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Distinction among eight opiate drugs in urine by gas chromatography-mass spectrometry

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Abstract

Opiates are commonly abused substances, and forensic urine drug-testing for them requires gas chromatographic-mass spectrometric (GC-MS) confirmation. There are also medical reasons to test urine for opiates, and confirmation procedures other than GC-MS are often used for medical drug-testing. A thin-layer chromatographic (TLC) method distinguishes morphine, acetylmorphine, hydromorphone, oxymorphone, codeine, dihydrocodeine, hydrocodone, and oxycodone in clinical specimens. In certain clinical circumstances, GC-MS confirmation is requested for opiates identified by TLC, but, to our knowledge, no previous report examines all of the above opiates in a single GC-MS procedure. We find that they can be distinguished by GC-MS analyses of trimethylsilyl (TMS) ether derivatives, and identities of 6-keto opiates can be further confirmed by GC-MS analysis of methoxime (MO)-TMS derivatives. Inclusion of deuterium-labeled internal standards permits identification of the opiates in urine at concentrations below the TLC cutoff level of 600 ng/ml, and the GC-MS assay is linear over a concentration range that spans that level. This GC-MS procedure has proved useful as a third-stage identification step in a medical drug-testing sequence involving prior immunoassay and TLC.

Keywords: Medical drug-testing; Morphine; Acetylmorphine; Oxymorphone; Hydromorphone; Codeine; Dihydrocodeine; Hydrocodone; Oxycodone

1. Introduction

Opiate analgesics are commonly abused substances, and clinical laboratories are often asked to identify them in urine [1–6]. Forensic urine drug-testing involves immunoassay screening and gas chromatographic-mass spectrometric (GC-MS) confirmation [3,5,6]. The requirement for

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GC-MS confirmation derives in part from the fact that assay results may be used against the subject from whom the specimen was obtained in adversarial proceedings that result in loss of livelihood or criminal charges. Such forensic urine drug-testing is generally limited to a restricted set of opiates, including, for example, morphine, codeine, and acetylmorphine [7].

Urine testing for opiates may also be performed for medical reasons [8], such as evaluation of patients with altered states of consciousness or monitoring efficacy of drug rehabilitation efforts. Results of medical testing procedures are ostensibly intended to facilitate diagnostic or therapeutic decisions which benefit the patient. Because there is no adversarial relationship between patient and ordering physician or testing laboratory, factors such as rapidity of testing, expense, and ease of performance may cause confirmation procedures other than GC-MS to be used in medical drug-testing. In addition, physicians often request identification of a wider range of opiates than those included in standard forensic urine drug-testing batteries.

When combined with immunoassay screening and derivatization, thin-layer chromatography (TLC) provides a relatively rapid second-stage identification method that can distinguish among eight clinically encountered opiates in urine specimens, including codeine, morphine, acetylmorphine, dihydrocodeine, oxymorphone, hydrocodone, oxycodone, and hydromorphone [9]. While this approach is adequate for most medical drug-testing circumstances, identification of an opiate other than one known to be prescribed for a patient may cause physicians to make decisions that are contested by the patient. Evidence of non-compliance with drug rehabilitation efforts, for example, may jeopardize a patient's receipt of an organ transplant. In such cases, physicians may request additional confirmation of an opiate identified in a medical drug-testing battery.

This creates the need for a third-stage identification step with greater structural specificity than TLC and at least equivalent sensitivity. In principle, GC-MS satisfies these requirements. GC-MS has been applied to several members of the battery of eight opiates identified above [4–6,10–16], but we are unaware of a previous report that examines all of these opiates in a single GC-MS procedure. We have determined whether each member of this battery can be distinguished by GC-MS analyses at concentrations that yield a positive result on TLC.

2. Materials and methods

2.1. Materials

Morphine, codeine, hydromorphone, hydrocodone, dihydrocodeine, oxymorphone, and oxycodone were obtained from Alltech (Deerfield, IL) and [$^2H_3$]morphine, [$^2H_3$]codeine, and 6-acetylmorphine from Radian (Austin, TX). *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL) and pyridine from Sigma (St. Louis, MO). SPEC PLUS VC MP3 extraction microcolumns were obtained from Toxi-Lab (Laguna Hills, CA) and methoxyamine·HCl from Supelco (Bellfonte, PA).

2.2. Sample extraction

Urine samples containing known concentrations of standard opiates were prepared and extracted as previously described [9]. In brief, opiate standards were added to blank urine at desired concentrations as methanol solutions and extracted with SPEC PLUS VC MP3 solid-phase columns. For quantitative analyses, 750 μl of an internal standard solution containing [$^2H_3$]morphine and [$^2H_3$]codeine (1000 ng/ml each) was added to each 3 ml urine specimen. Solid-phase extraction columns were equilibrated with 0.5 ml conditioning solvent (0.6 ml glacial acetic acid in 200 ml methanol). Urine samples (3 ml) were then applied and aspirated through the column under vacuum (15 mmHg). The column was then washed once with conditioning solvent (0.5 ml) and aspirated under vacuum. Opiates were eluted from the column by applying 1 ml of elution solvent (0.2 ml NH_4OH (6 mol/l) in 9.8 ml ethyl acetate), which was collected into a 5 ml silanized conical glass tube.

2.3. Sample derivatization

The extracted opiates were concentrated to dryness under air at 40°C. Samples were reconstituted in pyridine (100 μl) and the derivatization reagent, BSTFA (100 μl). Samples were then heated (75°C, 30 min), concentrated to dryness, and reconstituted in methylene chloride (50 μl). After centrifugation ($2000 \times g$, 5 min) the liquid phase was (5 μl) analyzed by GC-MS. In some cases, 6-keto opiates were converted to methoxime (MO) derivatives with methoxyamine·HCl in pyridine (100 μl, 0.5%, w/v) for 30 min at 75°C. This was followed by conversion to TMS derivatives.

