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Short communication

Solvent-enhanced microwave-assisted derivatization following solid-phase extraction combined with gas chromatography–mass spectrometry for determination of amphetamines in urine

Li-Wen Chung[a], Geng-Jhih Liu[a], Zu-Guang Li[a,b], Yan-Zin Chang[c], Maw-Rong Lee[a,*]

[a] Department of Chemistry, National Chung Hsing University, 250 Kuo Kuang Road, Taichung 402, Taiwan, ROC
[b] College of Chemical and Materials Engineering, Zhejiang University of Technology, Hangzhou City, Zhejiang Province, PR China
[c] Institute of Medical and Molecular Toxicology, Chung Shan Medical University, Taichung, Taiwan, ROC

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ABSTRACT

An approach using microwave-assisted derivatization (MAD) following solid-phase extraction (SPE) combined with gas chromatography–mass spectrometry (GC–MS) was developed to determine amphetamines in urine samples. The parameters affecting the derivatization efficiency – including microwave power and irradiation time – were investigated. Besides, solvent is thought critically important to MAD. Derivatization performance was studied using various solvents and compared with the performance obtained without solvent. Derivatization efficiency was clearly found to be enhanced by the presence of solvent. The highest derivatization efficiencies were obtained in ethyl acetate (EA) under microwave power of 250 W for 1 min. Calibration curves for all amphetamines were linear over a range from 1 to 1000 ng/mL, with correlation coefficients above 0.9992. The intra-day and inter-day precision were less than 15%. The applicability of the method was tested by analyzing amphetamine-abusing subjects urine samples. Accordingly, the solvent-enhanced MAD-GC–MS method appears to be adequate for determining amphetamines in urine.

1. Introduction

The most readily available amphetamines are amphetamine (AM) and methamphetamine (MA) [1]. They belong to the group of drugs called central nervous system stimulants which increase alertness, competitiveness, and aggression [2]. They are also associated with psychosis, paranoia, violence and increased stroke risk [3]. Amphetamines use is dramatically increasing worldwide and constitutes a serious social problem. The United Nations Office on drugs and crime estimates 25 million people used amphetamines stimulants globally in 2004 [4]. To successfully analyze large numbers of samples, clinical and forensic laboratories require rapid analysis methods.

Gas chromatography–mass spectrometry (GC–MS) has been the primary method for determining the presence of amphetamines [5–7]. Derivatization prior to GC–MS is used to improve chromatographic properties and assist formation of characteristic ions for identification via mass spectra [8,9]; these derivatization procedures typically are time-consuming and laborious. Microwave is a form of electromagnetic energy that substantially penetrates into reaction vessels, directly imparting energy to molecules, thereby reducing energy transfer time [10]. Therefore, microwave-assisted derivatization (MAD) cannot only save time, but also make the procedure more efficient.

Microwave-assisted derivatization has been recognized as a powerful technique and its application has increased considerably in recent years [11–14]. Among these studies, Thompson et al. [12] and Peter et al. [13] used MAD for determination of amphetamines, but the derivatization process is still critical in the consideration of solvent. A solvent-free system, which was used by Thompson et al. [12], is particularly attractive due to ease of handling and environmental friendliness; in addition, no solvent stripping would be required. However, solvent plays a very important role in derivatization. Li et al. used solvent to optimize the derivatization and the results suggest that solvent could accelerate the reaction and enhance analyte response [15]. Li et al. described suitable solvents to reduce derivatization time, which is completed quantitatively within 15 s in acetone, while in other solvents reaction requires more than 1 h [16]. Because derivatization is solvent-dependent, the solvent effect's impact on MAD should be considered. To date, few studies have addressed solvent effects on MAD performance. Thompson et al. [12] used 1-chlorobutane as MAD solvent; even so, the solvent was considered only for extraction not derivatization of amphetamines in MAD. Deng et al. [14] have made reference to

* Corresponding author. Tel.: +886 422851716; fax: +886 422862547.
E-mail address: mrlee@dragon.nchu.edu.tw (M.-R. Lee).

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this important matter. The authors selected acetonitrile (ACN) as the solvent for MAD based on its higher dipolar moment compared to other two solvents; however, additional experiments would be required to verify this as the optimal choice. Hence, further study is needed to determine the solvent effect on maximum MAD efficiency.

