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Short communication

Development of a high-performance liquid chromatography method to monitor the residues of benzimidazoles in bovine milk

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ABSTRACT

A reversed-phase high-performance liquid chromatography with ultraviolet (UV) detection was developed that can determine 11 benzimidazole (BZDs) and 10 metabolites of albendazole, fenbendazole and mebendazole in bovine milk. Samples were extracted with acetonitrile and purified by mixed-mode cation exchange (MCX) solid phase extraction cartridges. LC separations were performed on a C_{18} column with gradient elution using acetonitrile and ammonium acetate solution. The UV-detection was set at 292 nm. The method is very sensitive to each analyte with limits of quantification (LOQs) of lower than 10 $\mu g\,kg^{-1}$. The recoveries of the BZDs and their metabolites spiked in milk were more than 78% with between-day relative standard deviation values less than 16% at the concentration of 10, 50 and 100 $\mu g\,kg^{-1}$. The method developed has been successfully applied to monitoring real samples containing BZDs, which demonstrated that it is a simple, fast and robust method, and could be used as a regulatory toll to determine the residues of BZDs in milk.

1. Introduction

Benzimidazoles (BZDs) (Fig. 1) including thiabendazole analogues and benzimidazole carbamates have a bicyclic ring system in their structures in which benzene is fused to the 4 and 5 positions of the heterocycle (imidazole) [1]. They are anthelminthic agents widely used for the treatment of parasitic infections in food-producing animals including cows due to their high efficiency [2]. The extensive use of BZDs in lactating cows could result in residues of them in milk and dairy products, which is harmful to consumers owing to their teratogenic and embryotoxic properties [3,4].

The marker residues of most BZDs are defined as the sum of the parent or/and its persistent metabolites [5,6]. For the effective control the residues of BZDs in foods, methods capable of detecting the complete range of marker residues of them should be applied. However, it is difficult to develop and maintain a robust method that covers all BZDs because they differ greatly in terms of physical and chemical properties. LC–MS/MS is most widely used for analysis of BZDs [7–10]. However, mass instrument are still quite expensive and not readily available to chemists in most laboratories. HPLC is the most widely applied method to detect the residues of veterinary drugs with naturally ultraviolet or fluorescence chromophores in foods. Fluorescence is more sensitive and selective [11,12], but does not have the same range of applicability as UV. As a result, UV is the most widely applied detector [13–18]. A HPLC method determining 10 BZDs in milk [18] was developed. However there are no published reports for simultaneously measuring 21 BZDs in milk samples by HPLC until now.

The sample preparation is the crucial procedure in an analysis. Solid phase extraction (SPE) is generally applied to clean milk samples. However, the use of reversed-phase octadecyl silica [9,12,13,15] and strong cation exchange (SCX) cartridges [17] in SPE does not have enough extraction efficiency for milk samples and can only extract a few BZDs.

The objective of the present work was to develop a HPLC method with UV-detection for the determination of 21 BZDs in bovine milk. A simple sample preparation and cleaning-up applied with MCX cartridge has been established. The method is simple, fast, robust, and suitable for monitoring the residues of BZDs in milk samples.

2. Experimental

2.1. Drugs and reagents

Analytical standards of albendazole (ABZ), thiabendazole (TBZ), fenbendazole sulphoxides (oxfendazole, OFZ), mebendazole (MBZ), and carbendazim (MBC) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cambendazole (CAM), 5-hydroxy thi-

