, social and forensic perspective for years because of the broad time detection window after intake in comparison to urine or blood. However, the correct and reliable interpretation of opiates findings in an authentic hair sample requires optimalisation and standardisation of decontamination and incubation procedures. Comparing various published methods, we have found some variability in them and no unequivocal recommended procedure for starting with a method directly. Therefore, various combinations of solvents, of various polarity, as washing solvents were tested for removing opiates from the external surface of real hair samples. The yields of opiates from these washings were compared with the yields from the interior of the hair matrix after digestion with various procedures. The opiates after digestion were cleaned up from resulting solution on extraction columns with mixed solid-phase and analysed by GC–MS in standard EI mode after silylation. The efficiencies of neutral (Söerensen buffer, pH 7.4), acid (0.1 *M* HCl) and basic (1 *M* NaOH) digestion of the hair matrix were evaluated and the relative recoveries for morphine, codeine, dihydrocodeine and hydrocodone were compared. As it is very problematic to imitate the reference hair sample with a specific amount of analytes incorporated inside, which can be used for calibration to get a close estimate of the quantities of analytes inside the solid authentic sample, the total digestion of a hair sample in basic medium was considered to be a very important reference basis for quantitative determinations. The ratios of hydrolysis of labile 6-acetylmorphine or acetylcodeine were tested and evaluated in practical routine conditions of acid or neutral digestion of hair. Comparing the three methods of incubation of authentic hair samples, the methods using 1 *M* NaOH or 0.1 *M* HCl yielded higher recoveries of total equivalents of morphine or codeine, whereas the incubation in Söerensen buffer allowed the reflection of real ratios of labile metabolites and/or parent compounds in an original sample. This method has been shown to be capable of detecting hydrocodone in hair with other opiates concomitantly and to indicate the drug abuse pattern of a person at various time intervals in the past.

Keywords: Washing; Incubation; Hair analysis; Opiates

1. Introduction

Hair analysis to detect drug abuse has been a subject of interest from a clinical, social and forensic perspective because of the broad time detection window after intake in comparison to urine or blood, providing information about the history of drug abuse with respect to an individual [1–6]. However, possible external hair contamination, cosmetic hair treatment, individual hair pigmentation, irregular

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speed of growth of hair from various anatomical parts of the body can complicate the interpretation of analytical results [7–9]. Nevertheless the stability of a specimen, non-invasive sampling and broad time detection window are considered to be advantageous.

The mechanism whereby a drug is incorporated into the hair matrix has been a subject of permanent discussion. Drugs may be incorporated into the hair interior by diffusion from blood or diffusion from body sweat or sebum. Three important factors control the drug amount bound to protein inside the hair: melanin affinity, lipophilicity and basicity of a drug. For example, drug concentration in pigmented hair is higher than in non-pigmented, and cocaine and 6-monoacetylmorphine amounts in hair tend to be higher than benzoylecgonine and morphine ones [10–14].

In order to avoid false-positive results due to passive external contamination, a standardized washing procedure is required before the digestion of the hair matrix. Various methodological approaches have been proposed as incubation procedures, some of them working with hair cut into pieces of about 1 mm, some of them working with hair ground finely [15,16]. In the case of hair digestion in an alkaline medium (1 *M* NaOH), heroin, acetylmorphine or acetylcodeine are decomposed to morphine or codeine totally [17]. During incubation in an acid medium (0.1 *M* HCl), a significant portion of the acetylated opiates is hydrolyzed as well [17]. Minimum yields of hydrolysis of acetylated opiates can be achieved by incubation in a neutral medium, as in methanol [17] or Söerensen buffer, pH 7.4 [18]. It is very problematic to imitate the reference hair sample with a specific amount of analytes incorporated inside, which can be used for calibration purposes with the aim of getting a close estimate of the real quantities of analytes bound inside an authentic solid sample. In this context, the total dissolution of the solid hair matrix in an alkaline medium, which has been spiked with reference substances, has been recommended [17].

