TECHNICAL NOTE

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Improved GC/MS Analysis of Opiates with Use of Oxime-TMS Derivatives

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ABSTRACT: An improved gas chromatographic/mass spectrometric (GC/MS) assay is described for the quantitation of codeine and morphine as trimethylsyl (TMS) derivatives. The TMS derivatization of ketone-containing opiates results in the formation of multiple derivatives. Some of these products have retention times close to those of codeine-TMS and morphine-TMS. When the keto-opiates are present in samples assayed for codeine and morphine in urine, they can interfere with the quantitation of these commonly targeted opiates. The assay was improved with the addition of a pre-BSTFA derivatization step, whereby hydroxylamine was used to convert the keto-opiates into the corresponding oxime derivative. These derivatives were then reacted with BSTFA to form the TMS ethers and TMS oxime derivatives. The oxime step enabled production of single derivatives for hydrocodone and hydromorphone. In addition, the retention times for the oxime-TMS derivatives were increased so that they no longer elute near the targeted drugs of codeine and morphine. The addition of the oxime step does not affect the sylation of codeine and morphine, and the accuracy and precision of this assay were unaffected.

KEYWORDS: forensic science, gas chromatography/mass spectrometry, opiates, oxime derivatization

Quantitative gas chromatography/mass spectrometric (GC/MS) methods for opiates have centered largely around codeine and morphine, due to their availability and widespread abuse, and because these drugs are included in the *Federal Guidelines* for workplace drug testing (1). More recently, detection of the heroin-specific metabolite, 6-monoacetylmorphine (6-MAM), has become important because this metabolite has become required as part of the *Federal Guidelines* for opiate confirmation, which was originally to be effective May 1, 1998 (2). Additionally, in non-Federal workplace testing and forensic applications, there is a need to confirm other opiates in urine such as hydrocodone, hydromorphone, oxycodone, and oxymorphone, because these drugs are also abused in select populations, such as healthcare workers (3). The College of American Pathologists (CAP) includes these keto-opiates within their Forensic Urine Drug Testing surveys of certified laboratories.

Although many GC/MS assays have been described for codeine and morphine, most have some limitations regarding their ability to detect and resolve all of the synthetic opiates. Different derivatization schemes have been used to address these limitations. Acetic anhydrides have been successfully used for morphine, codeine, and synthetic opiates, but both morphine and 6-MAM are converted to heroin in this method (4,5). Pentafluoropropionyl (PFP) derivatives are useful for codeine and morphine, but one author suggests that hydromorphone interferes with morphine quantitation (6), while another found that oxycodone coelutes with codeine (5). Although oxycodone-PFP and codeine-PFP have different fragmentation ions, coelution of drugs can produce interferences when one drug is present in much higher concentrations relative to the other (7). Other fluoroacyl and trimethylsilyl reagents have the potential to produce multiple mono-, di-, and triderivatives for the synthetic opiates, making quantitative analyses for these substances complex. Multiple derivatives are also produced through tautomerism of opiates that contain keto functional groups, which can be overcome by borohydride reduction (8).

An alternate GC/MS opiate assay was developed by Jones et al. (9) and Broussard et al. (10) involving the use of hydroxylamine to convert keto-opiates to oxime derivatives (11). The oxime product is further derivatized with BSTFA to form the corresponding TMS derivative by blocking the ability of these drugs to tautomerize. This reduces the number of multiple derivatives for keto-opiates, while prolonging the retention times such that they do not coelute and interfere with the targeted opiates of codeine and morphine. This paper describes in more detail the mechanism of the gas chromatographic interferences caused by the presence of keto-opiates using the conventional TMS derivatization procedure, and evaluates the performance of the oxime assay on urine samples submitted for toxicologic analysis.

Materials and Methods

Reagents—All reagents and solvents used were of analytical grade or better. Methanol, methylene chloride, isopropanol, and ethyl acetate were from Burdick and Jackson. Acetic acid, ammonium hydroxide, and sodium hydroxide were from Mallinckrodt. N,O,-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was from Pierce Chemical Co. β-

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glucuronidase (*Patella Vulgata*), 2,000,000 units/g solid and hydroxylamine hydrochloride were from Sigma Chemical Co. Samples were extracted using World Wide Monitoring (Clean Screen DAU, C8, 200 mg) minicolumns. Standards for codeine, morphine, hydrocodone, hydromorphone, oxycodone, and oxymorphone were obtained in methanol from Restek. The deuterated internal standards, codeine-d_3 and morphine-d_3 were from Isotek. Quality control materials were from Con-DOA (Diagnostic Products Corporation).

