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Disappearance of codeine, morphine and 6-MAM in hair after cessation of abuse

A. Suárez-García, I. Álvarez-Freire *, A.M. Bermejo-Barrera, P. Cabarcos-Fernández, M.J. Tabernero-Duque

Institute of Forensic Science, Forensic Toxicology Service, Faculty of Medicine, University of Santiago de Compostela, C/San Francisco s/n, 15782 Santiago de Compostela, Spain

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ABSTRACT

Research on the determination of drugs of abuse in hair has established that drugs can be detected in hair even long after cessation of use. The purpose of this study was to analyze hair samples from chronic opioid users who were beginning a controlled drug cessation program. The study population (n = 15) is involved in a drug rehabilitation program in Santiago de Compostela, Spain. Over a 6-month period, subjects provided hair samples at 2-month intervals, with the first sample collected on the day they began the program. Codeine, morphine, and 6-MAM were analyzed by GC/MS (LOQ = 0.2 ng/mg). Hair tresses were divided into 1 cm segments and analyzed for all analytes 0–1 cm corresponding to the proximal portion to the scalp Following cessation of opioid use, traces of codeine, morphine, and 6-MAM still remained in the newly growing hair segments for a specified period. After 2 months, still 27 % of the users tested positive, and at 4 months, 20 % were positive but only for 6-MAM. However, after 6 months of abstinence, the results were negative for all analytes.

1. Introduction

Opioids use remains a major concern around the world due to potentially severe health consequences. An estimated 31 million people worldwide consumed opiates, mainly heroin, according to most recent data [1]. Heroin is a semisynthetic opioid of morphine, which is an alkaloid naturally extracted from seed pod of the opium poppy plant. The chemical structure of heroin is like morphine with the addition of 2 acetyl groups (diacetylmorphine), which increase its permeability into the central nervous system [2].

Due to its short half-life of just a few minutes, heroin is rarely detectable in body fluids. After administration, the drug is rapidly metabolized to form 6-monoacetylmorphine (6-MAM), which is then converted to morphine, the predominant metabolite of heroin [3].

The presence of 6-MAM in urine is considered a definitive proof of heroin use since it cannot be produced by the metabolism of morphine or codeine. However, its detection window is limited to around 8 h after the drug is taken. This situation highlights the need to explore and develop new techniques for analysis, such as the use of different matrices [4]. Hair analysis has shown its importance in retrospective investigation of long-term substance use due to its wide detection window. The reason behind this is that substances present in the bloodstream are distributed and integrated into the hair matrix. Hair grows at a steady rate of approximately 1 cm per month, and once substances and their metabolites are incorporated into the hair matrix, they can persist without being metabolized or broken down for a significant period. The collection of hair is non-invasive, and it prevents adulteration because it can be done under direct supervision. Additionally, hair samples can be kept at room temperature for an extended period without the need for special transport or storage conditions [5]. Hair analysis offers other benefits, making it a valuable method for addressing various clinical and forensic concerns. These include identifying drug use [6], detecting prenatal exposure and monitoring withdrawal treatment, revoking driving privileges, and investigating causes of death [7] [8].

External contamination remains one of the most significant limitations in hair analysis for groups of drugs that are typically smoked or inhaled. Therefore, the removal of possible drugs that have been deposited externally is crucial during the analysis. Various washout methods have been suggested to address this issue [9,10]. Several studies have evaluated the effectiveness of these wash protocols, and the results have shown that they were effective enough, indicating that the

* Corresponding author.
E-mail address: ivan.alvarez@usc.es (I. Álvarez-Freire).

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Table 1
Linearity, limits of detection and quantification.

Substance	Equation	R^2	LOD (ng/mg)	LOQ (ng/mg)
Codeine	y = 0.1617x-0.0109	0.9979	0.15	0.2
Morphine	y = 0.1841x-0.0151	0.9982	0.15	0.2
6-MAM	y = 0.1178x-0.0072	0.9983	0.15	0.2

Table 2
Precision and accuracy intraday and interday.

