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Evaluation of chemical derivatization agents for the gas chromatographic-isotope dilution mass spectrometric determination of morphine and codeine in urine

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ABSTRACT

With the aid of gas chromatography (GC)-isotope dilution mass spectrometry (MS) and an ion-trap GC-MS instrument, twelve chemical derivatization (ChD) agents which showed different potentials in derivatizing morphine (Mo) and codeine (Co) were further evaluated in terms of ease and efficiency of the ChD, analyte-internal standard (IS) ion cross contribution, shelf-life of the derivative, and experimental conditions of the ChD to see which ChD agent was best suited for the title drug urinalysis. It turned out to be N,O-bis(trimethylsilyl)acetamide (BSA), a silylation agent. Thus, optimum data quality of the total analysis was achieved starting with 100- μ L BSA plus 100- μ L ethyl acetate being added to the extracts previously obtained upon the automated solid-phase extraction (SPE) of the urine specimen, and followed by incubation at 100℃ for 30 min. The GC-MS qualifier ions for Mo-BSA were m/z 401, 414 and 429, with m/z 429 being the quantifier ion; the qualifier ions for Co-BSA were m/z 234, 343 and 371, with m/z 371 being the quantifier ion. Under optimal experimental conditions, the average recovery of Mo and Co as their BSA-derivatives from five serial spikes was 73.5%, with all individual recoveries exceeding 60%. The regression calibration curves for urinary spikes were typically linear within 50-1000 ng/mL, with correlation coefficients consistently exceeding 0.99. The limits of detection (LODs) and quantitation (LOQs) for electron impact (EI), positive ion chemical ionization (PCI) and negative ion chemical ionization (NCI) mode analyses were exclusively below 50 ng/mL no matter which definitions of the limits were adopted; that is, all were much lower than the legal cutoff, 300 ng/mL. The coefficients of variance (CVs) calculated for the triplicate quantitations of four BQC (blind quality control) samples ranged 6.8-14.2% for Mo, and 5.0-9.2% for Co. The precisions calculated for triplicate quantitations of a real-case specimen which TDx has screened as "opiates--high" were 7.0% for Mo and 7.1% for Co.

Keywords: Urine Drug Testing, Morphine, Codeine, Chemical derivatization, Gas Chromatography-Mass Spectrometry (GC-MS), Solid Phase Extraction (SPE)

Introduction

It was in 1993 when over three hundred kilograms of illicit heroin was seized in Chia-Yih area that the ROC government delivered a declaration against illicit and abused drugs. Since then a series of policies directed towards a drug-free society have been promoted. In 1997, an act called "Drug-Free Regulations" was enacted by the Legislative Yuan. It is that law that allows for collecting urine drug testing samples from specified people forcibly.

Currently, all the protocols worldwide of forensic urine drug testing include a screening immunoassay (IA) or thin-layer chromatography (i.e., the commercially marketed "Toxi-Lab" system) followed by a confirmatory gas chromatography-mass spectrometry (GC-MS)

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of those samples that are screened positive. So far as the GC-MS test is concerned, criteria adopted for evidential drug identification and quantification require the appearance of the monitored ions at correct retention times with acceptable intensity ratios among these ions. The retention times and ion intensity ratios observed in the test sample are compared with those established by an (or a set of) authentic calibration standard(s) incorporated in the same analytical batch [1]. Most often isotope dilution GC-MS employing deuterium-labeled analytes (i.e., isotopic analogs of the analytes) as the internal standards (ISs) are the choice of method. Meanwhile, most protocols require that some sort of chemical derivatization (ChD) be performed so as to conform to GC environment, improve GC separation, enhance GC-MS detection, and assist structural elucidation of the analytes [2-4]. Our previous works on the GC-MS determination of amphetamines and ketamines have all been in accordance with these criteria and have all achieved satisfactory results [2-4]. It should be noted, however, that improperly performing the ChD and GC-MS analysis may cause artifacts, unresolved peaks, or poor instrumental responses, and result in erroneous conclusions. The many possibilities for the drug user to have initially ingested the suspect drug of various forms, from various sources and by various means, both legal and illegal, further complicate both the chemical analysis and the data interpretation [5-7]. Considering the fact that the respective analytes and ISs will all go through the chemical/physical processes and undertake the isotope dilution GC-MS analysis, the quality of the analytical results relies on a sound sample pretreatment (including ChD), an effective GC-MS methodology, and a critical data evaluation process. The important roles of the ChD agent and the selected monitoring ions of the derivatized analytes/ISs cannot be over-emphasized.

Morphine (Mo) and codeine (Co) are frequently the primary analytes found in blood and urine and are used as indications of previous administration of opiates, including heroin, Mo, Co, poppy seeds, etc [5]. The cutoffs adopted by the U.S. Department of Human Health Service (DHHS) and Department of Defense (DoD) drug testing programs for positive opiates are 300 ng/mL for Mo and/or Co. In Taiwan the law entitled "Essentials of Drug-Testing Laboratory Accreditation and Management" also specifies the same values. The validity of such interpretations, however, has been questioned in light of the observations that these same analytes can appear in urine as a result of legal ingestion of certain drug products or other items, such as cough syrups and poppy seed containing materials. ElSohly et al. reviewed some thirty reports on this issue and drew four general guidelines to help differentiate the possible source of Mo and Co in urine [5]. Also, for the exact confirmation of heroin ingestion, the US Substance Abuse and Mental Health Services Administration (SAMHSA) added a string to the existing heroin cutoff; namely, a positive urine heroin test requires at least 2000 ng/mL of Mo and 10 ng/mL of 6-acetylmorphine (6-AM) be found simultaneously. To meet the increasingly strict legal requirements, most attention of the analyst concerning the technical aspects should be paid to the mutually related ChD and GC-MS steps if isotope dilution method is adopted to help secure the analytical data quality.