In the paper presented, various combinations of solvents of various polarity as washing solvents were tested for removing opiates from the external surface of real hair samples. The yields of opiates from these washings were compared with the yields from the interior of the hair matrix after digestion with various procedures. The opiates after digestion were extracted on columns with mixed solid-phase Spec Plus 3ML DAU and analysed by GC–MS in standard EI mode after a modified procedure of silylation [19] with respect to the potential presence of hydrocodone abused in the form of illegal ‘‘Brown liquid’’. Hydrocodone has not been an ingredient in any registered medicine in our country for more than 30 years. Nevertheless, ‘‘Brown’’ has been produced illicitly from pharmaceuticals containing codeine for many years, and has been abused by opiate addicts as an alternative to heroin. The efficiencies of neutral (Söerensen buffer, pH 7.4), acid (0.1 *M* HCl) and basic (1 *M* NaOH) digestion of the hair matrix were evaluated and the relative recoveries for morphine, codeine, dihydrocodeine and hydrocodone were calculated. The ratios of hydrolysis of labile 6-acetylmorphine or acetylcodeine were tested and evaluated in practical routine conditions of acid or neutral digestion of hair. Some examples of opiate findings in authentic hair samples of some addicts have been demonstrated. The published data about hydrocodone evidence in hair are scant [20] and we are presenting some of them.

2. Experimental

2.1. Reagents and analytical reference standards

Solvents (methanol, isopropanol, acetone, dichloromethane, *n*-hexane) and other reagents were of analytical grade quality. Extraction discs Spec Plus 3ML DAU (Ansys Technologies), were provided by DRG (Brno, Czech Republic). Silylating reagents MSTFA, ammonium iodide, and 1,2-ethanedithiobis-(trimethylsilane) were purchased from Fluka Chemie (Buchs, Switzerland). Reference standard substances used are listed in Table 1.

2.2. Hair samples for examination: decontamination procedures

A bulk of blank hair, necessary for method development and validation, was obtained from a hairdresser’s shop. The absence of opiates was

Table 1
Nomenclature and origin of reference standard substances

Abbrev.	Substance	M_r	Provider
DHC	Dihydrocodeine tartrate	451.5	Napp Research Centre, Cambridge, UK
COD	Codeine phosphate	406.4	UNDCP, Vienna, Austria
HC	Hydrocodone tartrate	449.5	Sigma, St. Louis, MO, USA
MORP	Morphine hydrochloride	375.8	UNDCP, Vienna, Austria
ACOD	Acetylcodeine base	341.5	UNDCP, Vienna, Austria
6MAM	6-Acetylmorphine hydrochloride	363.8	UNDCP, Vienna, Austria
M-D3	*N*-Methyl-D3-morphine	306.4	Lipomed, Arlesheim, Switzerland

verified. The hair, with potential drug content, was provided from autopsies, from addicted persons undergoing therapeutic treatment and from persons prosecuted by the police. In the initial phase of testing, the hair of addicts was decontaminated by washing a 300-mg sample in an ultrasonic bath with a sequence of solvents (5 min each) according to four procedures:

(A) 20 ml dichloromethane, 15 ml acetone, 15 ml methanol, 10 ml methanol;

(B) 20 ml isopropanol, 15 ml acetone, 15 ml methanol, 10 ml methanol;

(C) 20 ml dichloromethane, 15 ml isopropanol, 15 ml methanol, 10 ml methanol;

(D) 20 ml *n*-hexane, 15 ml acetone, 15 ml methanol, 10 ml methanol.

The last washing solvent was tested with GC–MS analysis for residual content of opiates.

2.3. Digestion of hair matrix

Washed dried hair was finely cut into approximately 1-mm sections and digested by these alternative procedures in parallel:

(1) Sonification 50 mg sample in 4 ml Söerensen buffer, pH 7.4, at 50 °C 5 h.

(2) Heating 50 mg sample under stirring in 1 ml 0.1 *M* HCl at 55 °C 16 h.

(3) Heating 50 mg sample in 1 ml 1 *M* NaOH at 80 °C 1 h.

2.4. Isolation of opiates from digested hair, clean up, derivatization

pH value of prepared extracts after acid or alkali digestion was adjusted to 7.0. In case of a remaining solid matrix, extracts were separated and internal standard, deuterated morphine 50 ng in 10 μl methanol, was added. Clean up procedure on Spec Plus 3ML DAU discs was applied:

(a) disc conditioning with 0.2 ml methanol and 0.2 ml phosphate buffer, pH 6;

(b) sample application;

(c) washing with distilled water 0.5 ml, 0.1 *M* acetic acid 0.5 ml, methanol 0.5 ml;

(d) drying with air under vacuum 5 min;

(e) elution with 1.5 ml dichloromethane–isopropanol–25% NH_4OH (80:20:2);

(f) evaporation to dryness;

(g) reconstitution in 100 μl silylating mixture;

(h) silylation 80 °C 20 min; and

(i) 1 μl portion of the silylated extract was injected into GC system.

