Determination of Amantadine Residue in Honey by Solid-phase Extraction and High-performance Liquid Chromatography with Pre-column Derivatization and Fluorometric Detection

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Amantadine (AMA) is an anti-viral drug used in apiculture to protect honeybee against the sacbrood virus (*Morator aetatulae*). This study described a reliable high-performance liquid chromatographic (HPLC) method for analyzing AMA in honey using a solid-phase extraction (SPE) cartridge (Plexa PCX) for purification, 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) as a pre-column derivatization agent, and fluorometric detection (λ_{ex}=470 nm, λ_{em}=530 nm). The chromatographic separation was performed on an XDB C18 column (150×4.6 mm i.d.) using 0.1% trifluoroacetic acid/acetonitrile (35∶65, *V*/*V*) as the mobile phase at a flow rate of 1.0 mL•min^{-1} with a run time of 20 min. Under these optimal conditions, a linear relationship was observed in the range of 0.025—1.0 μg•mL^{-1} with a good correlation coefficient (0.998) and low limit of detection (0.0080 μg•g^{-1}), the recoveries were all above 90%, and the intra-day and inter-day precision (RSD) ranged from 3.4%—5.1%.

Keywords amantadine, honey, solid-phase extraction, high-performance liquid chromatographic (HPLC), fluorometric detection

Introduction

Amantadine (AMA) is a derivative of adamantane and has been clinically used for the treatment of influenza, hepatitis C, parkinsonism, and multiple sclerosis.[1-3] AMA is also used to treat avian influenza in the poultry industry because of its antiviral activity. but AMA-resistant influenza variants have been widely distributed.[4-6] Most avian influenza viruses are zoonotic, and the emergence of AMA resistance may lead to the lost of efficacy of AMA on both avian and human viruses. To preserve the effectiveness of anti-viral drugs, such as AMA, for treating and preventing influenza infections in humans, some countries have taken measures to prohibit the use of these drugs in poultry.[7]

Amantadine, either alone or in conjunction with other natural substances, protects honeybee (*Apis mellifera*) from infection by sacbrood virus (*Morator aetatulae*), such as the apiculture medicine "Antiviral 628".[8] The sacbrood virus infects the honeybee larvae, resulting in a failure to pupate and death.[9] In China, AMA was recommended for use in pollution-free bee products production by Ministry of Agriculture as standard practice (NY5138-2002) until 2005 as it began to be prohibited in the poultry industry. AMA is still used by some beekeepers due to lack of more efficient substitute drugs against honeybee sacbrood disease. Therefore, the detection of AMA residue in honey has been involved in routine quality control for bee products in China because the improper use of AMA in apiculture can generate a considerable amount of residues. In addition, China has a large amount of honey to be exported to other countries every year, and the chemical residue has become the major technical trade barrier to protect the international market. In particularly, Japanese government issued positive list system for agricultural chemicals remaining in foods in 2006, which means that the agricultural chemicals whose maximum residue limits (MRLs) have not been established should not be detected in this food, such as the AMA in bee products.[10] But until now, there is no selective and sensitive method developed and validated for the determination of AMA in bee products.

AMA molecule does not exhibit a distinct absorption in the ultraviolet region above 200 nm because of the absence of chromophores, so the direct UV spectrophotometry cannot be used for its determination. Although the direct analysis of AMA in serum samples with high performance liquid chromatography (HPLC)-tandem mass spectrometry has been reported,[1] it cannot be used for routine detection in most laboratories because of the expensive equipments needed. Therefore, derivation of

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AMA is commonly adopted before it is analyzed by HPLC, gas chromatography, capillary electrophoresis, potentiometry, and fluorometry. Several derivatizing reagents have been intensively studied for HPLC analysis of AMA, including *o*-phthalaldehyde and 1-thio-*β*-*D*-glucose,[11] (2-naphthoxy) acetyl chloride,[12] anthraquinone-2-sulfonyl chloride,[13] dansyl chloride,[14] 1-fluoro-2,4-dinitrobenzene,[15] and 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F).[16] Among those, NBD-F selectively reacts with the primary and secondary amino groups and has been successfully used for the analyses of many compounds.[14,17-19]

In this paper an analytical method was developed to analyze AMA residue in honey. This method based on SPE procedure to extract AMA from honey samples, and the extracted AMA was derivatized with NBD-F. In the last, the derivatives were analyzed by HPLC with a fluorimetric detector.