So far as ChD is concerned, there are scattered papers in the literature which discuss and/or compare the performance of different ChD agents towards Mo and Co [8-12]. However, the number of kinds of agents they employed was somewhat limited. In addition, most of those studies did not use the prevalent isotope dilution method. Chen et al. compared pentafluoropropionyl anhydride (PFPA), heptafluorobutyric anhydride (HFBA), N-Methyl-N-bis-trifluoroacetamide (MBTFA), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and trifluoroacetic anhydride (TFA) [11]. The former two gave derivatives which produced too few secondary and tertiary fragment ions to make enough number of qualifier ions (usually 3) available, which in turn made the peaks of Mo and normorphone derivatives overlap in the selective ion chromatogram. MBTFA was inappropriate in that its ChD reaction with the IS used, naolorphine, yielded an unstable derivative. In contrast, the latter two gave derivatives showing three prominent qualifier ions whose intensities were stable for as long as 48 hours. Grinstead et al. simply compared ordinary acetic anhydride with PFPA [12]. While acetic anhydride-derivatized opiates were all able to offer qualifier ions of acceptable responses, the 6-AM stemming from the ChD reaction of acetic anhydride with Mo would be confused with any pre-existing 6-AM, making the quantification of Mo questionable. On the other hand, Co-PFPA showed appreciable tailing in the chromatogram. In the present paper, a more detailed comparison accompanied by isotope dilution GC-MS methodology is made on the ChDs of the most common opiates analytes, Mo and Co, with twelve ChD agents, including one alkylation, four silylation, and seven acylation agents. This quantitative evaluation is in terms

of analyte-internal standard (IS) ion cross contribution, stability of the derivative, and experimental conditions of the ChD to see which ChD agent was best suited for the title drug urinalysis.

Experimental

Materials

Racemic d,l-morphine (Mo; 1 mg/mL in methanol), d, l-codeine (Co; 1 mg/mL in methanol), d,l-morphine-d3 (Mo-d3; 0.1 mg/mL in methanol) and d,l-codeine-d3 (Co-d3; 0.1 mg/mL in methanol) were purchased from Cerilliant Co., USA. Two stock solutions were prepared and stored at below 0℃ prior to use: solution A containing 10 μg/mL each of Mo and Co in methanol; solution B containing 10 μg/mL each of Mo-d3 and Co-d3 in methanol.

N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was from Lancaster Synthesis Co., UK; N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), N,O-bis (trimethylsilyl)acetamide (BSA), N-methyl-N-(t-butyldimethylsilyl)-triflouoroacetamide (MTBSTFA), and N-Methyl-N-bis-trifluoroacetamide (MBTFA) from Sigma Chem Co. USA; trifluoroacetic anhydride (TFA), heptafluorobutyric anhydride (HFBA) and pentafluorobenzoyl chloride (PFBC) from Acros Organics Co., USA; pentafluoropropionyl anhydride (PFPA) and pentadecafluoroctanoyl chloride (PFOC) from Aldrich Chem Co., USA; trimethylammonium hydroxide (TBH) from Riedel de Haen Co., Germany; acetic anhydride from Osaka Chem Co., Japan. Pyridine was from BDH Co, UK; concentrated hydrochloric acid, acetic acid, ammonium acetate, and ammonium hydroxide from Yakuri Pure Chem. Co., Japan; methanol and ethyl acetate from Tedia Co., USA. All of the above agents and solvents were in analytical or reagent grade and were directly used without further purification.

Solid-phase extraction

All urinary samples were prepared using an automated Zymark Rapidtrace™ SPE Workstation equipped with up to ten ISOLUTER SPE cartridges (130-mg HCX adsorbent; 3-mL sample capacity). The general SPE procedure is as follows: To the test tube containing 2 mL of urine sample was added 60 μL of the foregoing solution B and 0.5 mL of 9N HCl. The mixture was subjected to acidic hydrolysis in a boiling water bath for 20 min, and allowed to cool to ambient temperature. To the mixture was added ca. 1 mL of 1M ammonium acetate buffer (pH~8) and ca. 0.5 mL of concentrated ammonia water to reach pH 7.5~8.5. On the other hand, the SPE cartridge was first conditioned with 1 mL of methanol flowing at 2mL/min, followed by 1 mL each of D.I. water and ammonium acetate buffer flowing at 2 mL/min. The sample solution was then introduced to the cartridge at a flow rate of 2 mL/min. After washing with 2 mL each of D.I. water, 0.1 M acetic acid and methanol, respectively, the cartridge was purged with nitrogen gas for 0.5 min to dryness. The analytes were finally eluted using 2 mL of a combined solvent (ethyl acetate : methanol : ammonia water = 70 : 25 : 5 v/v) which flew at 1.5-mL/min.

General procedure of ChD for the comparative evaluation of twelve ChD agents

To a screw-cap topped derivatizing tube was added 100 μL each of the foregoing solutions A and B. The combined solution was purged with nitrogen gas to dryness. To the residue was added 100 μL of appropriate ChD agent. The mixture was shaken for 1 min, incubated at 100℃ for 30 min, allowed to cool to ambient temperature, transferred to a concentration tube, and purged with nitrogen gas to dryness. More EA was added to make up a 200- μL solution. A 2- μL aliquot of this solution was injected for the GC-MS analysis.

Gas chromatography-mass spectrometry

The GC-MS analyses were carried out using a Finnigan MAT GCQ™ instrument equipped with a data processing system. The ion-trap GC-MS was first operated in electron impact full scan mode (GC-EIMS full scan; 50-700 amu) to look into the fragmentation nature (i.e., to pick up likely qualifier and quantifier ion candidates) of the derivatized analytes, and then in selected ion monitoring (GC-EIMS SIM) mode accompanied by extracted ion chromatograms (EIC) to further evaluate the qualifier and quantifier ions and run the formal analysis. The GC column used was a Rtx-5 capillary column (30 m * 0.25 mm I.D, 0.25 μm film thickness). The GC was operated in the splitless mode (i.e., purge off) when performing injection with the aid of a Finnigan CTC A200S autosampler, but 1 min later the purge valve was turned on. The injector temperature was 250℃. The column temperature was programmed from 100 to 250℃ at 20℃/min, with the final temperature held for 12.

5 min. Helium of 99.995% purity was used as the carrier gas at a linear flow-velocity of 35 cm/sec. Effluents from the GC column was transferred via a transfer line held at 280℃ to a 70-eV EI ionization source held at 200℃. In performing GC-EIMS SIM quantitation of the analyte, the calibration curve was produced by plotting the peak-area ratio (analyte : IS) against the concentration of the analyte in the fortified sample. The peak-area ratio used was the mean of triplicate analyses.

