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Review

Gas chromatographic–mass spectrometric procedures used for the identification and determination of morphine, codeine and 6-monoacetylmorphine

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Abstract

An overview of the analysis of opiates by gas chromatography–mass spectrometry (GC–MS) is presented. The review is focused on the hydrolysis, extraction and derivatization procedures most widely used for the identification and determination by GC–MS of legal and illegal opiates in various biological fluids.

Contents

1. Introduction

Opioid analgesics comprise a large group of substances. Some compounds have long been used for their therapeutic qualities as analgesics (morphine) or antitussive agents (codeine and the semi-synthetic derivatives, dihydrocodeine, oxycodone, etc.). Opiates are also found in opium poppy seeds, an ingredient of bakery products. With the rise in the use of illegal drugs there is increasing pressure to identify illegal drug consumption. Consequently, toxicology laboratories, especially those testing for substance abuse, must have specific and sensitive techniques to discriminate between the legal and illegal intake of opiates.

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Different methods have been developed for the detection and determination of opiates in body fluids, including thin-layer chromatography [1,2], gas chromatography with flame ionization [3,4] or electron-capture detection [5,6] and liquid chromatography [7–9]. However immunological methods are now widely adopted as the initial screening test to detect opiates in urine because they have adequate sensitivity and pretreatment of samples is not required [10,11]. These immunoassays are reliable for differentiating specimens containing opiate metabolites above cut-offs specified in the Mandatory Guidelines for Federal Workplace Drug Testing Programs, Notice Fed. Reg. 53 (1988) 11970 (300 ng/ml for total morphine and 25 ng/ml for free morphine). Some radioimmunoassay (RIA) kits are highly specific for free morphine [12], but generally the immunoassays are not very specific as legal and illegal opiates give substantial cross-reactivity. Therefore, presumptive positive specimens need to be retested and gas chromatography coupled with mass spectrometry (GC–MS) has been designated as the only acceptable confirmation technique [13].

This review is focused on sample preparation, derivatization and GC–MS procedures commonly used for confirmatory analysis and determination of opiates. The number of protocols proposed in the literature is relatively limited and numerous minor variations have been described, rendering the choice of a method difficult.

2. Opiate metabolism

The opiate substances the most frequently detected and determined in biological fluids are codeine, morphine and 6-monoacetylmorphine (6-MAM), a metabolite of heroin.

2.1. Morphine

In man, morphine metabolism depends largely on the route of drug administration. After oral administration, morphine is quickly absorbed from the gastrointestinal tract and is rapidly conjugated in the cells of the intestinal mucosa and in the liver, hence no free morphine appears in the plasma [14]. After an intravenous injection, the morphine level rises to a maximum in the plasma during the first 5 min and declines rapidly during the next 12 h. However, it can still be found in plasma 48 h after injection [15]. A half-time of 1.9–3.1 h and a detection time of 10–44 h have been established [16]. The mean half-life for free morphine is reported to range from 4.3 to 8.1 h and that for conjugate morphine is between 6.4 and 9.7 h [17,18].

In plasma, morphine is partly bound to proteins, preferentially to albumin [19,20]. The binding to albumin explains why morphine is still found in plasma 48 h after injection. The distribution of morphine to the tissues, principally the liver, kidneys, lungs and brain, then proceeds very rapidly.

Morphine is converted into the 3-glucuronide (M3G) and to a lesser extent into the 6-glucuronide (M6G) and 3,6-diglucuronide (M3,6G). More than 50% of the administered morphine is eliminated as M3G [21,22]. The level of M6G in the urine could reach 10% of that of M3G [22]. M6G has potent analgesic activity [23]. Physiologically, this metabolite does not accumulate in plasma, but it may be present in the plasma of patients with renal failure, resulting in side-effects [24] such as respiratory depression [25] or brain syndrome [26].