Apparatus—A Hewlett-Packard (HP) 5890A gas chromatograph 5970B mass selective detector was used throughout. An HP ultra 1 capillary gas chromatographic column (12 m × 0.2 mm × 0.33 μm film thickness) was used. The injector temperature was 275°C and was operated in the splitless mode. The oven was set at 130°C and ramped at a rate of 15°C/min to 260°C, and then to 280°C at 40°C/min. The carrier gas was ultra high-purity helium, operated at a flow rate of 1 mL/min. The sample volume was 1 μL. The mass selective detector was operated in the electron ionization (EI) mode and mass spectra were collected from 70 to 540 amu at 1.4 scans/s.

Procedure—The procedure described by Jones et al. (9) was modified by incorporating the oxime derivatization step with an enzymatic hydrolysis, instead of using an acid hydrolysis, and a separate oxime derivative step. Thirty microliters of codeine-d_3 and morphine d_3 (30 μg/mL) were added to a 3 mL aliquot of urine as internal standards. The samples were simultaneously reacted with 200 μL of 10 g/dL hydroxylamine and hydrolyzed with 5000 U/mL β-glucuronidase by incubation at 65°C for 2 h. After the tubes were cooled, the pH of the sample was adjusted to approximately 6 with 0.1 mol/L sodium hydroxide (NaOH) and added to a solid-phase extraction column. This is followed in sequence by washes of 2 mL water, 2 mL acetate buffer (100 mmol/L, pH 4.5), and 3 mL methanol. After the columns were dried, the opiates were eluted with methylene chloride:isopropanol:ammonium hydroxide (78:20:2 v/v/v) into centrifuge tubes. The eluates were evaporated to dryness and derivatized with the addition of 75 μL BSTFA with 1% TMCS and 75 μL of ethyl acetate. To ensure complete derivatization, the samples were reacted at 65°C for 20 min. Selected ion monitoring mode was used to quantitate codeine and morphine in the presence of hydrocodone, hydromorphone, oxycodone, and oxymorphone. The monitored ions were *m*/*z* 371 (quantitation ion), 343, and 234 for codeine, and *m*/*z* 374 and 346 for d_3-codeine. The corresponding ions for morphine were *m*/*z* 429 (quant ion), 414 and 401, and 432 and 404.

Experimental—Twenty-three urine samples that were positive for opiates by the immunoassay screening procedure (EMIT II, Syva Co., San Jose, CA) were extracted using the oxime derivatization procedure. Quantitative results for codeine and morphine were compared against an identical extraction procedure in which the hydroxylamine reagent was omitted. These specimens were assayed using selected ion monitoring mass spectrometry (MS). Codeine and/or morphine were detected in 17 of these specimens. Keto-opiates were present in some of these specimens, as well as in the remaining six that contained no codeine or morphine. To determine the effect of the oxime step on the chromatographic retention times, a mixture of opiates was also assayed using full-scan MS analysis. The limits of detection (LOD) and quantitation (LOQ) for codeine and total morphine were determined by the dilution method (12); urine controls (CON-DOA, Diagnostic Products Corporation, Los Angeles, CA) containing codeine and morphine were serially diluted with drug-free urine to concentrations of 30, 60, 90, and 120 ng/mL, and tested. The LOQ was the opiate concentration by which all of the analysis criteria were met (ion ratios, retention times, chromatographic symmetry, and quantitation within 20% of the expected value). The LOD was the opiate concentration by which 85% of samples met these criteria. The linearity was evaluated using four calibrators at concentrations of 100, 300, 750, and 1500 ng/mL for both opiates. A correlation coefficient of >0.9990 was deemed acceptable. The within-run and between-run precision was measured using the CON-DOA human urine-based quality control materials. Urine samples submitted to the toxicology laboratory at Hartford Hospital were split and extracted with and without the oxime step. Quantitative results were compared by linear non-weighted regression analysis. The error between methods was taken as the difference and is expressed as a percentage.