Complementary mass spectra were also acquired in the positive and negative ion chemical ionization full-scan modes (GC-PCIMS full-scan and GC-NCIMS full-scan) as well as in the MS-MS mode (tandem in "time" rather than in "space"). Complementary calibration curves were also plotted in the GC-PCIMS SIM and GC-NCIMS SIM modes accompanied by EIC chromatograms. The operation conditions were the same as those for the EI mode except that instead of using a 70-eV EI ionization source methane was used as reagent gas and was ionized under 100 eV.

Results and Discussion

Preliminary tests on twelve ChD agents

The remarkable efficacies of ChD of amphetamines and ketamines have previously been demonstrated and validated [2-4]. Based on a similar theory or reasoning, it shall be relatively safe to make the same assumptions for opiates. The following sections serve as a further validation. Of the twelve ChD agents tested (i.e., one alkylation, four silylation, and seven acylation agents), five acylation agents (i.e., TFA, MBTFA, HFBA, PFPA, and acetic anhydride) and three silylation agents (i.e., MSTFA, BSTFA, and BSA) yielded detectable ChD products. The chemical structures of these eight agents are shown in Fig.1. The total ion current chromatograms (TICs) obtained for the five derivatized analyte-IS pairs are displayed in Fig.2. Since MSTFA, BSTFA and BSA give the same TMS-substituted derivative, we simply choose BSA which has resulted in the strongest response as the representative. Similarly, TFA and MBTFA give the same TFA-substituted derivative, and we choose TFA as their representative. Due to steric hindrance or different reaction mechanism at all, PFOC, PFBC (for acylation), MTBSTFA (for silylation), and TBH (for alkylation) gave no detectable derivatives.

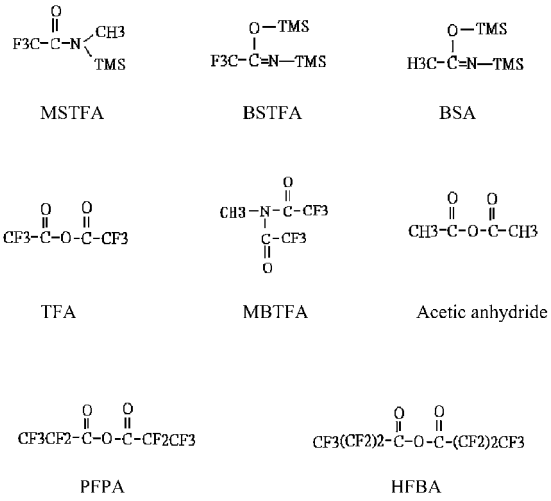

Fig.1 Chemical structures of the eight ChD agents that are capable of derivatizing morphine and codeine.

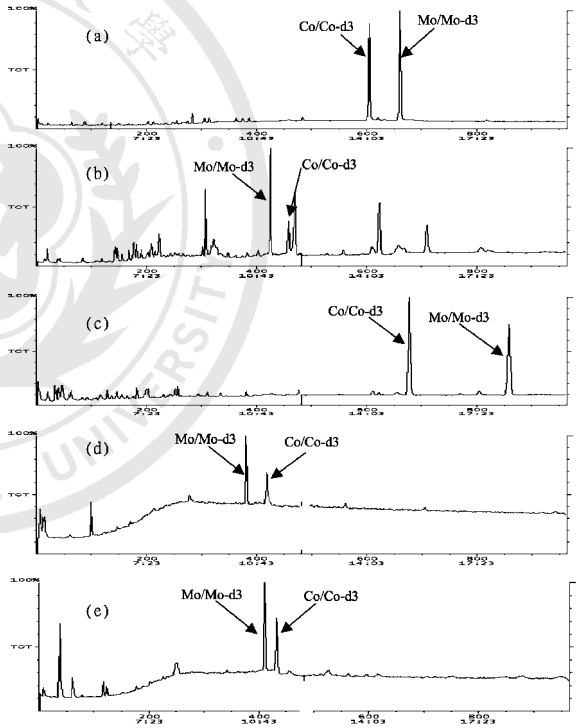

Fig.2 GC-EIMS full-scan TIC chromatograms obtained for the five derivatized Mo/Mo-d3 pairs and five derivatized Co/Co-d3 pairs: (a) with MSTFA, BSTFA and BSA; (b) with TFA and MBTFA; (c) with acetic anhydride; (d) with PFPA; (e) with HFBA. It should be noted that MSTFA, BSTFA and BSA gave the same TMS-substituted derivative, while TFA and MBTFA gave the same TFA-substituted derivative.

Evaluation of eight ChD agents with respect to the selection of qualifier and quantifier ions of the derivatives

Judging from our previous experience in analyzing amphetamines and ketamines, satisfactory GC resolution between Mo (or Co) and its deuterium-labeled IS may need the use of an IS labeled with more than nine deuterium atoms [2,3]. Being more available, however, d3-labeled ISs were used throughout the present study. This along with the demand of rapid analysis in practice did lead to inadequate separation between the derivatized analyte and IS peaks in the GC-MS full-scan TIC chromatogram. Fortunately, through more appropriate selection of SIM qualifier and quantifier ions, the essential or effective resolution (as opposed to the poor "superficial" resolution in the full-scan TIC chromatogram) and hence the accuracy and precision for the GC-EIMS and GC-PCIMS analyses can still be secured. Having gone through a detailed evaluation process according to that described by Liu for the quantitative determination of pentobarbital [1-3], the determined qualifier and quantifier ions for the GC-EIMS analyses of the five Mo-derivatives and five Co-derivatives, the SIM ion intensities and the percentages of the SIM ion intensities contributed by the corresponding ISs were listed in Table 1. Some other candidate ions that have finally been ruled out are also shown. Although silylation agents do not offer the most responsive quantifier ions, they do generate enough number of far above-average responsive, characteristic yet least interfered both qualifier and quantifier ions. Of the three silylation agents, BSA has been the most representative. Displayed in Figs.3 through 6 are the "pure" mass spectra of the respective BSA-derivatized analytes and ISs (i.e., each spectrum obtained in a separate run upon an aliquot containing just one derivative) acquired from the corresponding EI full-scan TICs. Shown in Fig.7 are the chemical structures of TMS-Mo's and TMS-Co's qualifier and quantifier ions.