About 5–10% of administered morphine is converted into the 3-ether sulphate [27,28] and 3–5% into normorphine [29]. Codeine was previously reported as a metabolite of morphine [14,15] but recently Mitchell *et al.* [17] demonstrated unequivocally the absence of codeine as a metabolite of morphine.

The water-soluble conjugates are mainly excreted via the kidneys and very little is eliminated via bile and faeces [15].

2.2. Heroin

The route of heroin administration is generally intravenous, resulting in a transient high drug

concentration in the blood. Heroin quickly disappears from the blood, its half-life being estimated to be *ca.* 2 min [30,31]. Heroin is rapidly deacetylated, first to 6-MAM, which is further hydrolysed to morphine. The pharmacological effects of heroin and 6-MAM are equipotent. 6-MAM is rapidly excreted in the urine within 1–4 h whereas the peak of free morphine occurs within 4 h (half-life 0.6 h) and that of total morphine within 8 h [18]. Hence the detection time is a very important parameter for interpreting results. The detection time for free morphine is substantially shorter than that for total morphine.

2.3. Codeine

Codeine is generally administered orally. The mean half-life of codeine in plasma ranges from 1.6 to 2.4 h according to the dose administered [18]. It is extensively metabolized in the liver, mainly by conjugation with glucuronic acid, and minor routes involve N-demethylation to norcodeine (about 10%) and O-demethylation to morphine. Codeine and its metabolites are eliminated in the urine.

Fig. 1 summarizes the main routes of opiate biotransformation. It is noted that morphine is a metabolite of both heroin, an illegal drug, and codeine, used in prescription medication. The evaluation of the percentages of urinary metabolites of opiates and the presence of M6M, which is solely attributed to heroin, allow the differentiation of heroin abuse from the consumption of legal drugs [13]. Table 1 gives the urinary percentages of opiate metabolites after administration of heroin, codeine and morphine.

3. Assays of opiates by GC–MS

3.1. Choice of samples

Many biological specimens can be used for substance abuse testing. Each type has advantages and disadvantages with respect to its availability and the information that its analysis can supply.

Saliva is readily available but has low drug concentrations and the drug level rapidly declines [34,35]. Hair is also easily available but requires sample pretreatment [36,37]. Tissues [38,39] and vitreous humor [40] are sometimes used. However, blood (or serum or plasma) and urine are the specimens preferentially used for the detection and determination of opiates. A blood sample often offers the advantage of acquiring the parent drug, but its collection requires invasive venous puncture and the drug concentrations decline rapidly. Blood samples are preferred for the follow-up of analgesic treatments. However, urine is generally accepted as the specimen of choice for drug abuse testing or doping analysis. Its collection is non-invasive, the volume obtained can be large and the concentrations of drugs or metabolites are often high, but the drug concentration can vary widely with dose absorbed, time elapsed since administration, etc. [13]. Hence the choice of sample depends on the aim of the analysis.

3.2. Hydrolysis

The opiates are partly conjugated with glucuronic acid and sulphate prior to urinary excretion. Hence total opiates can be recovered after hydrolysis. However, 6-MAM, a marker of heroin abuse, is degraded by acid hydrolysis and morphine can also be partially destroyed [41], so many laboratories do not routinely hydrolyse samples.

Urine is hydrolysed with concentrated hydrochloric acid at 115–120°C [15 p.s.i.; 1 p.s.i. = 6894.76 Pa] for 15 min [42,43,44] or with various concentrations of β-glucuronidase at 37°C for 1 h [45,46] or 24 h [47]. β-Glucuronidase has also been used in combination with arylsulphatase for 1 h at 60°C [40]. The hydrolysis can be performed with *Helix pomatia* juice for 2 h at 56°C [48] or 24 h [49]. In a recent study, acidic hydrolysis was compared with enzymatic hydrolysis and a highest codeine metabolite recovery was obtained with enzymatic hydrolysis [50] even though it is known that β-glucuronidase cannot completely hydrolyse codeine 6-glucuronide. To overcome problems relating to hydrolysis, in our