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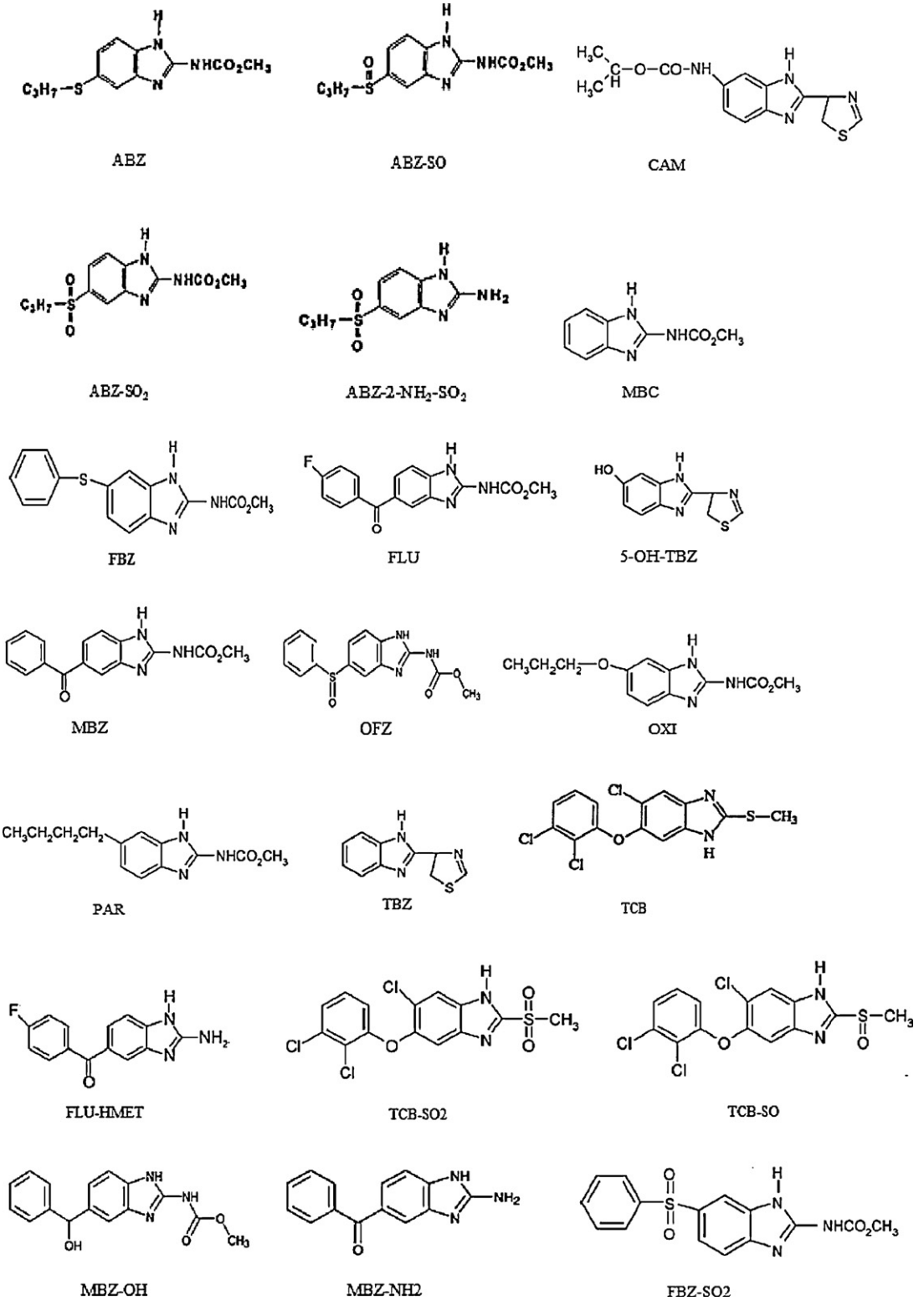

Fig. 1. Chemical structures of benzimidazoles.

abendazole (5-OH-TBZ), oxibendazole (OXI), flubendazole (FLU), fenbendazole (FBZ), and triclabendazole (TCB) were obtained from Dr. Ehrenstorfer (Gmbh, Augsburg, Germany). Albendazole sulphoxides (ABZ-SO), albendazole sulphone (ABZ-SO_2), albendazole-2-amino-sulphone (ABZ-NH_2-SO_2), amino mebendazole (MBZ-NH_2), hydroxy mebendazole (MBZ-OH), fenbendazole sulphone (FBZ-SO_2), hydroxyl-flubendazole (FLU-HMET) triclabendazole sulphoxide (TCB-SO), and triclabendazole sulphone (TCB-SO_2) were purchased from Witega (Belin, Germany). Parbendazole (PAR) was purchased from USP (St. Rockville, MD, USA).

