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Evaluation of Isotopic Analogs as Internal Standard for Quantitative Determination of Urinary 6-Acetylmorphine by Gas Chromatography/Mass Spectrometry

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ABSTRACT

This study examined the accessibility of using 6-acetylmorphine-d_3 (6-AM-d_3) as the internal standard and trifluoroacetic anhydride, pentafluoropropionic anhydride, and hexamethyldisilazane as the derivatizing agents in the quantitative analysis of 6-acetylmorphine (6-AM) by full scan mode and selective ion monitoring (SIM) mode gas chromatography / mass spectrometry (GC/MS). In this study, fragments with minimal interference between the analyte and the internal standard were evaluated and selected for quantitative determination. Calibration curves and precision analyses were further performed using these selected ions. In the full scan mode, there was a certain interference between the mass spectra of trimethylsilyl derivative of 6-AM and those of 6-AM-d_3 at some fragment ions. This requires that there be carefull selection of fragment ions for quantitative determination due to the potential cross-contribution and subsequent inaccurate results. On the other hand, 6-AM-d_3 might be suitable as the internal standard in quantitative analysis of the trifluoroacetyl or pentafluoropropionyl derivative by GC/MS since the interference between the mass spectra of these derivatives of 6-AM and those of 6-AM-d_3, was minimal. In the SIM mode, results were similar with those of the full scan mode. The suitable fragments were thus selected for the calibration curve and precision analysis by SIM GC/MS. Excellent linearity was obtained over the concentration range of 50-1000 ng/ml of 6-AM for their TFA, PFP, and TMS derivatives. Good precision was also obtained from within-run and between-run CVs for 6-TFA-AM, 6-PFP-AM and 6-TMS-AM at a concentration of 200 ng/ml. 6-AM-d_3 was demonstrated to be a suitable standard in quantitative analysis of TFA-, PFP-, or TMS- derived urinary 6-AM in SIM mode GC/MS analysis.

Key words: **6-Acetylmorphine, GC/MS, derivative.**

INTRODUCTION

Although the concentration ratio of urinary morphine/codeine has been widely adopted as a guideline for identification of heroin abuse, some limitations remain in the differentiation of heroin abuse from codeine, morphine abuse or from the ingestion of poppy seed [1~3]. The presence of 6-

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acetylmorphine (6-AM) in urine has been proposed to be a specific marker for heroin abuse [3~5]. Nalorphine and its deuterated analog of the analyte have been adopted as the internal standards, and trifluoroacetic anhydride (TFAA), pentafluoropropionic anhydride (PFPA), and hexa-methyldisilazane (HMDS) have been selected as the derivatizing agents in the quantitative analysis of 6-acetylmorphine (6-AM) in urine by GC/MS [6~10].

Previous studies have evaluated the accessibility of the deuterated analog as the internal standards of commonly abused drugs, such as amphetamine [11], benzoylecgonine [12], morphine, and codeine [13]. In this study, the mass spectra of trifluoroacetylated (TFA), pentafluoropropionylated (PFP) and trimethylsilylated (TMS) derivatives of 6-AM along with its isotopic analog 6-AM-d_3 are presented and their spectrometric data critically evaluated to identify ions suitable for SIM in GC/MS analysis. Furthermore, the standard curves and the precision of analysis of TFA, PFP and TMS derivatives of urinary 6-AM were also evaluated according to the ion intensity ratios of these selected ions of the analyte/isotopic analogs verses various concentrations of 6-AM in urine to verify the accessibility of 6-AM-d_3 as the internal standard in GC/MS analysis.

MATERIALS AND METHODS

I. *Standards, Reagents and Controls*

Standard 6-AM and 6-AM-d_3 (0.1 mg/ml in methanol) were purchased from Radian (Austin, TX, USA). Trifluoroacetic anhydride (TFAA) and pentafluoropropionic anhydride (PFPA) were purchased from Pierce (Rockford, IL, USA). Hexamethyldisilazane (HMDS) were purchased from Chem Service (West Chester, PA, USA). Chloroform, ethyl acetate, pyridine, dichloromethane, isopropyl alcohol, disodium hydrogen phosphate, sodium dihydrogen phosphate dihydrate, sodium acetate trihydrate, and glacial acetic acid were purchased from Merck (Darmstadt, Germany). 25 % Ammonia water was purchased from Riedel-de Haen (Seelze, Germany). Solid phase extraction cartridge Clean Screen CSDAU203 was purchased from World Monitoring (Bristol, PA, USA).

