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Determination of Morphine in Human Hair by GC/MS Method

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Determination of Morphine in Human Hair by GC/MS Method

Summary : *In this study morphine was determined in hair obtained from two persons suspected of heroine abuse by Gas Chromatography-Mass Spectrometry (GC/MS). Morphine were extracted by solid-phase extraction (SPE) and derivatized with N,O,-bis (Trimethylsilyl) trifluoroacetamide (BSTFA) + 1% Trimethylchlorosilane (TMCS). The derivatized extracts were injected into the GC/MS which operated in the selected ion monitoring (SIM) mode. Linearity was observed in our working range with "R^2" value of 0.9983. The recovery rate of the extraction method was 91.8% for morphine. 45.19 and 12.5 ng morphine/100 mg hair were determined in sample A and sample B respectively using this method.*

Key words: *Morphine, hair, GC/MS*

GC/MS metodu ile insan saçında morfin tayini

Özet : *Bu çalışmada eroin kullandığından şüphelenilen iki kişinin saçında GC/MS metodu kullanılarak morfin tayini yapılmıştır. Morfin, katı faz tüketimi yöntemi ile ekstre edilmiş ve BSTFA + %1 TMCS ile derivatize edilmiştir. Derivatize edilen ekstre SIM modu ile çalışan GC/MS'e enjekte edilmiştir. Çalışma aralığımızda linearite gözlenmiş ve R^2 değeri morfin için 0.9983 olarak bulunmuştur. Bu yöntem kullanılarak numune A ve B de sırası ile 45.19 ve 12.5 ng morfin (100 mg saç'da) tayin edilmiştir.*

Anahtar kelimeler: *Morfin, saç, GC/MS.*

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INTRODUCTION

Heroin abuse continues to represent a major health problem for the general world population. Diagnosis, treatment and prevention of drug use require objective means of identification of drug users[1]. Currently, the most frequently used biological specimens for the identification of drug use are blood, saliva and urine. There are certain advantages associated with each specimen. However, drugs are cleared rapidly from these fluids. Therefore blood, saliva and urine analyses provide short-term historical records of drug exposure[2]. In recent years hair has for emerged as a more useful biological specimen for identifying the metabolites of drugs. The toxicological analysis of hair, which has been used during the last decade to identify the presence of drugs and their metabolites in drug abusers, has certainly opened new horizons in the area of "drug testing". Since hair retains the drug in a relatively inert matrix, it provides a long-term record of drug use in contrast to the biological specimens mentioned above[2,3]. Consequently, hair analysis appears to provide a more comprehensive means of drug detection[2,3].

Hair analysis for drug abused is a relatively new and unexplored field, and numerous questions remain regarding the validity of hair testing. The mechanisms involved in the incorporation of drugs

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into hair should be understood more precisely. One of these mechanisms is thought to be passive diffusion[3,4,5,6]. Drugs enter hair by passive diffusion from the blood stream into the growing cells at the base of the hair follicle. The second mechanism is described the incorporation of drugs and metabolites into hair after formation of the hair shaft. According to this mechanism drugs and metabolites are incorporated into hair via secretions of the apocrine and sebaceous glands[4]. After hair has emerged from the skin, external contamination could be evaluated as a third mechanism involved in drug incorporation into hair. Since false positive results are caused by passive exposure (external contamination) the third mechanism become more important than the others[3,4]. Therefore, preliminary treatment (washing procedure) of hair to minimize the effect of possible external contamination appears to be important step in the determination procedures.

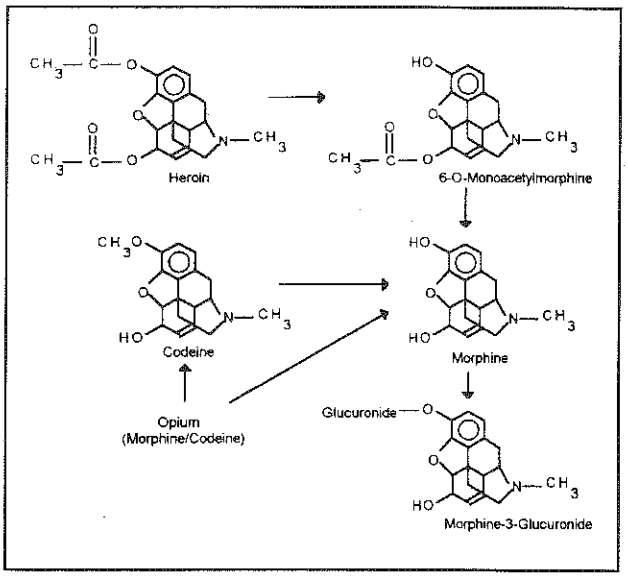

Figure 1. Metabolic pathways of opiates

The metabolic pathways of opiates are shown in Fig.1. Following intake, heroin is rapidly deacetylated to 6-O-monoacetylmorphine, which is then further hydrolysed to morphine[1,2]. Codeine has often been found in the urine of persons taking illicit heroin but this is not a metabolite of heroin. Rather it is the result of deacetylation of acetylcodeine, which is often found as an impurity in illicit heroin[2,7,8,9].

