An HPLC Method for Determination of Azadirachtin Residues in Bovine Muscle

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Abstract

A high-performance liquid chromatography (HPLC) method for the determination of azadirachtin (A and B) residues in bovine muscle has been developed. Azadirachtin is a neutral triterpene and chemotherapeutic agent effective in controlling some pest flies in horses, stables, horns and fruit. The actual HPLC method uses an isocratic elution and UV detection. Liquid–liquid extraction and solid-phase purification was used for the clean-up of the biological matrix. The chromatographic determination of these components is achieved using a C18 analytical column with water–acetonitrile mixture (27.5:72.5, v/v) as mobile phase, 1 mL/min as flow rate, 45°C column temperature and UV detector at 215 nm. The azadirachtin peaks are well resolved and free of interference from matrix components. The extraction and analytical method developed in this work allows the quantitation of azadirachtin with precision and accuracy, establishing a lower limit of quantitation of azadirachtin, extracted from the biological matrix.

Introduction

Azadirachtin (AZD) is a tetranortriterpenoid characteristic of Meliaceae especially of the Neem tree (*Azadirachta indica*) native of India. It is found in the bark, leaves, fruits, and especially in the seeds (1–3). In the extract approximately 18 compounds were identified but it is AZD was found in highest concentration. AZD is composed of at least nine closely related isomers. The types A (Figure 1) and B are dominant, with isomer A accounting for 83% and B 16% (4–6).

AZD is a chemotherapeutic agent effective in controlling some pest flies in horses, stables, horns, and fruit (7–10). This drug interrupts the life cycle of flies by inhibiting the development of the eggs, larvae, or pupae and by blocking the molting of larvae or nymphs, and inhibiting mating and sexual communication (11–14). Currently the oily extract of neem (neem oil) is applied on the skin of sheep as a precautionary measure for controling flies' attacks on production (1,15).

According to national (Agricultural and Livestock Service of Chile, SAG) and international (European Commission) standards, the drug residues used in meat and/or viscera which are imported or exported must be declared, although there are no limits set for them, as is the case with AZD (16–18).

Several analytical methods have been reported for the determination of AZD mainly in fruits. These reports include the use of colorimetric determination (19), spectrophotometry (20), high-performance liquid chromatography (HPLC) diode array detection (21–23), HPLC-mass spectrometry (24–27), and Enzyme-Linked Immunosorbent Assay (28).

The novelty of the present work is to develop and validate an easy method for the extraction and determination of residues of AZD from bovine muscle using HPLC with UV detection.

Experimental

Instrumentation

The HPLC system consisted of a Model L-6200 A pump, a Model L-7250 LaChrom autosampler, and a programmable L-4250 UV–vis absorbance detector (Merck, Darmstadt, Germany). Chromatographic analysis was performed using a Symmetry RP-18 (5 µm, 250 mm × 4.6 mm i.d.) column from

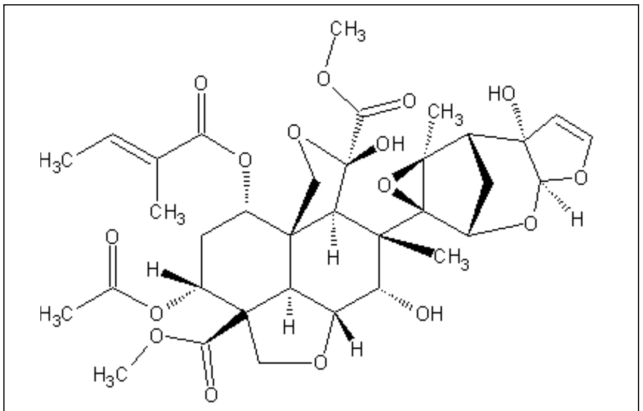

Figure 1. Structure of azadirachtin.

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Waters. Degassing of the mobile phase was achieved by continuous helium sparking in the solvent reservoirs. Data recording was carried out by D7000-HSM software (Merck). An Ultraturrax (Ika, Wilmington, NC) blender was used to homogenize samples.

Reagents and materials

AZD was donated by The Dharamsi Morarji Chemical Company (Mumbai, India) and carbamazepine, used as internal standard, was purchased from Laboratorio Bagó (Santiago, Chile). Both compounds were used as received. All solvents and reagents used were of reagent grade or HPLC quality. Water was purified and bidistilled from quartz apparatus and passed through a Milli-Q system from Millipore. All solvents and solutions for HPLC analysis were filtered through a 0.45-µm GVWP Millipore filter (Billerica, MA).