II. *Preparation of Standard Solutions and Buffers*

6-AM or 6-AM-d_3 standard solution 1μg/ml was prepared by dissolving 0.5 ml of 100μg/ml 6-AM or 6-AM-d_3 standard in 50 ml of methanol. 6-AM or 6-AM-d_3 urine standard 5μg/ml was prepared by dissolving 0.5 ml of 100μg/ml 6-AM or 6-AM-d_3 standard solution in 10 ml of urine. 6-AM urine standards 50, 100, 200, 300, 500, and 1000 ng/ml were prepared by dissolving 0.1, 0.2, 0.4, 0.6, 1.0, and 2.0 ml of 5μg/ml 6-AM urine standard in 10 ml of urine, respectively.

Phosphate buffer (pH 6.0, 100 mM) was prepared using disodium hydrogen phosphate (1.70 g) and sodium dihydrogen phosphate dihydrate (12.14 g) in 1 l solution which was adjusted to pH 6.0 with 1N NaOH or 1N HCl. Acetate buffer (pH 4.5, 100 mM) was prepared using sodium acetate trihydrate (2.93 g) and glacial acetic acid (1.62 ml) in 0.5 l solution.

III. *Evaluation of Full Scan and SIM Mass Spectra Data*

Prior to GC/MS analysis, 1 ml of 6-AM and 1ml of 6-AM-d_3 standard solutions were evaporated to dryness at room temperature and were trifluoroacetylated, pentafluoropropionylated and trimethylsilylated respectively. For trifluoroacetylation, both residues were dissolved with chloroform (200 μl), derivatized with TFAA (100 μl), shaken for 30 seconds, heated at 65℃ for 15 minutes, dried by nitrogen gas at room temperature, and reconstituted with chloroform (50 μl). For pentafluoropropionylation, both residues were dissolved with 2, 2, 3, 3, 3,- pentafluoro-1-propanol (25 μl), derivatized with PFPA (50 μl), shaken for 30 seconds, heated at 95℃ for 15 minutes, dried by nitrogen gas at room temperature, and reconstituted with ethyl acetate (50 μl). For trimethylsilylation, both residues were dissolved with pyridine (50 μl), derivatized with HMDS (50 μl), shaken for 30 seconds, heated at 100℃ for 40 minutes

and stood to room temperature for GC/MS analysis.

GC/MS analysis was performed using HP 5890 series II Gas Chromatograph interfaced to HP 5970 mass selective detector (MSD) with splitless mode. The gas chromatograph was equipped with a 25-m HP Ultra I capillary column (0.20 mm ID; 0.33 μm film thickness). Helium was used as the carrier gas with a flow rate of 0.6 ml/min. The oven temperature was run by two programs. The first program was initially maintained at 150℃, followed by increasing to 250℃ with a rate of 10℃/min and maintained at 250℃ for 5 minutes. The second program was increased to 280℃ with a rate of 10 ℃/min and maintained at 280℃ for 5 minutes. The injector and detector temperatures were maintained at 250℃ and 280 ℃, respectively.

Full-scan and SIM mass spectra of 6-AM or 6-AM-d_3 were evaluated according to the cross-contributions between the major fragment ions of the analyte and the corresponding ions of the isotopic analog in terms of relative ion intensities and their percentages on the mass spectral data of 6-AM or 6-AM-d_3.

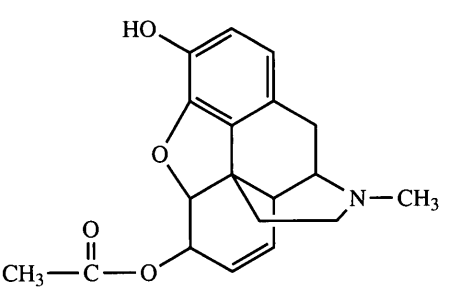
IV. *Calibration Curve Analysis*

1 ml of 50, 100, 200, 300, 500, and 1000 ng/ml 6-AM urine standards were pipetted into six 10 ml test tubes respectively. 40 μl of 5μg/ml internal standard solutions, 2 ml of 100 mM phosphate buffer (pH 6.0) were added into each test tube, and mixed thoroughly. Each of the six solid phase cartridges was sequentially conditioned in advance by 3 ml of methanol, 3 ml of water and 1 ml of 100 mM phosphate buffer (pH 6.0). Each of six prepared standard solutions were passed through the conditioned solid phase cartridge with a flow rate of 1~2 ml/min, and rinsed with 2ml of water, 2ml of 100 mM acetate buffer (pH 4.5), and 3 ml of methanol respectively. Each cartridge was vacuumed for 5 minutes at 10 inches of Hg, and eluted with 3ml of freshly prepared dichloromethane / isopropyl alcohol / 25 % ammonia water (78/20/2) solution. Each elute was collected and evaporated to dryness by a nitrogen gas flow at room temperature. Then SIM mode GC/MS analysis was performed by means of the same procedures mentioned above. Finally calibration curve analysis for each derivative of 6-AM and 6-AM-d_3 was presented and evaluated according to the ratios of the relative ion intensities of the selected corresponding ions of the derivatives versus concentrations of 6-AM urine standards.