2.4. GC-MS analyses

Aliquots (5 μl) of derivatized samples were injected (Hewlett-Packard Model 7673A autosampling device) in splitless mode into a Hewlett-Packard Model 5890 gas chromatograph interfaced with a Hewlett-Packard 5970A electron-impact quadrupole MS controlled by a Hewlett-Packard RTE-A data system. GC analysis was performed on a 30 m DB-5 column (0.25 mm I.D., 0.25 μm film thickness, Alltech) with helium as the carrier gas. Injection port and transfer line temperatures were 250 and 280°C, respectively. Initial GC oven temperature was 130°C. Starting 1 min after injection, the oven temperature was increased (10°C/min) to a final temperature of 270°C. Source temperature was 200°C. Mass spectrometry was performed in electron impact mode. Full-scan spectra were acquired over m/z range 50–550. Quantitative analyses were performed in selected ion monitoring mode.

2.5. Thin-layer chromatographic analysis of patient specimens

Triplicate extracts were prepared as above from each urine specimen which tested opiate positive by immunoassay (CEDIA, Boehringer Mannheim). One extract was analyzed directly by TLC, the second by TLC after treatment with methoxyamine·HCl, and the third saved for GC-MS analysis. After adding a TOXI-LAB application disk, the first extract was concentrated to dryness. The second extract was concentrated to dryness, reconstituted in 100 μl of 0.5% (w/v) methoxyamine·HCl in pyridine, and incubated (30 min, 75°C). Ethyl acetate (1 ml) and 1 ml 50% (w/v) dibasic potassium phosphate buffer were then added and the mixture shaken (10 min) and centrifuged ($2000 \times g$, 5 min). The upper phase was removed, and, after adding a TOXI-LAB application disk, concentrated to dryness. TOXI-LAB application disks containing derivatized and underivatized samples, respectively, were applied to TOXI-LAB TLC plates, which were developed in methylene chloride-isopropanol (88:12, v/v), allowed to dry, and treated with concentrated sulfuric acid. Rf values for morphine (0.34), acetylmorphine (0.79), dihydrocodeine (0.51), and codeine (0.66) are unaffected by exposure to methoxyamine [9]. Oxycodone, oxymorphone, hydromorphone, and hydrocodone exhibit Rf values of 0.92, 0.83, 0.47, and 0.66, respectively, before treatment with methoxyamine and values of 0.97, 0.88, 0.66, and 0.76, respectively, after treatment [9].

3. Results

The target set of opiates included four morphine congeners (Fig. 1) and four codeine congeners (Fig. 2). Each compound that contained an hydroxyl group was converted to its trimethylsilyl (TMS) ether. The opiates were then analyzed by GC-MS, and Figs. 3 and 4 illustrate electron impact (EI) mass spectra obtained for morphine and codeine congeners, respectively. The molecular ion (M) is abundant in each spectrum and unique to each compound in this set. Each spectrum also contains fragment ions that distinguish among the eight compounds, such as those resulting from loss of methyl radical (M-15) from TMS derivatives.

Fig. 5 illustrates GC-MS analysis of a mixture of the eight derivatized opiates. Compounds represented by chromatographic peaks were determined from their mass spectra and GC-MS analyses of single components. Of the eight tested opiate derivatives, only morphine-TMS and hy-

dromorphone-TMS failed to separate on GC, but these compounds are distinguishable from their mass spectra. Morphine and hydromorphone also exhibit distinguishable Rf values on TLC [9], and the two compounds are not confused in the identification sequence used in our medical drug-testing procedure in which TLC precedes GC-MS analyses. In addition, conversion of hydro-

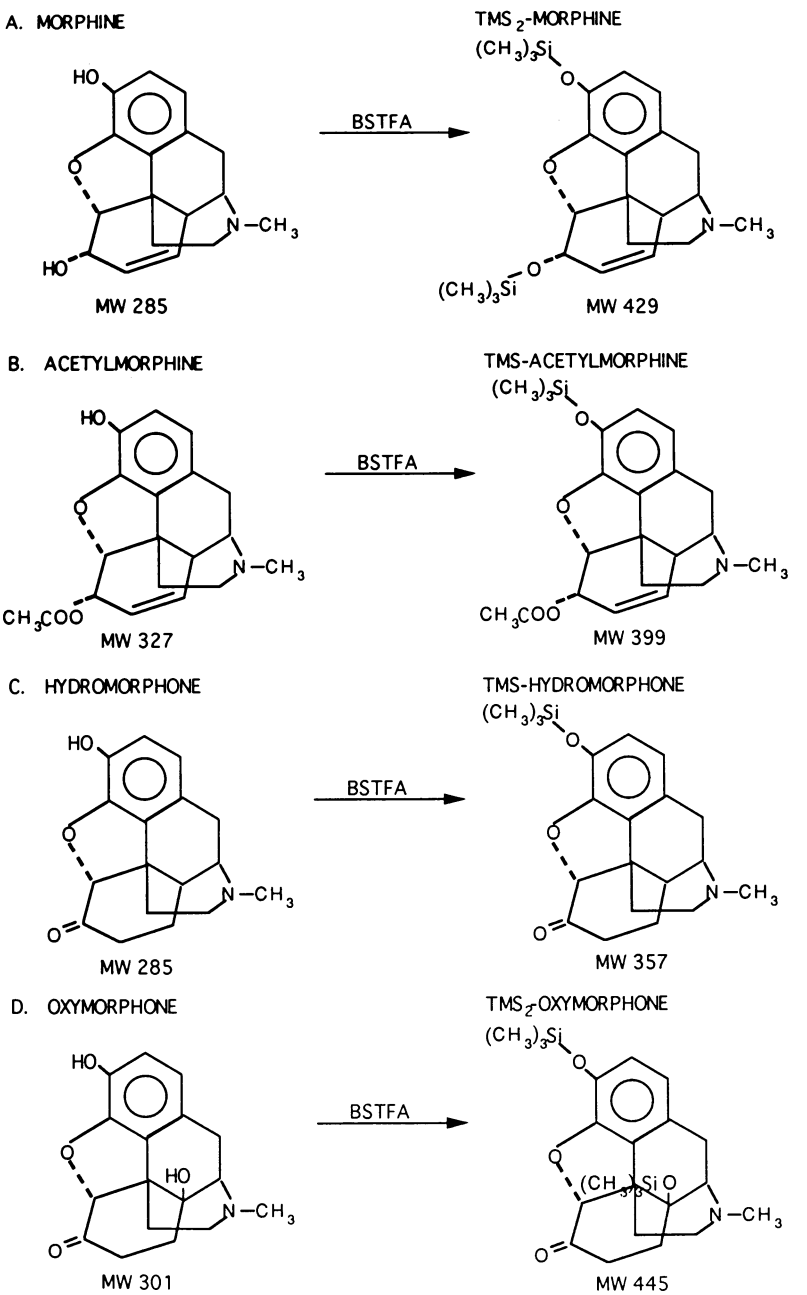

Fig. 1. Structures of morphine, its congeners, and their trimethylsilyl ether derivatives.