2.5. GC–MS method

The instrument used was an HP GC–MSD 6890-5973, splitless injector at 250 °C, auxiliary 270 °C, capillary HP5-MS 30 m×250 μm×0.25 μm, carrier gas He at constant flow 1 ml/min, oven temperature programmed from 85 °C 2 min, 30 °C/min to 220 °C, then 3 °C/min to 260 °C, then 15 °C/min to 280 °C, then held 3.5 min, time of analysis 25 min. MSD was working either in standard electron impact scan mode in the range 45–550 *m*/*z* or in SIM mode. Quantification was performed with internal standard method in SIM mode with ions monitored as given in Table 2.

Analytes in a sample were identified using comparison with the retention times and relative abundancies of monitored ions with standards. Standard calibration curves were obtained by adding calcu-

Table 2
Selected ions (m/z) monitored in GC–MS analysis: underlined ions are quantifying ions

Analyte	m/z
DHC	373, 358, 315, 282, 236
COD	371, 356, 343, 196
HC	371, 356, 313, 282
M-D3	432, 417, 404
MORP	429, 414, 401, 324, 236
ACOD	341, 298, 282, 229, 204
6-MAM	399, 340, 324, 287, 204

lated amounts of reference standards in methanol mixture to 50 mg decontaminated finely cut blank control hair to imitate specified concentration values of each substance, zero value included. These spiked samples were digested and calibrations were prepared separately for each digestion procedure, which was also used for authentic hair samples in an identical manner.

3. Results

3.1. Brief comparative evaluation of three digestion methods

At first, the stabilities of standard substances of acetylated opiates and hydrocodone under real conditions of hair digestion in three manners described above have been tested at two levels, 50 and 250 ng of each substance. Hydrocodone has been found stable and no loss due to any way of incubation has been noticed. The relative losses of original amount of both acetylated substances by hydrolysis in alkali media were 100%. The decomposition degrees of both substances in 0.1 *M* HCl were between 70 and 86% and with poor reproducibility. The stability of these substances during digestion in Söerensen buffer was acceptable with reproducible losses 7 and 18% of 6-monoacetylmorphine and acetylcodeine.

Calibration curves were prepared separately for each digestion procedure at five levels: 0, 0.5, 1, 2, and 5 ng/mg. Calibration related to digestion with Söerensen buffer involved all analytes tested, the ones related to acid and alkali hair digestion omitted labile acetylated opiates (acetylcodeine and acetylmorphine). Linearity was found in the whole calibration range with regression coefficients in the ranges: 0.9659–0.9977, Söerensen buffer; 0.9837–0.9899, 0.1 *M* HCl, 0.7107–0.9992, 1 *M* NaOH. Comparison of signal-to-noise ratio values after different digestion procedures of hair spiked to the level 0.5 ng/mg are presented in Table 3, indicating the higher chromatographic background after alkali digestion of hair.

Table 3
S/N test in spiked hair samples at the level 0.5 ng/mg digested with various methods

Opiate	S/N		
	Söerensen buffer	0.1 *M* HCl	1 *M* NaOH
DHC	27	18	13
COD	31	40	17
HC	30	23	–
MORP	13	5	5
ACOD	5	–	–
6MAM	26	–	–

A pool of hair of known opiate addicts has been prepared and repeatability of opiate assays using each of the three different digestion procedures has been tested and results are presented in Table 4. In this pool of authentic hair the amounts of opiates based on calibration related to digestion with Söerensen buffer have been determined as codeine 0.5 ng/mg, morphine 2.5 ng/mg, acetylcodeine 2.0 ng/mg, 6-monoacetylmorphine 11.4 ng/mg. The repeatability results of the assay based on hair digestion with 0.1 *M* HCl was very poor.

These preliminary comparative results, the best stability of acetylopiates and therefore the best assay reproducibility and relative low chromatographic background using hair incubation in Söerensen buf-

Table 4
Repeatability of opiate assay in a 50 mg sample of a pool of hair of known opiate addicts digested using various methods

Opiate	RSD (%) $n=6$		
	Söerensen buffer	0.1 *M* HCl	1 *M* NaOH
COD	3	23	5
MORP	6	5	3
ACOD	3	17	–
6MAM	7	63	–

fer initiated the more detailed validation of the assay with this way of hair digestion.