Water was purified with a Milli-Q Plus apparatus (Millipore, Bedford, MA, USA). 2,6-Di-tert-butyl-4-methylphenol (BHT) was purchased from Sigma–Aldrich. 1% BHT was prepared by dissolving 1 g of BHT in ethyl acetate and making up to 100 ml. Methanol and

acetonitrile were HPLC-grade and obtained from Fisher (Bar-Bel, France). Other solvents of analytical reagent grade including glacial acetic acid, ammonium acetate, ammonium hydroxide, hydrochloric acid and anhydrous sodium sulphate were supplied by Shanghai Guoyao Company (Shanghai, China). The cartridge used for SPE was Oasis MCX (60 mg, 3 ml, Waters Corp., Milford, MA, USA). The other cartridge tested were SCX cartridge (500 mg, 3 ml, Agilent, Milford, HA, USA), Oasis HLB (60 mg, 3 ml, Waters Corp.).

2.2. Standard solutions

Individual stock standard solutions (100 $\mu g\,ml^{-1}$) of all analytes were made by dissolving each pure standard in methanol. A 1.0 $\mu g\,ml^{-1}$ mixed standard fortification solution was prepared by combining 1.0 ml of each stock standard and dilute to 100 ml with methanol. Stock and mixed standard fortification solution were prepared every 3 and 1 months and were stored at or below −20 °C.

2.3. Sample preparation

Milk (5 g) was transferred into a 50 ml centrifuge tube and spiked with the analytes. 1 ml 1% BHT solution, 10 ml acetonitrile and 1 g anhydrous sodium sulphate were added to the sample, and the mixture was vortexed for 1 min. After sonicated for 5 min at room temperature, the sample was centrifuged at 8470 × *g* for 10 min. The supernatant was transferred and evaporated to dryness in a water bath at 40 °C. The residues were re-dissolved in 3 ml acetonitrile, vortexed for 1 min and sonicated for 2 min. 3 ml 0.1 M hydrochloric acid was added and re-vortex-mixed.

The MCX cartridge was pre-conditioned with 3 ml methanol and 3 ml water. The entire extracts (6 ml) were loaded onto the SPE column. The column was washed with 3 ml 0.1 M hydrochloric acid and 3 ml methanol, then dried by purging air at the rate of 10 ml min^{-1} for 5 min. The analytes were eluted with 6 ml ammonium hydroxide–methanol (5:95, v/v) and evaporated to dryness in a water bath at 40 °C. The dry residue was dissolved in 1.0 ml acetonitrile–0.02 M ammonium acetate aqueous solution (10:90, v/v). The sample was shaken for 1 min and centrifuged at 10,200 × *g* for 10 min. The resulting solution was injected into HPLC systems.

2.4. HPLC-UV analysis

HPLC analysis was on a Waters 2695 HPLC system coupled with UV detector. The chromatographic separation was accomplished on a Waters XTerra C_{18} column (250 × 4.6 mm, 5 μm) maintained at 40 °C. Mobile phase component A was 0.02 M ammonium acetate, and component B was acetonitrile. The mobile phase gradient profile was as follows (t in min): $t_{0'}$, A = 80%, B = 20%; $t_{50'}$, A = 55%, B = 45%; $t_{60'}$, A = 30%, B = 70%; $t_{70'}$, A = 15%, B = 85%; $t_{75'}$, A = 15%, B = 85%; $t_{76'}$, A = 80%, B = 20%; $t_{80'}$, A = 80%, B = 20%. When the retention time was from 0 to 50 min, the flow rate was 0.9 ml min^{-1}, and from 50 to 80 min, the flow rate was 1.0 ml min^{-1}. The injected volume was 50 μl. The UV detector was set at a wavelength of 292 nm for all the compounds.