Table 1 Qualifier/quantifier ions and candidate ions selected for the GC-EIMS analyses of the morphine/codeine derivatives as well as the percentages of the respective SIM ion intensities contributed by the corresponding ISs.

Derivatizing agent	Derivatized analyte	Selected ion-pair for derivatized analyte-IS[a] (m/z)	Full-scan relative intensity of analyte-IS ion (%)	SIM analyte ion intensity[b] and % contribution by IS[c, d]
MSTFA BSTFA BSA	TMS-Mo	429* (432)	100;100	243601[e] (0)
		414 (417)	33.5;33.5	89680[e] (0)
		401 (404)	50.0;53.3	113078[e] (0)
		287 (290)	30.0;36.7	58298[e] (0)
		220 (223)	53.3;55.0	94476[e] (10)
MBTFA TFA	TFA-Mo	477 (480)	26.3;26.7	375874[f] (0.8)
		364* (367)	100;100	1977807[f] (0.4)
		267 (270)	6.67;6.67	176011[f] (5.1)
Acetic anhydride	Acetyl-Mo	369 (372)	51.7;81.3	207486 (0.4)
		327* (330)	100;100	474403 (0)
		310 (313)	51.5;53.1	238404 (0)
		268 (271)	48.3;71.9	268102 (0)
		204 (207)	18.9;50.0	53265 (0)
PFPA	PFP-Mo	577 (580)	67.2;40.0	754803 (0)
		430 (433)	3.0;3.6	32580 (0)
		414* (417)	100;100	1160158 (0)
		267 (270)	9.4;13.6	55652 (0)
HFBA	HFB-Mo	677 (680)	6.3;9.5	153009 (0)
		464* (467)	100;100	2001110 (0)
		267 (270)	10.2;9.5	143687 (0)
MSTFA BSTFA BSA	TMS-Co	371* (374)	100;100	177756[g] (0)
		343 (346)	31.0;24.6	58996[g] (0)
		234 (237)	41.4;38.6	82999[g] (0)
		178 (181)	32.8;38.6	28620[g] (46)
		146 (149)	31.0;31.6	58721[g] (0)
MBTFA TFA	TFA-Co	395 (398)	55.2;61.5	426115[h] (0)
		282* (285)	100;100	579513[h] (0)
		267 (270)	13.8;15.4	135946[h](0)
Acetic anhydride	Acetyl-Co	341* (344)	100;100	458766 (0)
		282 (285)	93.1;91.5	504216 (0)
		267 (270)	20.7;13.5	128470 (19)
		229 (232)	34.5;32.6	103149 (5)
		204 (207)	27.6;36.5	76230 (9)
PFPA	PFP-Co	445 (448)	36.8;34.6	310665 (0)
		282* (285)	100;100	806819 (0)
		267 (270)	17.5;13.8	80909 (0)
HFBA	HFB-Co	495 (498)	41.2;56.1	503081 (0)
		282* (285)	100;100	1011304 (0)

[a] Ions with the m/z value underlined are qualifier ions and those asterisked quantifier ions.
[b] Low level of contribution may be "not detected" under relatively high "threshold" or "zero" settings.
[c] Whenever more than one ChD agent give the same derivative, the ion intensities entered are for the one that has the highest quantifier ion intensity.
[d] All data were obtained by injecting 2 μL of the derivatives solution that stemmed from 1 μg each of the pure analyte and IS.
[e] The ion intensities entered are for the BSA-derivatized Mo..
[f] The ion intensities entered are for the TFA-derivatized Mo..
[g] The ion intensities entered are for the BSA-derivatized Co..
[h] The ion intensities entered are for the TFA-derivatized Co..

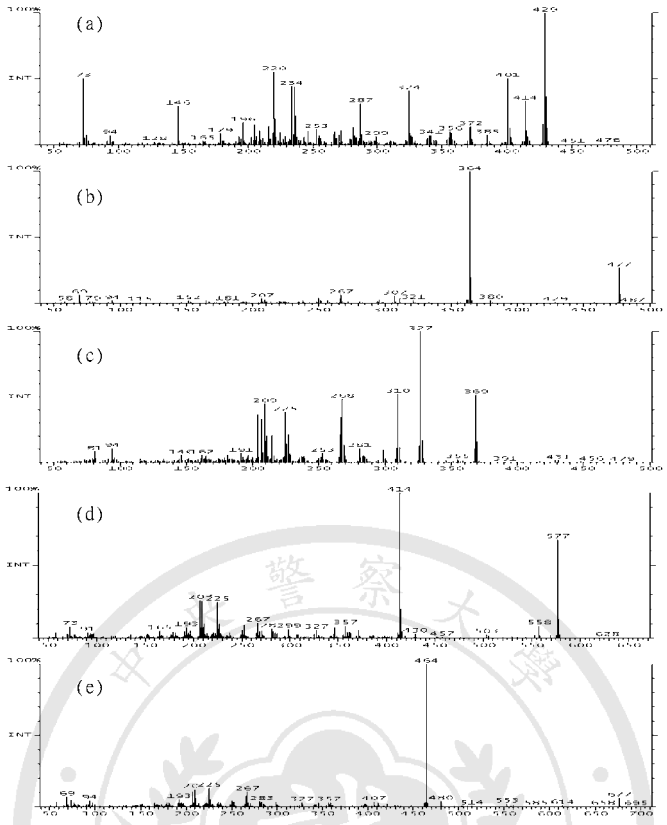

Fig.3 EI mass spectra of: (a) TMS-Mo; (b) TFA-Mo; (c) Acetyl-Mo; (d) PFP-Mo; (e) HFB-Mo.

