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Original article

Anthelmintics residues in raw milk. Assessing intake by a children population

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Abstract

Anthelmintics, such as benzimidazoles and probenzimidazoles, are veterinary drugs used against endoparasites in food producing animals. A number of these drugs are considered responsible for embryotoxicity and teratogenicity. The residue levels of Albendazole, Febantel, Fenbendazole, Mebendazole and some of their metabolites (Albendazole sulphoxide, Albendazole sulphone, Fenbendazole sulfone) were assessed in 123 (42 goat, 69 sheep, 12 bovine) raw milk samples collected from all farms throughout Southern Greece. Sample analysis was performed by HPLC with Diode Array Detector. A high percentage (27.6%) of the samples examined was found to be positive for the investigated compounds. In 14 samples (11.4%), the residues' concentration exceeded the established Maximum Residue Limits.

Estimated Daily Intakes were calculated for a population of 723 children aged 10-12 years. Data on milk consumption were obtained by personal interview through a 7-day food frequency questionnaire. The maximum Estimated Daily Intakes for the anthelmintic residues, concerning raw milk, did not exceed the current Acceptable Daily Intake.

Key words: anthelmintics, benzimidazoles, milk, residue intake

Introduction

Several anthelmintics, such as benzimidazoles and probenzimidazoles, are used for the prevention of animal infestations caused by nematodes, cestodes and trematodes (McKellar and Scott 1990). Among the most popular benzimidazoles are Albendazole (ABZ) and Mebendazole (MBZ). Albendazole sulphoxide (ABZ-SO) is a metabolite of ABZ, which is also marketed as an anthelmintic on its own and can be further oxidized to Albendazole sulphone (ABZ-SO₂) (EMEA 1999). Febantel (FEB) is a probenzimidazole with similar use (EMEA 2004a), which is further metabolized *in vivo* to Fenbendazole (FBZ), a ben-

zimidazole anthelmintic also. Fenbendazole sulfone (FBZ-SO₂) is a metabolite of FBZ (EMEA 2004b). Despite their beneficial use, embryotoxicity, teratogenicity and other adverse effects in a variety of animal species can be caused by benzimidazoles or their metabolites (Higa et al. 1992, EMEA 2001, 2004a,b,c, Ramirez et al. 2001). Thus, considerable attention has been focused on the threat to human health stemming from consumed food of animal origin such as milk, cheese and butter. Maximum Residue Limits (MRLs) in certain products of animal origin, including meat and milk, but not for other dairy products, have been established by the European Union (EU 2010). For ensuring conformity with regu-

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lations and providing a check on compliance with good veterinary practices, monitoring of benzimidazole residues in food is needed. The need for more intensive residue controls becomes stronger considering several studies (Friar and Reynolds 1991, Fletouris et al. 1997a, Rose et al. 1997) which indicate that benzimidazoles are not degraded after microwave and oven-baking, storage at -18°C for three to eight months and after cooking. Furthermore, Cooper et al. (2011) refer no major losses for residues of ABZ, MBZ or FBZ, after roasting of meat and liver (40 min at 190°C) or shallow frying (muscle 8-12 min, liver 14-19 min) in a domestic kitchen. Consequently, conventional cooking hardly protects consumers against the ingestion of residues of anthelmintic veterinary drugs in these foods. Accordingly, the estimation of residues intake through certain food items consumption becomes a necessity to ensure that the Acceptable Daily Intakes (ADIs) of the drugs are not exceeded. To the best of our knowledge, only one previous study has investigated the occurrence of benzimidazoles in raw milk in Greece (Tsiboukis et al. 2010). In international level, Takeba et al. (2000) have published results concerning Triclabendazole (TCB) residues in bovine raw milk from Tokyo area. Concerning the residual intake of ABZ, ABZ-SO, ABZ-SO₂, MBZ, FBZ, FBZ-SO₂ and FEB through milk consumption, no previous studies have been performed in Greece or elsewhere. Blasco et al. have assessed the intake of the benzimidazoles carbendazim (2002, 2005, 2006) and thiabendazole (2006), through fruit consumption in Spain. The aim of the present study was to determine concentration levels of ABZ, ABZ-SO, ABZ-SO₂, MBZ, FBZ, FBZ-SO₂ and FEB in raw milk samples (of bovine, goat and sheep origin) collected from animals in Southern Greece during spring and autumn of 2008. An assessment of children's daily intake of these compounds was attempted by utilizing data concerning milk consumption in Greece. Possible public health concerns are discussed.