In this study the hair samples were hydrolysed with NaOH, extracted by using SPE columns, the extract was derivatized with BSTFA, 1% TMCS and the determination was performed by using the GC/MS method. The recovery and reproducibility of results obtained using this method were compared with previous studies. Moreover the presence of morphine in hair obtained from 2 persons suspected of heroin abuse was investigated in order to test the validity of the present method.

Materials and Methods

Chemicals and Reagents: Morphine was extracted using Bond Elut Certify solid-phase extraction columns (Varian Sample Preparation Products, Harbor City, CA). Morphine-D_3 was used as the internal standard in GC/MS. Morphine and the deuterated internal standard mentioned above were supplied by Radian Corporation, Austin, TX. Derivatization was carried out with BSTFA [N,O,bis (trimethylsilyl) trifluoroacetamide] with 1% TMCS (trimethylchlorosilane) which was supplied by Pierce. The other chemicals were supplied from Merck.

Morphine-D_3 was obtained as solutions at concentrations of 100 µg/ml. Stock internal standard solution was prepared by diluting to give a final concentration of 2.5 ng/µl. Morphine was also obtained as solutions at concentrations of 1 mg/ml and was also diluted to give a concentration of 1.0 ng/µl. These final solutions were used as stock standard solutions.

0.15 M Phosphate Buffer, pH 9: Solution A: 26.69 g Na_2HPO_4 was added to a 1.0 l volumetric vial and the volume was brought up to 1.0 l with deionized (DI) water. Solution B: 20.40 g KH_2PO_4 was added into a 1.0 l volumetric vial and the volume was brought up to 1.0 l with DI water. 80 ml of solution A was measured and adjusted to pH 9 with solution B.

0.1 M Phosphate Buffer, pH 7: 13.609 g KH_2PO_4 was added to a 1.0 l volumetric vial and the volume was brought up to 1.0 l with DI water. The pH was adjusted to 7 with 1.0 M KOH.

0.1 M Acetate Buffer, pH 4.5: 80 ml of DI water and 570 µl of glacial acetic acid were added to a 100 ml

volumetric vial and vortexed. The pH was adjusted to 4.5 with 1.6 ml of 1.0 M KOH. Finally the volume was brought up to 100 ml with DI water.

Instrumentation: Analyses were performed on a Hewlett Packard Model 5790 A series Gas Chromatograph and 5970 A series mass selective detector (MSD) with a 12 m x 0.2 mm x 0.33 µm film HP Ultra-2 crosslinked 5% Ph Me Silicone capillary column. Column pressure: 10 psi, Septum purge flow: 0.8 ml/min, Split vent flow: 20 ml/min, column flow: 1.0 ml/min, Column temp.1: 110°C (time: 0.3 min, increment: 15°C/min) Column temp.2: 275°C (time: 20 min), Injector temp.: 250°C, Interface temp.: 275°C, Injection mode: splitless, Dwell Time: 50 ms.

Selected ions: Morphine-TMS m/z 429, 287, 324; Morphine-D_3-TMS m/z 432, 290. The selected ion ratios for quantification were m/z 429/432[7].

Washing procedure of hair samples: All the hair samples were cut as finely as possible and put into large test tubes. The blank hair was cut at first to prevent contamination. 5 ml of phosphate buffer (pH 9) was added into the tubes and washed for 2 times. Phosphate buffer was discharged carefully after the washing procedure had been completed. The same washing procedure was repeated with 6 ml of methanol (two times) and the hair samples were left to dry at 70°C.