Concentration (ng/mg)	Intraday CV (%)	Interday CV (%)	Intraday (%RSD)	Interday (%RSD)
Codeine				
0.2	10.97	13.2	6.45	5.95
1	3.27	6.19	10.82	9.45
5	10.37	3.25	8.13	7.83
Morphine				
0.2	3.53	12.15	10.3	11.1
1	2.74	3.26	7.22	5.42
5	0.93	5.9	6.97	8.03
6-MAM				
0.2	2.85	6.38	12.65	10.05
1	4.62	4.47	0.59	2.15
5	9.20	6.15	4.87	6.12

Table 3
Extraction recovery percentages.

Extr. Rec. (n = 5)	0.2 ng/mg	5 ng/mg
Codeine	91.6	86.3
Morphine	101.6	83.0
6-MAM	88.1	84.1

interpretation of the results is rarely affected by external contamination [11].

Drug incorporation into hair can occur through several mechanisms, such as drugs excreted in sweat or sebum seeping into the hair, drug diffusion from blood vessels that supply the hair follicle, or drugs in the surrounding environment that deposit on the hair strand. However, the exact process of how the drugs are incorporated to the hair fibers is not well understood. Therefore, researchers are currently studying the chemical properties of drugs that bind to hair in order to gain a deeper understanding of how these drugs are incorporated into the hair [12].

Hair growth occurs in a cycle that consists of three stages: anagen, catagen and telogen. The anagen phase is the active growth phase and can last for up to 6 years. About 85 % of the scalp hair is in the anagen phase at any given time. During the telogen phase, which is a resting stage, the hair follicle stops growing and remains inactive until a new hair grows and the old one falls out. This stage accounts for approximately 15 % of the hair on the scalp. The catagen phase is a transitional phase that lasts less than 3 weeks and is not easily noticed. After drug exposure, it is unlikely that a substance will be found in the same location on all hairs because, in contrast to anagen hair, the section of telogen hair where the drug is present will not move away from the scalp. [13]. In chronic heroin users, studies have shown that its metabolites can be detected in higher than expected concentrations for several months after they stop using the drug [14]. This may be due to the aforementioned hair follicle growth patterns.

One possible explanation for why people who have stopped using drugs may still test positive for them in their new hair, is that the drugs and their metabolites can remain in the body's hair follicles for an extended period. Furthermore, it is possible that substances deposited on the scalp can serve as a storage site for these drugs, leading to their continued presence in the hair after the individual has stopped using them. These factors may contribute to positive drug test results even after the cessation of drug use [4,15]. Since scalp hair strands often contain old hair in a telogenic or stationary phase. Hair in the telogen phase, which makes up around 10–15 % of a typical hair strand, can remain in this phase for up to 6 months [13]. It is likely that hair in the telogen phase can serve as an indicator of past drug consumption.

The aim of this research is to assess how long it takes for opioids (6-MAM, morphine, and codeine) to become undetectable in hair. To achieve this, a method based on the analysis of hair by segments was developed. Hair samples from people who entered a drug cessation program were used. The goal was to determine the total time it took for opiates to completely disappear from the hair of people who stopped using. This investigation can help determine whether individuals are compliant with the therapy program.

2. Material and methods

2.1. Reagents and reference standards

To develop the present study, solutions of 6-MAM, 6-MAM-d_3, morphine, morphine-d_3, codeine and codeine-d_3 in methanol (100 µg/mL) were obtained from Cerilliant® (Round Rock, TX, U.S.).

Pronase E, 1, 4-dithiothreitol (DTT), Chlorotrimethylsilane (TMCS), N-methyl-tert-butylsilyltrifluoroacetamide (BSTFA), acetic acid, gradient-grade methanol, and ammonium hydroxide were provided by

