Acetylcodeine as a Marker of Illicit Heroin in Human Hair: Method Validation and Results of a Pilot Study

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Abstract

Acetylcodeine (AC), which is an impurity of illicit heroin synthesis, was suggested as a marker of heroin abuse. A procedure for simultaneous quantitation of 6-monoacetylmorphine (6-MAM), which is the major metabolite of heroin, morphine, codeine, and AC in hair was developed. Fifty-milligram hair samples were incubated in 0.01M HCl overnight at 60°C. The resulting hydrolyzed solutions were extracted by an automated solid-phase extraction procedure and drugs were analyzed by gas chromatography–mass spectrometry in selected ion monitoring mode (SIM). This required prior derivatization with propionic anhydride. Different validation parameters, such as linearity, intra-assay accuracy, extraction recoveries, and limit of quantitation, were described. Seventy-three hair samples from heroin abusers and 43 hair samples from subjects who had completed a heroin-maintenance program were analyzed. AC was detected in 92% of the first sample group and in only 12% of the second sample group. In the two groups, about 98% of AC-positive samples were found. These results prove that AC can be considered as a suitable marker of illicit heroin use, along with 6-MAM detection.

Introduction

Several studies have analyzed opiates in hair (1,2) to prove the illicit consumption of heroin and distinguish heroin use from codeine (COD) or morphine (MOR) use. In fact, hair analysis has become a useful tool in toxicological studies because it makes long-term information (several months) on drug consumption available, which is not possible with urine analysis.

Until now, heroin exposure was proved by detection of 6-acetylmorphine (6-MAM), the major metabolite found in hair (3). The difficulty was to find a method that did not transform this compound into MOR, particularly in cases of simultaneous consumption of MOR or COD.

Heroin-maintenance programs have existed for some time, and it has been proven that the heroin used for this purpose does not contain acetylcodeine (AC), a manufacturing impurity found only in illicit heroin. This is why this compound could become a very interesting marker of illicit heroin use. However, AC in heroin is only reported in a range of 1 to 15% (4). Therefore, in order to detect AC at such low concentrations, a sensitive gas chromatography–mass spectrometry (GC–MS) method allowing the simultaneous quantitation of 6-MAM, COD, MOR, and AC in hair was developed. This method was validated and applied to hair specimens from patients who had participated in a heroin-maintenance program and from heroin abusers.

First, we tried to establish a relationship between the administered heroin dose and the concentration of the different opiates found in hair. Second, we simulated a parallel consumption of illicit heroin by administering heroin with varying percentages of AC to volunteer patients in a heroin-maintenance program. This allowed for an estimation of the number of times illicit heroin has to be taken before AC can be detected in hair.

Materials and Methods

Chemical reagents and instrumentation

The solvents, methanol, methylene chloride, and isopropanol; the hydrochloric acid; phosphate and acetate buffer; and pyridine were supplied by Merck (Darmstadt, Germany). Propionic anhydride was provided by Aldrich (Gillingham, England).

6-MAM, COD, MOR, and nalorphine were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA) and Sigma (Buchs, Switzerland), and AC was from Lipomed (Arlesheim, Switzerland). Hair samples were pulverized in a ball mill provided by Retsch (Schieritz, Hauenstein, Switzerland).

Automated extraction was performed on an ASPEC (Gilson Medical Electronics, Villiers-le-Bel, France). Isolute HCX cartridges were provided by IST (Hengoed, U.K.) and used for the extraction.

Hair sample analyses were carried out with a Hewlett-Packard (HP) 5890 GC equipped with an HP 5988 MS operating in electronic impact mode with an energy of 70 eV.

Materials for examination

Soaked hair was prepared according to the procedure de-

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scribed by Edder et al. (5): 50 mg of blank hair (without drugs) was added to an aqueous solution of drugs that was stirred overnight with a magnetic stirrer. This hair was then rinsed with water and methanol to eliminate adsorption of drugs.

Hair samples were obtained from subjects enrolled in a medical heroin treatment program called "Prove program" organized by the Federal Office of Public Health and from illegal heroin abusers. The subjects of the Prove program received heroin in a range of 120 mg to 750 mg per day. Samples were collected at least three months after the beginning of treatment. Two cases were excluded from the study because the quantity of collected hair was not sufficient for analysis.