OASIS HLB cartridges (Waters, Millford, MA) were tested for the isolation of the AZD from endogenous interference from bovine muscle.

Preparation of standards and stock solutions

Stock solution of AZD in methanol–water (90:10) was prepared by weighing on a microbalance 5 mg of compound, dissolved in 5 mL of solvent in an ultrasonic bath at room temperature to get a clear solution and adjusted to 10 mL with solvent in a volumetric flask. Thus, the prepared solution contains 420 µg/mL of AZD A and 80 µg/mL of AZD B in methanol–water (90:10). The internal standard (carbamazepine) was dissolved in methanol–water (90:10) to give a concentration of 2 mg/mL. All solutions were light protected, prepared daily, and stored in a refrigerator in amber glass vessels. The standard and stock solutions were not filtered because presence of interference peak signals or retention of the analytes were previously assessed using the same filter used to filter the samples (results not showed).

Sample preparation and extraction

Bovine muscle was purchased fresh and cut into small pieces; 5 g of sample were suspended in 1 mL of water and ground into a homogeneous sample using a meat grinder. The samples were kept frozen at –18°C. In order to apply the method of standard addition, increasing volumes of AZD standard were spiked into 5 g samples, which were homogenized for 1 min at 24000 rpm. Then, the mixture was extracted with 10 mL dichloromethane–isopropanol (95:5). The mixture was stirred for 1 min at 24000 rpm, allowed to stand for 5 min in ultrasonic bath, and finally centrifuged at 3000 rpm for 5 min. The organic phase obtained was isolated, filtered and evaporated under nitrogen stream in a water bath at 37°C. The residue was dissolved in 500 µL of methanol–water (90:10) and filtrated with a 0.45-µm GVWP Millipore filter.

HPLC-UV analysis

Twenty microliter aliquots of the sample extracts were injected into the Symmetry RP-18 column, and the mobile phase was pumped at a flow rate of 1 mL/min. Injections of standard solutions of AZD were made at regular intervals during the run. The absorption was detected at 215 nm. All experiments were carried out at a column temperature of 45°C, a critical factor for optimal determination. Recovery of the analyte was calculated by comparison of standards with extracts of fortified samples.

Results and Discussion

Extraction efficiency

In order to achieve optimal separation of these compounds from the bovine muscle matrix, different parameters were checked: dilution and buffering of the samples, washing solutions, pH and the composition of the elution solvents.

First, the efficiency of AZD extraction from an aqueous solution was evaluated. The solvents evaluated were dichloromethane, hexane and chloroform (29). The mixture consisted of dichloromethane with isopropanol (95:5) which gave optimal recovery of AZD. Using this same mixture of solvents for the extraction of AZD from fortified samples of bovine muscle, a recovery of 100% was obtained. To compare the extraction capacities of these organic solvents under different pH values, the pH in water solutions was increased from 2 to 10. The influence of pH on the extraction showed no significant differences. The AZD solution finally used was the aqueous solution pH 6. All experiments were performed at room temperature (20°C). In addition, the effect of the concentration of AZD on the extraction efficiency of dichloromethane with isopropanol (95:5) was studied. The results show that an increase of analyte concentration from 25 µg/mL to 75 µg/mL did not change the extraction efficiency. These procedures were performed in triplicate for each concentration used.

Several types of cartridges for solid-phase extraction (SPE) were tested in order to obtain satisfactory values for the recovery of AZD. However, the purification on SPE columns is feasible only for extraction of AZD from aqueous solutions, but not for extraction from meat, because the cartridge is blocked. Moreover, the sample should not be worked in propylene tubes because the AZD may be adsorbed in this material. Also, only glass lids were used, because the plastic seals could interfere with the chromatographic analysis. However, Swinnex syringes and micropipette tips used in this work are compatible with the samples.

Chromatographic analysis

The HPLC method described here was developed for quantitation of AZD A and AZD B in bovine muscle following the optimal chromatographic parameters (30).