3.2. Validation of digestion method with Söerensen buffer

A more detailed method of calibration has been prepared for this purpose. Fifty mg finely cut blank hair were spiked six times at the levels 0.1, 0.2, and 1.0 ng/mg and two times at the levels 0.5, 10, and 20 ng/mg. Linearity was found in the whole calibration range tested with regression coefficients for individual compounds between 0.9851 and 0.9995. Assay repeatability of individual opiates at the levels 0.1, 0.2, and 1.0 ng/mg are given in Table 5 together with evaluation of chromatographic signal to noise (S/N) values.

The S/N results allowed to determine values of limits of detection (LOD) for individual compounds using the criterion of minimum required value of S/N to be at least 3. Under this conditions the LOD value was 0.1 ng/mg for dihydrocodeine, codeine, morphine and 6-monoacetylmorphine, 0.2 ng/mg for hydrocodone and 0.4 for acetylcodeine (Table 5). The repeatability results were used to determine the individual values of limits of quantification (LOQ) using the criterion of maximum acceptable coefficient of variation 12%. This requirement led to the

Table 5
Repeatability, S/N test, regression coefficients of calibration of opiates in spiked hair samples digested with Söerensen buffer

Opiate	Spiked concentration (ng/mg)	Repeatability RSD (%) $n=6$	S/N	LOD (ng/mg)	LOQ (ng/mg)	Regression coefficient R^2
DHC	0.1	11.8	3			
	0.2	10.9	7			
	0.5	–	27			
	1.0	10.8	–			
	0–20			0.1	0.1	0.9851
COD	0.1	n.q.	3			
	0.2	11.9	6			
	0.5	–	31			
	1.0	10.6	–			
	0–20			0.1	0.2	0.9899
HC	0.1	n.q.	2			
	0.2	12.1	4			
	0.5	–	30			
	1.0	9.9	–			
	0–20			0.2	0.2	0.9946
MORP	0.1	25.5	4			
	0.2	11.9	6			
	0.5	–	13			
	1.0	6.7	–			
	0–20			0.1	0.2	0.9995
ACOD	0.1	n.q.	1.4			
	0.2	100	2			
	0.5	–	5			
	1.0	27.7	–			
	0–20			0.5	n.q.	0.9879
6MAM	0.1	11.8	3			
	0.2	9.7	5			
	0.5	–	26			
	1.0	5.4	–			
	0–20			0.1	0.1	0.9958

n.q., not quantified.

Table 6
Examples of opiate amounts extracted from authentic hair samples (ng/mg) using digestion method with Söerensen buffer

Person	Amount (ng/mg)				
	COD	HC	MORP	ACOD	6MAM
1	0.1	n.d.	0.9	0.2	1.1
2	0.6	n.d.	3.5	3.7	21.3
3	0.1	n.d.	0.5	0.3	0.6
4	0.4	n.d.	1.8	3.0	14.0
5	0.1	0.7	0.3	0.7	0.5
6	0.1	n.d.	0.9	0.4	4.8
7	0.2	n.d.	0.7	0.9	2.8

n.d., not detected.

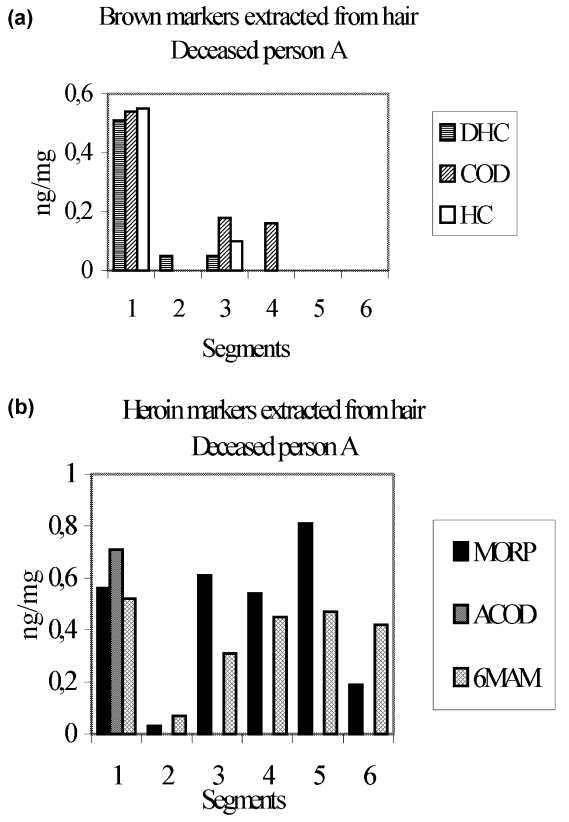

Fig. 1. Hair segment analysis for opiates in a case of fatal overdose (person A, 1.5-cm segments numbered in order from the scalp). Extracted amounts (ng/mg) after digestion with Söerensen buffer.