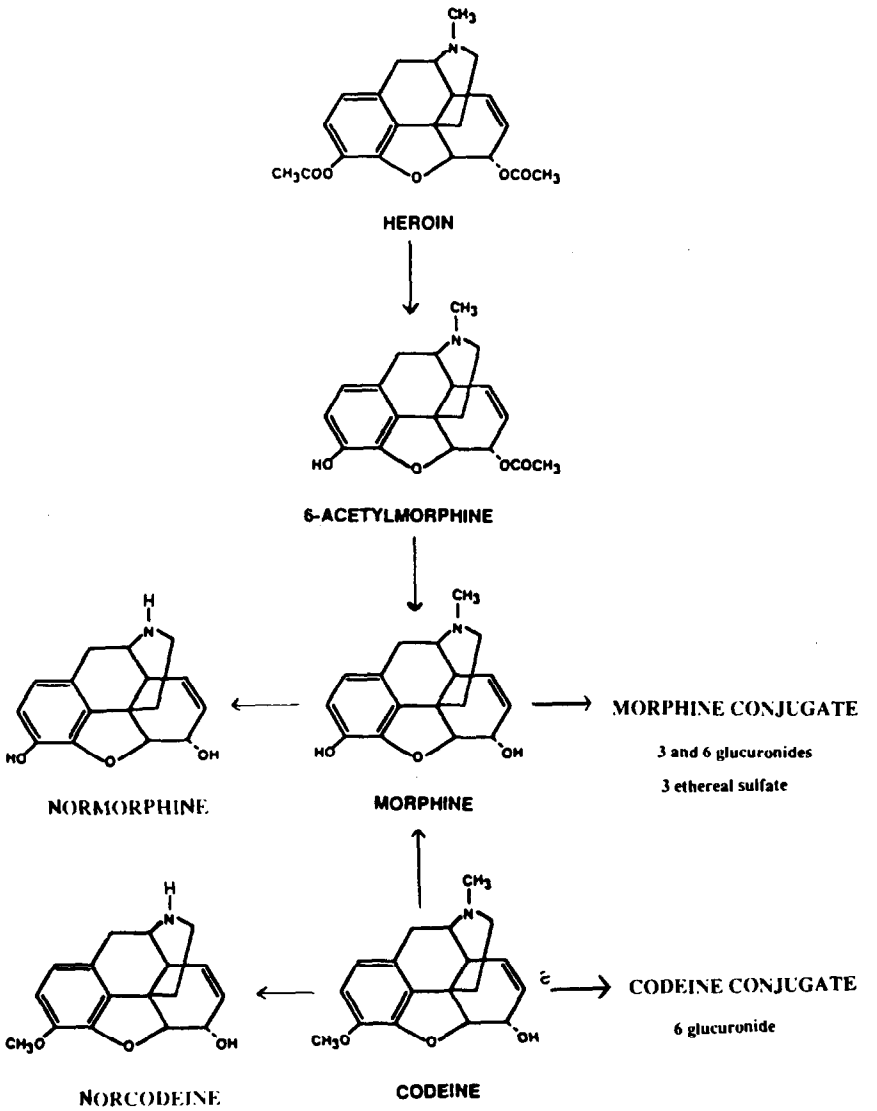

Fig. 1. Main steps of opiate biotransformation.

laboratory we usually assay both acid-hydrolysed and unhydrolysed urine.

3.3. Extraction

The opiates are extracted from biological fluids or tissue homogenates prior to detection and determination. Extraction with organic solvents is frequently used. In this case the samples are previously made alkaline (pH ≈ 9) with 1 *M* ammonia solution [3], 1.5 *M* sodium carbonate buffer [46], a mixture of 12 *M* sodium hydroxide and 7.3 *M* ammonium chloride [43] or borate buffer [45]. Then they are extracted with organic solvents: chloroform–2-propanol (9:1) [46,51,52], (4:1) [49] or (3:1) [38], dichloromethane–methanol (9:1) [3], dichloromethane–2-propanol (9:1) [47], isobutanol–dichloromethane (1:9) [43,53], ethyl acetate [39], or toluene–dichloromethane–isobutanol (6:3:1) [54].