Materials and methods

Chemicals and reagents

Memantine hydrochloride (MEM, ≥98.0% purity), amantadine hydrochloride (AMA≥99.0% purity), and NBD-F (≥98.0% purity) were purchased from Sigma-Aldrich (USA). Analytical grade trichloroacetic acid (TCA), phosphoric acid, chloridric acid, boric acid, ethylene diamine tetraacetic acid disodium salt (disodium EDTA), sodium hydroxide and ammonium hydroxide (25%) were obtained from Sinopharm Chemical Reagent (China). Chromatographic grade trifluoroacetic acid, methanol, and acetonitrile were obtained from Fisher (USA). All other chemicals used were of analytical grade. A Milli-Q® water purification system (Millipore, Bedford, MA, USA) was used to obtain deionized water.

Standard solutions and calibration curve

AMA and MEM (internal standard, IS) was quantitatively placed (100 mg each) into separate 100 mL volumetric flask, dissolved in 30 mL deionized water, and diluted with the same solvent to obtain stock solutions with a concentration of 1.0 mg•mL^{-1}. They were then diluted with water to obtain the standard working solutions. Stock solutions were stored at 4 ℃ and used for no longer than 3 months, whereas working solutions were freshly prepared before analysis.

The calibration curve of the sample was obtained by diluting the AMA working solutions with 0.1 mol/L borate buffer solution (containing 1 mmol/L disodium EDTA, adjusted to pH 8.0 by 0.1 mol/L NaOH) to produce the following concentrations: 0.025, 0.050, 0.10, 0.20, 0.40 μg•mL^{-1} and 1.0 μg•mL^{-1}.

Equipments

SPE cartridges Oasis WCX (150 mg, 6 mL, batch no: 186002498, Waters, USA) and Plexa PCX (60 mg, 3 mL, batch no: 12108603, Varian, USA), were applied and compared to extract analytes from honey samples to obtain the satisfactory recovery.

HPLC An Agilent 1200 HPLC machine equipped with an Agilent G1321A fluorescence detector and an Agilent Eclipse XDB C18 (150 mm × 4.6 mm i.d.) column were used for chromatographic analysis. The fluorescence detector was operated at an excitation wavelength of 470 nm and an emission wavelength of 530 nm. For quantification of chromatographic peaks, the Chem32 software was used.

Laboratory equipments A homogenizer apparatus (Vortex-5, Haimen Qilinbeier Instruments Co., Ltd, China.), an ultrasonic bath (KQ218, Kunshan Ultrasonic Instruments Co., Ltd, China, with 150 W output and 25 kHzfrequency), a centrifuge (2K15, Sigma, Germany), and a re-circulating water bath (HH-W, Jintan City Kaiyuan Experiment Instrument Factory, China) were used for the pretreatment of honey samples.

Analytical procedure

Sample preparation Honey samples were purchased from local supermarkets in Beijing in 2009. For honey fortification, 2.0 g of honey was weighed, heated in a 45 ℃ water bath for 20 min, and left to cool for 15 min. Appropriate amounts of AMA standard solutions were added to obtain a series of fortified samples (0.025, 0.10, and 0.20 μg•g^{-1}). The mixtures were homogenized and then processed in the extraction step.