In this study, solvent was assessed to optimize MAD conditions for determining amphetamines in urine. The impact on the derivatization efficiency – of microwave power and irradiation time – was also systematically investigated. The parameters were varied to assess their effect on linear range, limit of detection (LOD), limit of quantification (LOQ) and precision. Finally, the solvent-enhanced MAD technique was applied to quantify AM and MA in urine samples.

2. Experimental

2.1. Chemicals and reagents

Amphetamine and methamphetamine were obtained from Cerilliant (Round Rock, TX, USA). Methamphetamine-d5 (MA-d5) as an internal standard was purchased from Cerilliant. Heptafluorobutyric anhydride (HFBA) was from Supelco (Bellefonte, PA, USA). Analytical grade acetonitrile, ethyl acetate (EA), hexane, methanol and dichloromethane were obtained from Merck (Darmstadt, Germany) and used without further purification. Potassium dihydrogenphosphate was bought from Riedel-de Haën (Seelze, Germany). Acetic acid and phosphoric acid were obtained from TEDIA (Fairfield, OH, USA). Ultra-pure water (18.2 MΩ cm) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Artificial urine was purchased from Hycor Biomedical (Garden Grove, CA, USA). The stock solution was prepared by mixing AM and MA at a concentration of 100 μg/mL in methanol and stored at 4 °C. This stock solution was further diluted to yield an appropriate working solution of artificial urine. A pH 6 buffer solution was prepared with 0.1 M potassium dihydrogenphosphate, adjusted to pH 6 with 1.0 M phosphoric acid. Urine samples obtained from amphetamine-abusing suspects were supplied by the Institute of Medical and Molecular Toxicology at Chung Shan Medical University, and kept frozen at −30 °C. A 5 mL urine sample containing 50 μL of a 10 μg/mL MA-d5 solution was diluted with 3 mL of pH 6 buffer solution for solid-phase extraction (SPE).

2.2. SPE procedure

An SPE cartridge with 200 mg of a drug of abuse phase, consisted of octyl and strong cation exchange mode, was purchased from Chrom Expert (Sacramento, CA, USA); a 12-fold Visiprep SPE manifold was sourced from Supelco. The extraction procedure was recommended by the manufactory [17]. Prior to extraction, the cartridge was conditioned sequentially with 6 mL of methanol and 6 mL of pH 6 buffer solution. Urine samples were then loaded into the cartridge and the interferences washed off using 3 mL of ultra-pure water, 3 mL of 0.1 M acetic acid and 3 mL of methanol successively. The cartridge was then dried under full vacuum for 2 min, and the analytes eluted using a 3 mL mixed solvent solution of dichloromethane:2-propanol:hydrochloric acid (60:40:1, v/v/v). The eluate was collected in a 5 mL glass vial and evaporated to dryness at 55 °C under a gentle stream of nitrogen. The recovery was tested and obtained 93.28% for AM and 103.55% for MA.

2.3. Microwave-assisted derivatization

A Discover System microwave apparatus (CEM, Matthews, NC, USA) was used with a maximum microwave power of 300 W. Following the SPE, 100 μL of solvent, together with 100 μL of HFBA, was added to the vial for microwave irradiation. Following irradiation, the vial was cooled and the solution evaporated to dryness under a gentle stream of nitrogen. The dry residues were redissolved in 100 μL EA. 1 μL of the solution was injected into the GC–MS system for analysis.

2.4. GC–MS conditions

GC–MS analysis was performed using a CP-3800 GC with Varian 4000 MS (Walnut Creek, CA, USA). A 1079 injector was maintained at 250 °C in splitless mode. Separations were conducted using a capillary column DB-5 MS with 30 m length, 0.25 mm inner diameter, and 0.25 μm film thickness (Agilent, Palo, CA, USA). Helium (99.999%) was used as carrier gas at a constant flow of 1 mL/min. The GC oven was initially set at 80 °C for 1 min, programmed to 220 °C at a rate of 15 °C/min, and then maintained at 220 °C for 2 min [6]. The GC–MS interface, external source and ion trap temperatures were set at 250, 200 and 200 °C, respectively. Mass spectra were obtained using the electron ionization mode. A mass range of *m*/*z* 40–300 was scanned to verify the analytes. The following ions were monitored for each derivative; ions selected to quantify the derivatives are underlined: *m*/*z* 91, 118, 240 for AM-HFBA; *m*/*z* 118, 210, 254 for MA-HFBA; *m*/*z* 213, 258 for MA-d5-HFBA. Selected ion storage mode was performed to quantify the analytes with multiplier offset voltage at 280 V and filament emission current at 230 μA.

