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Short communication

Multi-residue analysis of avermectins in swine liver by immunoaffinity extraction and liquid chromatography–mass spectrometry

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Abstract

A multi-residue LC–MS method was developed to determine avermectin residues in swine liver. Abamectin and ivermectin (22,23-dihydroabamectin) were extracted, and cleaned up by immunoaffinty columns with immobilized anti-avermectin polyclonal antibodies. The cleaned samples were separated by high-performance liquid chromatography (HPLC) with a C_8 column and determined by negative-ion atmospheric pressure chemical ionization (APCI) mass spectrometry (MS) using selective ion monitoring (SIM) of $[M\text{-}H]^-$. Recoveries of abamectin and ivermectin from fortified samples at 5–100 $\mu g\ kg^{-1}$ levels ranged from 74 to 94% and from 65 to 87%, respectively. The limits of detection were 5 μg of abamectin or ivermectin in 1 kg sample. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Avermectins; Abamectin; Ivermectin

1. Introduction

Avermectins (AVMs) are a class of very potent drugs used in agriculture for treatment of a broad spectrum of parasitic diseases [1]. Avermectin B_1 is the main component in abamectin (ABM) which is approved for use in several animal species. The reduction product in position $-C_{22}=C_{23}-$ of abamectin is named ivermectin (IVM) which has even higher activity and broader spectrum [2]. The structure of ABM (B_1) and IVM (H_2B_1) are shown in Fig. 1. Abamectin B_{1a} and ivermectin H_2B_{1a} are not less than 80% in fermentation product or reduction of fermentation product, and their homologues ABM B_{1b} and IVM H_2B_{1b} are not more than 20%, so only ABM B_{1a} and IVM H_2B_{1a} are monitored in this study. ABM and IVM are the most common AVMs used in agriculture. The maximum residue limit (MRL) of ABM or IVM in swine liver (target tissue) is 15 $\mu g\ kg^{-1}$ [3].

AVM residues are usually determined using solid-phase extraction techniques and liquid chromatography with fluorescence detection after dehydration of the molecule to form an aromatic fluorescent moiety [4–7]. Immunoaffinity chromatography cleanup of AVMs is an alternate method of sample preparation. Li et al. obtained polyclonal anti-abamectin antibodies by immunizing New Zealand

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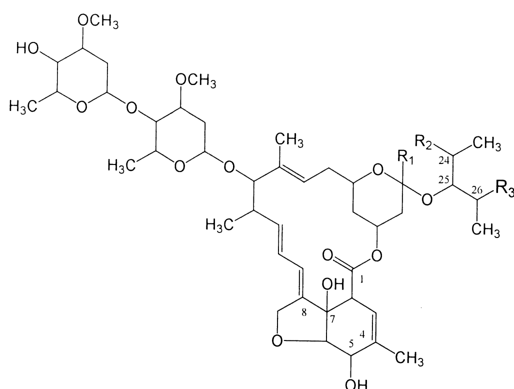

Abamectin B1a:	R1R2 = —CH=CH—	R3=C2H5
Abamectin B1b:	R1R2 = —CH=CH—	R3=CH3
Ivermectin H2B1a:	R1R2 = —CH2–CH2—	R3=C2H5
Ivermectin H2B1b:	R1R2 = —CH2–CH2—	R3=CH3

Fig. 1. The structure of abamectin and ivermectin.

rabbits with 4″-*O*-hemisuccinoylavermectin B_1-bovine serum albumin and developed an immunoaffinity column cleanup procedure for analysis of IVM in swine liver [8], ABM in cattle tissue [9] and IVM in sheep serum [10]. The polyclonal anti-abamectin antibodies can recognize both ABM and IVM [11], therefore, it is possible to cleanup ABM and IVM for multi-residue analysis by immunoaffinity columns.

Mass spectrometry (MS) is the preferred technique for confirmation of suspect residues due to its inherent specificity and sensitivity. With the development of HPLC–MS, several groups have reported successful confirmation of avermectins using this technique. Heller et al. [12] confirmed IVM residue in bovine milk and liver using particle beam LC–MS with negative ion chemical ionization. Turnipseed et al. [13] developed a multi-residue LC–MS method with negative-ion APCI to confirm ivermectin, doramectin, eprinomectin and moxidectin in several food matrices. It would be helpful for residue analysis if the high specificity of an immunoaffinity column cleanup procedure was combined with highly specific and sensitive LC–MS. In this paper, we describe an off-line method for multi-residue analysis of ABM and IVM in swine liver with immunoaffinity cleanup and LC–MS.