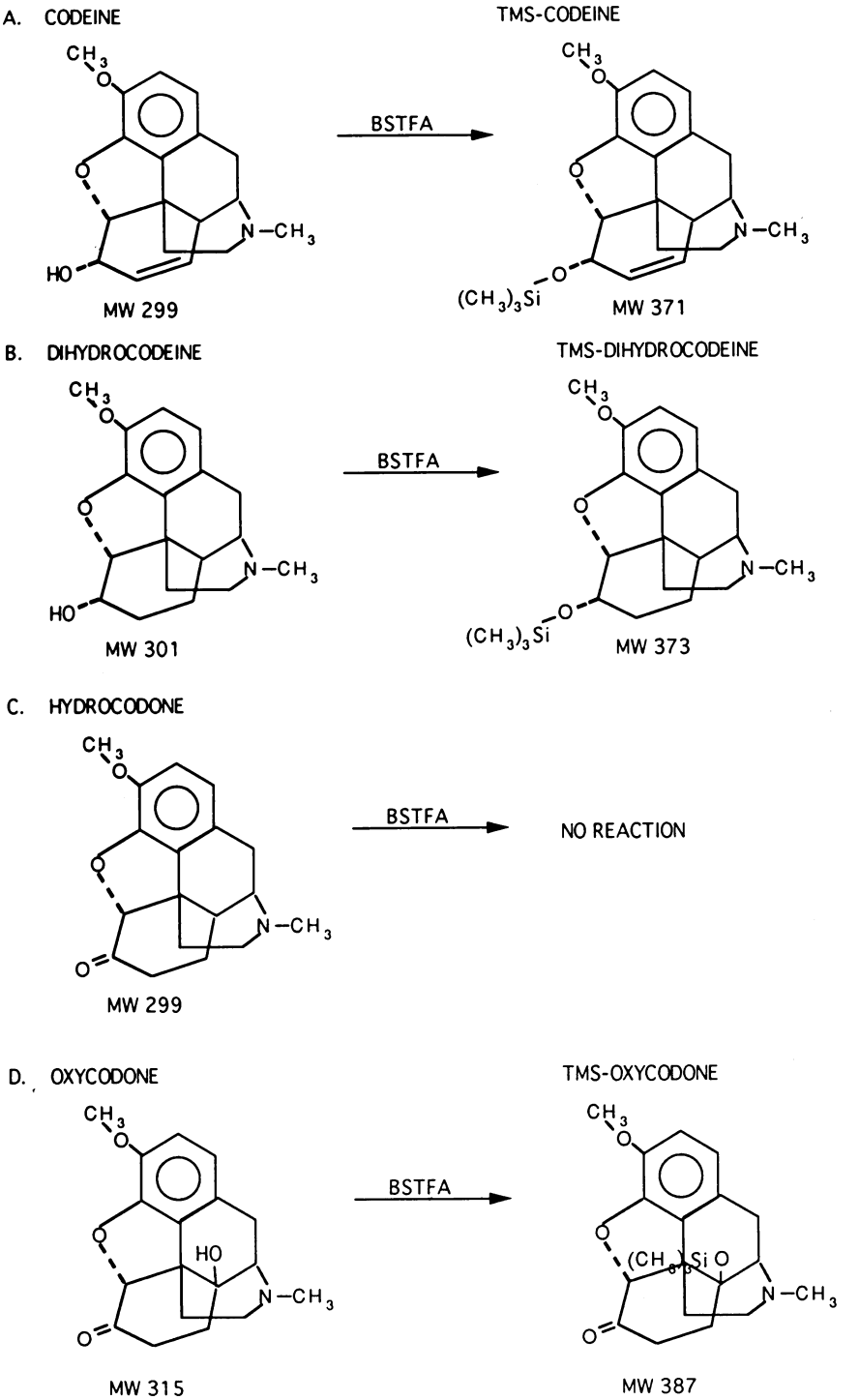

Fig. 2. Structures of codeine, its congeners, and their trimethylsilyl ether derivatives.