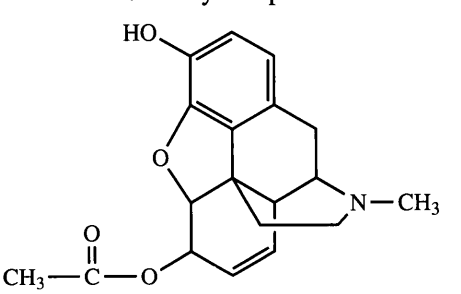
RESULTS AND DISCUSSION

The isotopic analog of an analyte is often considered the most suitable internal standard for quantitative analysis and is widely used in the determination of drugs and drug metabolites in biological specimens [11~13]. Selective ion monitoring of the analyte and the isotopic internal standard, or their derivatives, followed by the evaluation on the ion intensity ratio in the calibration standard and the test sample, provide the basis for a quantitative GC/MS analytical process.

The similarities between analyte and its isotopic analog in chemical properties and mass spectrometric fragmentation compensate for the possible errors derived from the loss of the analyte during sample preparation and the variation



6-Acetylmorphine



6-Acetylmorphine-d_3

Figure 1. Structures of 6-acetylmorphine and 6-acetylmorphine-d_3.

during GC/MS operations. However, not all commercial isotopic analogs can be used as internal standards since the analyte and the internal standard may not be adequately separated. In general the isotopic analog must generate at least one (preferably two or three) ions relatively free from cross-contribution by the analyte. There must also be at least three ions from the analyte that are relatively free from cross-contribution by the isotopic internal standard. If these two requirements are not met, the quantitative results may become unreliable [14].

Figure 1 shows the structures of 6-AM and 6-AM-d_3 which indicate that 6-AM-d_3 is distinguished from 6-AM in the N-methyl group on which three hydrogens were all substituted by deuterium. Figures 2-4 present the full-scan mass spectra of 6-TFA-AM, 6-PFP-AM, 6-TMS-AM, and their corresponding isotopic analogs i.e., 6-TFA-AM-d_3, 6-PFP-AM-d_3 or 6-TMS-AM-d_3. These spectra indicate that the mass differences between the major ions of 6-TFA-AM, 6-PFP-AM, or 6-TMS-AM and those of their corresponding isotopic analog, are all three amu. So 6-AM-d_3 might be adopted as the internal standard for urinary 6-AM in GC/MS.

The full-scan mass spectral data of 6-TFA-AM and 6-TFA-AM-d_3 are presented in Table 1. The results indicate that the full-scan mass spectrum of 6-TFA-AM-d_3 shows 0 % for m/z 311, 364 and 423 which are designated for 6-TFA-AM.; that of 6-TFA-AM shows 0 % for m/z 314,