This study was conducted following the guidelines for the protection of human subjects and each volunteer provided informed consent. Table I summarizes subject characteristics.

Table I. Characteristics of the Two Groups

Group	Male	Female	Age years mean (ranges)	Heroin dose mg/day mean (ranges)
Medical heroin consumers (n = 44)	32	12	33 (21–52)	460 (120–750)
Illegal heroin consumers (n = 74)	54	20	32 (18–50)	1200 (100–4000)

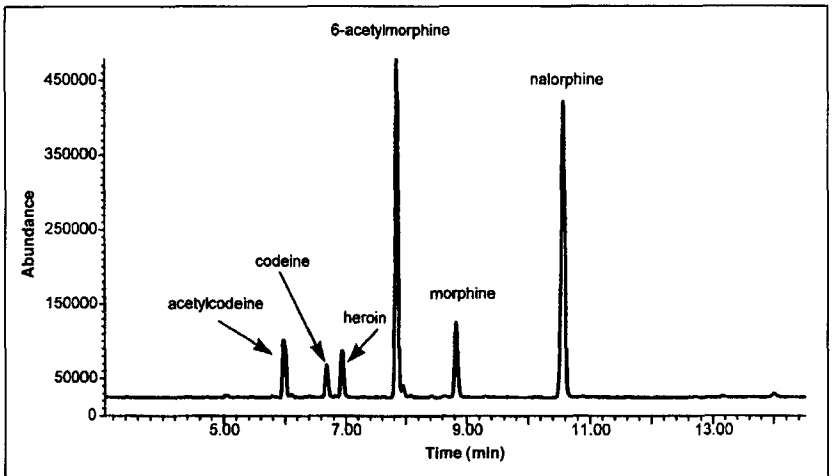

Figure 1. Limited full-scan GC–MS chromatogram of hair sample from an illicit heroin consumer. COD = 0.9 ng/mg, 6-MAM = 10.4 ng/mg, MOR = 3.1 ng/mg, AC = 0.8 ng/mg.

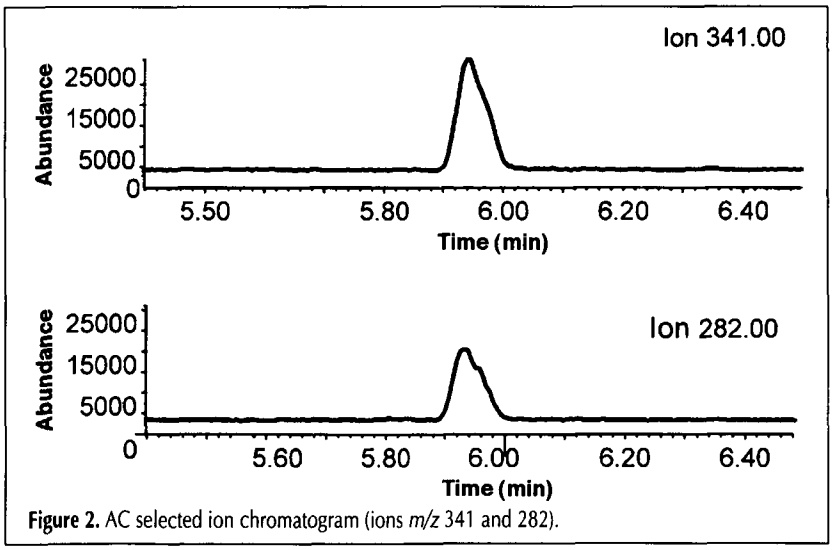

Figure 2. AC selected ion chromatogram (ions m/z 341 and 282).

Hair decontamination

Before analysis, all of the hair obtained from a patient was washed successively with 5 mL of methylene chloride, 5 mL of water, and 5 mL of methanol. This step is very important to eliminate possible external contamination. The hair was dried for a few minutes at 60°C in a heating block and pulverized for 5 min at 70 cycles/s in a ball mill.