Materials and Methods

Sampling

During spring and autumn of 2008, one milk sample was taken from each farm existing in southern Greece. Finally 123 milk samples were collected. Sampling was conducted in nine prefectures of Achaia, Iliia, Korinthia, Arcadia, Argolida, Messinia, Lakonia, Fokida and Attica. The milk samples concerned goat, sheep and cattle farms producing and supplying milk to the markets and dairy food producers. No samples were taken from farms producing

milk for domestic use only. All farms were officially monitored by the veterinary authorities and were approved for the production and distribution of raw milk. Raw milk samples were taken directly from the frigorific tank on the farm immediately after the milking and before the collection of milk by bulk tankers. Namely, they were collected as pooled samples from the whole animals' milking in the farm and not from selected animals. The samples were placed in sterilized polyethylene boxes and transported the same day into an isothermal container at 4°C at the Laboratory of Public Health (Medical School, University of Patras) and stored at -20°C until analysis.

Sample preparation

Analysis of milk samples was based on the method described by Su et al. (2003) previously applied in our laboratory (Tsiboukis et al. 2010). In brief, 5 mL of milk were mixed with 1 mL buffer solution (saturated NaHCO₃ pH 10), 1 mL BHT (6-di-tert-butyl-4 methylphenole) and 15 mL acetonitrile, shaken for 5 min and centrifuged at 3500 rpm for 5 min. The supernatant was transferred into a separation funnel. The procedure was repeated once more. The mixed supernatants, along with 30 mL acetonitrile saturated with n-hexane, were shaken for 5 min and the acetonitrile layer was collected into a concentration bottle in which 5 mL of 1-propanol were added. The solution formed was evaporated to dryness at 40°C using a rotary evaporator and reconstituted with 5 mL of 10% methanol. For the clean-up procedure, the reconstituted residue was passed through a LiChrolut RP-18 (40-63 mm) cartridge, preconditioned with 5 mL of methanol and then with 5 mL of 10% methanol. The concentration bottle was washed twice with 4 mL acetonitrile and the solution was passed through the cartridge. The formed eluate was collected in a different concentration bottle and was evaporated to dryness at 40°C using the rotary evaporator. The residue was reconstituted with 2 mL solution of acetonitrile-methanol-0.02M sodium dihydrogen phosphate (1:1:3, v/v) and filtered through a 0.2 mm cellulose filters.

HPLC analysis

Albenazole (ABZ 98%), Febantel (FEB 99%) and Mebendazole (MBZ 98%) were purchased from Neochema (Bodenheim, Germany), Fenbendazole (FBZ 99.0%), Albenazole sulfoxide (ABZ-SO 98.5%) and Albenazole sulfone (ABZ-SO₂ 99%) were purchased from Laboratory Dr. Ehrenstorfer (Augsburg,

Table 1. The analytical effectiveness of method at different levels of milk samples fortification.

Analyte	LOD ($\mu\text{g} \cdot \text{L}^{-1}$)	LOQ ($\mu\text{g} \cdot \text{L}^{-1}$)	Recovery Range %	RSD % (n=12)	Measurement uncertainty
MBZ	10	30	89.4-95.2	3.2-7.8	$0.028 \cdot C^a + 1.095$
ABZ	15	45	82.2-88.2	4.0-8.1	$0.032 \cdot C + 0.760$
ABZ-SO	10	30	97.2-107.6	4.2-8.5	$0.040 \cdot C + 1.250$
ABZ-SO ₂	10	30	95.0-98.8	2.5-6.5	$0.023 \cdot C + 1.020$
FEB	5	15	84.3-90.1	5.0-7.6	$0.043 \cdot C + 0.488$
FBZ	15	45	85.4-89.1	5.9-11.2	$0.051 \cdot C + 0.903$
FBZ-SO ₂	15	45	91.7-96.4	3.6-6.5	$0.033 \cdot C + 0.770$

^a Concentration level ($\mu\text{g} \cdot \text{L}^{-1}$)

Germany). Finally, Fenbendazole sulfone (FBZ-SO₂), with purity greater than 99%, was purchased from Witega Laboratorien Berlin-Adlershof GmbH. All substances were in solid phase. Intermediate standards of $50 \text{ mg} \cdot \text{L}^{-1}$ for each compound were prepared by appropriate dilution in pure methanol for ABZ, ABZ-SO, ABZ-SO₂, FBZ and FBZ-SO₂, in dimethyl sulfoxide for MBZ and in acetonitrile for FEB. Mixed working standards containing the 7 compounds at concentrations of 20, 50, 100 and $2000 \mu\text{g} \cdot \text{L}^{-1}$ were prepared in acetonitrile-methanol- NaH_2PO_4 (1:1:3) solution. The determination of the compounds of interest was achieved by an HPLC system (Varian 9010 equipped with diode array detector Polychrom 9065). The separation column used was a Pursuit 5 mm C18 $250 \cdot 4.6 \text{ mm}$. The gradient profile is reported in a previous study (Tsiboukis et al. 2010). The flow rate was $1 \text{ mL} \cdot \text{min}^{-1}$ and the injection volume was $50 \mu\text{L}$.

