

Collaborative Study on the Determination of 7-Aminoflunitrazepam in Urine by GC-MS

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ABSTRACT

A previously published gas chromatography-mass spectrometry (GC-MS) method for the analysis of 7-aminoflunitrazepam (7-amino-FM₂) in urine was subjected to an inter-laboratory collaborative study. The method was rigorously defined in terms of performance requirements, yet allowed some degrees of flexibility to individual analysts. The collaborative study followed the guidelines provided by the Association of Official Analytical Chemists International (AOAC International). Each of nine drug-abuse urine testing laboratories in Taiwan participated in the study to analyze 3 samples in duplicate. The repeatability relative standard deviation (RSD_r) and the reproducibility relative standard deviation (RSD_R) of the measurements of 7-amino-FM₂ in urine were determined to be less than 9.91% and 14.84%, respectively. The method showed acceptable reproducibility, as evidenced by HORRAT values of 0.6 to 0.7. The method was thus proposed to be used by the testing laboratories in Taiwan.

Key words: 7-aminoflunitrazepam, GC-MS, urine, inter-laboratory study

INTRODUCTION

Flunitrazepam (FM₂) is a derivative of 7-nitro-1,4-benzodiazepine. 7-aminoflunitrazepam (7-amino-FM₂) is the main metabolite of FM₂ in urine. FM₂ is extensively used for treating insomnia and frequently abused. It is classified as a Schedule 3 drug under the Controlled Drugs Act in Taiwan. In Taiwan, over 1200 abuse cases of 7-amino-FM₂ had been reported since 2002 and more than 90 cases were reported positive in urinary test⁽¹⁾.

Several immunoassay⁽²⁻⁴⁾ and gas chromatography-mass spectrometry (GC-MS)⁽⁴⁻⁹⁾ protocols have been used to study the excretion profile of 7-amino-FM₂ ingestion under controlled conditions. This paper reports the result of a collaborative study designed to validate a GC-MS method for the measurement of the 7-amino-FM₂ in urine. The analytical protocol was described in the literature⁽⁹⁾, with the exception that individual analysts were given some degrees of flexibility while maintaining certain performance criteria. Specifically, minimum performance criteria and system suitability test (the precision of the analysis must not exceed a predefined value)⁽¹⁰⁾ should be made, but analysts were encouraged to use their routine analytical system (e.g., instrument, injector, and column) and to use individual judgment in adjusting the operating conditions.

MATERIALS AND METHODS

I. Reagents

7-amino-FM₂ (100 µg/mL in acetonitrile) and 7-amino-FM₂-d₇ (100 µg/mL in acetonitrile) were from Cerilliant (Austin, TX, USA). N-Methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA) was from Sigma (St. Louis, MO, USA). Ethyl acetate was from Merck (Darmstadt, Germany). NaHCO₃ (7.57 g) and Na₂CO₃ (6.35 g) were dissolved in 70 mL and 20 mL of double deionized water, respectively. These two solutions were mixed and then 66% KOH was added to make 100 mL of carbonate buffer solution (1.5 M, pH = 9.0). Individual stock solutions containing 5 µg/mL of 7-amino-FM₂ and 5 µg/mL of 7-amino-FM₂-d₇ in methanol were prepared. Working solution of 2 µg/mL of 7-amino-FM₂ was subsequently prepared. Blank urine specimen was collected from laboratory personnel and the urine was tested negative for the 7-amino-FM₂. A five-point standard curve at the concentrations of 80, 100, 120, 150, and 200 ng/mL was made by linear least-square regression analysis of the ratio of the peak area of 7-amino-FM₂ to the peak area of the internal standard, 7-amino-FM₂-d₇.