Sample extraction and clean-up Aliquots of blank honey (2.0 g) and fortified samples were mixed with 160 μL IS and 20 mL of 1% (*w*/*v*, 1 g/100 mL) TCA in a 100 mL transparent glass beaker and homogenized by stirring. Samples underwent an ultrasonic-assisted extraction for 10 min, followed by centrifugation at 4 ℃ and 10000 g for 10 min in 50 mL centrifuge tubes. The supernatants were collected.

Half of the supernatants (11 mL) for each sample were passed through the SPE cartridge, which had been pre-conditioned with 3 mL methanol and 3 mL water, followed by washing with 3 mL water and 1 mL methanol and then eluted with 6 mL of 25% ammonia/methanol (1∶20, *V*/*V*). The flow rate was lower than 0.5 mL•min^{-1} under gravity. The eluates were vortexed for 5 s and evaporated to dryness at 50 ℃ under a stream of nitrogen. The residues were re-dissolved in 1.0 mL borate buffer solution and prepared for derivatization.

Derivatization NBD-F was selected as the derivatizing reagent in this study, and the procedure referred to Higashi (2005) with slight modification.[16] Briefly, each 400 μL solution for the calibration or extracted sample solution was transferred to an HPLC brown glass vial, and then 100 μL NBD-F solution (21.8 mmol/L in acetonitrile, stored at −20 ℃, restored to room temperature before use) was added in. The mixture was allowed to react for 12 min at 60 ℃ and then placed on ice to stop the derivatization reaction. Ice-cold 0.1 mol/L HCL (200 μL) was immediately added to the brown glass vial, and 40 μL of the derivatized sample

was injected into the HPLC instrument for analysis.

Chromatographic condition The mobile phase consisted of 0.1% trifluoroacetic acid/acetonitrile (35∶65, *V*/*V*), which was filtered using a 0.45 μm filter (Millipore) and degassed with ultrasonic treatment before use. The derivatives were eluted from the column at 30 ℃ at a flow rate of 1.0 mL•min^{-1}.

Method validation Parameters for AMA validation included linearity, linear range, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ). For linearity validation, six calibration solutions were analyzed in five replicates. Calibration curves were constructed by linear regression of the peak area ratios of AMA to the internal standard versus AMA concentrations.

Both intra-day (daily) and inter-day (day-to-day) precision assessment for the established AMA analysis method were conducted. Intra-day precision was estimated by replicating analysis (n=6) of blank honey sample fortified at 0.10 μg•g^{-1}. Inter-day precision was estimated by replicating analysis of blank honey sample fortified at three concentrations (0.025, 0.10, and 0.20 μg•g^{-1}) on different days.

Accuracy was estimated by means of the recovery at three fortification levels (0.025, 0.10, and 0.20 μg•g^{-1}), with each concentration analyzed in triplicate. The LOD and LOQ were defined as the concentration of standard solutions with threefold and tenfold signal-noise ratios, respectively.

Application of the method to real honey samples

The validated method was used for AMA determination with 12 honey samples, including three acacia honey varieties, two rapeseed honey varieties, two chaste tree twig honey varieties, two Chinese date honey varieties, two citrus honey varieties, and one mixed honey variety derived from different floral species.

Results

Optimization of sample extraction conditions

The AMA retention capacities of Oasis WCX and Plexa PCX SPE cartridges were compared. The AMA standard solution was diluted with water for loading on the equilibrated Oasis WCX, and diluted with 1% TCA for loading on Plexa PCX cartridges respectively. Analytes were eluted with 2% (*V*/*V*) formic acid-methanol solution (2%, *V*/*V*) for the Oasis WCX cartridge and with 25% ammonia/methanol solution (1∶20, *V*/*V*) for the Plexa PCX cartridge. Results indicate that AMA could be retained in both cartridges, while the Plexa PCX cartridge was recommended for use in the following study because it is cheaper than the Oasis WCX cartridge.