Results and Discussion

The combination of BSTFA and TMCS, without the addition of the oxime pre-derivatization step, produced a single trimethylsilyl (TMS) derivative at the hydroxy position of codeine, and a single 2TMS derivative at both hydroxy positions for morphine. For the

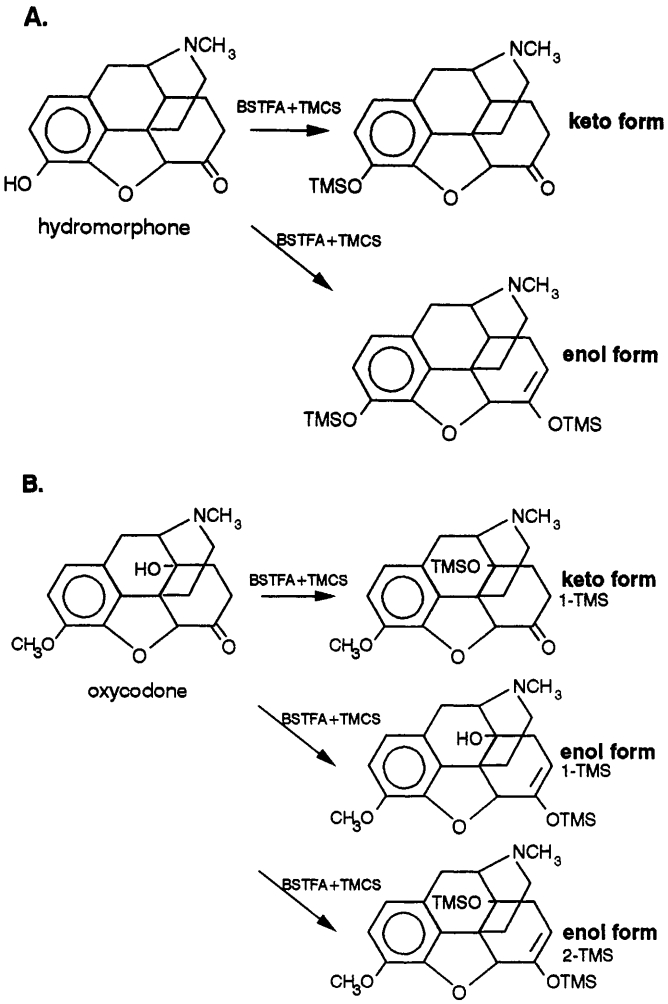

FIG. 1—*Derivatization reaction between BSTFA and TMCS and (*a*) hydromorphone and (*b*) oxycodone.*

heroin-specific metabolite, 6-monoacetylmorphine (6-MAM), only one TMS derivative is produced corresponding to the single hydroxy position. The keto-opiates produced multiple derivatives because they contain a keto functional group that can be present in either the keto or enol form. In the keto form, hydrocodone does not have active hydrogens and does not derivatize with BSTFA. In the enol form, however, a 1TMS derivative is produced. For hydromorphone, either a 1TMS or a 2TMS derivative will form, depending on the state of the keto group (as shown in Fig. 1*a*). Oxycodone and oxymorphone contain an additional hydroxy functional group at carbon 14 that is capable of derivatization by BSTFA. This particular hydroxy group does not completely derivatize with BSTFA under the conditions described in the Materials and Methods section. Therefore, for oxycodone, four different compounds are possible with BSTFA: one underivatized form, and three possible derivatives (two different 1TMS, and one 2TMS), as shown in Fig. 1*b*. Four different TMS derivatives are produced for oxymorphone (one 1TMS, two 2TMS, and one 3TMS).

Figure 2 shows the gas chromatogram of an unextracted mixture of these opiates, and Table 1 summarizes the 14 different compounds and derivatives that are possible with BSTFA + TMCS. The 1TMS derivative of hydrocodone (peak 3) elutes just after codeine (peak 2), while the 2TMS derivative of oxycodone (peak 7) elutes near morphine (peak 8). None of the other forms present GC detection problems with codeine and morphine, but may contribute to problems if non-coeluting internal standards are used for these drugs.