2.5. Method validation

The LOD and LOQ values of each analyte were considered as the concentration giving a signal to noise ratio of 3 and 10, respectively. Standard calibration curves were prepared using the mixed standard solution at concentration levels from 20 to 2000 $\mu g\,l^{-1}$. The method was further tested by matrix-match calibration curves which were made by fortified with the analytes at each of six concentrations from 10 to 200 $\mu g\,kg^{-1}$. The spiked samples were performed with complete extraction and purification procedure. The calibration curves were calculated using the linear least squares regression analyses of the peak area to concentration ratios. Accuracy and precision of the analytical method were calculated by the determination of six replicates of blank samples fortified at three different spiked levels (10, 50 and 100 $\mu g\,kg^{-1}$) over a period of 3 days. Recovery was performed by comparing the analytical results of extracted BZDs from fortified whole samples with matrix-match calibration curves. Intra-day precision was conducted on the same day. Inter-day precision was determined by repeating the study on 3 consecutive days.

For the study of specificity, 20 blank milk samples originating from different lots were analysed. The analytes were identified by matching peaks retention times with the values of the corresponding standard analysed under the same experimental conditions.

3. Results and discussion

3.1. Optimization of the HPLC conditions

Acetonitrile and methanol are usually used in the mobile phase for reversed-phase (RP) HPLC separation of various compounds. Therefore these two reagents were tested in this study. According to the structure of BZDs, the pH of the mobile phase may be a critical factor in achieving the chromatographic separation of BZDS studied. Several mobile phase additives such as acetic acid and ammonium acetate were comprehensively investigated. The maximum sensitivities and satisfactory separation of all analytes were achieved when using 0.02 M ammonium acetate buffer (pH 5) and acetonitrile as mobile phase. The gradient was optimized to provide the maximum separation possible in a minimum time period. Fig. 2 shows the LC-UV chromatogram of standard solution at the 20 $\mu g\,l^{-1}$ level.

3.2. Optimization of sample preparation

3.2.1. Selection of clean-up condition

The procedure of purification was necessary for animal matrices being rich in protein. SPE is a technique used to clean-up the analytes by using different cartridges. There were some papers reported to clean-up BZDs using C_{18} cartridges [9,12,13,15] and SCX cartridges [17]. According to the properties of the BZDs, Oasis HLB cartridges, SCX cartridges and Oasis MCX mixed-mode cartridges were studied with blank whole samples. When used HLB cartridges, there did not appear to give cleaner extracts and high recovery. After the SCX cartridges clean-up procedure, it was found to have no interference, but the recovery was still low. Because Oasis MCX mixed-mode cartridges have both hydrophobic and cation exchange retention properties, better recovery than with SCX cartridges might be obtained. The results showed that Oasis MCX was superior to the other cartridges in terms of good recovery and little matrix interference. During elution step, different solvent including methanol, acetonitrile and combinations of them with ammonium hydroxide were tested. The results showed that 6 ml ammonium hydroxide–methanol (5:95, v/v) provided the highest recovery and was finally selected for subsequent LC-UV analysis.

3.3. Validation of the analytical method

The LOD and LOQ of the examined BZDs extracted from milk were 3 and 10 $\mu g\,kg^{-1}$. The linearity of the standard mixtures was good for all analytes within the range from 20 to 2000 $\mu g\,l^{-1}$, as proved by the correlation coefficients being greater than 0.999 for all curves. Matrix-matched calibration standard curves were used to quantify the target analytes in milk and to make the method as accurate as possible. The correlation of the coefficient values were above 0.99 for all analytes within the range from 10 to 200 $\mu g\,kg^{-1}$. All calibration curves indicated excellent method reliability that