The chromatographic parameters (retention time, peak shape and others) for the resolution of AZD A, AZD B in the presence of carbamazepine, (internal standard) were tested under several experimental conditions. A series of mobile phases with increasing polarity under an isocratic regime were employed, the effect of pH and buffer concentration were also tested at several temperatures (20, 25, 30, 35, 40, and 45°C). According to the results obtained, the best analysis, satisfactory resolution and relatively short analysis time were obtained when separation was performed on Symmetry RP-18 from Waters protected with appropriate guard column, at 45°C temperature, using a mobile phase consisting of acetonitrile–water 27.5:72.5 (v/v). The best

compromise between resolution and analysis time was obtained with this mobile phase. The flow rate was 1 mL/min and UV–vis detection of AZD A and AZD B were performed at 215 nm. The near UV detection wavelength set at 215 nm was considered an optimal relationship between the sensitivity of the AZD and interferences present in bovine muscle samples. The retention times of carbamazepine, AZD A and AZD B were 20.3, 37.2, and 41.8 min, respectively.

Figure 2, HPLC shows that the isocratic resolution provides an optimal separation of the AZD A and B in bovine muscle.

Chromatographic performance data for a typical run are presented in Table I.

Retention factor (k') was defined as $(t_R - t_0)/t_0$, where t_R is retention time of peak (min) and t_0 is void time (min). In this method, the void time was 3.5 min. Retention factor in the range of $0.5 < k' < 20.0$ is desired to clearly separate the first peak from void time and to avoid a higher retention time for the last band. Retention factor of 4.8, 9.6, and 10.9 were found for carbamazepine (internal standard), AZD A, and AZD B, respectively, indicating a satisfactory separation for these compounds.

Tailing is defined as $W_{0.05} / 2\ t_W$, where $W_{0.05}$ is peak width at 5% of peak height (min) and t_W is distance between peak front and peak retention measured at 5% of the peak height (min). The tailing factor for carbamazepine, AZD A, and AZD B were all near to 1.0.

Resolution (R_s) is the distance between the peak centers of two component peaks dividided by the average base width of the peaks, $Rs = (t_2 - t_1) / 0.5\ (W_1 + W_2)$.

Resolution of 2.0 or greater is desired for critical band pair. Critical resolution of 9.9 and 2.6 were observed between carbamazepine–AZD A and AZD A–AZD B peaks, respectively.

Table I. HPLC Chromatographic Performance Data of the Method

Analite	Retention time (min)	Tailing	Retention Factor	Resolution*
Carbamazepine	20.3	1.3	4.8	9.9
Azadirachtin A	37.2	0.9	9.6	2.6
Azadirachtin B	41.8	1.1	10.9	

* between critical band pair.

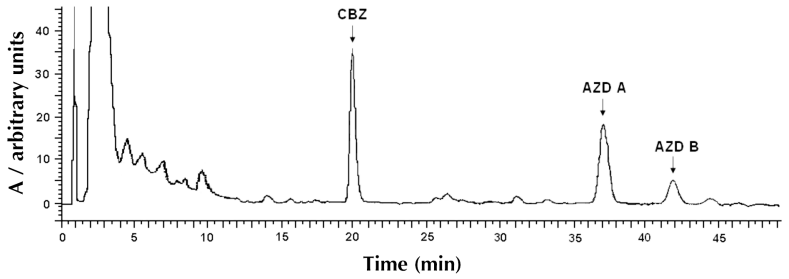

Figure 2. HPLC chromatogram of fortified sample with CBZ = carbamazepine (20 µg/g muscle), AZD A = azadirachtin A (52 µg/g muscle), and AZD B = azadirachtin B of bovine muscle (11 µg/g muscle).

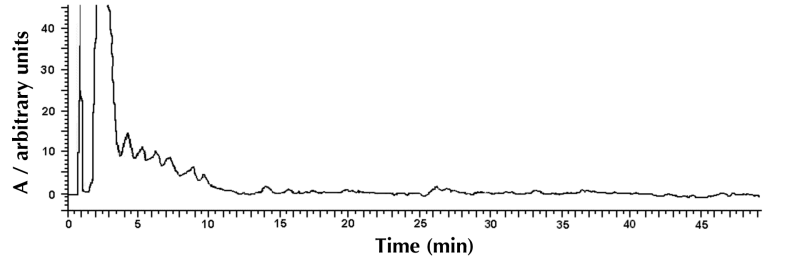

Figure 3. HPLC chromatogram of blank bovine muscle monitored at 215 nm.