Figure 3 illustrates the derivatization reactions when hydroxylamine is added during the hydrolysis step, and prior to derivatization with BSTFA + TMCS. Codeine, morphine, and 6-MAM are unaffected by hydroxylamine, as they do not contain keto-functional groups. The hydroxylamine produces a stable oxime derivative for keto-opiates, which are unable to enolize, and a single 1TMS derivative is formed for each of hydrocodone and hydromorphone (see Fig. 3*a*). Two derivatives each are formed for oxycodone and oxymorphone, reflecting the incomplete derivation

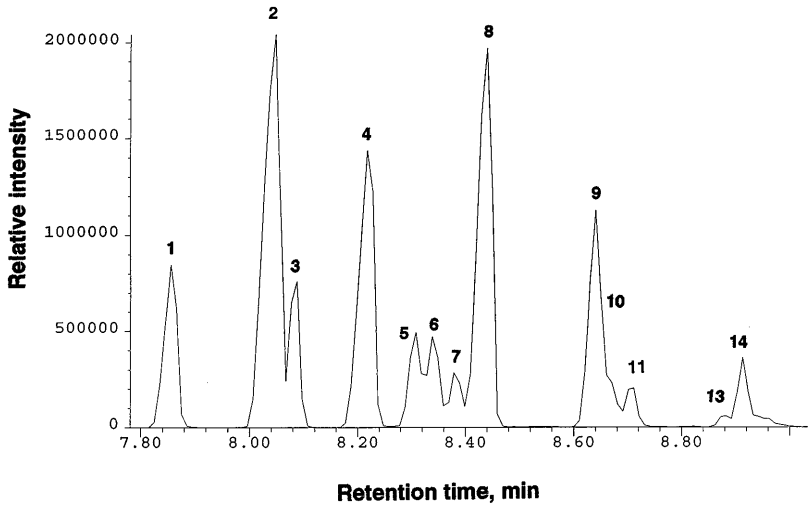

FIG. 2—Gas chromatographic analysis of a standard mixture of codeine, morphine, hydrocodone, hydromorphone, oxycodone, and oxymorphone, without the oxime derivative reaction. 1: underivatized hydrocodone; 2: 1TMS-codeine; 3: 1TMS-hydrocodone; 4: 1TMS-hydromorphone; 5: underivatized oxycodone; 6: 2TMS hydromorphone; 7: 2TMS oxycodone; 8: 2TMS morphine; 9: 1TMS-oxymorphone; 10: 1TMS-oxycodone (enol form); 11: 1TMS-oxycodone (keto form); 12: 3TMS oxymorphone; 13: 2TMS oxymorphone (enol form); 14: 2TMS oxymorphone (keto form).

TABLE 1—*Molecular ions of TMS and oxime-TMS derivatives of natural and synthetic opiates.*

Drug	TMS Derivative Form (GC Peak)*	mol. ion	RRT†	Oxime-TMS Derivatives Form (GC Peak)‡	mol. ion	RRT
Codeine	1TMS (2)	371	1.000	N/A (1)	N/A	1.000
Morphine	2TMS (8)	429	1.049	N/A (2)	N/A	1.049
Hydrocodone	underiv. (1)	299	0.976	1TMS (3)	386	1.068
	1TMS (3)	371	1.005			
Hydromorphone	1TMS (4)	357	1.021	2TMS (4)	444	1.088
	2TMS (6)	429	1.036			
Oxycodone	underiv. (5)	315	1.032	1TMS (6)	402	1.121
	1TMS (enol) (10)	387	1.078	2TMS (5)	474	1.125
	1TMS (keto) (11)	387	1.079			
	2TMS (7)	474	1.041			
Oxymorphone	1TMS (9)	373	1.074	2TMS (8)	460	1.134
	2TMS (enol) (12)	445	1.104	3TMS (7)	532	1.143
	2TMS (keto) (13)	445	1.112			
	3TMS (14)	517	1.083			

* GC peaks correspond to those shown in Figs. 2 and 3, respectively.
† RRT = retention time relative to codeine.
‡ N/A = not applicable.