Preparation of standards and samples: The standards were prepared shown below;

Blank: 100 mg blank hair + 20 µl (50 ng) I.S. + 1 ml 0.1 N NaOH

25 ng: 100 mg blank hair + 20 µl (50 ng) I.S. + 25 µl (25 ng) Morphine + 1 ml 0.1 N NaOH

50 ng: 100 mg blank hair + 20 µl (50 ng) I.S. + 50 µl (50 ng) Morphine + 1 ml 0.1 N NaOH

100 ng: 100 mg blank hair + 20 µl (50 ng) I.S. + 100 µl (100 ng) Morphine + 1 ml 0.1 N NaOH

Sample A: 100 mg hair + 20 µl (50 ng) I.S. + 1 ml 0.1 N NaOH

Sample B: 100 mg hair + 20 µl (50 ng) I.S. + 1 ml 0.1 N NaOH

Blank, standards and samples were hydrolysed in 1 ml of 0.1 N NaOH for 24 hours at 60°C and the protein matrix of the hair was destroyed during this incubation. When this procedure was completed, 5 ml of 0.1 M phosphate buffer (pH: 7) was added to each tube and the pH was adjusted to 7 with 0.6 N HCl. All the tubes were finally centrifuged (3000 rpm, 7 min).

Extraction procedure: Blank, standards and samples mentioned above were passed slowly through the Bound Elute Certify columns after the columns had been activated by passing sequentially through 3 ml of methanol and 3 ml of DI water. To rinse the columns, 3 ml of DI water, 2 ml of 0.1 M acetate buffer (pH 4.5) and 3 ml of methanol were also passed sequentially through the column and dried under full vacuum for two minutes. 2 ml methylene chloride: isopropanol (80:20) with 2% ammonium hydroxide was used to elute the analytes through the columns and the eluents were evaporated under a stream of nitrogen at 40 C in a water bath. The residues were reconstituted with 100 µl of BSTFA with 1 % TMCS in a suitable screw capped tubes and placed in a heating block at 70°C for 1 hour. After cooling, 2 µl of the derivatized solutions was injected into the GC/MS.

Linearity: 25 µl (25ng), 50 µl (50 ng) and 100 µl (100ng) of morphine were added to the drug free solutions containing 100 mg of blank hair (as described in the preparation of standards). These hair samples were extracted and derivatized in the described manner. The ion ratios for quantitating the analyte were plotted against their respective concentration. The plots were subjected to linear regression analyses (Fig. 2).

Recovery Studies: Drug-free hair was used to prepare standard morphine solution of 25 ng/100mg hair. The standard solution was extracted in the previously described manner, with the exception that no internal standards were added to the blank hair solution prior to extraction. After extraction and prior to derivatization, 20 µl of deuterated internal standard (morphine-D_3) was added to the extract. The derivatized extract was analyzed under the described conditions. The area ratio of the extracted drug to the respective internal standard was compared to the corresponding area ratio of unextracted

25ng morphine standard. The recovery results are the mean of three determinations (Table 1).

Reproducibility studies: Reproducibilities of the analyses were determined by calculating coefficients of variation. Morphine added (25 ng/100 mg and 100 ng/100 mg) drug free hair standard solutions were analyzed in 5 separate runs under the described conditions (Table 2).

Limit of detection (LOD): The limit of detection was determined according to the IUPAC method[10].

RESULTS AND DISCUSSION

In this study the solid-phase extraction (SPE) and the BSTFA + 1% TMCS derivatization techniques were used simultaneously in combination with GC/MS method. This combination is very rapid and provides excellent linearity, recovery and reproducibility for routine laboratory use. In our previous study the advantages of this combination were described in detail[11]. The limit of detection for morphine was calculated as 0.1 ng/mg hair by this method.

Linearity: As shown in Figure 2, the "R^2" value of morphine was calculated as 0.9983. The y-intercept was 0.0752 for morphine. These results provided us with an adequate quantification in our working range.

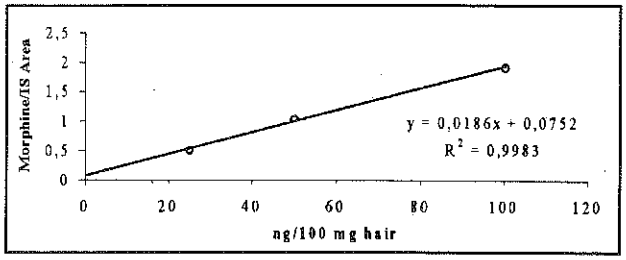

Figure 2. Peak area ratios versus concentrations of morphine in hair.