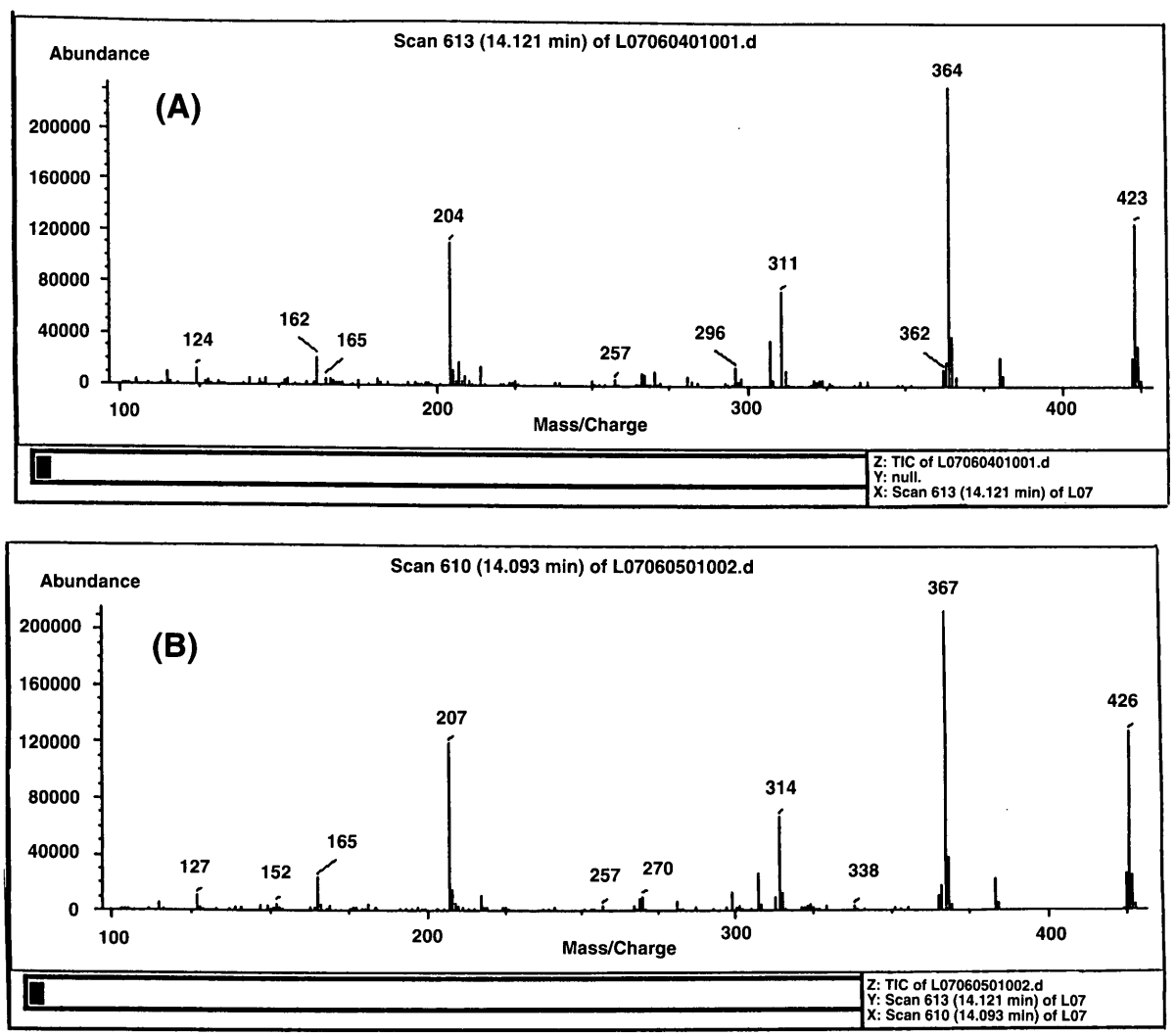


Figure 2. Mass spectra of trifluoroacetyl derivatives of 6-acetylmorphine (A) and 6-acetylmorphine-d_3 (B).

367 and 426 which are designated for 6-TFA-AM-d_3. 6-TFA-AM and 6-TFA-AM-d_3 appear to be mutually free of interference. Thus 6-AM-d_3 may be suitable for SIM data acquisition of 6-AM if TFAA was adopted as the derivatizing agent. Fragment ions of m/z 364, 423 and 311 are selected for SIM data acquisition of 6-TFA-AM ; m/z 367, 426 and 314 are selected for SIM data acquisition of 6-TFA-AM-d_3.

SIM mass spectral data produced from the selected ions of 6-TFA-AM and 6-TFA-AM-d_3 are shown in Table 2. The table indicates the interference of 6-TFA-AM-d_3 to 6-TFA-AM at m/z 311, 364 and 423 are 0.5 %, 0.2 %,and 0.0 %, respectively and that of 6-TFA-AM to 6-TFA-AM-d_3 at m/z 314, 367 and 426 are 0.5 %, 0.4 %, and 0.4 %, respectively. They appear to be low or free of interference between 6-TFA-AM and 6-TFA-AM-d_3. Thus 6-TFA-AM-d_3 is suitable for SIM data

Table 1. Full-scan mass spectral data of 6-TFA-acetylmorphine and 6-TFA-acetylmorphine-d_3

Ion (m/z)	Relative intensity, %	
	6-TFA-acetylmorphine	6-TFA-acetylmorphine-d_3
423	54.6	0.0
364	100.0	0.0
311	31.6	0.0
426	0.0	59.8
367	0.0	100.0
314	0.0	31.6