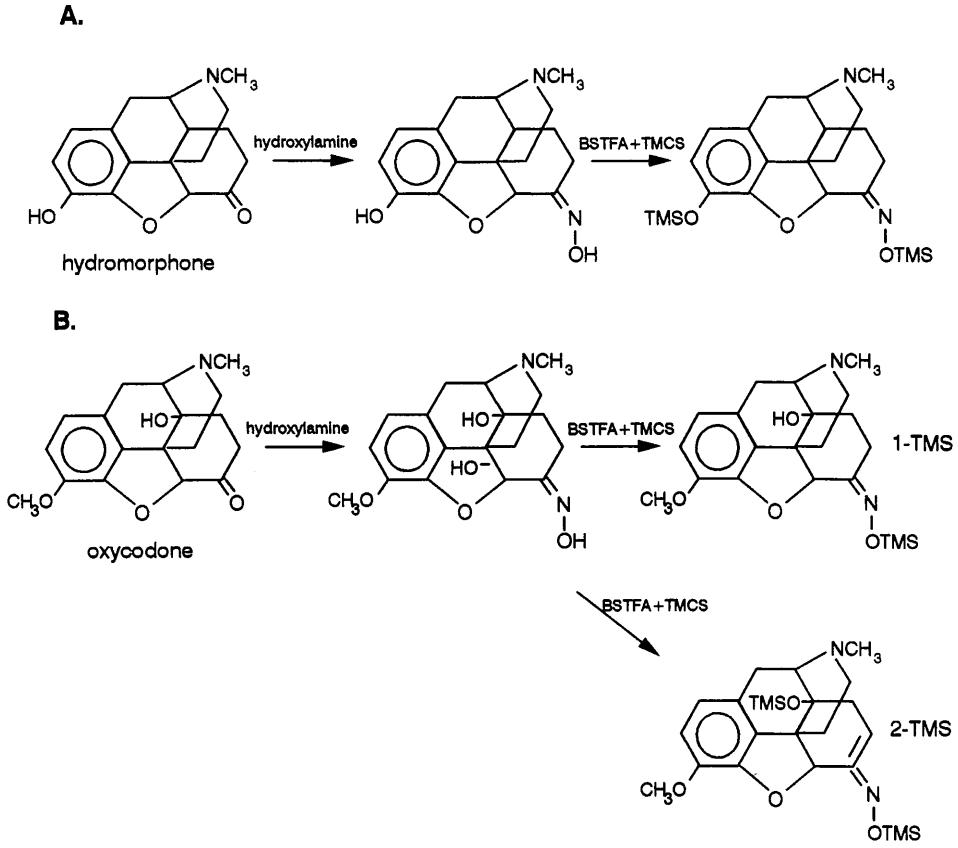

FIG. 3—*Derivatization reaction of BSTFA + TMCS after reaction with hydroxylamine for:* (a) *hydromorphone and* (b) *oxycodone.*

reaction at the hydroxy position of carbon position 14 (Fig. 3*b*). Figure 4 illustrates the gas chromatogram of the same opiate mixture presented in Fig. 2. As shown, the number of derivatives for the keto-opiates in reduced from 14 to 8. More importantly, the retention times of these derivatives are greater relative to the non-oxime TMS derivative, and they no longer have the potential to interfere with the quantitation of codeine and morphine.

The analysis of urine samples containing keto-opiates is even more difficult, when considering that multiple derivatives are also possible for metabolites of these drugs. For example, 6-oxymorphol is a metabolite of oxymorphone (13), and will react with BSTFA to form both 2TMS and 3TMS derivatives. Oxycodone will metabolize to oxymorphone, and to noroxycodone, which has the potential of four additional derivatives (with tautomerism and the incomplete derivatization of the hydroxy group at carbon position 14). Similarly, hydrocodone produces norhydrocodone, hydromorphone, hydrocodol, and hydromorphol, each with the potential to produce multiple derivatives. Use of the oxime derivatization step reduces the number of possible derivatives formed for the keto-opiate metabolites (e.g., noroxycodone).

A quantitative comparison between the oxime-TMS and non-oxime-TMS derivative procedures is shown in Fig. 5. Most of the quantitative results are within 20% of each other, indicating no significant difference by usual forensic standards between the derivatization methods. The regression between oxime-TMS and non-oxime TMS returned a y-intercept of 10.7 ng/mL, slope of 0.913, and a correlation coefficient of 0.976 for $n = 16$ (range 34 to 1507 ng/mL). The assay has a limit of detection of 60 ng/mL for codeine and morphine, and a limit of quantitation (LOQ) of 90 ng/mL for these drugs. The LOQ is within the acceptable 40% of cutoff limit (120 ng/mL) required for SAMHSA-regulated specimen retesting protocols. When the cutoff concentration for opiates increases to 2000 ng/mL, the LOD and LOQ limits will not be an issue. The regression curve of area response versus opiate concentration (ng/mL) for determination of linearity was $y = 0.056x + 0.0030$, $r = 0.99979$ for codeine, and $y = 0.037x + 0.0014$, $r = 0.99980$ for morphine. The within-run ($n = 10$) precision for the low control (360 ng/mL) was 1.7 and 1.5% for codeine and morphine, respectively. The between-run precision was 4.8 and 5.0%, respectively, for the low control.