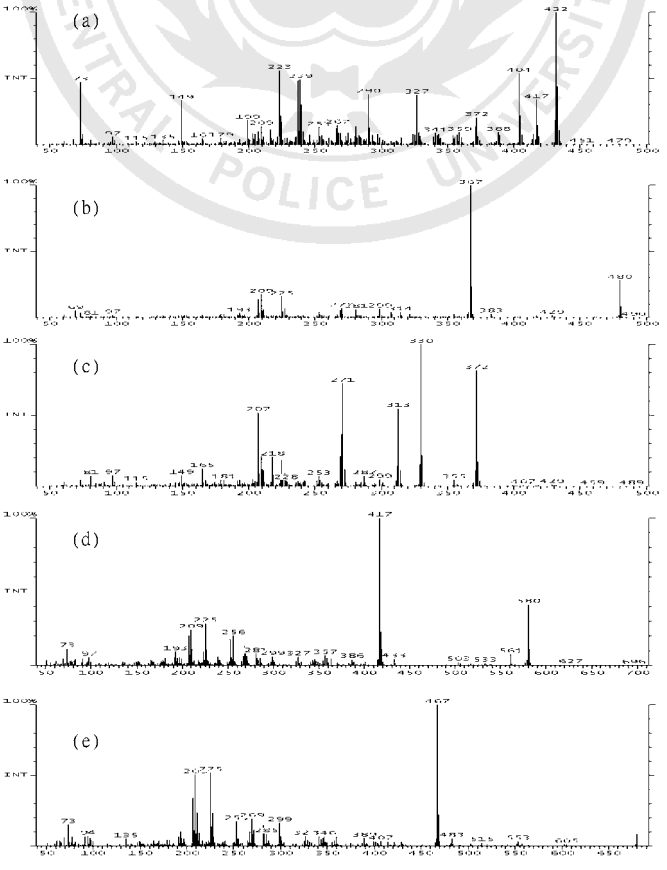

Fig.4 EI mass spectra of: (a) TMS-Mo-d3; (b) TFA-Mo-d3; (c) Acetyl-Mo-d3; (d) PFP-Mo-d3; (e) HFB-Mo-d3.

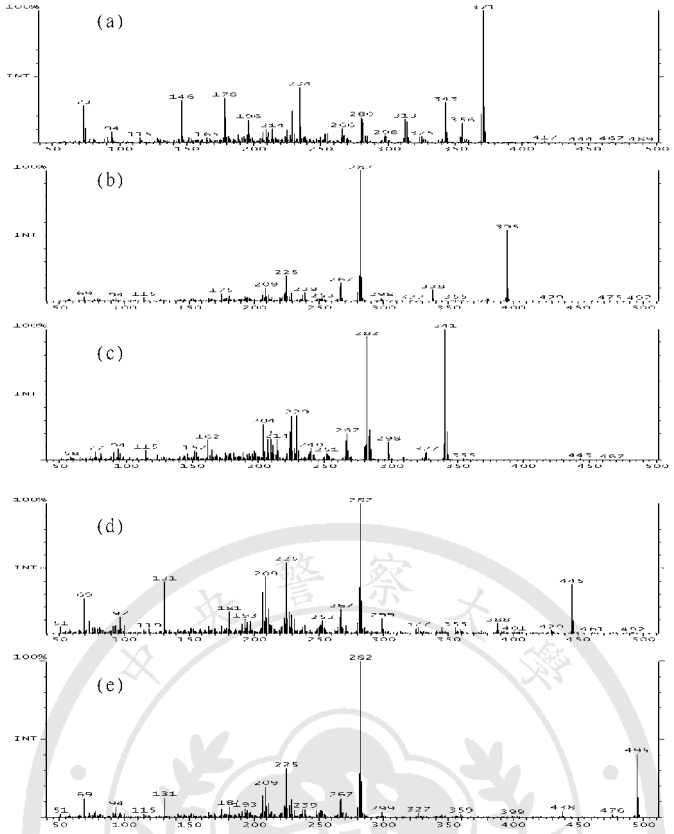

Fig.5 EI mass spectra of: (a) TMS-Co; (b) TFA-Co; (c) Acetyl-Co; (d) PFP-Co; (e) HFB-Co.

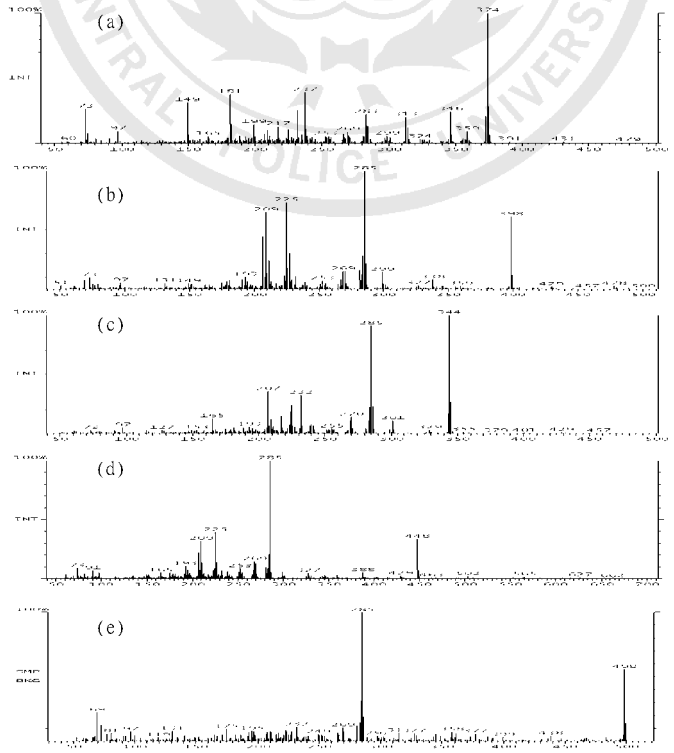

Fig.6 EI mass spectra of: (a) TMS-Co-d3; (b) TFA-Co-d3; (c) Acetyl-Co-d3; (d) PFP-Co-d3; (e) HFB-Co-d3.

