

Determination of Ivermectin Residues in Bovine Liver Samples by ELISA and HPLC with Fluorescence Detection

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ABSTRACT

Background and Objectives: Ivermectin is an anti-parasitic medication frequently used in many food-producing animals. This study aimed to investigate the level of ivermectin residue in liver samples collected from slaughterhouses in Qazvin Province, Iran.

Methods: Overall, 88 bovine liver samples were randomly collected and analyzed for detection of ivermectin residues. The samples were analyzed for ivermectin contamination by enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC). The samples were extracted using liquid-liquid extraction procedure for the ELISA. Solid phase extraction using a C18 column followed by fluorescence-derivatized with 1-methylimidazole and trifluoroacetic anhydride in acetonitrile were used for the HPLC assay. Recovery values obtained from the HPLC method ranged from 81.3 to 92.5%, with a relative standard deviation of 6.7-12.2%.

Results: First, all samples were screened by the ELISA method. Based on the results, 16 samples (18.2%) contained no detectable levels of Ivermectin residue, while Ivermectin was found in 72 samples (81.8%). In addition, 22 of the positive samples (30.55%) contained high Ivermectin level (>50 ppb). Analysis of the samples by the HPLC method showed that eight samples (9.09%) contained ivermectin levels above the maximum residue limit.

Conclusion: This study demonstrates the presence of different levels of Ivermectin residue in bovine liver samples collected from the Qazvin Province in Iran. Therefore, regulatory authorities should ensure proper withdrawal period before slaughter of the animals.

Keywords: Ivermectin, Cattle, Liver, ELISA, HPLC.

INTRODUCTION

Ivermectin is a member of a group of naturally occurring macrocyclic lactones known as avermectins, which exhibits broad-spectrum anti-parasitic activity. It consists of a mixture of two homologues containing 22, 23-dihydro-2,6-dimethyl-2,3-dioxoleno[2,3-b]pyridine B_{1a} and 22, 23-dihydro-2,6-dimethyl-2,3-dioxoleno[2,3-b]pyridine B_{1b} (1). Highly sensitive analytical assays are required to determine the amount of drug residues in different matrices. In recent years, a number of chromatographic methods have been developed for determination of veterinary drug residues in different matrices such as liver, muscle, milk, serum, plasma and salmon (2-5). In addition, anti-ivermectin antibodies have been successfully used in immunoassay methods (6, 7). These immunoassay methods are more promising screening techniques for residue analysis compared to conventional physicochemical methods. Since no study has reported the level of Ivermectin in bovine liver samples in Iran, this study aimed to evaluate the Ivermectin residue in bovine livers in Qazvin Province, Iran. Liver was chosen as the target matrix since it contains the highest concentration of Ivermectin residue. Furthermore, maximum residue limit (MRL) for Ivermectin in liver has been reported to 100 ng/g (8, 9). High performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) were used to determine Ivermectin concentrations in liver samples. This study aimed to investigate the level of ivermectin residue in liver samples collected from slaughterhouses in Qazvin Province, Iran.

MATERIAL AND METHODS

In this study, 88 cattle liver samples (weighing 100g each) were obtained from slaughterhouses in the Qazvin Province between October and December 2014, using stratified purposeful sampling. Liver samples were transferred to a laboratory under suitable conditions, and analyzed immediately after preparation.

All chemicals used are of HPLC grade, unless stated otherwise. Ethyl acetate, methanol, acetonitrile, 1-methylimidazole and trifluoroacetic anhydride (99%) were purchased from Sigma-Aldrich (Chemical Co., St. Louis, Mo, USA). Sodium chloride, sodium sulfate anhydrous, glacial acetic acid

(Darmstadt, Germany). Ivermectin standard was obtained from Sigma Co. (St. Louis, MO, USA). The liquid-liquid extraction method was carried out for the ELISA. Briefly, one gram of homogenized liver samples was agitated using a vortex mixer with 1 ml of 0.5 M NaCl and 1 ml of 100% methanol. After vortexing for 1 minute, 5 ml of ethyl acetate was added to the mixture. After mixing for 10 minutes, the mixture was centrifuged at 2000 x g for 5 minutes. Then, 4 ml from the supernatant was transferred to a glass tube. Ethyl acetate was evaporated at 50 °C under a mild stream of nitrogen. The residue was dissolved in 100 µl of acetonitrile, and centrifuged at 2000 x g for 5 minutes. At the last step, 10 µl of the supernatant was diluted with 190 µl of sample dilution buffer, and then 50 µl of the mixture was used for ELISA. The ELISA technique was performed using EuroProxima Ivermectin ELISA kit (EuroProxima, Netherlands) according to the manufacturer's instructions. First, 50 µl from the antibiotic standards and the Ivermectin extracts was added to the bottom of each well in duplicate. Then, 25 µl of conjugate solution (ivermectin-HRP) and 25 µl of antibody solution were added to all wells. Addition of the stop reagent (containing 1 M sulphuric acid) in the presence of the considered antibiotics led to a color change from blue to yellow. The results were analyzed by photometry at 450 nm. It has to be noted that the limit of detection (LOD) for the kit was 4ng/g. Two grams of homogenized cattle liver samples was transferred into a 15 ml polypropylene tube. After adding 0.5 mL of methanol and 4 mL of acetonitrile, the tube was vortexed for 1 minute. The mixture was centrifuged at 4800 RPM for 10 minutes, and 2 ml of the supernatant was transferred to a new tube. Two mL of deionized water was added, and the tube was shaken gently. The supernatant was loaded into a C18 solid-phase cartridge at flow rate of 0.2 mL/min. The cartridge was previously activated by 5 mL of acetonitrile and 5 mL of acetonitrile/water (3/7, v/v, containing 0.1% triethylamine). The tube was washed with 1 mL of acetonitrile/water and passed through the C18 solid-phase cartridge for 1 min. The sample solution was extracted with 1 mL of acetonitrile/water (9/1, v/v) and collected in a tube containing 0.3 g of sodium sulfate