This procedure restricts interferences from keto-opiates in the targeted analyses of codeine and morphine. Although not tested in this study, the use of the oxime procedure also provides a mechanism for a more direct quantitation of keto-opiates. This is particularly true for hydrocodone and hydromorphone, as single derivatives are formed.

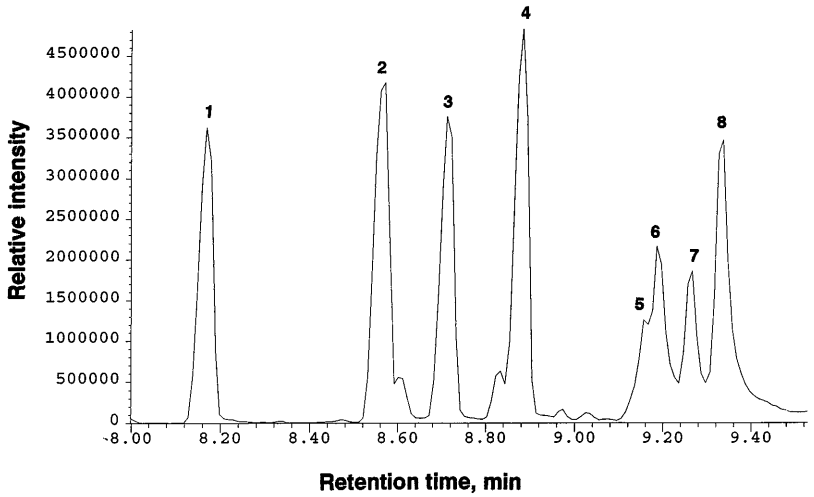

FIG. 4—*Gas chromatographic analysis of a standard mixture of codeine, morphine, hydrocodone, hydromorphone, oxycodone, and oxymorphone, with the oxime derivative reaction. 1: 1TMS-codeine; 2: 2TMS-morphine; 3: oxime-1TMS-hydrocodone; 4: oxime-2TMS-hydromorphone; 5: oxime-2TMS-oxycodone; 6: oxime-1TMS oxycodone; 7: oxime-3TMS-oxymorphone; and 8: oxime-2TMS-oxymorphone.*

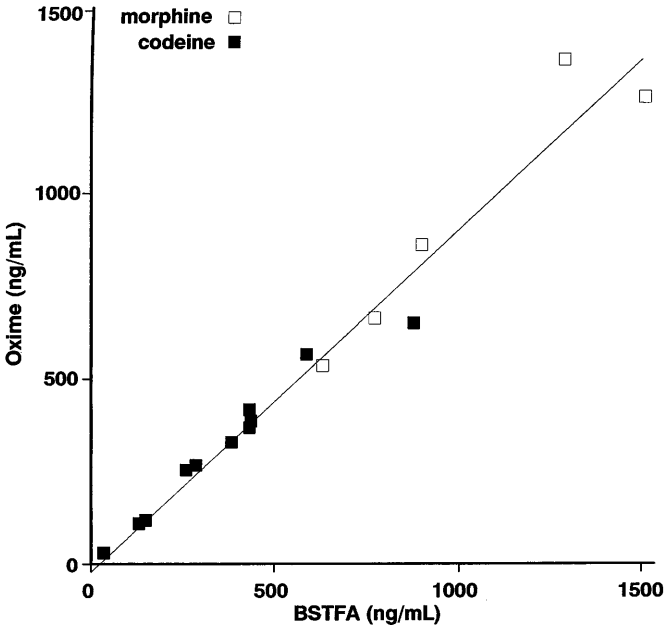

FIG. 5—*Combined analytical correlation of the oxime-TMS vs. the non-oxime TMS procedure for the quantitation of codeine and morphine from urine samples submitted for forensic toxicology testing. (Note that neither codeine nor morphine undergoes oxime derivatization.)*

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