II. Extraction and Derivatization

The analytical protocol was performed on a GC/MS method with liquid-liquid extraction as described by

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Wang *et al.*⁽⁹⁾. One hundred microliter of internal standard solution (100 µg/mL of 7-amino-FM₂-d₇), 1 mL of carbonate buffer solution and 5 mL of ethyl acetate were added to 2 mL of the urine samples. The mixtures were shaken for 5 min and centrifuged at 1500 rpm for 5 min. Finally, the upper layers of the mixtures were transferred to clean test tubes. The extraction solvent was evaporated to dryness under a gentle stream of nitrogen at room temperature. 7-Amino-FM₂ in the dried extract was then converted to its TMS-derivative using MSTFA as the derivatization reagent.

III. GC-MS Procedures⁽⁹⁾

An Agilent 6890 GC/5973N mass selective detector system was used to acquire full-scan and selected ion monitoring (SIM) mass spectrometric data. An HP-5MS capillary column (30 m × 0.25-mm i.d., 0.25-µm film thickness) was used with the following conditions: splitless injection; Helium flow rate, 0.8~1.2 mL/min; injection port, 260 °C; interface, 280 °C; column oven programming: starting at 150 °C and holding for 1 min, increasing to 300 °C at 20 °C/min, then holding for 3.5 min. The following parameters were used for injecting samples into the GC-MSD system: sample size, 2 µL; injection mode, splitless; injector purge-off duration, 1 min. Ions selected for 7-amino-FM₂ (as TMS-derivative) were *m/z* 355, 326, and 327; the corresponding ions for 7-amino-FM₂-d₇ (as TMS-derivative) were *m/z* 362, 333, and 334 (quantification ions are underlined). Limits of quantitation and limits of detection are 5.0 and 2.5 ng/mL, respectively.

IV. Collaborative Study

The procedures for the preparation of solutions and analyze were written as a form of standard operation procedure (SOP). Nine drug-abuse urine testing laboratories in Taiwan participated in the collaborative study. All of the testing laboratories passed their own quality control and were accredited by the National Bureau of

Controlled Drugs, Department of Health, Executive Yuan, Taiwan. Each collaborator received a reference standard of 7-amino-FM₂, an internal standard of 7-amino-FM₂-d₇, a derivatizing reagent of MSTFA, and test samples (80, 100, 150 ng/mL in duplicates) of 7-amino-FM₂-spiked urine. Concentrations of the test samples were unknown to the collaborators. The collaborators also received a set of instructions regarding the SOP and a report form for recording results. They were asked to follow the SOP to analyze the samples and to describe specific operational parameters of the instrument system used and to submit the report forms along with the chromatograms. Each laboratory was encouraged to use one's routine analytical system (e.g., instrument, injector, and column) and to use individual judgment in adjusting the operating conditions.

V. Statistical Analysis⁽¹¹⁾

The statistical terms used are those given by the Association of Official Analytical Chemists International (AOAC International), including (a) repeatability (intra-laboratory) standard deviation (*S_r*), (b) repeatability relative standard deviation (*RSD_r*), (c) reproducibility (inter-laboratory) standard deviation (*S_R*), (d) reproducibility relative standard deviation (*RSD_R*), and (e) HORRAT values. The acceptability of reproducibility of the method was assessed on the basis of HORRAT values. Moreover, the Cochran and Grubbs tests were used for outliers.

Cochran test is used to remove the extreme individual values from a set of laboratory values (1-tail test at a probability value of 2.5%). Grubbs tests are used to remove the laboratories with extreme averages. Grubbs tests include single value test (2-tail; *P* = 2.5%) and pair value test (2 values at the highest end, 2 values at the lowest end, and 2 values, one at each end, at an overall *P* = 2.5%). HORRAT value is the ratio of observed *RSD_R* to predicted *RSD_R* (predicted *RSD_R* = $2C^{-0.1505}$, *C* is the estimated mean concentration). HORRAT value between 0.5 and 1.5 may be taken to indicate that the performance