2.5. Method validation

Artificial urine was spiked with 1, 10, 100, 250, 500, 750 and 1000 ng/mL of AM and MA, then analyzed with the corresponding procedure to generate calibration curves. These calibration curves were plotted with peak area ratio (analyte to internal standard) as a function of the concentration. The LOD and LOQ were defined as the concentrations with signal-to-noise ratios of 3 and 10, respectively [18]. The precision of assay was evaluated with quality control (QC) samples generating intra-day and inter-day variability data. The QC samples were prepared at three different concentrations of low, medium and high, which were 1, 500 and 1000 ng/mL, respectively. For intra-day precision, five QC samples were analyzed in 1 day. For inter-day precision, five QC samples were analyzed on consecutive days.

3. Results and discussion

3.1. Optimization of microwave-assisted derivatization parameters

3.1.1. Selection of solvent

Three solvents, namely ACN, EA and hexane, were assessed in an effort to find the optimal MAD conditions. The selection of ACN was based on its polarity [14], EA was a common solvent of heating derivatization [6], and hexane was usually discussed in fast derivatization [15,16]. The derivatization efficiency in a solvent system was investigated and the results compared with those of a HFBA alone system. In Fig. 1, the peak area of derivatives was improved by the addition of solvent, indeed, by up to three orders of magnitude as compared to the HFBA alone system using EA on MA-HFBA, for the same reaction time. This result is in agreement with that described by Li et al., in which solvent was shown to enhance analyte response in derivatization [15]. The presence of solvent was also shown to increase the derivatization efficiency within a short time using MAD.

The derivatization efficiency was slightly elevated when using hexane, and much higher in ACN and EA, compared to the HFBA

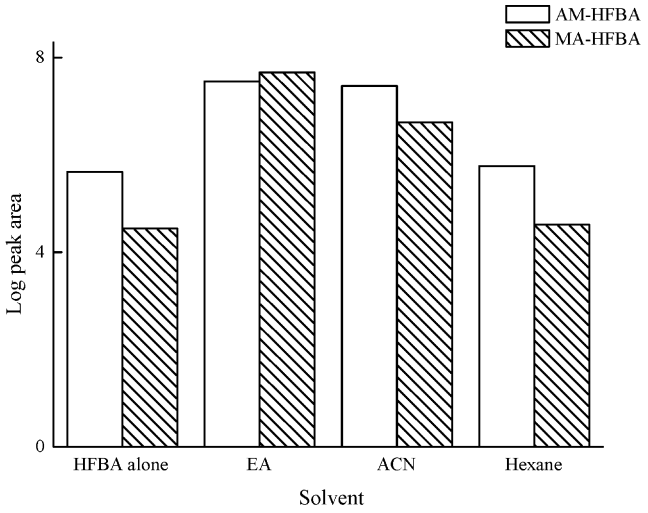

Fig. 1. Effects of solvent on peak area of AM-HFBA and MA-HFBA. Experimental conditions: 500 ng/mL of amphetamines; microwave power of 250 W; irradiation time of 30 s.