Selectivity

Selectivity of method was assessed by the absence of any interference in the elution times of both AZD (A and B) and internal standard in the same chromatographic run as shown in blank chromatograms (Figure 3). To check the selectivity of the method six different samples of bovine muscle were analyzed, after being pre-treated as described above. The comparison of chromatograms of blank (Figure 3) and fortified samples (Figure 2) demonstrates that no interference was detected from endogenous substances with the AZD (A and B) and carbamazepine. The retention time for AZD A, AZD B, and carbamazepine in the chromatogram of fortified sample is similar to that obtained for standard solutions of these three compounds in methanol–water (90:10).

Robustness

Robustness of the method was checked by applying the proposed HPLC method with mobile phases with different pH values and ionic strength, detection at different wavelengths and variations in temperature of the chromatographic column.

Relative error was calculated by comparing pH value of the mobile phase from 3 to 7.4 and to those obtained by changing the detection wavelength from 215 to 240 nm. pH and ionic strength produced no major changes in the chromatograms. Peak areas at wavelengths over 225 nm were much smaller than at 215 nm, affecting the sensitivity of the method. Resolution between AZD A and AZD B was highly dependent on temperature. Temperatures below 45°C did not allow good resolution. Robustness study was performed in fortified samples with 100 µg AZD A /g muscle and 19 µg AZD B /g muscle, and the solutions contained 315 µg/mL of AZD A and 60 µg/mL of AZD B in methanol–water (90:10).

Linearity and range

Linearity, accuracy, precision, and method quantitation and detection limits tested to ensure method suitability were fully tested for determination of AZD A and B. For the evaluation of linearity, a linear regression model in a standard curve with six concentrations between 11.44 µg/g muscle and 343.05 µg/g muscle was used for AZD A (Figure 4) and six concentrations between 2.46 µg/g muscle and 73.5 µg/g muscle was used for AZD B (Figure 5). In accordance with the Food and Drug Administration's recommendations for evaluation of linearity, a plot of concentration versus signal and

the mean relative error (RE) of the interpolated concentration of the quality control standards were taken into consideration. The following criteria were taken into account to assess linearity: the values for the medium- and high-quality control samples should be within 15% of the actual value, only low quality control samples could be within 20%, four to six quality control samples fulfilled the same criteria and a correlation coefficient ≥ 0.95 was considered adequate.

For determination of the analytical linearity of the preparation procedure, a calibration diagram was constructed using carbamazepine as internal standard compound.

The linear dependence peak area of AZD A/peak area of carbamazepine obtained with determination coefficient (0.9982) is the following:

$$\frac{Area\ AZD\ A}{Area\ carbamazepine} = 0.0724 \times AZD\ A\ (\mu g/g\ muscle) + 0.5616$$

For AZD B, the following result was obtained:

$$\frac{Area\ AZD\ B}{Area\ carbamazepine} = 0.1126 \times AZD\ B\ (\mu g/g\ muscle) + 0.0582$$

with determination coefficient of 0.9963

Limits of detection and quantitation

There are at least four different ways to determine quantitation limits of analytes and signal-to-noise ratio (S/N) is one of the most commonly used procedures. This procedure has been used to determine quantitation limits of AZD (A and B). Minimum levels of AZD obtained after extraction process according to the protocol described above, were used in determining the limits of detection and quantitation. Signal-to-noise ratios were determined until a minimum S/N ratio of 10 was achieved. Using this method, a quantitation limit of 22 µg /g muscle was observed for AZD A and 11 µg /g muscle for AZD B. A detection limit (defined at signal to noise ratio of 3) of 10 µg/g muscle was also determined for AZD A and 2 µg/g muscle for AZD B. The limits of detection for AZD A and AZD B are shown in Figure 6.

Precision and accuracy

Precision was expressed as the coefficient of variation (CV) of the interpolated concentrations. A CV ≤ 15% for medium and high controls and ≤ 20% for low range is acceptable, indicating sufficient reproducibility for this method. Precision was required as part of each sequence run from the beginning of the sequence. Three injections of the target level of calibration were performed and the data were evaluated with the interpolated concentrations in the calibration curve. For each sample preparation two injections were performed.

The accuracy of the method was expressed as the relative error (RE) of the interpolated concentration of fortified bovine muscle samples at three concentration levels for each isomer. The guidelines of European Commission provide a range of 80–120% for these tests.

Coefficients of variation for three different concentrations of each AZD were evaluated (Table II). For each specific theoretical concentration the analytical recovery (µg/g muscle), standard deviation, coefficient of variation, and relative error for three independent samples were assessed.