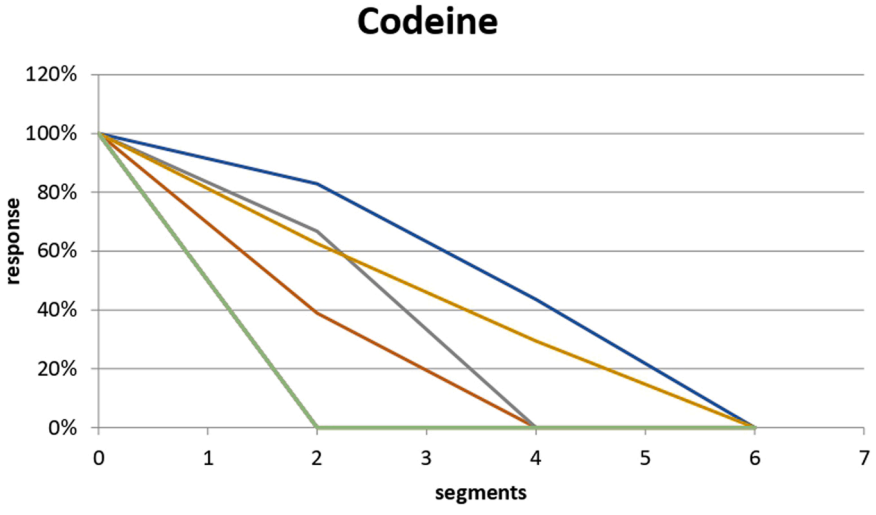

Fig. 1. Segmental response during six months after opioids discontinuation for fifteen subjects.

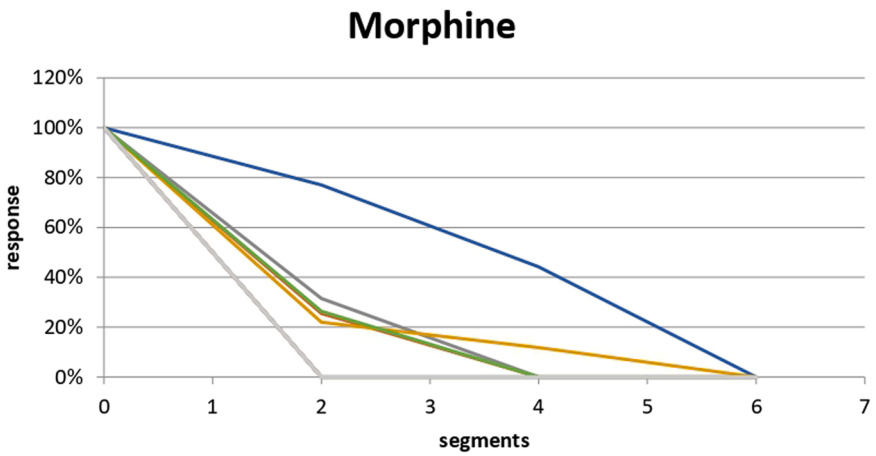

Fig. 2. Segmental response during six months of metabolite morphine for fifteen subjects.

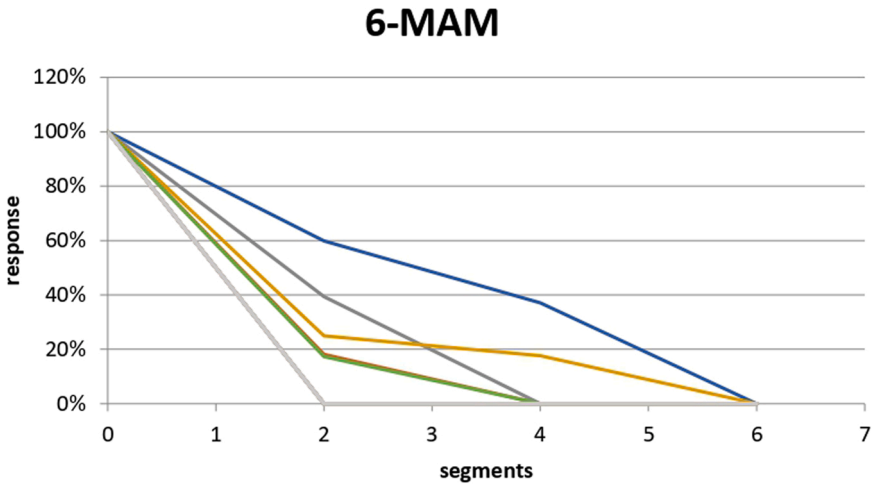

Fig. 3. Segmental response during six months of 6-MAM for fifteen subjects.