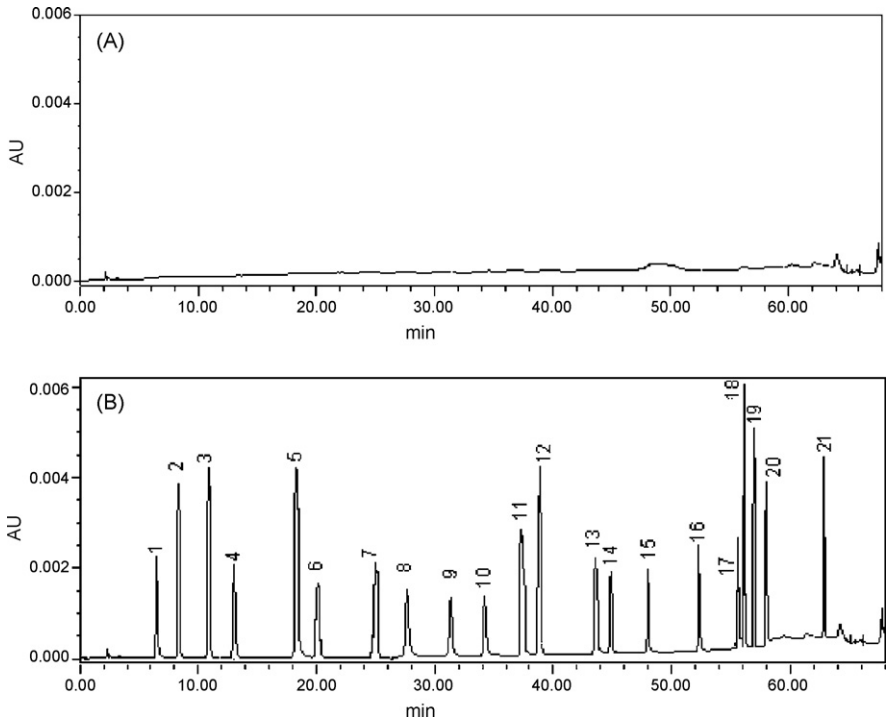

Fig. 2. LC-UV chromatograms of blank milk (A) and spiked with 10 μg kg^{-1} benzimidazoles (B).

could be used to accurately quantify trace amount of these analytes in milk.

The results of accuracy and precision data were summarized in Table 1. The mean recoveries of BZDs were ranged from 78% to 109%. The intra-day and inter-day values were all below than 16%, indicating a precise and accurate method for the determination of BZDs in milk.

After comparing with the background noise in various matrices, the results showed that there were no interference peaks that could be detected at the retention time of these target analytes (Fig. 2). It demonstrated that the method could be applied to monitor the residues of the different milk samples.

Table 1
The validation results of benzimidazoles in milk by LC-UV.

Compound	Recovery (%)	Intra-day RSD (%) $n = 18$	Inter-day RSD (%) $n = 54$
5-OH-TBZ	85–107	7–9	7–10
ABZ-NH_2-SO_2	95–106	5–9	7–11
ABZ-SO	91–105	4–8	5–10
MBC	92–108	6–9	6–10
TBZ	90–104	5–8	6–9
ABZ-SO_2	95–103	4–8	4–9
OFZ	88–108	6–10	7–10
MBZ-NH_2	91–110	5–9	5–10
MBZ-OH	89–101	4–9	5–9
FLU-HMET	85–105	5–11	7–10
FBZ-SO_2	88–108	8–12	8–14
CAM	91–96	5–7	5–7
OXI	86–98	6–9	5–10
MBZ	85–105	5–10	6–10
FLU	93–112	6–9	5–10
ABZ	95–102	5–7	6–10
PAR	83–108	5–8	7–10
FBZ	79–98	9–15	11–16
TCB-SO	80–103	8–11	9–14
TCB-SO_2	81–109	9–13	10–15
TCB	78–105	8–13	11–15

Note: The recovery, intra- and inter-day data showed are obtained in the analysis of the corresponding samples at 10, 50 and 100 μg kg^{-1}.

3.4. Application of the method

The developed method was applied to the analytes of 50 milk samples obtained from local dairy farmers. All samples were processed according to the method described. No detectable residues of the target analytes were found in any of the 50 samples.

4. Conclusions

A specific, sensitive and reliable multi-residues method for the determination of 21 BZDs in milk samples with HPLC-UV was developed. The method included a liquid–liquid extraction and a clean-up step by SPE. Polymeric Oasis MCX mixed-mode cation exchange cartridges made the method selective, reproducible, and fast. BZDs compounds were successfully separated by HPLC with the mobile phase of acetonitrile and ammonium acetate solution. The method has satisfactory validation characteristics with respect to specificity, accuracy, precision and sensitivity. Therefore, we conclude that this HPLC method is suitable for the routine determination of BZDs residues in milk.

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