LOQ value 0.1 ng/mg for dihydrocodeine and 6-monoacetylmorphine and 0.2 ng/mg for codeine, morphine. Acetylcodeine can be quantified with sufficient precision only at very high levels.

3.3. Assay of opiates in hair of known addicts

To apply careful washing procedures is a prerequisite to prevent false-positive results. As no significant difference was found between the four procedures tested, washing procedure ''A'' has been adopted throughout our study as convenient. The potential loss of analytes fixed in the inner part of hair due to washing was found as negliglible—in maximum less or few units of percentage related to extracted amounts fixed in hair interior which was taken as 100%.

The range of values of opiates determined in authentic hair samples of several addicts taken near roots and digested with Söerensen buffer are given as examples in Table 6. The sectional analyses of opiates in the hair of two persons (A and B) who died of opiate overdose, have been performed. Results related to person A are given in two parts of Fig. 1 (a, Brown abuse; b, heroin abuse). Results related to person B are given in Fig. 2. The segments were 1.5 cm in length, person A had long hair sufficient to take six segments, person B's hair was cut short, which allowed us to analyze only two segments.

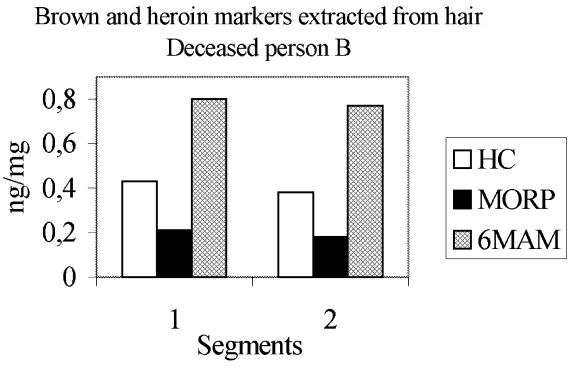

Fig. 2. Hair segment analysis for opiates in a case of fatal overdose (person B, 1.5-cm segments numbered in order from the scalp). Extracted amounts (ng/mg) after digestion with Söerensen buffer.

4. Discussion

Quantitative determination of opiates in hair is strongly dependent on the method applied to hair digestion and the absolute values determined may depend also on the blank hair matrix used for calibration. Therefore rather than absolute accurate true values related to the distribution of opiates fixed inside the hair, the relative amounts extracted by a standard method from several hair segments, can be of practical importance reflecting the time course of the possible drug career of an individual in the past.

The tests of stability of acetylated substances in various conditions used for hair digestion proved that in alkali media the labile acetylated opiates were decomposed completely and alkali digestion of hair allowed measurements of equivalents of morphine and codeine only. The acid digestion did not destroy the hair matrix fully, but significantly decomposed acetylated opiates (70–86%) and the reproducibility of this hydrolysis was poor. The decomposition of acetylated opiates in Söerensen buffer was minor (7–18%) and the reproducibility was better.

Comparing chromatogram backgrounds related to spiked hair samples and blank hair, the lowest one was after Söerensen buffer digestion, the highest one after alkali digestion, which had the impacts on the values of limit of detection. From this standpoint the digestion with Söerensen buffer was optimal. Using the assay with Söerensen buffer digestion, the LOD values of individual analytes were between 0.1 and 0.2 ng/mg, repeatability near the LOD approximately 12%. The linearity was verified in the range 0–20.0 ng/mg with regression coefficients between 0.985 and 0.999.

Data determined using the method based on hair digestion in mild conditions of Söerensen buffer at pH 7.4 best reflect the true ratio between analytes at the time of sampling. This method could be the method of choice, yielding information on potential opiate abuse in the time elapsed and preserving the specific markers of heroin abuse in their original form. This method described above has been shown to be capable of detecting hydrocodone in hair with other opiates concomitantly and distinguishing between various drug abuse patterns at different times. The results of sequential hair analysis of two forensic cases of fatal opiate overdose indicate the possible interpretation. Person A's heroin intake was regular with a reduction in intake approximately 2–3 months before death. The heroin intake was occasionally supplemented with ''Brown'', but in the last month before death ''Brown'' consumption was increased significantly with heroin intake remaining static. The results concerning person B indicated no difference in the intake of heroin and ''Brown'' at the two time intervals, which could be examined. The distribution of hydrocodone related to codeine and dihydrocodeine in hair could be of some interest in future studies.

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