Table 4
Concentrations of codeine, morphine, and 6-MAM in hair for positive cases.

		Months of reported abstinence			
		0	2	4	6
Cod (ng/mg)	n	15	15	15	4
	Mean ± SD	0.40 ± 0.20	0.36 ± 0.14	-	-
	Min-max	0.21–0.78	0.25–0.52	-	-
Mor (ng/mg)	n	15	15	15	5
	Mean ± SD	0.82 ± 0.33	0.43 ± 0.25	-	-
	Min-max	0.51–1.49	0.22–0.78	0.45	-
MAM (ng/mg)	n	15	15	15	5
	Mean ± SD	1.26 ± 0.38	0.41 ± 0.16	0.31 ± 0.15	-
	Min-max	0.91–2.48	0.29–0.68	0.21–0.42	-

Merck® (Darmstadt, Germany). TRIS-hydroxymethyl-aminomethane (TRIS) was supplied from Sigma-Aldrich® (Stemheim, Switzerland). Purified water was supplied using a Milli-Q water system from Millipore (Le Mont-sur-Laussanne, Switzerland).

2.2. Instrumentation

A gas chromatography-mass spectrometry (GC-MS) system was used in this research. It was composed by an HP 6890 GC from Hewlett-Packard® (Little Falls, DF, USA), equipped with an HP 7683B auto-injector from Agilent® (Las Rozas, Spain) and connected to an HP 5973 inert mass selective detector, also from Agilent®.

To separate the different compounds, a capillary column HP-5MS was used (crosslinked 5 % phenylmethylsiloxane, 30 m x 250 μm i.d., 0.5 μm film thickness). The samples (2 μL) were injected into the instrument using the splitless injection mode (2 min). The injection port was heated to 240 °C, while the temperature of the ion source was set at 300 °C. The initial temperature of the column started at 90 °C and hold for 1 min. Then, the temperature was increased at a rate of 30 °C per minute until it reached 235 °C, and it was held for 10 min. After that, the temperature was ramped again, but a slower rate of 15 °C per minute, until it reached 260 °C and held for 5 min. Finally, the temperature was increased to 300 °C for 5 min to clean the column.

The mass selective detector operated at 320 °C. The ion source was set to 250 °C and the quadrupole was kept at 100 °C. The mass analyzer worked by electron ionization (70 eV), in selected ion monitoring (SIM). For each analyte the following ions were selected: *m/z* 371, 178, 234 (codeine); *m/z* 374, 181, 273 (codeine-d_3); *m/z* 429, 414, 236 (morphine); *m/z* 432, 417,239 (morphine-d_3); *m/z* 399, 340, 287 (6-MAM); *m/z* 402, 343, 290 (6-MAM-d_3). The underlined ions were used for quantification.

2.3. Hair samples

The hair samples used in this study were obtained from people (n = 15) enrolled in a drug cessation program. Participants voluntarily provided hair samples by previously signing an informed consent to

Table 5
Levels of codeine, morphine and 6-MAM in the 0–1 cm proximal hair segment during six months from 15 opioid users.

Subject			Codeine (ng/mg) month				Morphine (ng/mg)				6-MAM (ng/mg)			
	Age	S	0	2	4	6	0	2	4	6	0	2	4	6
1	55	M	0.37	0.30	*	0	1.01	0.78	0.45	0	1.13	0.68	0.42	0
2	29	M	0.31	0	0	0	0.88	0.22	0	0	0.98	*	0	0
3	44	M	0.78	0.52	0	0	1.49	0.47	0	0	1.33	0.52	0	0
4	49	M	0.40	0.25	0	0	1.20	0.26	0	0	1.17	0.29	0.21	0
5	49	F	0.21	0	0	-	0.60	0	0	-	2.48	0	0	-
6	27	M	0	0	0	-	0.58	0	0	-	1.12	0	0	-
7	19	M	0	0	0	-	0.54	0	0	-	1.10	0	0	-
8	39	M	0	0	0	-	0.56	0	0	-	1.14	0	0	-
9	41	M	0	0	0	-	0.55	0	0	-	1.25	0	0	-
10	43	M	0	0	0	-	0	0	0	-	1.13	0	0	-
11	32	M	*	0	0	-	0.64	0	0	-	1.27	0	0	-
12	45	F	*	0	0	-	0.61	*	0	0	1.06	*	0	0
13	34	M	0.34	0	0	-	1.08	0	0	-	1.63	0	0	-
14	51	M	0	0	0	-	0.51	0	0	-	0,91	0	0	-
15	51	M	0	0	0	-	1.26	0	0	-	1.24	0	0	-