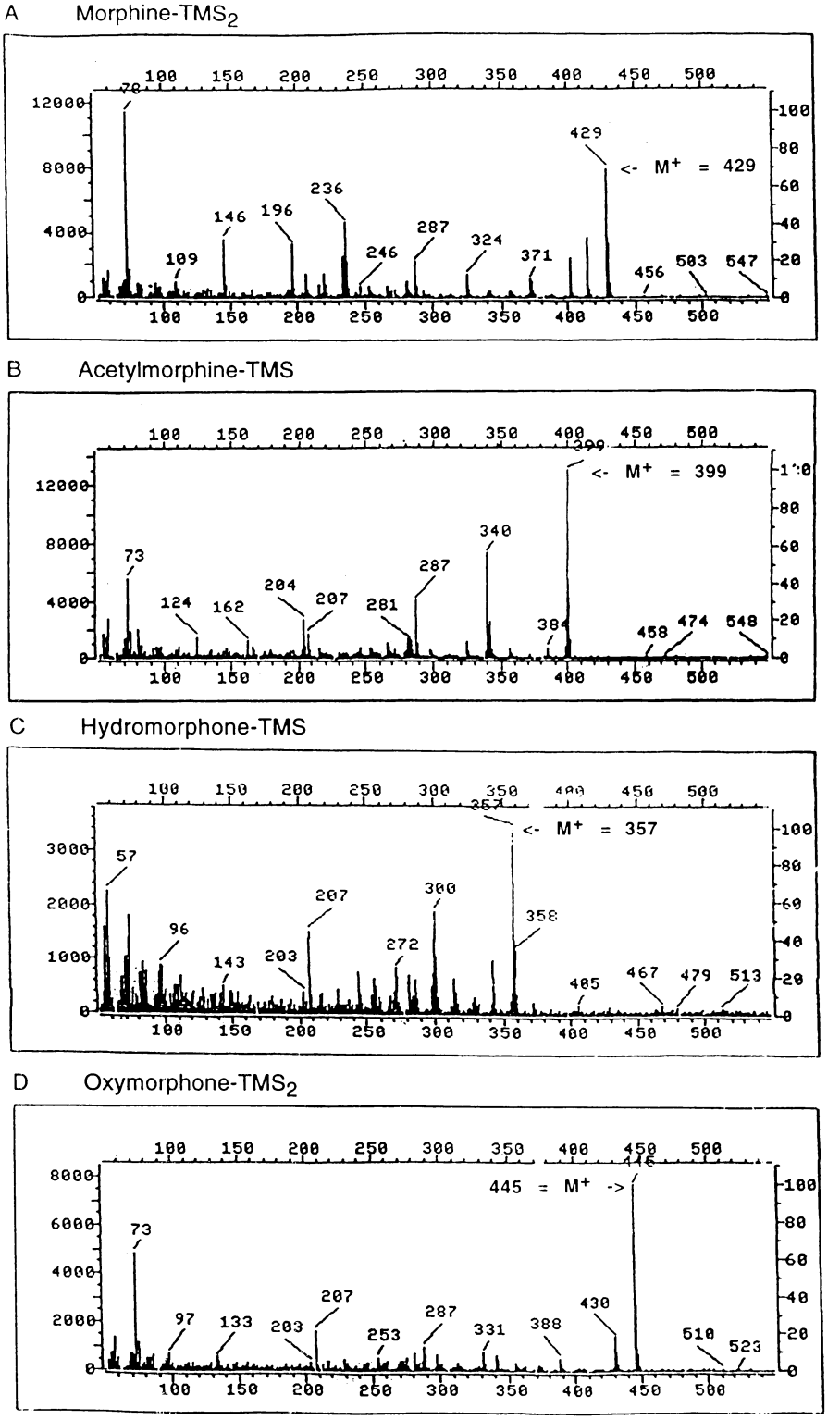

Fig. 3. Electron impact mass spectra of the trimethylsilyl ether derivatives of morphine and its congeners.

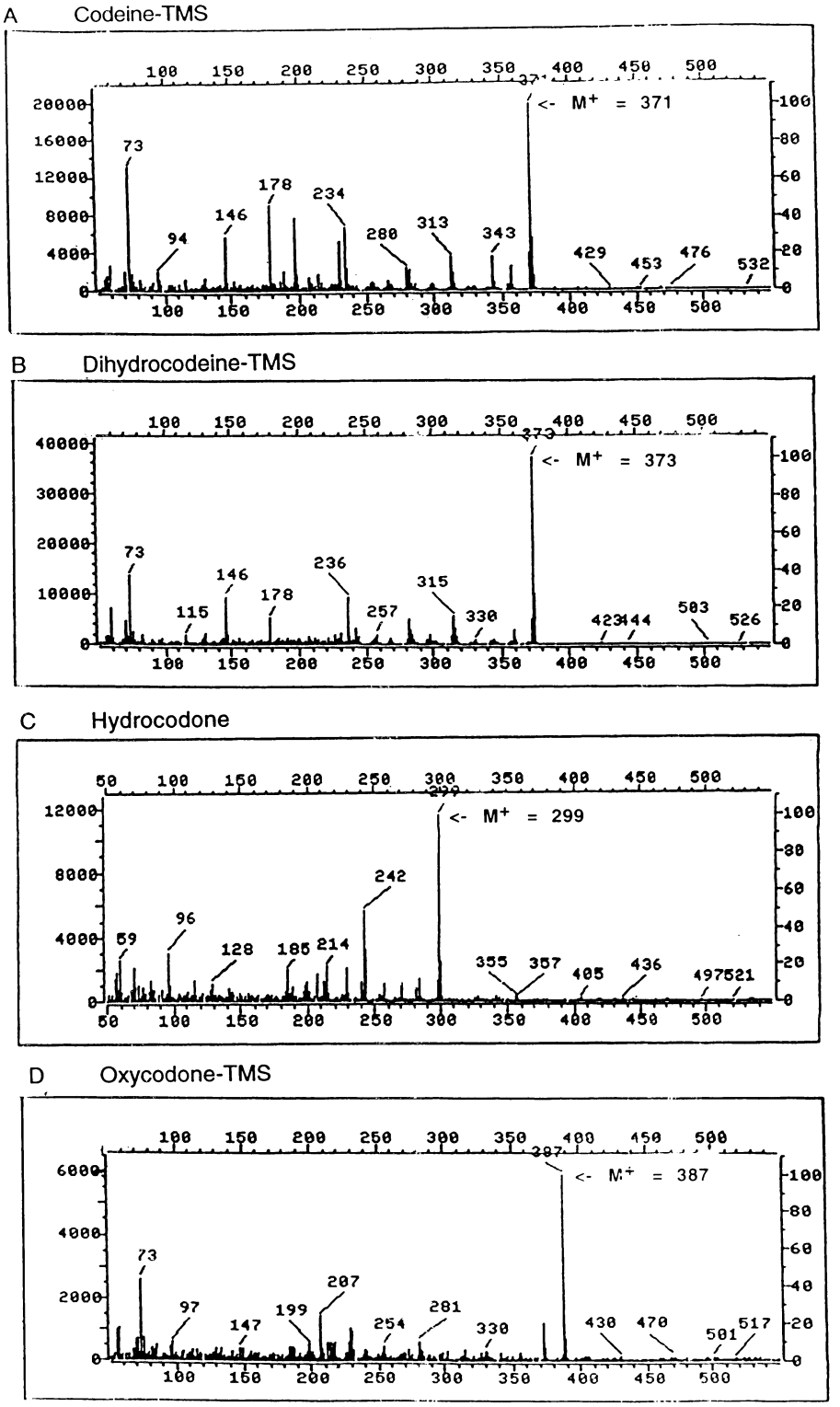

Fig. 4. Electron impact mass spectra of the trimethylsilyl ether derivatives of codeine and its congeners.