To obtain good elution results for AMA and IS, different volumes of elution solvent [25% ammonia/methanol (1∶20, *V*/*V*)] were analyzed. Results indicate that analytes were completely eluted with 6 mL 25% ammonia/methanol (1∶20, *V*/*V*) with total recoveries of approximately 100% (Table 1). The optimized conditions using Plexa PCX cartridge for extraction and clean-up of AMA and IS from honey are as follows: 1% TCA as extraction solvent, 3 mL water and 1 mL of methanol as wash solvents to remove the impurities, and 6 mL of 25% ammonia/methanol solution as elution solvent to obtain the analytes.

Table 1 Recoveries of AMA and IS at different elution volumes

Analyte	Recoveries (%) for different elution volumes (mL)						
	1	2	3	4	5	6	7
AMA	0	0	21.0	71.7	91.6	100	103
IS	0	11.5	62.2	94.8	98.3	101	98.4

Optimization of derivatization condition

The derivatization reaction with NBD-F was conducted in borate buffer (pH=8.0) medium with 1.0 μg•mL^{-1} AMA standard solution at 60 ℃ for different derivatization times (5, 8, 10, 12, and 15 min). Results indicated that the peak area increased with the extension of derivatization time in the range of 5 min to 12 min, while exhibited a decreasing trend as the time was longer than 12 min (Figure 1).

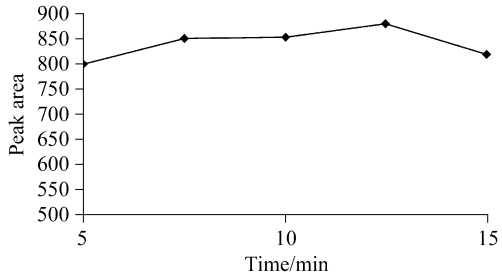

Figure 1 Peak areas of AMA derivatives under different derivatization times.

The storage stability of the mixture of derivatives of AMA (1 μg•mL^{-1}) and IS (0.4 μg•mL^{-1}) at room temperature was analyzed by measuring their peak areas every 2 h. As can be seen from Table 2, there were no significant differences in the peak areas ratios of AMA to IS at different detection times, suggesting that AMA and IS derivatives were stable for up to 12 h.

Optimization of chromatographic conditions

To optimize the chromatographic conditions, the mobile phase consisting of 0.1% trifluoroacetic acid/acetonitrile at three different ratios (20∶80, 30∶70, and 35∶65, *V*/*V*) were evaluated and compared. Results showed that the 20∶80 and 30∶70 ratios of mobile phases resulted in impurity peaks, and the AMA derivatives could not be absolutely separated. The best peak resolutions were obtained at a solvent ratio of 35∶65. Under this optimized condition, the retention times for the AMA and IS derivatives were around 9.5 min and 18.1 min, respectively. Representative chromatographs are shown in Figure 2.

Table 2 Stability of NBD-F derivatives of AMA and IS

Determination time/h	Peak area		Peak area ratio (AMA/IS)
	AMA (1.0 μg•g^{-1})	IS (0.40 μg•g^{-1})	
0	750	270	2.8
2	769	261	2.9
4	752	269	2.8
6	750	258	2.9
8	724	259	2.8
10	715	252	2.8
12	734	260	2.8

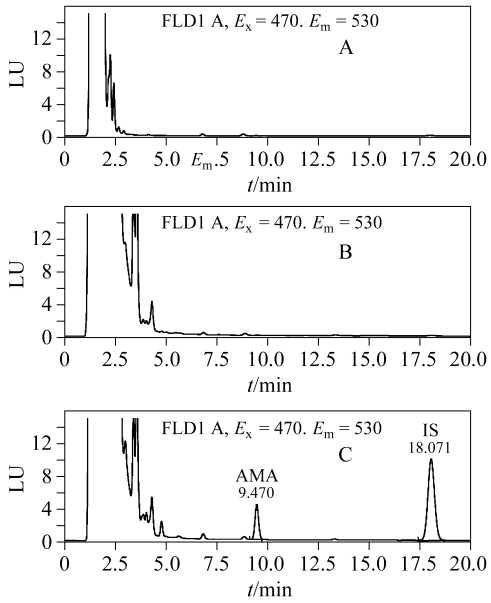

Figure 2 Chromatograms of the AMA and IS derivatives. (A) Blank solution without honey; (B) Blank honey; (C) Blank honey spiked with AMA (0. 10 μg•g^{-1}) and IS (0. 40 μg•g^{-1}).