2. Experimental

2.1. Reagents

ABM and IVM were provided by CAU Newtech Development (Beijing, PRC). A stock solution (ABM, 100 μg ml^{-1} of B_{1a} and 2.2 μg ml^{-1} of B_{1b}; IVM 100 μg ml^{-1} of H_2B_{1a} and 1.9 μg ml^{-1} of H_2B_{1b}) was prepared by dissolving in methanol, and stored at −20°C. A working solution was prepared by diluting the stock solution with methanol.

Methanol was LC grade purchased from Fisher Scientific (Fair Lawn, NJ, USA). The water was purified using a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA). Sepharose CL-4B (46–165 μm) was obtained from Pharmacia (Uppsala, Sweden), 1,1′-Carbonyldiimidazole (CDI, 97%) from Fluka (Buchs, Switzerland). All other reagents were analytical grade or better.

Phosphate-buffered solution (PBS) was prepared by dissolving 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KCl and 8.8 g NaCl in 900 ml of water. This solution was adjusted to pH7.4 with 2.0 mol l^{-1} NaOH, and diluted to 1 l with water. PBS containing 0.5 mol l^{-1} NaCl was prepared in the same way as above except 29.3 g of NaCl was used. PBS (0.5 mol l^{-1} NaCl)–methanol (9:1, v/v) and water–methanol (9:1, v/v) were degassed by ultrasonification after mixing.

2.2. Apparatus

The HPLC–MS system was composed of a Mariner time of flight (TOF) MS with a Sciex APCI interface (PE PerSeptive Biosystems, Framingham, MA, USA) and a HP1100 HPLC system with a quaternary pump, a variable wavelength detector, an online vacuum degasser and a 20 μl manual injector (Hewlett-Packard, Palo Alto, CA, USA). The ion polarity was negative. The nebulizer, curtain and auxillary gas were nitrogen with flow-rate 0.65, 0.5 and 2.5 ml min^{-1} respectively. The nozzle, quadrupole and spray chamber were heated to 180, 140 and 475°C respectively. The spray tip and nozzle potential were 5442 and 30 V. The acquisition speed was 4 s per spectrum. Other conditions are the same as ESI with negative ion detection when optimized with the standard oligodeoxythymidylate $p(dT)_6$.

(Skimmer 1 Potential, 10.01 V; Quadrupole DC Potential, 6.35 V; Deflection Voltage, −0.15 V; Einzel Lens Potential −29.00 V; Quadrupole RF Voltage 799.80 V; Push Pulse Potential, 475.04 V; Pull Pulse Potential, 224.92 V; Pull Bias Potential, 14.00 V; Acceleration Potential, 4000.02 V; Reflector Potential, 1549.99 V; Detector Voltage 2450.09 V). The LC column was a Zorbax Eclipse XDB-C8 0.46×15 cm (Hewlett-Packard, Palo Alto, CA, USA). The mobile phase was 85:15 methanol–water. The total run time was 18 min. At the end of each run, the content of methanol was increased to 100% for 3 min, and then equalized with mobile phase for another 3 min. The flow-rate was 0.5 ml min^{-1}.

Other apparatus were a homogenizer, Model AM-6 (Nihonseiki Kaisha, Tokyo, Japan), a Vortex Mixer, Model MVS-1 (Beide Instrument, Beijing, PRC), a rotary evaporator, Model RE-52A(Yarong Biochemical instrument, Shanghai, PRC), a shaking apparatus, Model SHZ-82 (Taicang Biochemical instrument, Jiangsu, PRC), a ultrasonic cleaner, Model KQ-100 (Kunsan Ultrasonic Instrument Co., Ltd., Jiangsu, PRC), a centrifuge, Model LD4-2A (Anting instrument, Shanghai, PRC), and a constant flow pump Model DDB-300 (Haitian Electronic instrument, Zhejiang, PRC). Glass columns for packing immunosorbent, 10×0.7 cm I.D., with a fritted disc (porosity 40–60 μm) sealed into the bottom and a 10 ml reservoir connected to the top with ground-glass joints were self-made.

2.3. Immunosorbent preparation

The polyclonal antibodies were prepared by immunizing New Zealand rabbits with 4′′-*O*-hemisuccinoylavermectin B_1-bovine serum albumin [11], and the immunoglobulin G (IgG) fraction of the antisera was purified by ammonium sulfate precipitation and diethylaminoethyl cellulose anion-exchange chromatography [9]. The immunosorbent was prepared by coupling antibodies to CDI-activated Sepharose CL-4B [8] and stored in PBS–0.02% sodium azide at 4°C.