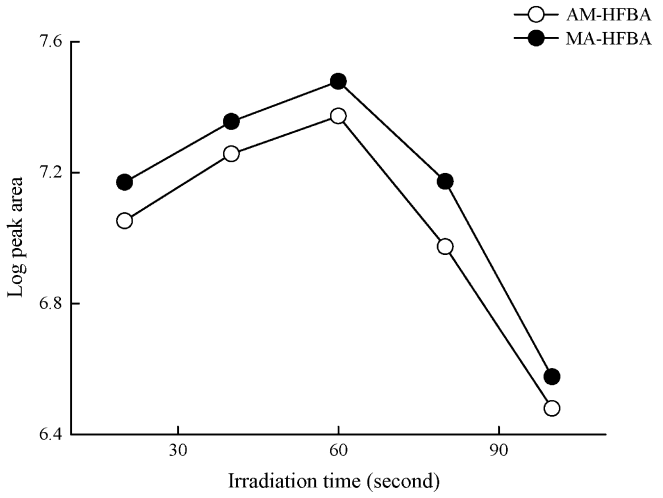

Fig. 3. Effects of irradiation time on peak area of AM-HFBA and MA-HFBA. Experimental conditions: 500 ng/mL of amphetamines; addition of EA; microwave power of 250 W.

alone system. In terms of enhancing derivatization efficiency, hexane is likely a weaker solvent than ACN and EA because of the low solubility of analytes and completely non-polar molecule of hexane [11]. Higher derivatization efficiency was obtained with EA than ACN, due to that the addition of EA fascinated the derivatization [10]. Further, it is noteworthy that with the use of EA as a solvent, the derivatization efficiency of MA-HFBA exceeded that of AM-HFBA, which is comparable to the previous literature [13]. Consequently, EA was chosen as the optimal solvent, of those studied, for MAD of AM and MA.

3.1.2. *Effects of microwave power and irradiation time*

Microwave power and irradiation time can affect derivatization efficiency [19], so they were studied in order to optimize reaction conditions. Fig. 2 shows the peak areas of derivatives with microwave power varying from 100 to 300 W. The derivatization efficiency increases as the microwave power increases, from 100 to 250 W, and then decreases at higher power, possibly due to degradation of the derivatives [20]. The highest derivatization efficiency was observed at 250 W and determined to be the optimal power level for further analysis.

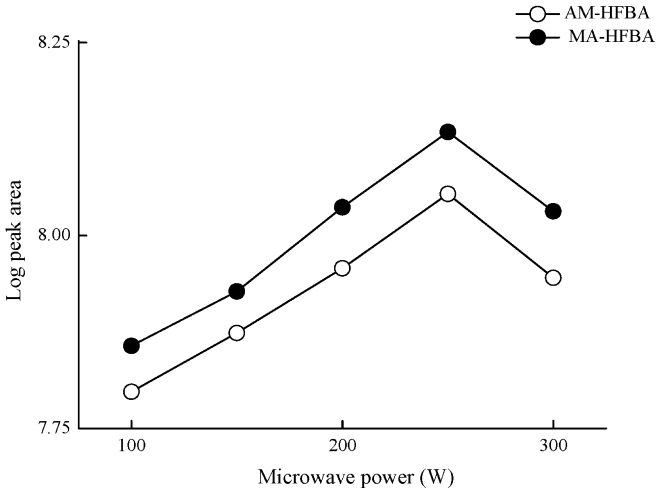

Fig. 2. Effects of microwave power on peak area of AM-HFBA and MA-HFBA. Experimental conditions: 500 ng/mL of amphetamines; addition of EA; irradiation time of 30 s.

To ensure optimal conditions and to minimize the assay time, the effect of varying the irradiation time, from 20 to 100 s, was assessed. As shown in Fig. 3, microwave irradiation raised the peak area of derivatives as time increased from 20 to 60 s. However, extending the irradiation time beyond 60 s led to lower derivatization efficiency based on the tolerances towards microwave treatment [20]. Therefore, the optimal irradiation time was determined to be 60 s for MAD. In EA, the microwave power of 250 W for 1 min was used; in the presence of 1-chlorobutane, microwave power was 340 W for 6 min [12] or in the absence of solvent was 440 V for 5 min was found to be optimal [13]. This indicates that when using EA, lower power and shorter timeframes are required for the effective application of MAD to identify the presence of amphetamines.