Validation of the method

A linear regression equation of $y=0.3582x-0.0025$ with a correlation coefficient of 0.998 was obtained from the calibration curve of six samples (0.025, 0.05, 0.10, 0.20, 0.40, and 1.0 μg•mL^{-1}). This indicates a very good linearity between the peak area ratios of AMA to the internal standard (y) versus AMA concentrations (x).

The LOD and LOQ of AMA in honey was 0.0080 μg•g^{-1} and 0.025 μg•g^{-1}, respectively. The precision and accuracy for intra-day and inter-day assays of AMA are shown in Table 3. In the intra-day assay, the relative standard deviation was 3.8%, and mean recovery was 97.3%. These variables were 3.4%—5.1% and 92.5%—97.3% in the inter-day assay. These results implied a good reproducibility of the proposed method.

Analysis of real samples

Results showed that the AMA residues in 12 tested honey samples were below the LOD of this method, but there is no interfering peaks appeared at the retention time of AMA and IS for all samples.

Discussion

The determination of AMA in pharmaceutical formulations and biological samples, such as urine and plasma, has been intensively studied. A variety of pretreatment and extraction methods have been used, with liquid-liquid extraction (LLE) method being the most common.[12,20] Initially, LLE methodology was used to extract AMA from honey. However, this yielded recoveries of only 10%—20%, maybe because of the honey samples with high content of sugar. Thus, the SPE method was used to improve the recoveries of AMA in honey in this study. Additionally, the LLE technique requires a large amount of toxic organic solvent, such as benzene and dichloromethane. Solid-phase extraction (SPE) is a simple, efficient, and solvent-reducing alternative that has been successfully used to analyze various chemical residues in honey samples.[21-23]

In light of the procedures for derivatization of AMA with NBD-F of Higashi *et al*.(2006),[16] we made a slight modification by dissolving the sample in borate buffed

Table 3 Precision and accuracy of determination of AMA in honey

Concentration/(μg•g^{-1})	Mean concentration/(μg•g^{-1})	Precision (RSD/%)	Mean recovery/%
Intra-assay (six replicates)			
0.10	0.097	3.8	97.3
Inter-assay (n=3 d, three replicates per day)			
0.025	0.023	5.1	92.4
0.10	0.097	3.4	97.3
0.20	0.19	4.7	95.0

instead of phosphate-buffered saline, and the results demonstrated that it works well and wiped off the process for preparing the phosphate-buffered saline.

The method described by Higashi *et al.*[16] used 0.1% trifluoroacetic acid/acetonitrile/ethanol (40 ∶ 40 ∶ 20, *V*/*V*/*V*) as mobile phase at a flow rate of 1.0 mL•min^{-1} and with fluorescence detection at an excitation wavelength of 470 nm and an emission wavelength of 530 nm. When these conditions were applied in our research, the longest retention times of the AMA and IS derivatives were obtained with 16.9 min and 30.2 min, respectively. So we optimized the chromatographic conditions and shortened the retention times.

In conclusion, the SPE method was applied to extract and remove AMA from honey successfully, and NBD-F was used to derivatize AMA for HPLC analysis in honey samples. This is the first method established for the determination of AMA in honey. This method was found to possess appropriate accuracy, precision, a low detection limit, and low cost. It was proven suitable for the routine analysis of AMA for the quality control of honey.

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