2.4. Column capacity determination

A relatively large amount of IVM (4000 ng IVM in 50 ml of PBS–methanol, 85:15, v/v) was drawn through an immunoaffinity column of 1.0 ml bed volume continuously at a flow-rate of 1.2 ml min^{-1} by gentle suction. The ivermectin-saturated column was washed with 30 ml of PBS (0.5 mol l^{-1} NaCl)–methanol (9:1, v/v) and then 30-ml of water–methanol (9:1, v/v). IVM was eluted with 3 ml of 100% methanol, and determined by LC–MS. The column was regenerated by washing with 5 ml of water and 10 ml of PBS, and stored in PBS–0.02% sodium azide at 4°C.

2.5. Sample preparation/immunoaffinity column cleanup

Partially thawed swine liver was minced, and homogenized with a homogenizer for 2 min at high speed. The homogenate was stored at −20°C.

5.0 g of thoroughly thawed homogenate was transferred to a 25 ml graduated test-tube, and 15.0 ml of methanol was added. The mixture was shaken thoroughly by hand and again by using a shaking apparatus for 1 h at medium speed. The sample was adjusted to a volume of 20 ml with methanol and shaken thoroughly. After centrifugation for 5 min at 2000 *g*, 10 ml of supernatant was collected and mixed with 40 ml of PBS. This solution was subjected to the immumoaffinity column cleanup procedure.

The sample solution was draw through an immunoaffinity column of 2.0 ml bed volume continuously at a flow rate of 1.2 ml min^{-1}, and then washed with 30 ml of PBS (0.5 mol l^{-1} NaCl)–methanol (9:1, v/v) and then 30 ml of water–methanol (9:1, v/v). IVM and ABM were eluted with 4 ml of 100% methanol. The eluent was evaporated to dryness at 40°C and the residue was redissolved in 1 ml of methanol with a Vortex mixer for 15 s. After filtration through a 0.45 μm filter, aliquots of 20 μl were used for LC–MS analysis.

2.6. Calibration curve and fortification

The standard calibration curve for ABM and IVM covered a concentration range from 10 to 500 ng ml^{-1}. Blank swine liver homogenates were fortified with 10–50 μl of ABM and IVM working solution at 5–100 μg kg^{-1} levels and mixed thoroughly. After 10–15 min, the sample was extracted, cleaned up and determined as described earlier. The levels of ABM and IVM in the sample were calculated with

the following equation: ABM (or IVM), μg kg^{-1} = $(2 \times C \times V)/W$. Here C (ng ml^{-1}) is the concentration of ABM (or IVM) in the final sample solution, determined from the standard curve, V (ml) is the volume of the final sample solution, and W (g) is the weight of the sample.

3. Result and discussion

3.1. Optimization of the LC–MS conditions

The difference between ABM and IVM is a double bond in a ring and this will not affect their behaviors in MS significantly, so we only studied the best ionization conditions of IVM and then applied them to ABM. There are two forms of atmospheric pressure ionization commonly used in HPLC–MS, one is electrospray ionization (ESI), and the other is APCI. In ESI positive ion detection mode, no peaks of $[M+H]^+$ were observed even as the concentration of acetic acid in the buffer solution was increased to 0.1 and 1%. Only three groups of peaks corresponding to $[M+NH_4]^+$, $[M+Na]^+$, $[M+K]^+$ were observed in the mass range from 300 to 1000. With a nozzle potential at 120 V, the solvent of 50:50 methanol–water with 0.01% ammonium acetate, the peaks of $[M+NH_4]^+$ had maximum sensitivity with a linear correlation between concentration and response over a broad range. However, when we prepared the sample according to reference [8] and determined IVM by MS, the peaks of $[M+Na]^+$ and $[M+K]^+$ in the mass spectra were much higher than in a standard sample. No matter whether we increased the volume of 80:20 methanol–water after absorption, or even changed PBS to ammonium acetate buffer in the sample preparation, the ratio of $[M+Na]^+$ and $[M+K]^+$ to $[M+NH_4]^+$ was still higher than for the standard. It is difficult to obtain the correct result using adduct ions like $[M+NH_4]^+$ for biological samples which contain Na^+ and K^+. In ESI negative ion detection mode, 90:10 methanol–water was found to be an appropriate solvent. At lower nozzle potentials, a high intensity of adduct ions was formed. $[M+Cl]^-$, $[M+HCOO]^-$, $[M+CH_3COO]^-$ and $[M+C_3H_5O_3]^-$. With increasing nozzle potential, the intensity of fragmental ions was increased, which corresponded to the loss of H, the loss of one or two H_2O, sugar moieties or retro-Diels-Alder fragment ions from $[M\text{-}H]^-$. When nozzle potential was 200 V, the highest intensity of $[M\text{-}H]^-$ was achieved, but the sensitivity was not high enough for the residue analysis.