The organic phase is evaporated and directly derivatized [46,49,55] or purified by the acid-

Table 1
Urinary metabolites of codeine, morphine and heroin expressed as percentage of administered dose

Metabolite	Codeine	Morphine	Heroin
Free	4.9–8.2 [18]	2–12 [14,15,17]	–
6-Glucuronide	25–56 [18]	<1–10% [21–23]	–
3-Glucuronide	–	20–74 [15,17,23]	–
3-Ether sulphate	–	0.5–10 [23,28]	–
6-Monoacetylmorphine	–	–	0.1–2.8 [27,29]
Morphine:			
Free	~0.1% [18]	–	3.1–7.7 [27,29,32]
3-Conjugate	2–9% [18]	–	34–67 [23,27,29,33]
Normorphine:			
Free	Traces	0.5–1.5	–
Conjugate	–	3–5	–
Norcodeine:			
Free	–	–	–
Conjugate	–	–	–

base method [3,38,43,48,53], by organic phase partitioning [54] or by the solid-phase method on cartridges containing cyanopropyl- or propylamine-modified silica [53]. Paul *et al.* [53] compared solid-phase and acid-base purification and preferred the latter method because it provides between-run consistency in drug recovery.

When a solid-phase extraction technique is used directly, the samples are first made alkaline and passed through a C_{18} reversed-phase column [40,56], C_{18} Bond Elut column [57–60] or Extrelut column [4]. The effect of sample pH on retention has been investigated. Huang *et al.* [59] found the recovery of compounds such as morphine, codeine and hydrocodone from urine to be independent of pH, whereas a better recovery for morphine, codeine and to a lesser extent M3G and M6G was obtained at pH 9 by Pawula *et al.* [61]. On increasing the pH, the ionization of the basic nitrogen group ($pK_a = 8$) is suppressed, thus making morphine and normorphine interact more with the C_{18} group, whereas the glucuronides remain completely ionized at high pH, so their retention times are not increased. The optimum pH for extraction of 6-MAM was found to be 8–8.5 [53]. The opiates are eluted from the column with dichloromethane–acetone (1:1) [56,58], chloroform–2-propanol (9:1) [52] or (4:1) [39], methanol [40], ethyl acetate [4] or dichloromethane–2-propanol (4:1) [50]. Solid-phase extraction gives the best sample purification [40], yielding a low GC–MS background which enhances the mass spectral characteristics and permits a better identification of drugs and metabolites, but it is more expensive than liquid extraction.

3.4. Derivatization

Some investigators do not derivatize the opiates before chromatography [6,62]. However, the underivatized opiates show poor chromatographic properties. The derivatization process converts the polar hydroxyl groups into a non-polar derivative, improving the chromatographic resolution and increasing the sensitivity. Several methods are available for obtaining derivatives: acetylation, propionylation (propionic anhydride) and the formation of trimethylsilyl or perfluoroester derivatives. Although the number of derivatizing agents described in the literature is relatively limited, there is great variability in the experimental conditions. The main protocols are summarized in Table 2.

Maurer and Pfleger [63] described a screening procedure for the detection of 56 opioids, analgesics and their metabolites. However, most reports concern methods for the identification of morphine, codeine and 6-MAM because the purpose is to identify illegal drug abuse. The

Table 2
Derivatizing agents and derivatization conditions described in the literature for the identification and determination of opiates