Table 1. GC-MS systems used by the collaborators

Lab.	Instrument	Column			
		Model	Length (m)	Diameter (mm)	Film (µm)
1	Agilent 6890/5973	Agilent, HP-5Ms	30	0.25	0.25
2	Agilent 6890/5973	Supileo, Equity-5	12	0.20	0.33
3	Agilent 6890/5973	Quadrex, UAC-1	15	0.25	0.50
4	Agilent 6890N/5973N	Agilent, J&W DB-5	15	0.25	0.25
5	Agilent 6890/5973	Agilent, HP-5Ms	30	0.25	0.25
6	Agilent 6890/5973N	Agilent, J&W DB-5	30	0.25	0.25
7	Finnigan GC 8000 top/Voyager	Restek, Rtx-5Ms	15	0.25	0.25
8	Finnigan GC/Polaris Q	Chrompack, CP-Sil 8CB-MS	30	0.25	0.25
9	Agilent 6890N/5973	Agilent, HP-5Ms	30	0.25	0.25

value for the method corresponds to good performance. The limits for the performance acceptability are 0.5 to 2.

RESULTS AND DISCUSSION

The diversity of instrument systems used by the collaborators is shown in Table 1. Other analytical conditions of the GC-MS system (eg. injection mode, column pressure, gas flow rate, ionization mode, interface temperature and electron energy etc.) were similar within the collaborators. Laboratory 1 and 2 experienced sample emulsion in the extraction step after adding ethyl acetate. For improvement, sample shaking was limited to 5 min or less. Most collaborators commented favorably on the method. The time required for the collaborators to complete the analysis of the samples in the study varied from one to three weeks.

The Cochran and Grubbs test for outliers, when applied to laboratory means for each sample, showed no outlier overall. The raw data and analysis of variance with each sample are shown in Tables 2 and 3. The

RSD_r values were 5.15~9.91% and the RSD_R values were 13.36~14.84%. HORRAT values ranged from 0.6 to 0.7. These data further indicate that the proposed GC-MS method for the determination of 7-amino-FM₂ in urine showed good inter-laboratory reproducibility.

CONCLUSIONS

The collaborative study of the GC-MS method for the determination of 7-amino-FM₂ in urine has demonstrated good inter-laboratory reproducibility. The method was proposed to be used by the drug-abuse urine testing laboratories in Taiwan.

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Table 2. Laboratory analysis results for the determination of 7-amino-FM₂ in urine by GC-MS

Lab.	7-amino-FM ₂ (ng/mL) ^a					
	A ₁	A ₂	B ₁	B ₂	C ₁	C ₂
1	69	68	82	84	125	121
2	72	66	89	89	117	116
3	88	94	104	105	183	157
4	81	83	96	104	138	146
5	90	71	104	105	141	136
6	92	79	99	99	146	158
7	83	83	104	104	156	160
8	101	101	109	123	177	170
9	78	103	114	138	171	180
Mean	83.8	83.1	100.1	105.9	150.4	149.3
SD	10.1	13.8	9.9	16.6	23.0	21.6

^aA₁ and A₂ are blind duplicate pairs of spiked urine samples (spike concentration: 80 ng/mL), B₁ and B₂ are blind duplicate pairs of spiked urine samples (spike concentration: 100 ng/mL), C₁ and C₂ are blind duplicate pairs of spiked urine samples (spike concentration: 150 ng/mL).

Table 3. Inter-laboratory study results for the determination of 7-amino-FM₂ in urine by GC-MS

Added (ng/mL)	Found (ng/mL)	No. of lab ^a	S _r	RSD _r (%)	S _R	RSD _R (%)	HORRAT
80	83.4 ± 11.7	9 (0)	8.27	9.91	11.92	14.28	0.6
100	102.9 ± 13.4	9 (0)	6.87	6.68	13.75	13.36	0.6
150	149.9 ± 21.7	9 (0)	7.72	5.15	22.24	14.84	0.7

^aEach values is the number of laboratories retained after elimination of outlier; each value in parenthesis is the number of laboratories removed as outliers. S_r: repeatability standard deviation; RSD_r: repeatability relative standard deviation; S_R: reproducibility standard deviation; RSD_R: reproducibility relative standard deviation.

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