M: Male; F: Female.
* Concentration value between LOD and LLOQ.

participate in the research. Compliance with detoxification treatment was demonstrated by periodic urine analysis.

The study's protocol was approved by The *Clinical Research Ethics Committee of Galicia, Spain*. Hair samples were collected every two months during six months, starting from the patients' enrollment in the program. The samples were collected from the posterior vertex area, 0–1 cm segment proximal to the scalp was analyzed. The first hair segment (sample 0 in Table 5) was taken on the same day as enrollment, representing drug use before abstinence and the concentration was considered to set 100 % response. Subsequent samples were taken at two-month intervals (samples 2,4 and 6 in Table 5). The samples collected had not undergone any cosmetic treatments, and patients were instructed not to apply any of that to their hair during the study. To eliminate external contamination, the hair was decontaminated by washing with a 0.1 % solution of neutral soap (Tween 20) and distilled water. After drying at 40 °C, the hair was cut into 1-mm segments and 50 mg aliquots were weighed.

2.4. Sample preparation

The process of analyzing hair for the presence of 6-MAM, codeine and morphine involved a previously validated protocol [16]. After an enzymatic hydrolysis process, the drugs were extracted from the hair using SPE as the extraction technique. Polymeric reversed phase cartridges Strata-X 33 μm (60 mg; Phenomenex®, UK) were used. Once the analytes were separated, the eluate was dried up using a stream of nitrogen gas in a heating block set at 40 °C. The extract obtained contains the substances of interest.

The extract needed a posterior derivatization step (BSFTA-TMCS (99:1), 40 μL) The sample was then heated at 100 °C for 20 min and then injected into the GC/MS system for further analysis and detection of the analytes.

3. Method validation

The method was rigorously tested and validated following the guidelines set by the FDA [17]. Linearity, limits of detection and quantification, intraday and interday precision and accuracy, and recovery were evaluated.

3.1. Linearity

To assess linearity, 50 mg aliquots of drug-free hair were doped with standard solutions of morphine, codeine, and 6-MAM at different concentrations: 0.2–0.5–0.8–1–2–4–5 ng/mg (ISTD: 5 ng/mg). The calibration curves were repeated 7 times. The samples were extracted following the previously described procedure. Subsequently, a simple linear regression analysis was conducted on the aliquots. Table 1 shows the calibration plots of the analytes. The coefficients of correlation were found to be higher than 0.99 for the concentration range studied. This indicates the reliability and accuracy of the calibration plots.

3.2. Sensitivity

The method's sensitivity was assessed by determining the limit of detection (LOD) and the lower limit of quantification (LLOQ). To establish the LOD, a practical approach was employed where a range of hair samples containing diminishing quantities of the substances of interest were examined. The LLOQ represents the lowest concentration of the analytes that can be reliably and accurately quantified. (Table 1).

3.3. Accuracy and precision

The precision and accuracy of the measurements were assessed through both interday and intraday assays. For interday evaluation, six determinations were performed for each concentration on different days. Intraday precision and accuracy were determined by preparing and analyzing five replicates at three concentration levels (low, medium, and high) on the same day. Precision was quantified as the coefficient of variation (CV) of the measured values, calculated as the ratio of the standard deviation to the mean, multiplied by 100. Precision values were expected to be below 15 % for all concentrations, except for the LLOQ, where a 20 % precision was deemed acceptable. (Table 2).