Method or derivatives	Sample	Derivatizing agent	Volume of derivatizing agent (μl)	Conditions		Solvent for reconstitution of derivatives	Ref.
				Temperature (°C)	Time (min)		
Acetylation	Purified products	Acetic anhydride–pyridine (1:1)	100–400	50–100	15–50	Chloroform, acetone, acetyl acetate	43,55,64
	Urine						
	Vitreous humor	Acetic anhydride–pyridine (2:1)	150	60	30	–	40
	Urine	[$^2H_6$]Acetic anhydride–pyridine 1:1	200	60	15	Ethyl acetate	46
	Urine	Acetic anhydride–4-dimethylamino-pyridine (9:1)		50	15–30	–	44
Propionylation	Urine	Propionic anhydride–pyridine (1:1)	100	50–70	30	Toluene	53
Trimethylsilyl derivatives	Urine	BSA	25	60	60	Chloroform	49
	Urine	BSTFA–acetonitrile (1:2)	80	60	15	Butyl acetate	3[a]
	Urine	BSTFA–TMCS (99:1)	25–50	Room temperature to 70	15–30	Ethyl acetate, dichloromethane	46,64,65
Trifluoroacetyl derivatives	Urine	TFA	50–100	60	20–30	Dichloromethane, ethyl acetate, chloroform	43,48[a],66
	Blood, pure compounds, hair	MBTFA	40–50	60–70	20–30	Dichloromethane	64,70
Pentafluoropropionyl derivatives	Blood, urine	PFPA	25–80	60–70	15–30	Ethyl acetate, dichloromethane, butyl acetate, heptane	3[a],43,47[a],56, 58,60,64,68
		PFPA–ethyl acetate (1:1)	100	90	15	Ethyl acetate	39
		PFPA–PFP-OH (2:1)	75	70–90	15–20	Ethyl acetate	55,67
Heptafluorobutyryl derivatives		HFBA	50–100	60–70	30	Dichloromethane	43,64,71[a]

Abbreviations: BSA = bis(trimethylsilyl)acetamide; BSTFA = bis(trimethylsilyl)trifluoroacetamide; HFBA = heptafluorobutyric anhydride; MBTFA = methylbistrifluoroacetamide; PFPA = pentafluoropropionic anhydride; TMCS = trimethylchlorosilane; TFA = trifluoroacetic anhydride. Detection by GC–MS except where indicated otherwise.
[a] Detection by GC.

determination of these compounds requires the addition of an internal standard to the samples before the extraction procedure. In general, nalorphine or corresponding deuterated products ([$^2H_3$]morphine, [$^2H_3$]codeine, [$^2H_3$]6-MAM) are used as internal standards, the latter permitting the recovery problem to be overcome. At least two ions are monitored for the identification of each opiate. The ions most often used for morphine are those at m/z 327 and 353 (acetylation), 364 and 477 (TFA), 414 and 577 (PFPA), 464 and 207 (HFBA); for codeine 282 and 229 (acetylation), 292 and 395 (TFA), 282 and 445 (PFPA), 282 and 495 (HFBA); for nalorphine 353 and 395 (acetylation), 390 and 503 (TFA), 440 and 603 (PFPA), 207 and 490 (HFBA); and for 6-monoacetylmorphine 204, 328 and 372 (acetylation), 364 and 423 (TFA), 414 and 473 (PFPA). Table 3 gives a more complete list of ions used for opiate identification.

The acetyl derivatives are stable for up to 72 h when stored at room temperature in ethyl acetate [46,56]. However, the acetylation protocol (70°C, 20 min) results in incomplete derivatization, and in addition to the major derivatization product diacetylmorphine a small amount of 3-monoacetylmorphine (3-MAM) is also produced [55].

The m/z 285 ion is found in the mass spectrum of both 3-MAM and [$^2H_3$]acetylcodeine, making this ion unsuitable as a specific ion for [$^2H_3$]codeine. Morphine and 6-MAM are both converted into diacetylmorphine, and therefore acetyl derivatives do not permit morphine and 6-MAM to be distinguished [55].