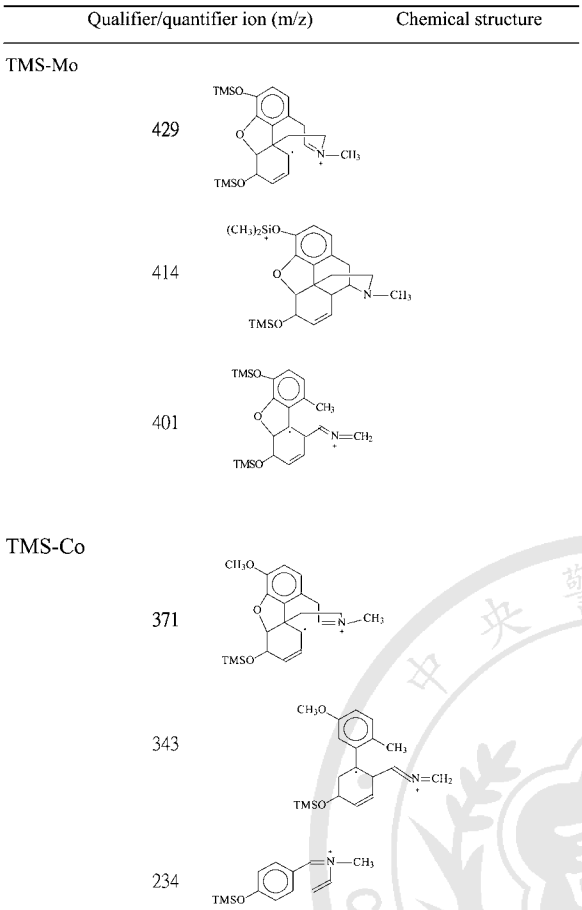

Fig.7 Chemical structures of TMS-Mo's and TMS-Co's qualifier/quantifier ions. Ions m/z 429 and 371 are the respective quantifier ions.

Comparison of eight ChD agents in terms of stability of derivatives

For this part of study, appropriate Mo- and Co-derivatives freshly prepared according to the ChD procedure described in the experimental section were dissolved with 200 μ L of ethyl acetate. Three 2- μ L aliquots of the solution were injected for the GC-EIMS analysis after 12, 36, 60 hours, respectively, of standing. In view of the precisions of the SIM analyte-to-IS peak height ratios, the acylation agent PFPA shows the best precision, with all the three coefficients of variance (CVs) below 4% (Fig.8). The silylation agent BSA ranks second, with all the three CVs below 5%. As to the instrumental response, BSA and acetic anhydride produce the highest SIM ion intensities that can remain six to seven orders of magnitude after 60 hours of standing of the derivatives.

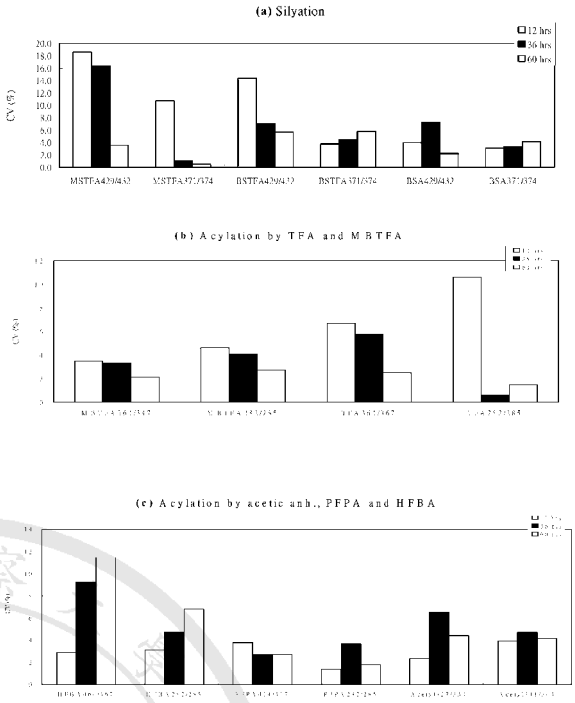

Fig.8 Relative stabilities of morphine and codeine derivatives in terms of the coefficients of variance (CVs) of SIM analyte-to-IS peak height ratios measured after 12 to 60 hours of standing of the derivatives.

Comparison of eight ChD agents in terms of GC retention times

Besides the above stated superficially unresolved analyte-IS pairs, the retention times shown in Table 2 indicate that TMS-substituted Mo and Co can be completely separated by a RT difference of 1 min or so in an acceptablly short running time using the above described temperature program, whereas all the acylation derivatives of Mo and Co except acetyl-Mo/Co are separated only by a RT difference of 0.5 min or so. Acetyl-Mo and acetyl-Co are well separated by a RT difference of 3 min, but the running time needed is relatively long.

Table 2 Retention times (RTs) of derivatized morphine and codeine.

Derivatizing agent	Morphine derivative	RT (min)	Codeine derivative	RT (min)
MSTFA BSTFA BSA	TMS-Mo	15.11	TMS-Co	14.05
TFA MBTFA	TFA-Mo	10.88	TFA-Co	11.51
PFPA	PFP-Mo	10.07	PFP-Co	10.79
HFBA	HFB-Mo	10.61	HFB-Co	11.15
Acetic anhydride	Acetyl-Mo	18.31	Acetyl-Co	15.47

Auxiliary GC-MS analysis using PCI and NCI

If necessary, GC-MS analysis using the relatively softer chemical ionization (CI) can provide both qualitative and quantitative information complementary to those given by the relatively harder EI mode. For the detection of opiates, PCI was found to produce instrumental responses nearly ten times those given by negative ion CI (NCI), being in agreement with our previous experience in analyzing amphetamines and ketamines. A comparative investigation of the ten derivatives also indicated that all PCI mass spectra looked relatively trivial and had prominent and characteristic M+1 and M+29 quasi-molecular ion peaks. However, the ones resulting from BSA-ChD (i.e., 429 + 1 = 430 and 429 +29 = 458 for TMS-Mo; 371 +1 = 372 and 371 + 29 = 400 for TMS-Co) were by far the most intense (Figs. 9 and 10). In contrast, all NCI mass spectra consistently show the quasi-molecular M − 2 peak. As to the MS/MS mass spectra, they all benefit from low background interference and can be used to help elucidate any ambiguous EI mass spectra.