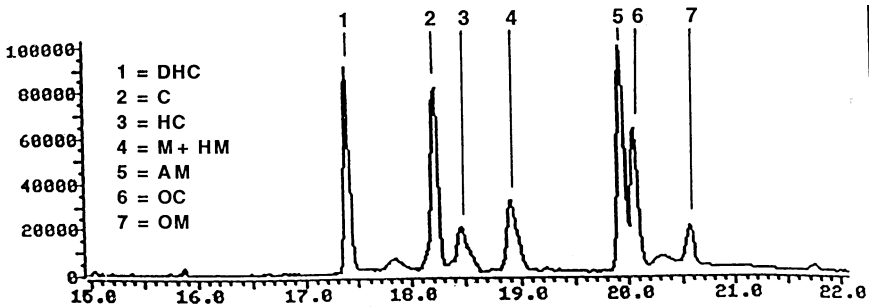

Fig. 5. Gas chromatographic-mass spectrometric analysis of a mixture of the trimethylsilyl ether derivatives of eight opiates. To a blank urine specimen, a mixture of opiates was added in methanol: dihydrocodeine (DHC, 1000 ng/ml), codeine (C, 1000 ng/ml). hydrocodone (HC, 500 ng/ml), morphine (M, 300 ng/ml), hydromorphone (HM, 500 ng/ml), acetylmorphine (AM, 1000 ng/ml) oxycodone (OC, 1000 ng/ml), and oxymorphone (OM, 500 ng/ml). After extraction and conversion to TMS derivatives, opiates were analyzed by GC-MS in full-scan mode (m/z 50-550). The ordinate is total ion current and abscissa GC retention time (min).

morphone to a methoxime (MO) derivative before conversion to a TMS derivative permits GC distinction between morphine-TMS and hydromorphone-MO-TMS (Fig. 6). The mass spectrum of hydromorphone-MO-TMS (Fig. 7) is also distinct from that of morphine-TMS (Fig. 3).

In addition to hydromorphone, the 6-keto opiates oxymorphone, hydrocodone, and oxycodone are readily converted to MO and then to TMS derivatives. Mass spectra of these derivatives contains the expected molecular ions and ions (M-31) reflecting loss of methoxy radical (Fig. 7). Conversion to MO derivatives can therefore be used as an adjunct to identification of 6-keto opiates. Two stereoisomers of the MO derivatives are produced for each compound (Table 1).

Quantitative studies were therefore performed with TMS derivatives of opiate mixtures to determine whether they could be identified at concentrations that produce a positive result on TLC. Mass spectra of TMS derivatives of the internal standards [$^2H_3$]morphine and [$^2H_3$]codeine contained prominent molecular ions and several fragment ions with m/z value three units higher than those of analogous ions in spectra of unlabeled compounds. The molecular ion is generally the most abundant ion in mass spectra of opiate TMS derivatives (Figs. 3 and 4), and it was selected as the quantitator ion—summarizes fragment ions for each target analyte that were selected for qualitative identification. Qualifier ions were required to co-elute with the quantitator ion and to exhibit an abundance relative to the quantitator ion that fell within 20% of that observed with 500 ng/ml reference standards (Table 2).

Fig. 8 illustrates GC-MS profiles for molecular ions of a mixture of TMS derivatives of each target analyte and the internal standards. The ion current tracing for m/z 374 displays both the molecular ion of [$^2H_3$]codeine-TMS and the [^{13}C]isotope of the molecular ion of dihydrocodeine-TMS, but this does not confound estimation of the relative abundance of codeine and dihydrocodeine because baseline GC resolution of these compounds is achieved. Hydromorphone-TMS (m/z 357), morphine-TMS (m/z 429), and [$^2H_3$]morphine-TMS (m/z 432) co-elute, but ions monitored for morphine-TMS and [$^2H_3$]morphine-TMS do not occur in the mass spectrum of hydromorphone-TMS because the m/z values of these ions exceed that of the molecular ion of hydromorphone-TMS.

Constant amounts of the [$^2H_3$]labeled internal standards were added to a series of blank urine specimens, and varied amounts of each target opiate were added as mixtures to individual specimens. After extraction, conversion to TMS derivatives, and GC-MS analysis, the area of the molecular ion current peak at the GC retention time for each target analyte was divided by that for the molecular ion current peak for the [$^2H_3$]labeled internal standard that eluted most closely to the target analyte. This area ratio was a linear function of opiate concentration over the

range of 150–1000 ng/ml for oxycodone and oxymorphone and of 150–1500 ng/ml for other tested opiates. This region of linearity spans the concentration (600 ng/ml) required to produce a positive result for the opiates on TLC [9].

The GC-MS procedure was then applied to a series of samples to which either 300 or 500 ng/ml of each opiate had been added, and measured values fell close to the target concentrations for each tested opiate (Table 3). These data suggest that the GC-MS procedure can confirm the presence of opiates at the level of 600 ng/ml required to produce a positive result on TLC [9]. The applicability of the GC-MS method to patient samples was examined by analyzing TLC-positive specimens submitted for medical drug-testing for opiates in which physicians requested additional confirmation because of patient denial of use of the identified opiates (Table 4). Good correspondence between the two methods was observed.

4. Discussion

This report indicates that eight opiate analgesics can be distinguished in urine by GC-MS at concentrations required to produce a positive result on TLC in medical drug-testing. Each of