anhydrous. After mixing the solution, the tube was centrifuged at 4800 RPM for 5 minutes. The supernatant was transferred to a tube and then air-dried with nitrogen. Later, 0.1 mL of anhydrous acetonitrile was added to elute for the derivatization reaction (10, 11). Two reagents used for derivatization reaction, were freshly prepared with anhydrous and light protected conditions. Reagent (1): 1-methylimidazole and acetonitrile anhydrous mixed gently (1:1, v/v). Reagent (2): trifluoroacetic anhydride and acetonitrile anhydrous mixed gently (1:1, v/v)(10). The mobile phase was a mixture of 940 ml acetonitrile in 60 mL of water. Before use, the mobile phase was gently mixed and degassed by sonication for 5 minutes. After filtration, the filtrate was used as the mobile phase (10). Analysis conditions were as follows: Fluorescence detector: agitation wavelength 365 nm, emission wavelength 470 nm, and flow rate: 1.2 mL/min. For recovery test, 0.1 ml of working standard solution at concentrations of 50, 100, 200 and 500 ppb was added to one gram of liver sample, separately. Spike samples were extracted, purified and derivatized according to the extraction method described earlier. The samples were auto-injected into the HPLC instrument (2475 multi-wavelength fluorescence detector, Waters Co., USA) and analyzed in triplicate. LOD of this method was 2 ng/g. A stock solution of 0.05 mg/ml Ivermectin was prepared in acetonitrile. For the recovery experiments, adequate amounts from the stock solution were mixed with anhydrous acetonitrile, and diluted in different concentrations of the working standard solution. Then, 0.1 mL of each working standard solution was individually placed in capped tubes, and 0.2 mL of the reagent 1 and 0.2 mL of reagent 2 were added to each tube. After vortex mixing for 2 minutes, 10 μ L of and triethylamine were purchased from Merck

glacial acetic acid was added to the tube. After vortex mixing for 1 minute, the tube was placed in a sand water bath (60 °C) for 30 minutes, and then in an ice bath (0 °C) for 5 minutes. The mixture was filtered using 0.45 μ m filter membranes. Individual standard was analyzed with HPLC under the conditions mentioned earlier. Finally, standard curves were plotted using means of the area under the curves. The Student t-test was used to evaluate the differences between samples analyzed by ELISA and HPLC. P-values less than 0.05 were considered statistically significant.

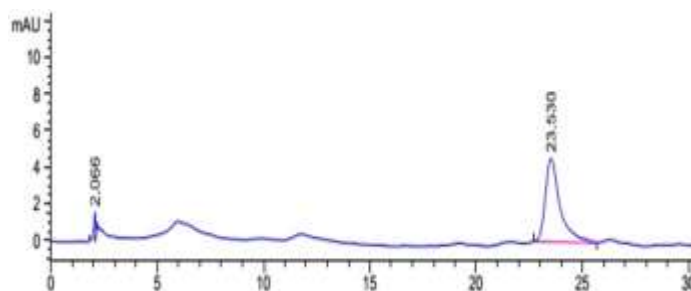
RESULTS

The analytical calibration curves were obtained with external standards based on injection of the standard solutions prepared in acetonitrile (Figure 1). Mean of values were used to obtain the analytical calibration curves by plotting the corresponding peak area using the analyte concentration (data not shown). The recovery rate and relative standard deviation (%RSD) of Ivermectin concentrations were analyzed in triplicate by different personnel at different times according to the mentioned procedures. The developed method had relatively good precision (Table 1). The LOD value for Ivermectin was found as 2.0 ng/g by determining the signal/noise ratio at 3:1. In this study, 23.8 % (n=21) of liver samples were taken from males and 76.2% (n=67) were from females. Among the 88 bovine liver samples, 16 (18.2%) contained non-detectable level of Ivermectin residue. Other samples contained different concentrations of Ivermectin residue (Table 1). Based on the results, 27 samples (30.7%) contained 0-10 μ g/kg ivermectin, 23 samples (26.2%) contained 11-50 μ g/kg Ivermectin and 14 samples (15.9%) contained 5-100 μ g/kg Ivermectin. Moreover, the concentration of Ivermectin in eight samples (9.09%) exceeded the MRL of 100 ng/g.