Extraction of hair samples

Because drugs are fixed inside the hair matrix, a hydrolysis procedure is required before extraction. About 50 mg of powdered soaked hair or real hair sample was placed in a 10-mL glass tube, and 1 mL of 0.01M hydrochloric acid was added. After incubation at 60°C for 12 h, the solution was neutralized with 1 mL of 0.01M NaOH and buffered with 1 mL of 0.1M phosphate buffer (pH 7.0).

After centrifugation at 4000 rpm for 5 min, the supernatant was transferred into a special glass tube for extraction.

The ASPEC system was programmed to extract the hair samples in the following steps: (1) column conditioning with methanol (2 mL); (2) column conditioning with water (2 mL); (3) dispensing samples on the column (3 mL); (4) rinsing column with water (2 mL); (5) rinsing column with pH 4 acetate buffer (1 mL); (6) rinsing column with methanol (2 mL); (7) drying column with air; (8) elution with methylene chloride/isopropanol/ammonia hydroxide (2 mL); and (9) addition of chromatographic standard nalorphine (50 µL of a solution at 20 µg/mL).

The complete procedure has already been described (6).

Derivatization

GC–MS analysis required a derivatization procedure by propionylation. One hundred microliters of pyridine and 100 µL of propionic anhydride (PA) were added to the extract and incubated for 30 min at 60°C. After evaporation under a nitrogen stream, the extract was reconstituted in 50 µL of ethyl acetate and placed in a sample vial for GC–MS analysis.

GC–MS method

Helium was used as the carrier gas with a capillary column (DB-5MS, 15 m × 0.25 mm × 0.25 µm, J&W Scientific, Folsom, CA). The temperature program was as follows: 170°C

maintained for 1 min to 240°C at 20°C/min, to 256°C at 2°C/min, to 270°C at 10°C/min, and held for 0.6 min. Injector temperature was 270°C and injection was made in splitless mode. The source and interface temperatures were 200°C and 280°C, respectively

Two microliters of the sample was injected into the GC–MS system, which was operating in selected ion monitoring mode (SIM). The electron multiplier voltage was set at + 200 V above the EI-tune voltage.

Table II. Linearity and LOQ for Each Opiate (n = 8)

	Linearity 0.1–20 ng/mg	LOQ (ng/mg)
Morphine	$y = 0.0011x + 0.021$ $R = 0.9967$	0.08
Acetylmorphine	$y = 0.0015x + 0.032$ $R = 0.9960$	0.1
Codeine	$y = 0.0011x + 0.038$ $R = 0.9948$	0.09
Acetylcodeine	$y = 0.0013x - 0.013$ $R = 0.9969$	0.09

Table III. Extraction Recovery and Intra-Assay Accuracy for Each Opiate (n = 6)

	Recovery %	Intra-assay accuracy mean conc. (ng/mg)	CV (%)
Morphine	85	5.8	4.9
		34.3	11.0
Acetylmorphine	99	4.7	8.6
		27.3	16.0
Codeine	93	4.8	8.2
		29.2	14.3
Acetylcodeine	66	5.1	10.1
		28.4	16.0

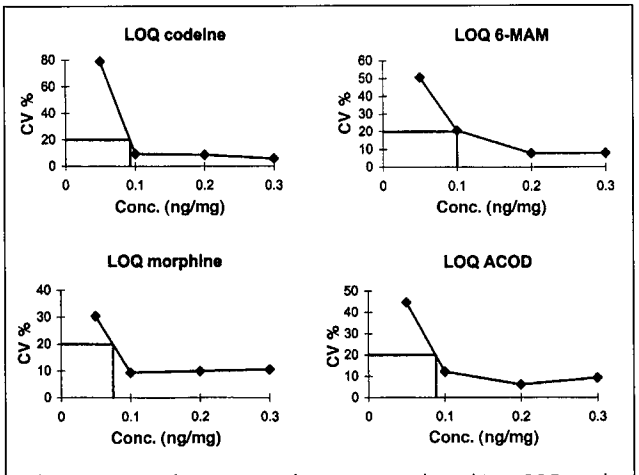

Figure 3. Limit of quantitation for MOR, acetylmorphine, COD, and AC.