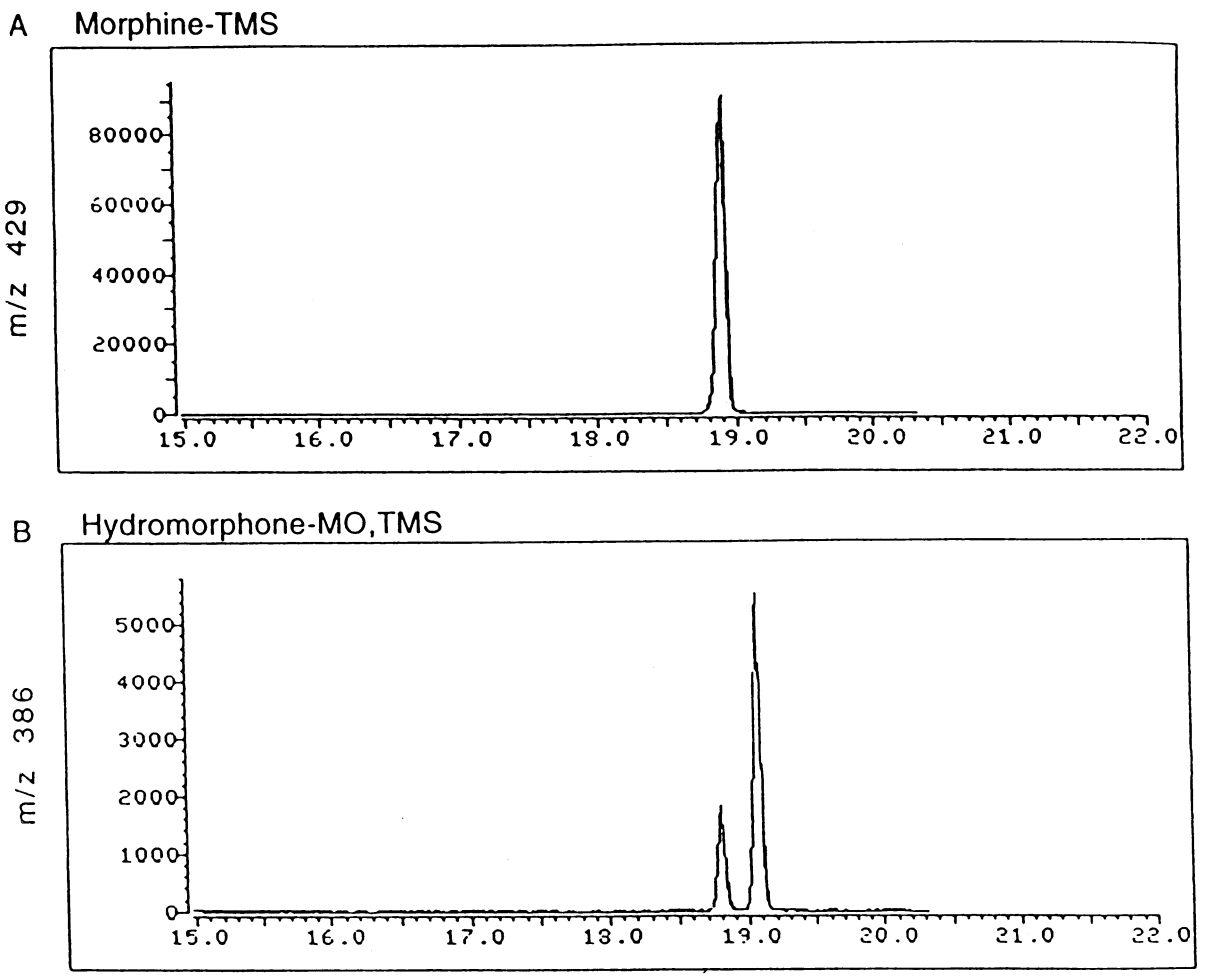

Fig. 6. Gas chromatographic-mass spectrometric analysis of the methoxime, trimethylsilyl ether derivative of hydromorphone.

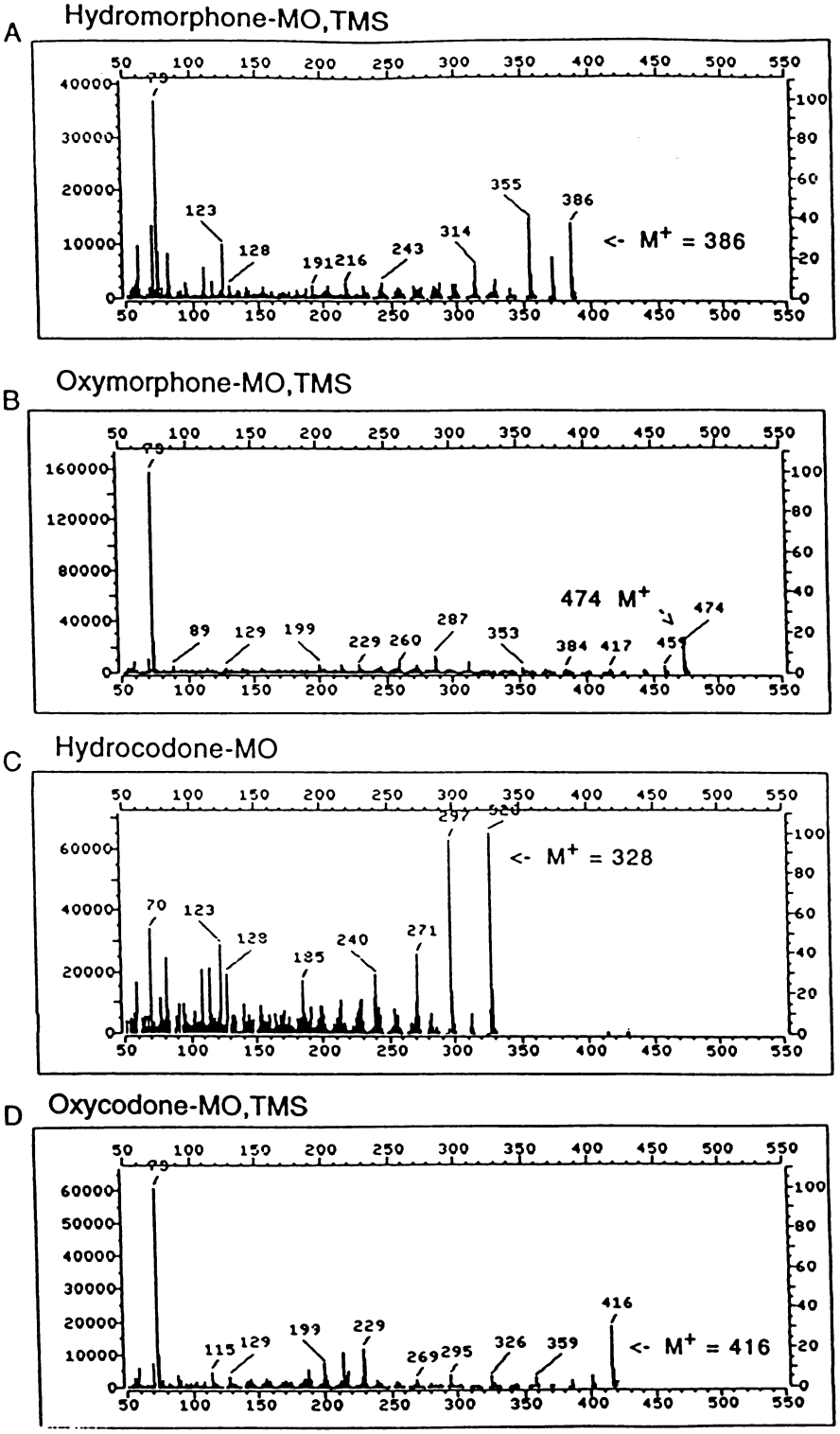

Fig. 7. Electron impact mass spectra of the methoxime, trimethylsilyl ether derivatives of 6-keto opiates.