Table 1- The recovery values and RSD of ivermectin from spiked bovine liver samples (n=3)

Ivermectin concentration (μ g/kg)	Mean \pm SD of recovery (%)	RSD (%)
500	81.3 \pm 5.5	6.7
200	84.6 \pm 8.1	9.5
100	89.6 \pm 3.5	3.9
50	92.5 \pm 11.3	12.2
10	86.3 \pm 9.4	10.8

Figure 1- HPLC chromatogram of ivermectin standard solution (100 ppb)

Table 2- Prevalence and Mean \pm SD of ivermectin residues in bovine liver samples

Ivermectin residue level ($\mu\text{g}/\text{kg}$)	Number of samples	Prevalence	Percentage of contamination	Mean \pm SD
ND	16	0.18	18.2	0
0-<10	27	0.3	30.7	4.41 \pm 2.15
11-50	23	0.26	26.2	28.4 \pm 8.13
51-100	14	0.16	15.9	73.3 \pm 13.49
>100	8	0.09	9.09	137.7 \pm 16.05

ND=not detectable

DISCUSSION

The sensitivity of immunoassays should be high enough to allow the detection of drug residues below the MRL suggested in the current guidelines (12). It is essential to use real matrices as main sites of drug metabolism for the detection of drug residues in raw animal products. Based on our previous study, the impact of matrix on the results should not be neglected, especially in the case of meat and other solid matrices that have difficult preparation procedures. Therefore, the recovery values should always be calculated. Liver has been used in several studies as the main matrix for evaluation of Ivermectin level in meat. This is because the concentration of Ivermectin is higher in the liver tissue compared to meat. Consumer risk of raw animal products in Iran is unknown. There are currently limited number of studies in Iran on this subject, which are mainly focused on drug residues in raw milk, honey and chicken. In this study, the number of samples that had non-conforming values or values above the MRL of Ivermectin was small (9.09%), and the majority of samples (91%) contained Ivermectin residues lower than the MRL. Generally, drug residue contamination of animal source food is rare in developed countries e.g. prevalence rate of <1% in Europe (13). Several studies in the US during 2005-2007 detected non-conforming levels of Ivermectin residue in samples from various

cattle breeds. Between 2006 and 2009, the Canadian national chemical residue monitoring program did not report any non-conforming Ivermectin residues in either Canadian or imported cattle samples (14). Meat exporting countries are responsible for protecting both public health and market access by complying with the international regulations. These countries should focus on minimizing the occurrence of non-conforming residues of all potentially harmful chemicals such as Ivermectin, while using all available resources efficiently.

The underlying reason for the non-conformity of Ivermectin residue in some beef and bovine liver samples could be the indiscriminate use of Ivermectin for its anti-parasitic effects in fattened calves. The frequency of using Ivermectin in cattle depends on the farmer's and veterinarian's perception of the likelihood of parasite infestation, which tends to be seasonal (15, 16). Since the amount of Ivermectin used at different times of the year is expected to be variable, the results of the present study are not representative of populations slaughtered throughout the year. Use of veterinary drugs in accordance with the manufacturers' recommendations and in compliance with good veterinary practice could prevent occurrence of residues exceeding the established MRLs in tissues (17). In this study, only a small number of

samples (9%) contained non-conforming Ivermectin residues. This is consistent with a main objective of the national veterinarian drug residue monitoring program, which is to verify that non-conforming residues are low and the health of consumers is protected (18). We also recommend development of monitoring programs for evaluation of anti-parasitic drug residues in raw livestock products and identification of risk factors associated with Ivermectin residue. The findings of the present study confirmed that liver samples collected from slaughterhouses in the Qazvin Province contain different levels of Ivermectin residue. Some of these samples are not suitable for consumption since they contain Ivermectin residues higher than the recommended MRL. Further studies are necessary to determine the presence of other drug residues in liver and tissue samples and evaluate the risk of these residues considering

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daily intake and other related factors.

CONCLUSION

The method used in this study is a simple and efficient technique for the analysis of Ivermectin residues in liver and meat samples. This new and faster extraction method benefits from using two derivatization reagents during the extraction and produces less effluent. Moreover, quantification with a fluorescence detector is highly selective and sensitive, allowing the detection of 2.0 µg/L of Ivermectin.

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CONFLICT OF INTEREST

I have no conflicts of interest to declare.

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