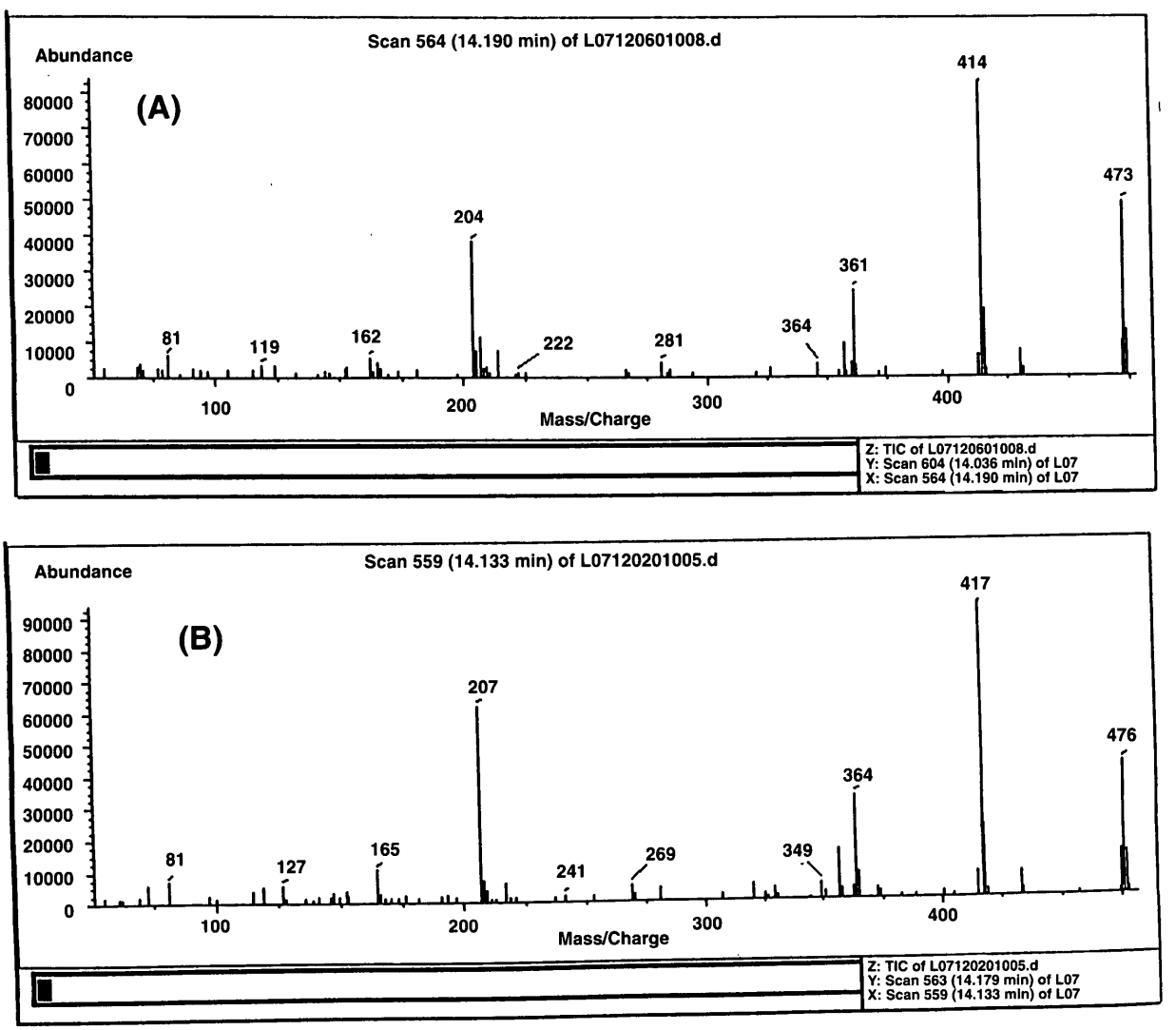


Figure 3. Mass spectra of pentafluoroproionyl derivatives of 6-acetylmorphine (A) and 6-acetylmorphine-d_3 (B).