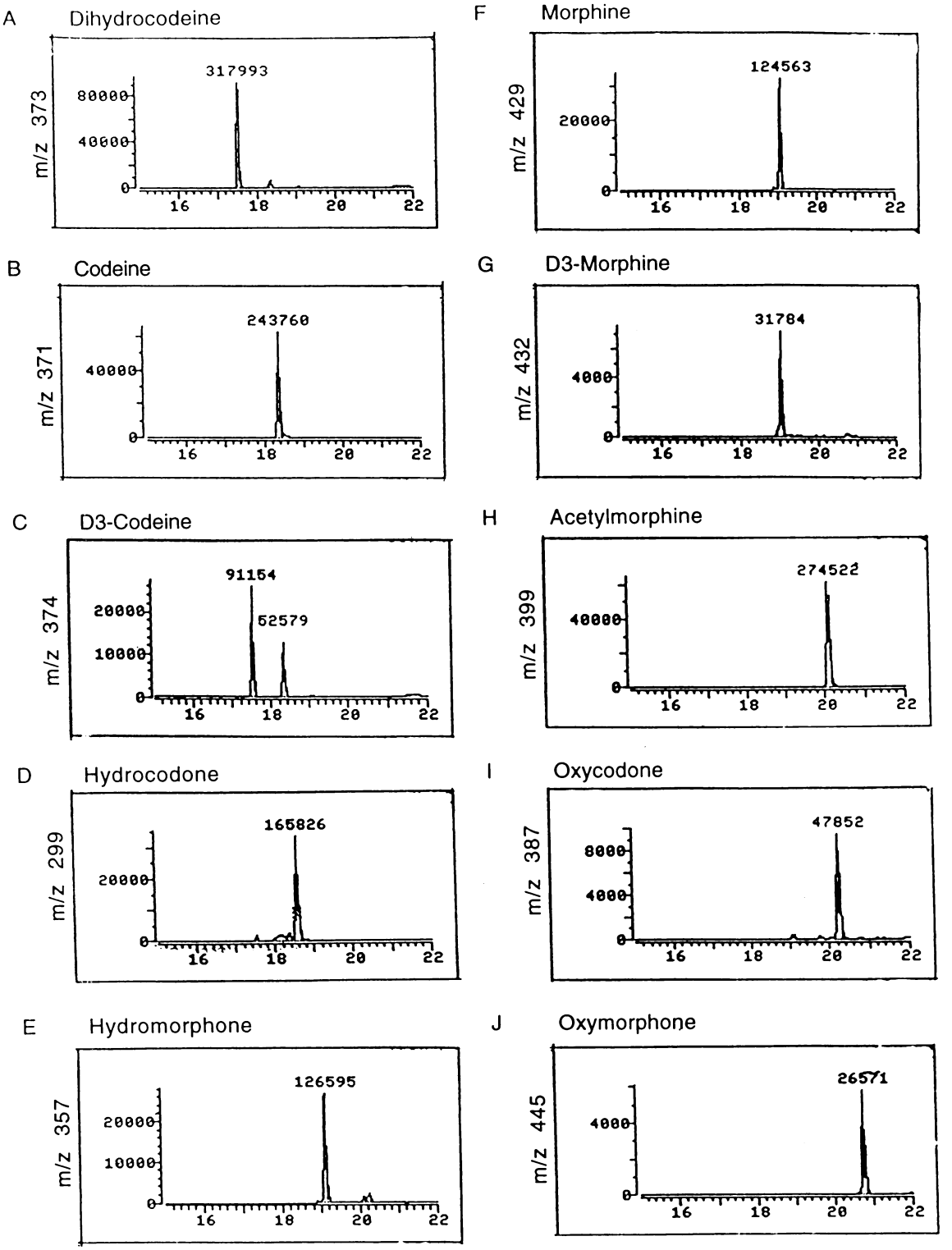

Fig. 8. (*Continued*)

Fig. 8. Gas chromatographic-mass spectrometric analysis in selected ion monitoring mode of trimethylsilyl ether derivatives of a mixture of eight opiates and internal standards [$^2H_3$]morphine and [$^2H_3$]codeine. A mixture of the specified opiates and two [$^2H_3$]-labeled internals standards was added to a blank urine specimen, extracted, converted to TMS derivatives, and analyzed by GC/MS with selected monitoring of molecular ions. The ordinate represents current for molecular ions and the abscissa GC retention (min).

Table 1
Gas chromatographic retention times and molecular ions of methoxime, trimethylsilyl ether derivatives of 6-keto opiates[a]

Opiate	TMS_x		Derivative x	MO, TMS_x		Derivative x
	RT (min)	M^+ (m/z)		RT (min)	M^+ (m/z)	
Hydrocodone	18.43	299	0	18.63	328	0
				18.94	328	0
Hydromorphone	18.90	357	1	18.75	386	1
				19.10	386	1
Oxycodone	20.00	387	1	19.65	416	1
				20.17	416	1
Oxymorphone	20.50	445	2	19.72	474	2
				20.28	474	2

[a] The indicated opiates were treated with trimethylsilylating reagent before (leftmost entries) or after (rightmost entries) conversion to methoxime (MO) derivatives and then analyzed by GC/MS. Tabulated values include the GC retention time (RT), the m/z value of the molecular ion (M^+), and the number of trimethylsilyl (TMS) groups incorporated into the derivative (x). Two stereoisomers were formed for each MO derivative.

these opiates is encountered in clinical specimens. Enterally administered formulations of dihydrocodeine (e.g. DHC-Plus), hydromorphone (e.g. Dilaudid), oxycodone (e.g. Percodan), and oxymorphone (e.g. Numorphan) are available as prescription analgesics in the USA. Codeine and morphine are also contained in prescription pharmaceuticals and in poppy seeds used to flavor bakery products [17], and ingestion of such foods can produce detectable quantities of codeine and morphine in urine [18–20]. The only known source of acetylmorphine in human urine is the illicit opiate heroin (diacetylmorphine), which is sequentially deacetylated to yield acetylmorphine and then morphine [6,10,15,21–24].Because of their similar structures, each of these opiates can yield a positive opiate immunoassay, although the concentration required varies [3,14]. Because of the ambiguity of a positive opiate immunoassay, physicians often request identification of specific opiates in urine submitted for medical drug-test-