Recovery: In this study a recovery rate of over 90% has been obtained for morphine, seen in Table 1. So far in many studies numbers of extraction procedures were performed in order to extract morphine from hair. However different extraction recoveries were reported in most of these studies. Mangin et al reported a recovery of over 75% for the opiates (morphine, codeine and 6-monoacethylmorphine)[7]. The use of liquid-liquid extraction instead of solid-phase extraction could be the main reason of the lower recovery rate in Mangin's study. Moeller et al also reported a recovery rate of 70% for morphine[12]. The Moeller's procedure and ours seem very similar to each other. The combination of solid-phase extraction and derivatization method was also used simultaneously in Moeller's study as in our study. However the different hydrolysation procedure, the use of different types of solid-phase extraction columns and derivatization agents might be the reason for the lower recovery. In this study the recovery obtained was higher than the studies mentioned above as seen in table 1.

Table 1. The results of the recovery studies

	Recovery (%)	
	Mean*	Range
Morphine	91.8	88.3 - 93.4

*The results are the mean of three determinations (0.25 ng/mg)

Reproducibility: An adequate reproducibility was provided by this method. The coefficient of variation (CV) for morphine can be seen in Table 2. Moeller reported a CV value of 4% for morphine in his study, which was very close to our results. The similarity of the CV values obtained in Moeller's and in the present study indicated that the procedures of both methods have provided a comparable reproducibility.

Table 2. The results of the reproducibility studies

Reproducibility								
Drug	Actual ng/100mg		Found* ng/100mg		S.D. ng/100 mg		C.V.	
Morphine	25	100	25.5 (24.3-28.4)	101.1 (97.6-103.5)	1.86	2.14	7.3	2.1

*The concentrations are the mean of five determinations

The chromatograms of blank hair and standards are shown in figure 3 and 4. In both samples morphine was determined in hair as seen in Figure 5 and 6.

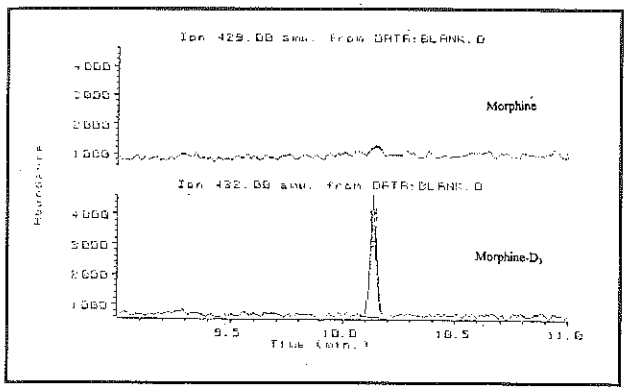

Figure. 3 SIM chromatograms of morphine-D_3 from blank hair.

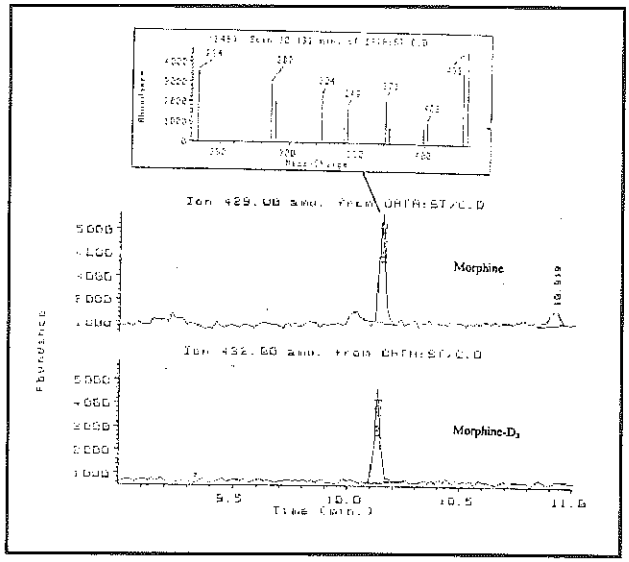

Figure. 4 SIM chromatograms of standards extracted from blank hair.