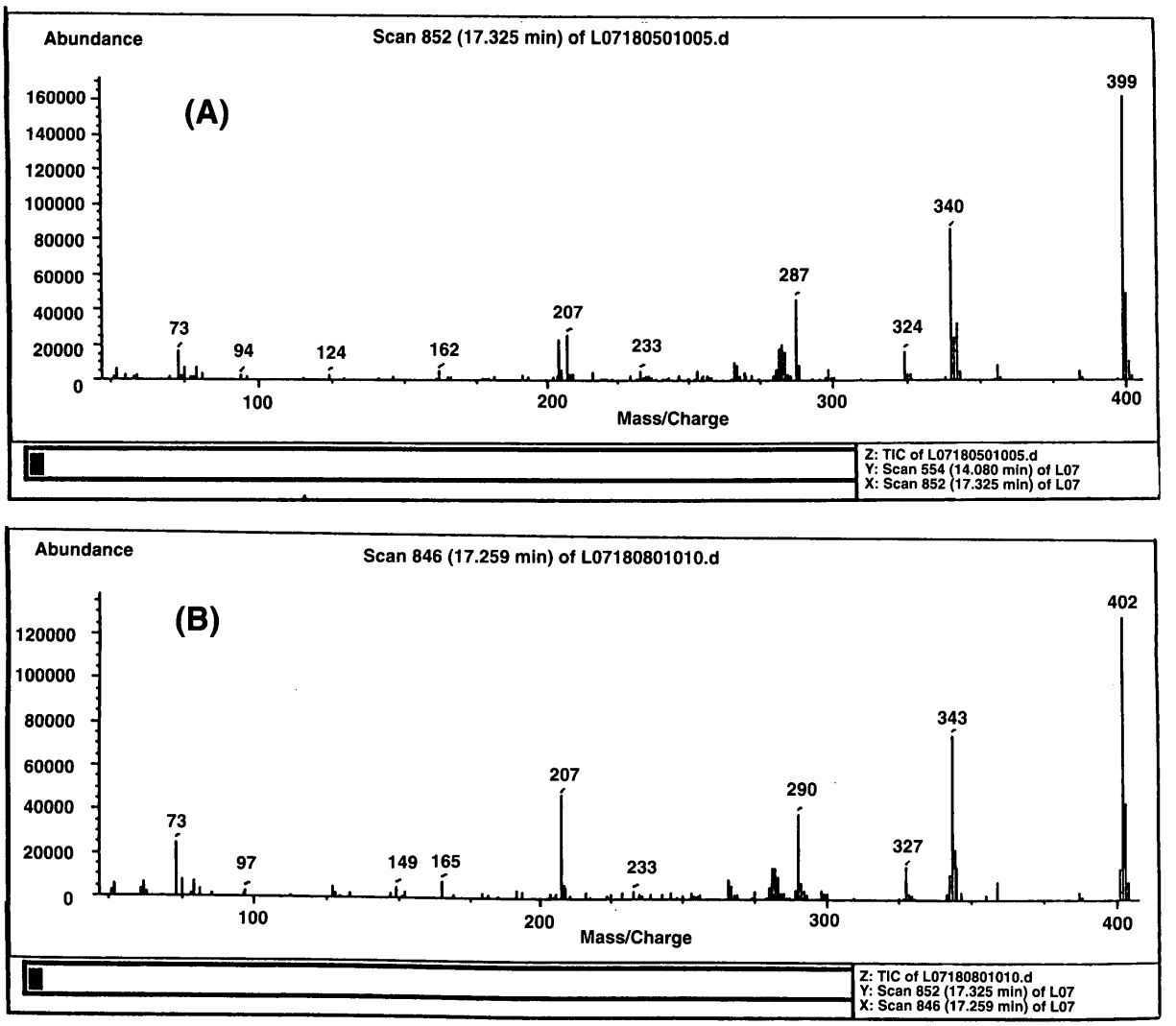


Figure 4. Mass spectra of trimethylsilyl derivatives of 6-acetylmorphine (A) and 6-acetylmorphine-d_3 (B).

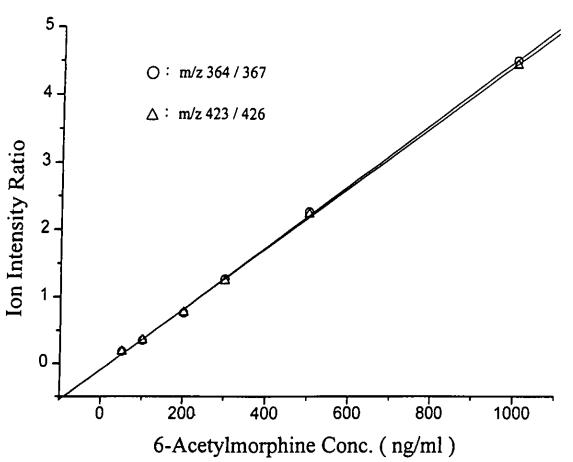


Figure 5. First order fit of 6-TFA-acetylmorphine calibration curve.

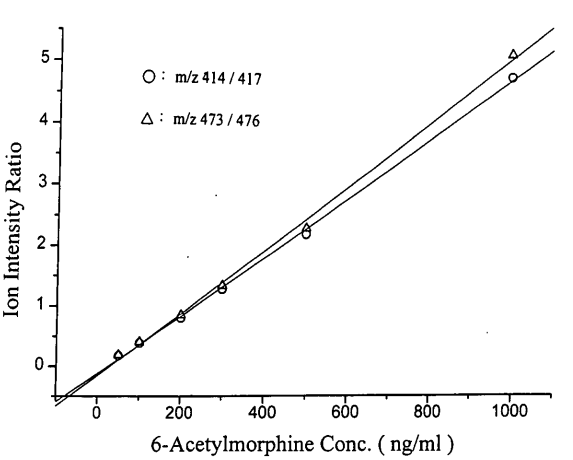


Figure 6. First order fit of 6-PFP-acetylmorphine calibration curve.