3.4. Recovery

The efficiency of extraction was determined by performing two separate batches (n = 5) of low and high control samples. In the first batch, the standard solution was added before SPE process, while in the second batch, it was added after SPE but before evaporation. To calculate recovery, the area ratios of the samples to which the standard was added before SPE were divided by those of the samples to which the standard was added after SPE. The average results are presented in Table 3.

4. Results and discussion

The purpose of this research is to know the time that elapses between

an opiate user stops consuming and opioids completely disappear from the new hair.

Although there is currently little evidence on the disappearance time of drugs in hair samples, some studies indicate that, in the case of cocaine, at least 6 months are necessary to ensure that the new hair (close to the head) is negative after beginning drug withdrawal [18]. Some authors suggest that for the hair of an opioid user to test negative (in the segment closest to the scalp) at least 3 months must pass [15]. Other studies suggest that they could be detected up to 4–5 months after the cessation of consumption [4].

The patients under study (n = 15) participated in an opiate withdrawal program that required their admission and permanent control (with periodic urine drug detection tests to confirm their abstinence). Using the methodology described above, hair samples were analyzed to determine the residual amounts of codeine, morphine, and 6-MAM present in abstinent individuals. It was found that after cessation of use, drug levels in the hair decreased significantly over time (as shown in Figs. 1–3). After the first two months in the program, most of the participants tested negative but still 27 % were still testing positive (Table 4).

In this work, 0.2 ng/mg has been selected as LLOQ to quantify the decreasing concentration of opioids in the consecutive segments studied. This cut-off of morphine, codeine, and 6-MAM was chosen because it is recommended by the Society of Hair Testing guidelines [19].

In this group of patients, it has been observed that after the first two months of abstinence, only 4 of the 15 analyzed tested positive for codeine and 5 for morphine and 6-MAM. After four months, only 2 subjects remained positives to morphine and 6-MAM. After 6 months, all patients tested negative for codeine, morphine, and 6-MAM. (Table 5).

These results coincide with those obtained in the previously mentioned study carried out by our laboratory with cocaine users who began their withdrawal, confirming that at least 6 months are necessary to ensure the complete disappearance of drugs in all cases [18].

Segmental hair analysis allows retrospective analytical study on hair samples [20,21]. This is particularly useful for conducting large-scale population studies and makes it possible for studies to cover long periods, spanning several months or even years [14]. This study provides valuable and fundamental information to ensure compliance with the cessation of patients who start their treatment. This is of particular importance in cases of legal implication, where the user claims to have stopped using and yet the hair samples give a positive result.

5. Conclusion

In the scientific literature consulted, there are few published works on the disappearance time of drugs from the hair in addicts who stop using drugs. In this study, 15 patients were followed from the beginning of their abstinence. The results showed that, although after 2 months in most cases the analysis of codeine, morphine and 6-MAM in the hair is negative, 6 months are necessary to ensure the complete disappearance of the drugs from the new growing hair.

Compliance with the ethical standards

The authors affirm that they do not have any conflicts of interest.

This research has involved the use of human biological samples, which has been conducted in strict adherence to all relevant legal requirements and in accordance with the ethical standards outlined in the Helsinki Declaration and the Council of Europe's recommendations on research involving human biological material. The study received approval from the Clinical Research Ethics Committee of Galicia before it commenced. The researchers themselves bear the legal responsibility for the samples, and they will not be shared with third parties or utilized in other research projects. All participating researchers have demonstrated their competence and expertise in conducting research projects.

Informed written consent was obtained from all participants involved in this study, and a copy of the consent form is available for reference if necessary. Anonymity was maintained during the handling of biological samples in the laboratory. Standard protocols for clinical investigations were followed to access only the data required for scientific research purposes. The processing of data adheres to the provisions of Law 3/2018 on the protection of personal data and digital rights guarantee developed in Spain by Regulation (EU) 2016/679 of the European Parliament and Council, dated April 27, 2016, concerning the protection of individuals with regard to the processing of personal data and the free movement of such data (repealing Directive 95/46/EC: General Data Protection Regulation).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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