The analytical recovery for all concentrations studied for AZD A is close to 100%. Coefficients of variation for AZD A were found to range between 1.3–2.8%, and relative error between –0.5 to 2.8%. Moreover, the analytical recovery for AZD B (85.2–101.9%) showed a variation greater than AZD A with a coefficient of variation between 0.4–2.9%.

The results obtained show the adequate precision and accuracy of this method.

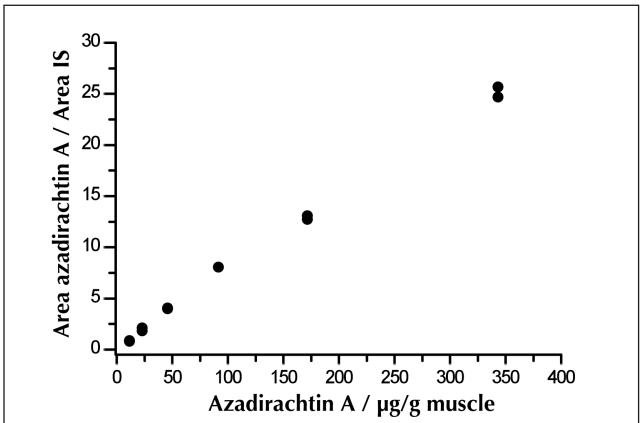

Figure 4. Response curve of azadirachtin A in bovine muscle samples.

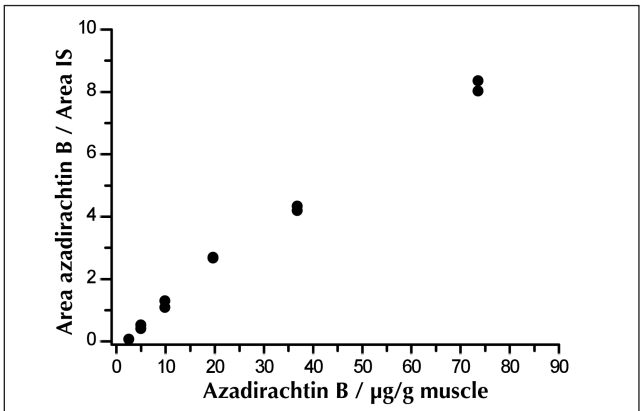

Figure 5. Response curve of azadirachtin B in bovine muscle samples.

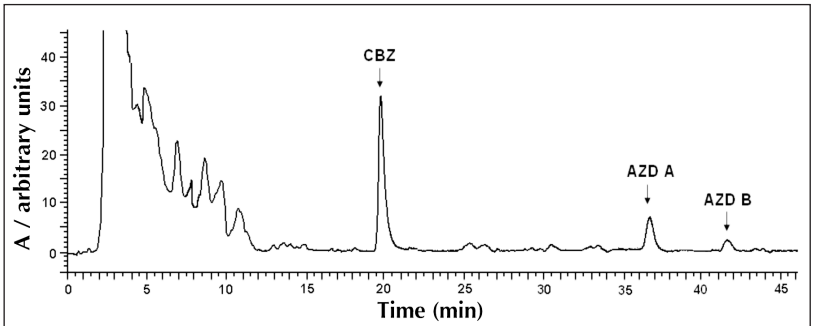

Figure 6. HPLC chromatogram of fortified sample with 20 µg/g muscle of CBZ, 10 µg/g muscle of AZD A, and 2 µg/g muscle of AZD B.

Table II. Summary of Method Precision and Accuracy Results

Compound	Theoretical conc. (µg/g muscle)	Recovery average conc.* (µg/g muscle)	Analytical recovery (%)	Relative error (%)	Coefficient of variation (%)
AZD A	45.5	45.7 (0.6)	100.4	–0.4	1.3
	170.6	171.5 (3.7)	100.5	–0.5	2.2
	343.2	343.0 (9.7)	99.9	0.1	2.8
AZD B	23.0	19.6 (0.1)	85.2	17.3	0.4
	37.1	36.8 (0.9)	99.2	0.8	2.4
	72.1	73.5 (2.1)	101.9	–1.9	2.9

*Based on three data points. Standard deviation is given in parentheses.

Conclusion

An isocratic reversed-phase HPLC method associated with UV–vis detection for determination of residues of AZD in bovine muscle has been developed and validated. The use of dichloromethane–isopropanol (95:5) as extraction reagent is effective for the extraction of AZD from this complex matrix. The chromatographic results show that the technique is simple, linear, sensitive, reproducible, precise, and accurate.

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