Detection was performed with the following ions: *m/z* 355 and 282 (COD); *m/z* 383 and 327 (6-MAM); *m/z* 397 and 341 (MOR); *m/z* 341 and 282 (AC); and *m/z* 423 and 367 (nalorphine).

Quantitation was based on the compound peak-area ratios versus the chromatographic standard, nalorphine.

Table IV. Opiate Concentrations in Hair of Patients Who Participated a Heroin-Maintenance Program

Number	Patient number	Morphine (ng/mg)	6-MAM (ng/mg)	Codeine (ng/mg)	AC (ng/mg)	Hair (cm)
1	16	2.6	4	0	0	3
2	17	2.8	2.1	0	0	3
3	18	5.2	2.8	0.1	0	3
4	19	3.2	1.9	0	0	5
5	20	8.9	4.2	0	0	3
6	21	4.2	2	0	0	3
7	22	7.6	4.7	0	0	3
8	23	3.7	1.7	0.1	0	3
9	24	6.3	3.1	1.5	0	3
10	25	4	1.1	0	0	3
11	26	4.6	3.7	0	0	5
12	27	6.2	1.4	0	0	2
13	28	3.3	2.5	0	0	6
14	30	2.8	3.8	0	0	4
15	34	2.1	0.8	0.1	0	3
16	35	7.8	5.3	0	0	3
17	36	6	3.7	0	0	3
18	37	0.8	0.2	0	0.1	3
19	38	7.1	5.9	0	0	3
20	39	9.6	5.5	0	0	3
21	40	1.6	0.6	0	0	3
22	41	14	5.4	0	0	3
23	42	2.3	0.3	0	0	3
24	43	4.3	3.8	0.9	0.3	3
25	44	1.6	5.1	0	0	3
26	45	4.5	2	0	0	3
27	46	6.3	4	0	0	3
28	47	9.4	4.7	0.1	0	3
29	48	10.8	6.5	0.3	0	3
30	49	4.6	3.3	0	0	3
31	51	13.1	5.2	0	0	3
32	52	15.1	3.3	0.1	0	3
33	54	1.9	0.6	0	0	3
34	55	1.9	0.8	0.1	0	3
35	56	4.4	1.6	0.1	0	3
36	58	0	0	0	0	4
37	59	4	1.3	0.1	0	4.5
38	60	9.3	9.4	0.2	0.1	3
39	61	5.4	1.7	0	0	4
40	62	6	2.2	0	0.1	4
41	63	2.7	1.1	0.1	0	3
42	64	2.5	0.9	0	0	3
43	65	6.6	37.3	1.2	3.4	3
mean		5.4	3.8	0.1	0.0	3.3
median		4.5	2.8	0.0	0.0	3.0
maximum		15.1	37.3	1.5	3.4	6.0
minimum		0.0	0.0	0.0	0.0	2.0
rate		97.7	97.7	32.6	11.6	

Study of AC elimination in hair

This study enrolled three volunteers (B, C, and D) who had participated in a heroin-maintenance program and agreed to take heroin containing a varying percentage of AC. In order to simulate parallel illicit consumption of heroin, they received heroin with a varying percentage of AC over 36 days as follows: between days 2 and 9, they received heroin with 9% AC; on days 12 and 13 they were given heroin with 5% AC; and between days 19 and 26, they ingested heroin with 3% AC. Hair was taken on the first day of the study and 10 days after the end of the study. All patients received their daily heroin in three separate doses (410–770 mg per day) with only the morning dose containing AC.

Validation protocol

Soaked hair. The soaked hair was analyzed by the entire procedure, and the five compounds were well separated within a run time of 15 min. Soaked hair proved to be a useful material for the validation process.

Linearity. Standard calibration was performed in hydrochloric acid (0.01M) with a linearity between 50 and 1000 ng corresponding to a range between 1 and 20 ng/mg of hair. Five concentrations were chosen for the calibration, and each of these points was done twice. These standards were analyzed by the complete procedure.