In APCI positive ion detection mode, we did not observe any adduct ions. Like previous researchers, we chose APCI with negative ion detection. When the nozzle potential was 30 V, the intensity of the predominant $[M\text{-}H]^-$ ion was maximum, and no other peaks of adduct ions were observed. The sensitivity of $[M\text{-}H]^-$ was high enough to provide an adequate detection limit, so only the isotopes of $[M\text{-}H]^-$ were used for the quantitative analysis of IVM and ABM. Because there were no interfering peaks near m/z 873 (IVM) and 871(ABM), the extracted ion chromatogram of 873±3 was used for quantitative analysis. Fig. 2 shows the negative ion APCI mass spectra of ABM and IVM. ESI-TOF MS can give relatively abundant information of accurate ion mass and isotope distribution, which are very helpful criteria for conformation, therefore, quantitative and qualitative data can be obtained in a run of LC–MS. It is important to separate IVM and ABM in LC because their mass difference is only two;

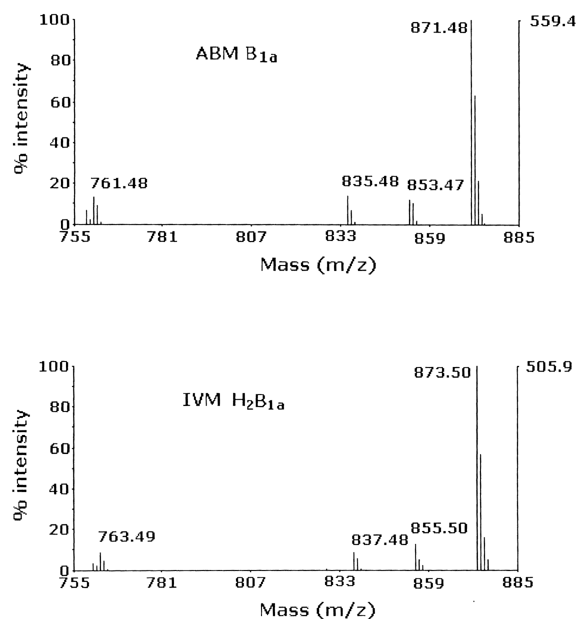

Fig. 2. The negative ion APCI mass spectrum of AVM B_{1a} and IVM H_2B_{1a}.

otherwise, the isotopes of ABM would overlap with the peaks of IVM. The C8 column proved to be suitable for separating IVM and ABM.

3.2. Immunoaffinity column cleanup

It is essential to degas the adsorption, washing and elution buffers; otherwise, the change of methanol content in the buffers may cause small bubbles in the absorbent which will drop the efficiency of the column.

The procedures used for immunoaffinity column cleanup were based on previous work [8], but some revision was made. In previous work, 1 ml of the immunoaffinity absorbent was washed with 40 ml of PBS (0.5 mol l^{-1} NaCl)–methanol (9:1, v/v) and 10 ml of water–methanol (8:2, v/v) after adsorption. Ten ml of water–methanol (8:2, v/v) could not thoroughly removed the salts which absorbed to MS, causing problems. So 30 ml of water–methanol (8:2, v/v) was used to remove salts. Reduction of the volume of PBS (0.5 mol l^{-1} NaCl)–methanol (9:1, v/v) from 40 to 30 ml did not affect the final result significantly.

The total column capacity of 1 ml of immunosorbent bed volume was 900 ng of IVM and tended to decrease in a way similar to the previous reports: 50% remained after 20 cycles of use over a period of 2 weeks.

Fig. 3 shows the extracted ion chromatograms of LC–MS of standard IVM and ABM, fortified sample and blank swine liver. The extracted ion chromatogram is essential for the quantitative analysis of the complex matrices, because it can remove the interference.