Table 2. SIM mass spectral data of 6-TFA-acetylmorphine and 6-TFA-acetylmorphine-d_3

Ion (m/z)	Intensity and % contribution by analog	
	6-TFA-acetylmorphine	6-TFA-acetylmorphine-d_3
423	5736340(0.0)	0
364	9820021(0.2)	24229
311	3170811(0.5)	15039
426	15896	4156265(0.4)
367	29530	7423444(0.4)
314	12010	2501846(0.5)

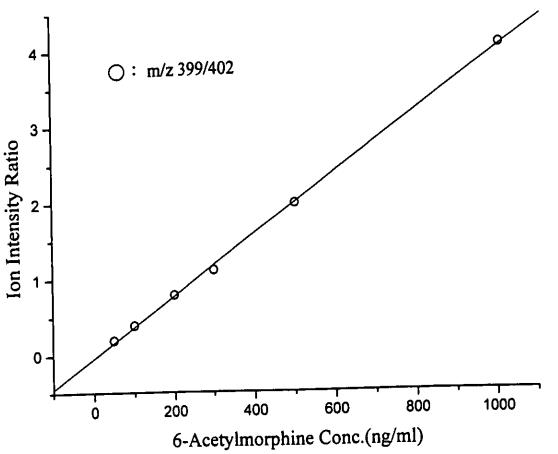


Figure 7. First order fit of 6-TMS-acetylmorphine calibration curve.

Table 3. Selecting appropriate isotopic internal standard according to selected ions and their cross-contribution (shows in parentheses) for GC/MS analysis of trifluoroacetyl (TFA), pentafluoropropionyl (PFP)and trimethylsilyl (TMS) derivatives of 6-acetylmorphine

Derivative		Analyte	Internal standard	Quantitative ion pair
TFA	Ion selected	311 364 423	367 426	
	(cross-contribution)	(0.5%)(0.2%)(0.0%)	(0.4%)(0.4%)	
	Ion ratio selected	311/364, 423/364	426/367	364/367, 423/426
PFP	Ion selected	361 414 473	417 476	
	(cross-contribution)	(0.5%)(0.5%)(0.0%)	(0.3%)(0.3%)	
	Ion ratio selected	361/414, 473/414	476/417	414/417, 473/476
TMS	Ion selected	287[a] 340 399	290 402	
	(cross-contribution)	(3.5%)(0.8%)(0.2%)	(1.1%)(0.0%)	
	Ion ratio selected	287/399, 340/399	290/402	399/402

[a]High cross-contribution

Table 4. Calibration curve analysis for GC/MS analysis of trifluoroacetyl (TFA) , pentafluoropropionyl (PFP) and trimethylsilyl (TMS) derivatives of 6-acetylmorphine

Derivative	Ion pair selected	Linear equation	Standard deviation	Coefficient of correlation
TFA	364/367	$y=4.6x10^{-3}x-0.0942$	0.0486	0.9996
	423/426	$y=4.53x10^{-3}x-0.0885$	0.0435	0.9997
PFP	414/417	$y=4.73x10^{-3}x-0.1210$	0.0645	0.9994
	473/476	$y=5.11x10^{-3}x-0.1557$	0.0993	0.9988
TMS	399/402	$y=4.08x10^{-3}x-0.0389$	0.0438	0.9996

Table 5. Precision for GC/MS analysis of trifluoroacetyl (TFA) , pentafluoropropionyl (PFP) and trimethylsilyl (TMS) derivatives of 6-acetylmorphine at concentration of 200 ng/ml

Derivative	Ion ratio selected	within-day	Between-day
TFA	364/367	0.0148	0.0305
	423/426	0.0261	0.0359
PFP	414/417	0.0346	0.0733
	473/476	0.0294	0.0608
TMS	399/402	0.0220	0.0279

acquisition and 6-AM-d_3 is suitable as the internal standard of GC/MS analysis of 6-AM if TFAA was adopted as the derivatizing agent. Suitable ions for SIM data acquisition for 6-TFA-AM are m/z 311, 364 and 423, and m/z 314, 367 and 426 for 6-TFA-AM-d_3, respectively. Suitable ion pairs for quantitative SIM GC/MS analysis of 6-TFA-AM /6-TFA-AM-d_3 are 364/367 and 423/426. By means of the same evaluation procedures, the results indicate that 6-PFP-AM-d_3 is also suitable for SIM data acquisition and 6-AM-d_3 is suitable as the internal standard of GC/MS analysis of 6-AM if PFPA was adopted as the derivatizing agent. When HMDS was adopted as the derivatizing agent, 6-TMS-AM contributed 10.4 % interference to 6-TMS-AM-d_3 at m/z 343, and 6-TMS-AM-d_3 contributed 3.5 % interference to 6-TMS-AM at m/z 287. Therefore, it is necessary to carefully select fragment ions for quantitative determination since some yield cross-contribution.