Limit of quantitation (LOQ). The LOQ was obtained by incorporated the drugs to the hydrolyzed blank hair with three different concentrations (0.1, 0.2, and 0.3 ng/mg) of the four compounds of interest. Eight replicated samples were analyzed for each concentration studied. LOQ was determined to be the concentration at which the relative standard deviation (RSD)

Table V. Opiate Concentrations in Hair of Illicit Heroin Users

Number	Patient number	Morphine (ng/mg)	6-MAM (ng/mg)	Codeine (ng/mg)	AC (ng/mg)	Hair (cm)	Number	Patient number	Morphine (ng/mg)	6-MAM (ng/mg)	Codeine (ng/mg)	AC (ng/mg)	Hair (cm)
1	77	8.9	45.0	2.7	0.0	5.0	40	116	1.9	5.0	1.0	0.3	3.0
2	78	0.4	3.4	0.1	0.4	12.0	41	117	9.3	7.9	0.1	0.1	3.0
3	79	0.5	3.0	0.9	0.5	7.0	42	118	3.7	2.4	0.5	0.2	3.0
4	80	1.4	1.2	0.4	0.0	2.5	43	119	0.9	1.0	0.5	0.1	3.0
5	81	2.3	4.0	0.4	6.0	3.0	44	120	0.9	3.7	0.1	0.5	3.0
6	82	0.9	5.6	0.4	0.7	3.0	45	121	3.7	1.1	0.1	0.1	14.0
7	83	1.7	6.3	0.8	0.5	3.0	46	122	2.0	0.3	0.1	0.1	3.0
8	84	0.6	1.2	0.3	0.2	5.0	47	123	1.9	1.2	0.1	0.1	3.0
9	85	0.5	0.3	0.0	0.1	5.0	48	124	1.3	0.0	0.0	0.1	11.0
10	86	4.0	5.2	2.5	3.5	1.5	49	125	53.7	51.4	1.1	2.8	4.0
11	87	3.1	10.4	0.9	0.8	8.0	50	126	5.0	0.4	0.3	0.1	3.0
12	88	0.5	1.0	0.2	0.2	3.0	51	127	9.2	1.1	0.3	0.3	10.0
13	89	1.0	1.3	0.4	0.2	4.0	52	128	1.5	0.9	0.4	0.2	8.0
14	90	1.9	3.1	0.8	0.5	3.0	53	129	4.7	6.5	1.5	0.4	10.0
15	91	9.6	26.9	3.2	3.3	3.0	54	130	0.1	0.1	0.1	0.0	3.0
16	92	1.0	1.0	0.8	0.2	3.0	55	131	5.3	4.5	0.2	0.3	11.0
17	93	2.4	7.8	1.0	0.6	3.0	56	132	2.2	2.5	0.3	0.3	13.0
18	94	1.7	3.3	0.9	0.6	3.0	57	133	4.2	0.3	0.2	0.2	7.0
19	95	0.6	1.2	0.5	0.4	3.0	58	134	0.5	3.4	0.4	0.5	12.0
20	96	24.9	64.8	15.1	10.5	3.0	59	135	0.4	1.7	0.8	0.2	7.0
21	97	2.4	6.8	1.0	0.7	3.0	60	136	1.1	7.8	0.6	1.0	11.5
22	98	0.3	0.0	0.2	0.0	3.0	61	137	0.7	3.4	0.3	0.4	9.0
23	99	9.5	17.6	5.7	1.6	3.0	62	138	0.3	1.3	0.3	0.2	3.0
24	100	4.8	4.6	1.0	0.4	3.0	63	139	5.3	36.3	1.3	3.2	10.0
25	101	7.0	24.1	2.4	1.4	3.0	64	140	0.6	3.0	0.7	0.4	8.0
26	102	4.0	6.3	1.2	0.5	3.0	65	141	0.0	1.5	0.0	0.2	11.0
27	103	2.4	4.9	0.4	0.6	3.0	66	143	1.1	3.9	0.4	0.5	7.0
28	104	1.7	2.5	0.2	0.1	3.0	67	144	11.2	23.4	2.4	2.0	4.0
29	105	3.6	8.5	1.7	0.7	3.0	68	145	0.0	5.2	0.0	0.5	12.0
30	106	1.8	1.5	0.5	0.2	3.0	69	146	1.5	7.2	0.8	0.8	5.5
31	107	1.2	2.7	0.6	0.3	3.0	70	147	2.0	2.5	0.4	0.3	11.0
32	108	4.1	1.5	0.2	0.1	3.0	71	148	0.0	2.5	0.0	0.0	7.0
33	109	2.4	1.8	1.5	0.2	3.0	72	149	1.0	4.4	0.4	0.5	9.0
34	110	4.2	16.0	1.4	1.5	6.0	73	150	0.0	1.2	0.0	0.0	5.0
35	111	0.7	1.9	0.6	0.3	4.5		mean	3.7	7.2	1.0	0.8	5.4
36	112	6.3	4.2	0.1	0.2	3.0		median	1.9	3.3	0.4	0.3	3.0
37	113	6.4	21.3	2.3	2.3	5.0		maximum	53.7	64.8	15.1	10.5	14.0
38	114	2.0	5.1	0.8	0.5	3.0		minimum	0.0	0.0	0.0	0.0	1.5
39	115	2.7	1.8	0.1	0.1	4.0		rate	94.5	98.6	94.5	91.8	