Table 2
Ions for selected monitoring in gas chromatographic-mass spectrometric analysis of trimethylsilyl ether derivatives of opiate drugs[a]

Opiate	Quantitator ion	Qualifier ions	
	M^+ (m/z)	m/z (ra)	m/z (ra)
Morphine	429	414 (32)	402 (21)
[$^2H_3$]Morphine	432	417 (35)	405 (37)
Hydro-morphone	357	342 (30)	300 (53)
Acetylmorphine	399	340 (58)	287 (32)
Oxymorphone	445	430 (20)	207 (25)
Codeine	371	343 (19)	234 (32)
[$^2H_3$]Codeine	374	346 (18)	237 (35)
Dihydrocodeine	373	315 (16)	236 (25)
Hydrocodone	299	284 (11)	242 (51)
Oxycodone	387	372 (21)	207 (30)

[a] The molecular ion was selected as quantitator ion, and two additional reasonably abundant ions in the mass spectrum were selected as qualifier ions. Qualifier ions are identified by m/z value and by their abundance relative to the quantitator ions (ra).

ing [9]. This may help to ascertain whether the compound(s) responsible for the positive immunoassay arise from an illicit drug, from dietary sources, or from a prescription pharmaceutical. In the last case, another question is whether the pharmaceutical was prescribed by the physician or obtained from illicit sources or from another physician by drug-seeking patients. To assist in such cases, a TLC method is used in our laboratory that distinguishes the eight opiates examined here at concentrations above 600 ng/ml [9]. Because patients may deny ingestion of the identified compound(s), physicians sometimes request additional confirmation.

The GC-MS assay described here has proved useful in such circumstances, and it is used as a third-stage identification step in a medical drug-testing sequence that includes prior immunoassay and TLC [9]. As presently constructed, the assay is not intended for formal forensic urine drug-testing, such as that sanctioned by the Substances Abuse Mental Health Services Administration (SAMHSA) [7]. Mandatory guidelines for federal workplace drug testing established by SAMHSA limit testing to codeine, morphine, and acetylmorphine and exclude other analytes examined here [7], but there is growing interest in testing additional opiates for medical purposes [25].

Many procedures for analysis of specific subsets of opiates have been developed that involve a variety of chromatographic modalities, derivatization schemes, internal standards, and detection methods [3–6,9–16,26–30]. Analysis of 6-keto opiates is complicated by keto-enol tautomers that may form separable derivatives, and analysis of oxime derivatives may be helpful [16]. In our testing sequence, 6-keto opiates are identified by the effect of methoxyamine on their TLC mobility [9], and the derivative so formed can also be converted to a TMS derivative and characterized by GC-MS. Opiates with a tertiary hydroxyl group, such as oxymorphone and oxycodone, exhibit detection limits that are higher than those for opiates with only secondary hydroxyl groups, and limited information is available on identification or quantitation of these compounds in human urine [14].

Because of the complexity of the opiate family and the differing needs to identify specific members in various testing circumstances, no single analytic scheme may be optimal for all purposes. Forensic drug-testing and medical drug-testing place different constraints on laboratories. We believe that, for the medical testing purposes described in this report, the sequence of immunoassay, TLC, and GC-MS permits identification of a set of eight opiates commonly encountered in clinical settings in the United States with a degree of certainty that can assist in making clinical decisions. This is the only report of which we are aware to examine all eight members of this set in a single testing procedure that includes GC-MS analysis of each member. The GC properties and full mass spectra of the twelve opiate derivatives reported here may be useful to others involved in analyzing this complex set of substances. Modifying our procedure by using alternate extraction methods [30] and including additional internal standards [16] might permit its adaptation to additional testing circumstances.

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Table 3
Quantitation of urine supplemented with the indicated analyte (ng/ml)[a]

Analyte supplemented (ng/ml)	300	500
Dihydrocodeine	295 ± 10.8	504 ± 17.5
Codeine	302 ± 8.4	498 ± 15.0
Hydrocodone	281 ± 26.7	521 ± 37.5
Morphin	296 ± 18.0	510 ± 29.5
Hydromorphone	315 ± 33.0	525 ± 50.0
Acetylmorphine	280 ± 47.7	515 ± 34.5
Oxycodone	314 ± 114	518 ± 97.5
Oxymorphone	331 ± 144	532 ± 94.5

[a] Blank signals and precision of measurements of opiate concentrations in urine by GC/MS. Internal standard [$^2H_3$]morphine and [$^2H_3$]codeine (250 ng/ml each) were added to a series of blank urine specimens to which either 300 or 500 ng/ml of each of the eight target opiates had been added. After extraction and conversion to TMS derivatives, opiates were analyzed by GC-MS. Tabulated quantities were determined by interpolation from a standard curve. Values represent means of ten replicates and are represented as mean ± SD.

Table 4
Thin layer chromatographic and gas chromatographic-mass spectrometric analyses of opiate immunoassay-positive patient urine specimens[a]

Patient	Opiates identified on TLC	GC-MS quantitation (ng/ml)
1	Codeine	Codeine (3431), morphine (65)
2	Morphine, acetylmorphine	Morphine (866), acetylmorphine (736)
3	Codeine	Codeine (858)
4	Hydromorphone	Hydromorphone (4166)
5	Codeine	Codeine (5765), morphine (147)
6	Oxycodone	Oxycodone (245)
7	Oxycodone	Oxycodone (1971)
8	Oxycodone	Oxycode (1399)
9	Morphine, acetylmorphine	Morphine (5872), acetylmorphine (75)
10	Acetylmorphine	Morphine (10 195), acetylmorphine (237)

[a] Triplicate extracts were prepared from opiate-immunoassay positive urine samples submitted for medical drug-testing. The first extract was analyzed directly by TLC, the second by TLC after derivatization with methoxyamine, and the third, to which [$^2H_3$]morphine and [$^2H_3$]codeine had been added, by GC-MS after derivatization with BSTFA.

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