Derivatization with BSTFA is quantitative and each opiate gives only one derivative. However, TMS derivatives of codeine and norcodeine co-elute and 6-MAM gives an additional peak, eluting at the retention time of morphine, which increases when the 6-MAM derivative is stored at room temperature for more than 3 h [3]. The TMS derivatives are known as to be moisture sensitive [64].

PFP derivatives are also sensitive to moisture, but no breakdown products are detected after storage for 24 h in good conditions [55]. The addition of PFPOH improves the yield of the derivatives. Christophersen *et al.* [3] obtained only one PFP derivative for each opiate with the derivatization protocol described (60°C, 15 min), whereas Paul *et al.* [43] found two derivatives for morphine (3,6-di-PFP-morphine and 6-PFP-morphine). In addition, the morphine and the 6-MAM can be clearly detected [56,58]. In spite of their disadvantages, acetyl and PFP derivatives are widely used for the identification and determination of opiates.

3.5. GC–MS procedures

Some investigators use chemical ionization mass spectrometry for the identification and determination of opiates with methane [49,42,61] or ammonia–methane (1:5) [57] as the reactant gas. However, in recent studies the electron impact mode is chosen, generally at 70 eV. The chromatographs are equipped with a 12- or 15-m fused-silica capillary column with apolar stationary phases of cross-linked dimethylsilicone, phenylmethylsilicone or 95% dimethyl–5% polysiloxane [46,56]. The oven temperature can be maintained in the isothermal mode at 230°C [52,45], but in general temperature programming is used with an initial temperature between 50°C [64] and 160°C [40] and a final temperature from 240°C [40] to 280°C [37], the rate of increase being from 10°C/min [31] to 50°C/min [64].

4. Conclusions

Various GC–MS methods have been described for the identification and determination of opiates. As morphine and codeine are conjugated before being excreted in the urine, hydrolysis is required to recover these two compounds totally. Acidic hydrolysis is more rapid and easier than enzymatic hydrolysis. However, 6-MAM can be destroyed during this process. The assays of opiates include an extraction step, first performed with organic solvents but now often replaced by solid-phase extraction. The latter technique has the advantage of decreasing the background noise, which improves the identification of the drugs. Further, this pro-

Table 3
Principal ions (m/z) used for the detection and identification of opiates

Product	Acetylation	Propionylation	TMS	TFA	PFPA	HFBA
Morphine	395, 375, 369, 353 310, 268, 216		429, 414, 369, 287, 268, 236	477, *363*, 263	*577*, *414*, 361, 357	677, 464, 210, 207
[$^2H_3$]Morphine	378, 372, 334, 330, 313			480, *367*	580, *417*	
Codeine	395, 353, *344*, 341, *282* 229		371, 234, 229	*395*, *282*	*445*, 283, *282*	495, 282, 225
[$^2H_3$]Codeine	344, *285*, 232			*398*, *285*		
Nalorphine	401, 395, 357, 353, 336		455, 414, 324, 260	503, 391, 390	*603*, 440, 207	491, 490, 207
6-MAM	372, 328, 204	384, *383*, 324	390, 340, 287, 204	423, *364*, 426, 367	*473*, 414, 361, 476, 417	
[$^2H_3$]6-MAM		*389*				
Hydrocodone	299, 242, 185					
Oxycodone	360, 257, 314, 298					
Hydromorphone:						
Monoacetyl	327, 285, 229			381, 325		
Diacetyl	369, 327, 310					
Oxymorphone	391, 221					
Ref.	44, 46, 52, 55, 64	53	64, 65	37, 43, 66	3, 34, 37, 43, 55, 56, 58, 60, 64, 67, 68	43, 64

The ions with m/z values in italics were the most commonly used for determination.

cedure reduces the handling of organic solvents, but it is more expensive.

The derivatization agents most often used are on the one hand an acetylating agent which gives stable derivatives and on the other PFPA, the derivatives of which are sensitive to moisture, and in some circumstances two peaks can be obtained; however, it gives a clean mass spectrum and good results for determination using the selected-ion monitoring mode.

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