3.2. *Method validation*

The linearity, LOD, LOQ and precision were investigated under the optimum MAD conditions applying SPE, followed by GC–MS. The correlation coefficient of AM is 0.9992 and that of MA is 0.9994 in the linear range. Linear response was found over a concentration range of 1–1000 ng/mL, consistent with reports in the literature, which indicate linear response over the ranges of 250–2500 ng/mL [12] and 5–1000 ng/mL [13]. The sensitivity was evaluated by determining the LOD and the LOQ. The LODs are 0.05 ng/mL for AM and 0.23 ng/mL for MA. The LOQs are 0.17 ng/mL for AM and 0.77 ng/mL for MA. This method was indicated good sensitivity comparing with the previous study, in which LOD was 20 ng/mL for AM, 5 ng/mL for MA and LOQ was 50 ng/mL for AM, 10 ng/mL for MA [21]. Precision in terms of intra-day and inter-day variation is expressed as a relative standard deviation (RSD) of the QC samples. As shown in Table 1, the RSDs range between 2.6 and 13.7% for intra-day precision. The RSDs range from 6.4 to 14.6% for inter-day precision. The RSDs are within 15% for all measurements, indicating the adequate precision of this method [22].

3.3. *Application*

The applicability of the new methodology was investigated for determination of AM and MA in urine samples obtained from amphetamine-abusing suspects. The chromatogram of the artificial

Table 1
Precision for three QC samples in urine ($n = 5$)

Compound	QC concentration (ng/mL)	Intra-day precision (RSD, %)	Inter-day precision (RSD, %)
AM	1	13.0	13.8
	500	11.2	12.4
	1000	7.5	7.1
MA	1	13.7	14.6
	500	5.8	10.1
	1000	2.6	6.4

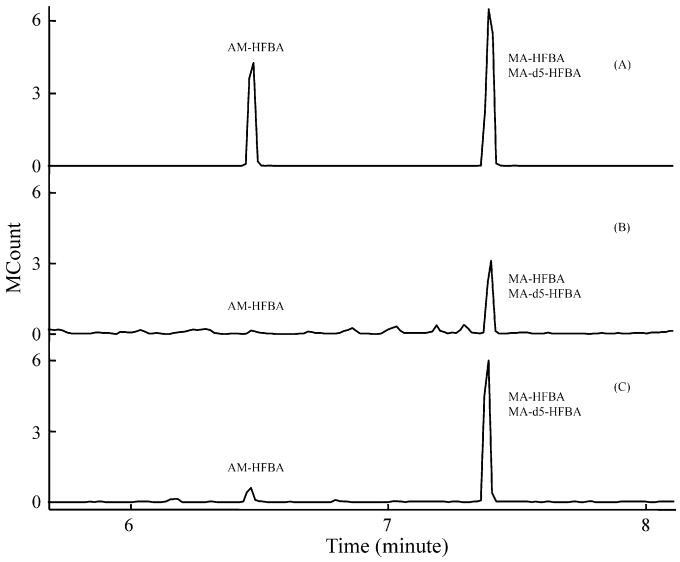

Fig. 4. Mass ion chromatograms of (A) the spiked 1000 ng/mL amphetamines in artificial urine, (B) U_1 sample and (C) U_2 sample.

urine sample spiked with 1000 ng/mL of amphetamines is depicted in Fig. 4A, and those of the actual urine samples are illustrated in Fig. 4B and C. Fig. 4A shows no interference peak, and in Fig. 4B and C, the peaks of other components are well separated from those of the analytes. In the chromatogram, amphetamines were found in the urine samples. The U_1 sample was found to contain AM at 14 ± 3 ng/mL and MA at 321 ± 47 ng/mL. The U_2 sample contained AM at 117 ± 25 ng/mL and MA at 638 ± 48 ng/mL. The accuracy of these results were verified by the Institute of Medical and Molecular Toxicology at Chung Shan Medical University, their results show the U_1 sample with a 13 ng/mL concentration of AM and a 315 ng/mL concentration of MA; the U_2 sample had 104 ng/mL AM and 688 ng/mL MA.

4. Conclusion

A rapid derivatization coupled with SPE and GC–MS analysis was developed for determination of amphetamines in urine. Solvent-induced MAD was successfully demonstrated in the presence of EA and ACN, showing results roughly 1000 times greater than that with a solvent-free system using HFBA. The optimal conditions employ EA as the solvent. Amphetamines can be derivatized with HFBA at microwave power of 250 W for 1 min, whereas greater microwave power and longer times are required without solvent. Given its impact on reducing the required time and power, EA is recommended to facilitate MAD for determining the presence of amphetamines in urine.

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