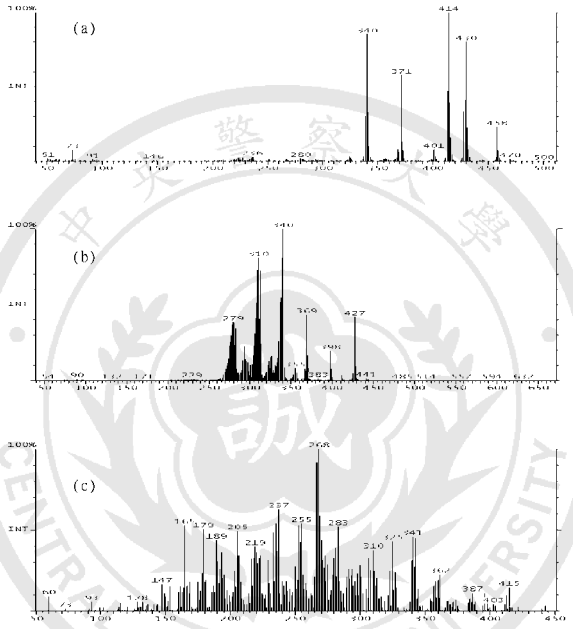

Fig.9 Mass spectra of TMS-morphine obtained in (a) PICI, (b) NICI, and (c) MS-MS (tandem in time) modes.

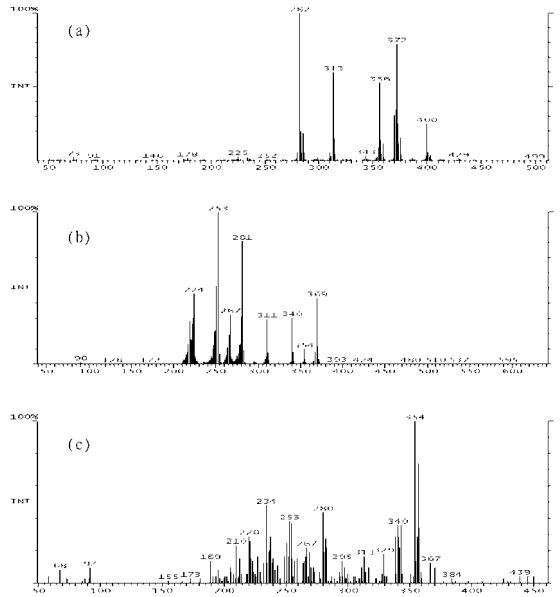

Fig.10 Mass spectra of TMS-codeine obtained in (a) PICI, (b) NICI, and (c) MS-MS (tandem in time) modes.

Optimization of experimental conditions for BSA-ChD

An overall consideration of the above shown results indicated that the silylation agent BSA should come the first as the choice of ChD agent for the Mo and Co to be analyzed. The BSA-ChD conditions might be thought to be crucial to the recovery (in terms of the SIM intensities of the quantifier ions) of the analytes. A series of experiments demonstrated that, generally speaking, reasonably higher ChD temperatures and longer ChD times would result in higher instrumental responses. The amounts of ChD agents (so long as in significantly excess amounts) or whether a catalyst such as pyridine had been added did not show appreciable effects on the responses. A fact of most concern has been that 100 μL each of BSA and ethyl acetate took the shortest time to purge to dryness, whereas the addition of carcinogenous pyridine took much longer time to purge. Thus the optimal ChD conditions would be 100- μL BSA plus 100- μL ethyl acetate being added to the extracts previously obtained upon the automated solid-phase extraction (SPE) of the urine specimen, and followed by incubation at 100℃ for 30 min.

Quantitation

Using BSA as the ChD agent, the five-point (50, 100, 300, 500, 1000 ng/mL) method calibration curves plotted for the GC-EIMS SIM analyses of Mo and Co in urine (equations: y = 0.0033x + 0.0911 for Mo; y =0.0025x + 0.1472 for Co) are both linear within 50~1000 ng/mL, with the correlation coefficients (r2) being 0.9914 and 0.9977, respectively. Likewise, the method calibration curves plotted for the GC-PCIMS SIM analyses of Mo and Co in urine (equations: y = 0.0038x − 0.0767 for Mo; y = 0.0039x + 0.0103 for Co) are also both linear within 50~1000 ng/mL, with the correlation coefficients (r2) being 0.9949 and 0.9952, respectively.

To be used in monitoring the instrumental performance and calculating the below discussed analyte recoveries for the sample preparation steps, the five-point instrumental calibration curves plotted for the GC-EIMS SIM analyses of authentic Mo and Co (equations: y = 0.4154x − 18.984 for Mo; y =0.3599x − 0.2986 for Co) are both linear within 50~1000 ng/mL, with the correlation coefficients (r2) being 0.9968 and 0.9989, respectively. The five-point instrumental calibration curves plotted for the GC-PCIMS SIM analyses of authentic Mo and Co (equations: y = 0.355x + 16.876 for Mo; y = 0.3649x + 5.4334 for Co) are also both linear within 50~1000 ng/mL, with the correlation coefficients (r2) being 0.9928 and 0.9957, respectively.

Limits of detection (LODs) and limits of quantitation (LOQs)

The method limit of detection, (M)LOD, and method limit of quantitation, (M)LOQ, were determined in this study by three definitions. Definition A is currently more prevalent in the forensic practice [13]. After serial analyses of urinary spikes containing 1000, 500, 250, 100, 50, 25, 10 ng/mL, etc., of Mo and Co, the respective lowest concentrations of the two analytes that analyzed accurately within ±30% of their respective target concentrations were designated as the respective LODs of the two analytes, the string being that two ion ratios of each BSA-derivative (i.e., Im/z 401/Im/z 429 and Im/z 414/Im/z 429 for TMS-Mo, and Im/z 234/Im/z 371 and Im/z 343/Im/z 371 for TMS-Co, taking GC-EIMS approach as the example) matched within ± 20% of those of the calibrators. In turn, the LOQs were the respective lowest concentrations of the two analytes that quantitated within ±20% of their respective target concentrations, the string being that the above stated two ion ratios of each BSA-derivative also matched within ± 20% of those of the calibrators. Whereas the four limits of linearity exceeded 4000 ng/mL, unfortunately, the four LOD's and four LOQs obtained by this definition were unable to go below 50 ng/mL due to negative intercepts on the y axis. In contrast, definition B by which the lowest concentration giving signal-to-(neighboring) noise ratio≧3 (10) is taken as the LOD (LOQ) is trivial, and the LODs/LOQs thus obtained were much lower than those by the other two definitions. Definition C is somewhat academic [14]. Nevertheless, its relevant data are presented in support of the practicability of the proposed drug-testing method. Here the two limits are defined as the analyte concentrations giving peaks in the SIM chromatogram with heights equal to the mean + N × standard deviation, where N = 3 for the LOD and 10 for the LOQ. The mean is the measured average of noises taken from a baseline region located far away from the analyte peak using a fortified sample. Accordingly, the standard deviation is the measured fluctuations of the noises. Thus, a 300-ng/mL Mo/Co fortified urine sample was pretreated and analyzed according to the above described procedure. The LODs and LOQs calculated for the respective quantifier ions using the equipped soft-

ware range from 4.8 to 30.6 ng/mL. In summary, PCI mode which have lower background can usuaally achieve lower LODs and LOQs than EI mode. Moreover, the generally low limits achieved in this study should sufficiently meet the requirements of most of the urine drug testing programs.