was equal to 20% (7). These LOQ values were then used as cutoffs in all the study.

Extraction recovery and intra-assay accuracy. Extraction recovery was determined by comparing the peak areas of an extracted hydrochloric acid solution with the peak areas of a methanolic solution, both at the same concentration (1 µg/mL). These solutions were prepared six times each.

Six replicates of soaked hair at low concentrations and high concentrations were analyzed through the complete procedure in order to determine intra-assay accuracy.

Results and Discussion

A specific method was developed for the quantitative determination of 6-MAM, COD, MOR, and AC. Figure 1 shows the GC–MS chromatogram of a hair extract from an illicit heroin user with the following retention times: 5.94 min, 6.64 min, 7.79 min, and 8.76 min for AC, COD, 6-MAM, and MOR, respectively. Figure 2 shows the selected ion chromatogram for AC. Heroin appears on the GC–MS chromatogram; in fact, the method allowed for the detection of heroin but was not validated for this compound because of its low extraction recovery (about 30%).

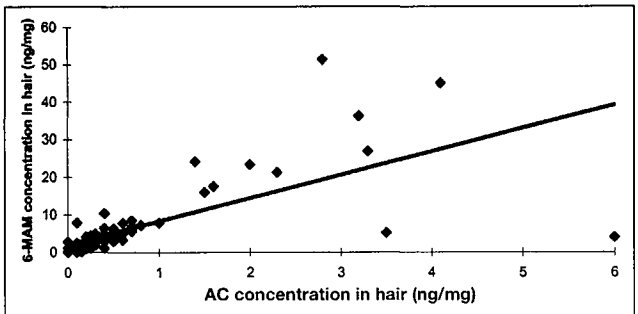

Figure 4. Relationship between 6-MAM concentration and AC concentration found in hair of heroin abusers ($y = 6.18x + 2.09$, $r = 0.68$).

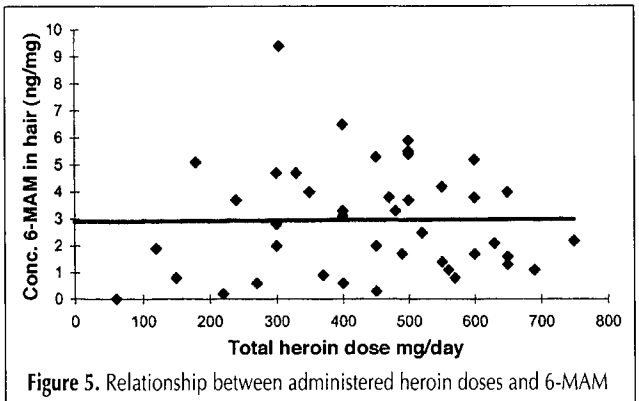

Figure 5. Relationship between administered heroin doses and 6-MAM concentration found in hair ($r = 0.01$).