The SIM mass spectral data of TFA, PFP and TMS derivatives of 6-AM and 6-AM-d_3 are summarized in Table 3. Suitable ions for 6-PFP-AM are m/z 361, 414 and 473, and those for 6-PFP-AM-d_3 are m/z 417 and 476. Suitable ion pairs (6-PFP-AM/6-PFP-AM-d_3) for quantitative determination are 414/417 and 473/476. Suitable ions for 6-TMS-AM are m/z 340 and 399, and those for 6-TMS-AM-d_3 are m/z 290 and 402. Suitable ion pair (6-TMS-AM/6-TMS-AM-d_3) for quantitative determination is only 399/402.

Finally calibration curves of 6-TFA-AM, 6-PFP-AM, and 6-TMS-AM according to these selected ion pair (s) are presented in Figure 5 to 7, respectively. Results of linear regression analysis for these calibration curves over the concentration range of 50 - 1000 ng/ml and the precision analysis at the concentration of 200 ng/ml of 6-AM are listed in Table 4 and Table 5. The results from 6-TFA-AM/6-TFA-AM-d_3 at 364/367 and 423/426 both have a good linearity (for m/z 364/367: $y = 4.6 \times 10^{-3} x - 0.0942$, $n = 6$, $SD = 0.0486$, coefficient of correlation = 0.9996; for m/z 423/426: $y = 4.53 \times 10^{-3} x - 0.0885$, $n = 6$, $SD = 0.0435$, coefficient of correlation = 0.9997) in the specified concentration range. The coefficients of variation in within-day assay at the concentration of 200 ng/ml of 6-AM at 364/367 and 423/426 are 0.0148 and 0.0261 (n = 3), and in between-day 0.0305 and 0.0359 (n = 3), respectively. The results from 6-PFP-AM/6-PFP-AM-d_3 at 414/417 and 473/476 and 6-TMS-AM/6-TMS-AM-d_3 at 399/402 also have a good linearity over the specified concentration range and a good precision in the specified concentration. Therefore, 6-AM-d_3 is suitable as the internal standard for GC/MS analysis when any of TFAA, PFPA or HMDS is chosen as derivatizing agent. However it is necessary to carefully select fragment ions for quantitative determination because since some may cause cross-contribution and thus produce inaccurate results.

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以氣相層析質譜分析方法及氘同位素藥物爲內部標準品應用於定量尿液中6-乙醯嗎啡之探討

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摘 要

本研究係探討以6-乙醯嗎啡（6AM）之氘同位素藥物6-AM-d_3爲內部標準品及不同衍生化試劑於氣相層析質譜儀分析尿液中6AM含量之適用性。本研究所採用之衍生化試劑爲trifluoroacetic anhydride （TFAA）, pentafluoropropionic anhydride （PFPA）及hexamethyldisilazane （HMDS），分別進行衍生化後，探討全掃描質譜圖（Full Scan）及選定離子監測（SIM）圖譜之離子碎片強度是否相互干擾，並以迴歸分析其實際應用在定量上之可行性。研究結果顯示，由全掃描質譜圖來分析，6-TMS-AM之質譜圖對於6-TMS-AM-d_3於m/z 287處有些許干擾；但以TFAA及PFPA爲衍生化劑時，並無明顯干擾。由選定離子監測質譜圖分析，6-AM-d_3與6-AM經TFAA及PFPA衍生化後之選定離子碎片相互干擾甚低；另，6-TMS-AM之質譜圖對於6-TMS-AM-d_3於m/z 343處有明顯干擾。本研究再分別以TFAA，HMDS及PFPA爲衍生化試劑，選擇適用之離子碎片爲定量用離子，進行迴歸分析，於50, 100, 200, 300, 500, 1000 ng/ml濃度範圍內，具有良好的線性關係。因此以TFAA，HMDS及PFPA爲衍生化試劑時，6-AM-d_3應可供作氣相層析質譜法分析尿液中6-乙醯嗎啡定量時的內部標準品。但以HMDS衍生化定量時，由於有明顯干擾，應慎選適用之離子。

關鍵詞：6-乙醯嗎啡，氣相層析質譜儀，衍生化試劑。