Table 3 Method limits of detection (LODs) and method limits of quantitation (LOQs) for the total analysis of morphine and codeine in urine via GC-EIMS and GC-PCIMS, respectively.

Ionization mode		Criterion[a]	Morphine (ng/mL)	Codeine (ng/mL)
EI	LOD	Bias $\leqq \pm 30\%$	50	50
		$S_m/N \geqq 3$	1.1	1.4
		$S_m \geqq \overline{X}_{bl}+3SD_{bl}$	30.6	12.5
	LOQ	Bias $\leqq \pm 20\%$	50	50
		$S_m/N \geqq 10$	3.5	4.7
		$S_m \geqq \overline{X}_{bl}+10SD_{bl}$	36.0	26.8
		Linear working range : 50~$\geqq$4000 ng/mL		
PCI	LOD	Bias $\leqq \pm 30\%$	50	50
		$S_m/N \geqq 3$	6.1	1.3
		$S_m \geqq \overline{X}_{bl}+3SD_{bl}$	7.5	4.8
	LOQ	Bias $\leqq \pm 20\%$	50	50
		$S_m/N \geqq 10$	14.2	4.3
		$S_m \geqq \overline{X}_{bl}+10SD_{bl}$	25.8	10.6
		Linear working range : 50~$\geqq$4000 ng/mL		

[a] Detailed definitions and criteria are given in the text.

Analyte recoveries indicative of the efficiency of SPE and BSA-ChD

In order to achieve an immunity to a wide variety of chemical and physical interferences and to improve the quantitative analytical quality, isotope-dilution method was employed in this study. As is described in the experimental section, known amounts of d3-labeled ISs were routinely added to the urine sample prior to performing SPE. Nevertheless, in addition to the full understanding of the instrumental performance, it is also informative to have insight into the actual efficiency of SPE and ChD.

Five 2-mL aliquots of urinary spikes containing 50, 100, 300, 500, 1000 ng/mL, respectively, each of Mo and Co were subjected, respectively, to SPE followed by BSA-ChD and GC-EIMS SIM analysis according to the procedure and conditions described above. The recoveries of Mo/Co were obtained by dividing the regressed concentrations of recovered Mo/Co (actually recovered as BSA-derivatives) by the originally spiked concentrations of Mo/Co. Thus, the mean recoveries (N = 3) calculated for the five aliquots are listed in Table 4. The fair to high recoveries indicate that the SPE and BSA-ChD steps are efficient.

Table 4 Recoveries calculated (N = 3) for the SPE and BSA-ChD of morphine and codeine from five urinary spikes.

Concentration	Morphine(%)	Codeine(%)	Average[b](%)
50 ng/mL	83.6	78.5	81.1
100 ng/mL	82.5	90.2	86.4
300 ng/mL	66.6	65.2	65.9
500 ng/mL	62.4	75.2	67.8
1000 ng/mL	60.7	73.9	67.2
Average[a]	71.2	76.6	73.5[c]

[a] Average recoveries calculated for the same analyte of different concentrations.
[b] Average recoveries calculated for different analyte of the same concentrations.
[c] Average recoveries calculated for all spikes.

Case study

The analytical scheme proposed in this report as a choice of confirmatory protocol for forensic urine drug testing was applied to the determination of Mo and Co in four blind-quality-control (BQC) and four real-case urinary specimens. While the latter have previously been screened by TDx (a fluorescence polarization immunoassay, FPIA) as "HIGH" for opiates, no information about the true concentrations for the former has been given. After getting through the above described analytical procedure and regression calibration in triplicate, the relevant data were shown in tables 5 and 6. Putting aside the concern about the accuracies and forensic interpretation of the drug levels, the four low Co outcomes for the real-case samples and all the small CV values (mostly below 10%) have validated the proposed analytical scheme as a competent confirmatory protocol for forensic urine opiates testing.

Table 5 Results obtained upon the total analyses (N =3) of four blind-quality-control (BQC) samples designated for morphine and codeine.

Analyte		Sample No.			
		M523-1	M523-2	M523-3	M523-4
Codeine	Mean	461.9	525.7	540.0	511.9
	SD	23.1	48.6	30.3	30.6
	CV(%)	5.0	9.2	5.6	6.0
Morphine	Mean	402.7	687.0	578.7	462.7
	SD	31.0	57.7	82.3	31.4
	CV(%)	7.7	8.4	14.2	6.8

Table 6 Results obtained upon the total analyses (*N* =3) of four real-case samples, all definitely involving opiates administration.

	TDx for opiates	GC-EIMS (ng/mL)	
		MO	CO
Run 1	High	2946.5	42.4
Run 2		3083.6	48.0
Run 3		2684.5	42.6
Mean		2904.9	44.3
SD		202.8	3.4
CV (%)		7.0	7.1

Conclusions

The results presented in this report demonstrated that SPE and BSA-ChD followed by isotope dilution GC-MS is a sound analytical scheme for the conclusive determination of Mo and Co in urine, and should meet the criteria adopted by the U.S. HHS and DoD (Department of Defense) drug testing programs. Although BSA only generate "among the most" GC-MS responsive opiates derivatives, it based on an overall consideration in terms of the ease for handling ChD process, lengths and differences of relevant RTs, shelf-life of the derivative, data quality of the analysis, compatibility between EIMS and CIMS mode, and accessibility and cost of the ChD agent does offer the most advantages over other ChD agents. In conclusion, the proposed scheme is simple, effective, reliable, and robust. It may serve as a confirmatory protocol for forensic urine drug testing.

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