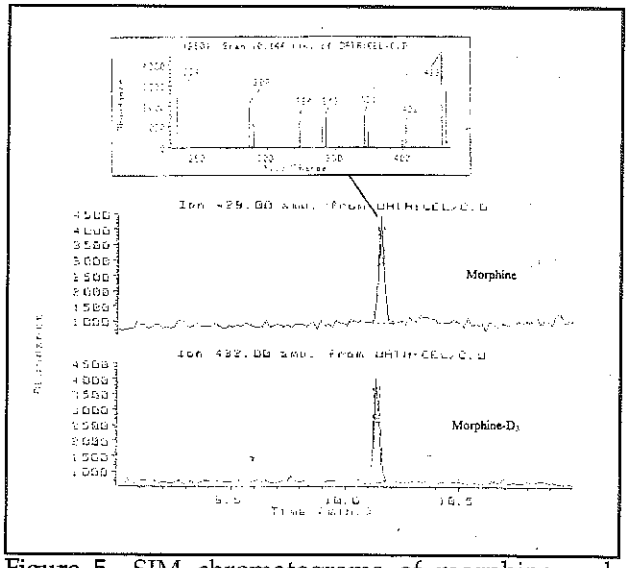

Figure. 5 SIM chromatograms of morphine and morphine-D_3 (Sample A).

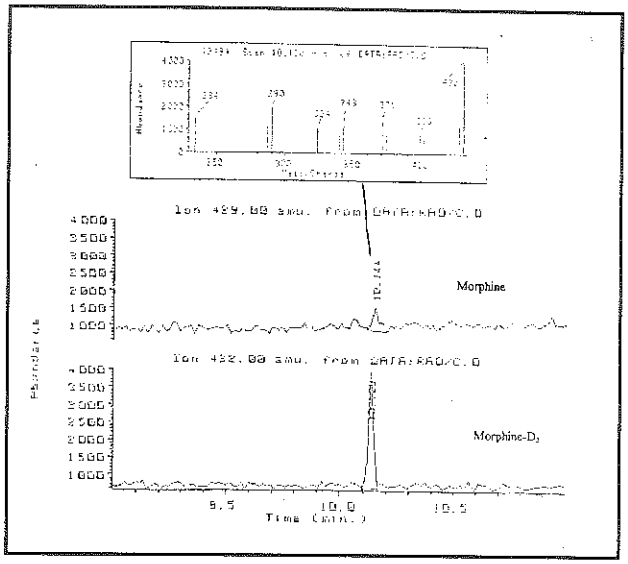

Figure. 6 SIM chromatograms of morphine and morphine-D_3 (Sample B).

45.19 and 12.5 ng morphine/100mg hair were determined in sample A and sample B respectively using the chromatograms mentioned above. However there were two main problems in evaluating the results. The first one was to differentiate the heroin and morphine users. Since heroin is rapidly deacetylated to morphine after intake, determination of morphine in hair is always possible in both heroin and morphine users[2]. The second one was to remove the external contamination of drugs. External contamination could be a potential route of entry into hair for drugs that are smoked such as heroin, cocaine and marijuana[2,3]. Therefore, an appropriate washing procedure should be performed before the extraction procedure to prevent false positive hair tests resulting from external contamination. However there is still no good agreement as to how effective various washing procedures are in removing these drugs from hair[7].

In recent years determining 6-monoacethylmorphine in hair has solved the first problem. This finding provides certainty in confirmation of heroin intake[2,7]. However in our study, due to the hydrolisation conditions, it was not possible to determine 6-monoacethylmorphine in hair, since 6-monoacethylmorphine hydrolyzed rapidly to morphine in strong alkaline conditions. Nev-

strong alkaline condition provides, better recovery[13,14,15] rates. Therefore we decided to use alkaline extraction conditions in this study. As could be seen in table 1 and 2, we determined adequate reproducibility and recovery rates for this method. However, differentiating the heroin and morphine users is unfortunately not possible using this method because of the alkaline hydrolysation conditions.

The external contamination emerged as a second problem in such studies. In fact any washing procedures can remove the externally contaminated drugs from hair completely[15]. Several decontamination procedures employing detergents and solvents have been examined in numbers of studies, and none of them completely decontaminated the hair[15,16,17]. Therefore it is a serious problem in distinguishing the users from the nonusers.

In our study morphine was determined in both hair samples as seen in Fig. 5 and Fig. 6. However the probability of an external contamination should be also considered especially in sample B because of the relatively low concentration of morphine. It is well known that vashing procedures can remove a considerable amount of the externally contaminated drug[15]. Therefore small quantities of drugs in hair could be a sign of an external contamination and should be evaluated more carefully to prevent possible mistakes.

According to our results we can say that sample A probably had used morphine or heroin in the past. However, it is not so easy to make a definite decision on sample B. We can only say that morphine is definitely present in sample B but it is probably a result of an external contamination. Consequently we should emphasize that external contamination of hair by drugs can easily occur and there are no quantitative measures that can distinguish between endogenous and exogenous exposure[18]. Any interpretation of hair analysis data should consider the possibility that the sample could have been externally contaminated.

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