Satisfactory validation data concerning the other opiates were achieved for linearity, recovery, and intra-assay accuracy. Table II gives the linearity and correlation coefficients obtained for these compounds as well as the LOQ ($n = 8$). Figure 3 shows a curve for the LOQ value. Extraction recovery and intra-assay accuracy are given in Table III. Therefore, a lower recovery for AC compared to the other compounds was noted and could be explained by a slight hydrolysis of AC to COD. The RSD for the intra-assay accuracy determination was generally below or equal to 16% for the two concentrations studied.

Seventy-three hair samples from heroin abusers and 43 hair samples from medical heroin users were analyzed by the described procedure. In one case in each group, the amount of hair sample was not sufficient for analysis and the patient was not taken into consideration. Quantitative results and the respective median, mean, and extreme values for all opiate analytes are given in Tables IV and V. The rate corresponds to the percentage positive for each compound. AC was detected in 92% of the illicit heroin samples and only in 12% of the medical heroin samples.

Generally, the parent drug is the predominant analyte detected in hair sample. However, in this study, about half of the samples have a COD concentration higher than the corresponding AC concentration. This could be explained either by a slight hydrolysis of AC into COD or by a simultaneous COD intake.

In a recent article, O'Neal and Poklis (4) found a positive relationship between AC and 6-MAM concentrations in urine with $r = 0.878$. Kintz et al. (8) observed a similar correlation in hair with $r = 0.915$ and an AC concentration about 8 times lower than the 6-MAM concentration. In our analyses, 6-MAM was detected in almost all the samples, and its concentration was higher than the AC concentration with a 6-MAM/AC ratio ranging from 1 to 20. Figure 4 shows a positive relationship between these concentrations with $r = 0.68$. The highest concentration of AC in hair (10.5 ng/mg) has been excluded because this point had an excessive weight in the statistical analysis. In their article, Kintz et al. (8) observed that hair did not test positive for AC when the 6-MAM concentration was below 1 ng/mg. In our analyses, even with a 6-MAM concentration of 0.3 ng/mg, a corresponding AC concentration up to 0.2 ng/mg could be measured. These results prove good method sensitivity.

However, there was no significant relationship between the administered heroin dose and the 6-MAM concentration in hair for the group that participated in a heroin-maintenance program (Figure 5).

Results concerning the study of AC elimination in hair are given in Table VI. The hair of the three volunteers patients (B, C, and D) collected before the beginning of the study were

Table VI. Acetylcodeine in Hair (ng/mg) after Consumption of Heroin Containing Varying Percentages of Acetylcodeine

	Morphine		6-Acetylmorphine		Codeine		Acetylcodeine	
	segment 1	segment 2	segment 1	segment 2	segment 1	segment 2	segment 1	segment 2
B	12	9.6	7.3	6.8	0.3	0	0.1	0
C	15	10.6	9.4	7.4	0.4	0.1	0.1	0
D	7.5	5.8	6.8	6.8	0.2	0.2	< 0.1	0

analyzed by the described procedure, and only 6-MAM and MOR were quantitated. COD and AC were not detected, so these results show that these patients had not consumed illicit heroin in the three months before the beginning of the study. The results given in Table VI represent hair samples collected 10 days after the end of the study. Segment 1 corresponds to the first centimeter from the root, and segment 2 is the remaining length to the tip.

AC and COD were detected in the first segment of hair samples of the three patients, and COD was even detected in the second segment for patients C and D. This observation indicates that hair growth varies from one individual to another. The hair growth rates for patients C and D were probably higher than 1 cm/month, which is generally accepted as a mean. Therefore, the presence of COD in the second segment should be explained by a higher hair growth rate.

As the aim of this study was to simulate parallel consumption of illicit heroin, it was concluded that repeated illicit heroin intake is necessary before AC and COD can be detected in hair.

Conclusions

A specific method was developed to detect and quantitate AC and other opiates in hair. Even though AC is found at low concentrations in hair because of its weak presence in illicit heroin, this procedure revealed illicit use of heroin at a high rate (> 90%).

6-MAM is certainly the best marker of heroin in hair (rate = 98%), but this study has demonstrated that AC is an interesting biomarker of illicit heroin consumption during the course of a heroin-maintenance program.

Other studies should be considered if results are to be compared with additional personal characteristics, such as hair color, melanin content, gender, and age, as these factors could influence opiate concentrations in hair.

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