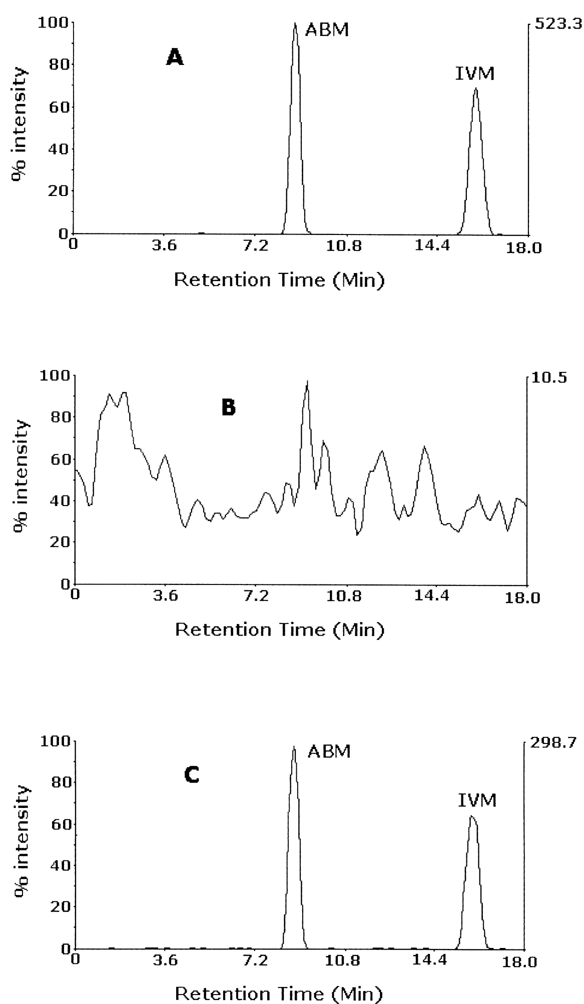

Fig. 3. The extracted ion chromatograms (873±3) of LC–MS, (A) 125 ng ml^{-1} ABM and IVM in methanol, (B) blank swine liver, (C) swine liver fortified with 50 μg kg^{-1} of ABM and IVM respectively.

3.3. Determination and fortification

The standard calibration curves for IVM and ABM (concentration vs. chromatographic peak area) were linear in the concentration range of 10–500 ng ml^{-1}. The linear equation for IVM is $y = 26.9x - 302.6$ ($r = 0.9996$, $n = 5$, SD for slope = 0.5 and SD for intercept = 74.7), and the equation for AVM is $y = 28.1x - 255.7$ ($r = 0.9980$, $n = 5$, SD for slope = 1.3 and SD for intercept = 186.3). (y is the chromatographic peak area, x is the concentration of IVM or AVM)

The results of fortification studies are shown in Tables 1 and 2.

Recoveries of IVM and ABM were 65–87% and 74–94% respectively. The limits of detection (S/N ratios at chromatogram peaks of ABM and IVM were 12.7 to 1 and 8.2 to 1 respectively) were 5 μg kg^{-1} in this study. This is one of the simplest methods for confirmation multi-residue of AVMs in animal tissues yet reported. It is sensitive and reliable enough for determining IVM and ABM residues in liver tissue. Liver is one of the most

Table 1
Recoveries of ivermectin from fortified swine liver

Added (μg kg^{-1})	Determined (μg kg^{-1})			Average (μg kg^{-1})[a]	Recovery (%)
0	–			–	–
5	4.1,	4.2,	4.9	4.4±0.4	87
20	12.2,	14.4,	15.7	14.1±1.8	71
50	24.5,	32.0,	38.8	31.8±7.2	64
100	63.6,	59.1,	72.6	65.1±6.9	65

[a] Values are mean±standard derivation.

Table 2
Recoveries of ABM from fortified swine liver

Added (μg kg^{-1})	Determined (μg kg^{-1})			Average (μg kg^{-1})[a]	Recovery (%)
0	–			–	–
5	4.8,	4.3,	4.9	4.7±0.3	94
20	18.1,	19.9,	19.8	19.3±1.0	97
50	35.1,	42.3,	47.0	41.5±6.0	83
100	77.1,	78.9,	66.8	74.3±6.5	74

[a] Values are mean±standard derivation.

difficult tissues to cope with because of interferences; therefore, this method should translate well to other matrices such as milk, muscle and serum. The recovery of IVM was lower than that of ABM because of the weak binding of IVM to the antibodies, and IVM was liable to be lost in the cleanup procedure. Because the activity of the antibodies was decreased during storage, 2 ml of immmunosorbent bed volume was used in sample preparation instead of 1 ml. Although the column capacity was enough theoretically when immunosorbent bed volume was 1 ml, the recovery of IVM was only 60% of that of ABM when the fortified concentrations were over 20 μg kg^{-1}. The large excess of immunosorbent is necessary for multi-residue cleanup when the bindings of haptens are different.

Acknowledgements

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