Method quality assurance

Single standard solutions were analyzed to obtain a library containing the UV spectra of ABZ, ABZ-SO, ABZ-SO₂, MBZ, FBZ, FBZ-SO₂ and FEB at wavelengths ranging from 190 to 367 nm. Standard curves were constructed at four concentration levels (20, 50, 100 and $2000 \mu\text{g} \cdot \text{L}^{-1}$). The confirmation of the presence of an investigated compound was achieved by comparing both retention time and spectra in a wavelength range of 220-344 nm. Matches only above 99% were accepted. Chromatograms of milk samples were presented at 292 nm. The signal to noise (S/N) ratio was set at four for the determination of the Limit of Detection (LOD), while the Limit of Quantification (LOQ) was $3 \cdot \text{LOD}$. Recoveries were determined at 4 spiking levels of the 7 substances in 5 mL of milk (0 as blank and 20, 50, 100 and $2000 \mu\text{g} \cdot \text{L}^{-1}$) in triplicate. Detection limits at 292 nm were estimated to be $5 \mu\text{g} \cdot \text{L}^{-1}$ for FEB, $10 \mu\text{g} \cdot \text{L}^{-1}$ for

MBZ, ABZ-SO and ABZ-SO₂ and $15 \mu\text{g} \cdot \text{L}^{-1}$ for ABZ, FBZ and FBZ-SO₂. Every 10 samples, a fortified milk sample at the level of $50 \mu\text{g} \cdot \text{L}^{-1}$ was used as a quality control check. Intra- and inter-day variability was 1-2% and 3-4%, respectively. Method performance data is provided in Table 1.

Milk consumption assessment

The milk consumption data concerning children population of Western Greece were obtained from a previous study (Roumelioti and Leotsinidis 2009) of our laboratory in order to calculate the Estimated Daily Intake (EDI). In brief, a validated seven-day food frequency questionnaire was used to assess the milk and other food items consumption of children aged from 10 to 12 years old. The filling in of this questionnaire was achieved by personal interviews which took place in elementary schools in the area of interest. These schools were randomly selected and the total number of personal interviews was 723.

Results

A total of 123 milk samples (42 goat, 69 sheep, 12 bovine) were tested for the seven anthelmintic residues. The number of positive samples and the frequency of residues occurrence per milk type is shown in Table 2. A percentage of 27.6% of milk samples was found to be positive for the seven anthelmintic substances. The concentrations of the anthelmintic residues in positive milk samples along with the corresponding MRLs and the number of violative samples, i.e. exceeding the MRLs, are presented in Table 3. It should be noticed that the MRLs concerning ABZ, ABZ-SO and ABZ-SO₂ are calculated as the sum of all three compounds expressed as ABZ (EMEA 2004c). For comparison purposes, the concentrations of the determined compounds are also presented in terms of $\mu\text{g} \cdot \text{kg}^{-1}$.

Table 2. Occurrence of anthelmintic residues in milk samples.

Milk Type	Samples n		Compound Frequency n (%)						
	Total	Positive (%)	FBZ	FBZ-SO ₂	ABZ	ABZ-SO	ABZ-SO ₂	MBZ	FEB
Goat	42	9 (21.4)	n.d. ^a	n.d.	n.d.	1 (2.4)	4 (9.5)	n.d.	6 (14.3)
Sheep	69	21 (30.4)	n.d.	n.d.	n.d.	2 (2.9)	8 (11.6)	1 (1.4)	12 (17.4)
Bovine	12	4 (33.3)	n.d.	n.d.	n.d.	n.d.	1 (8.3)	n.d.	3 (25.0)
Total	123	34 (27.6)	n.d.	n.d.	n.d.	3 (2.4)	13 (10.6)	1 (0.8)	21 (17.1)

^a not detectable

Table 3. Concentration of anthelmintic residues in positive milk samples and the corresponding MRLs.

Analyte	Samples n			Mean value (µg · L ⁻¹) (Range)	^a Mean value (µg · kg ⁻¹)	MRLs (µg · kg ⁻¹)
	Total n	Positive n (%)	Violative n (%)			
MBZ	123	1 (0.8%)	–	22.8	22.1	–
ABZ	123	0	–	21.1	18.8	100
ABZ-SO	123	3 (2.4%)	–	(12.0 – 37.9)	(as ABZ)	(as ABZ)
ABZ-SO ₂	123	13 (10.6%)	–	22.2		
				(11.1 – 70.5)		
FEB	123	21 (17.1%)	14 (11.4%)	20.7	20.1	10
				(5.0 – 71.8)		
FBZ	123	0	–			10
FBZ-SO ₂	123	0	–			10

^a Specific Gravity of milk: 1.030 kg · L⁻¹

Within the positive samples, three (2 goat, 1 sheep) contained simultaneously FEB and ABZ-SO₂, and were collected from Fokida, Argolida and Achaia during spring. One sample of sheep origin was recorded containing MBZ and FEB and was collected from Ilia during spring. All in all, out of the 34 contaminated milk samples, 21 contained FEB, 13 ABZ-SO₂, three ABZ-SO and one MBZ. ABZ, FBZ and FBZ-SO₂ were not detected in any sample. Apart from the one sample found with MBZ, other 13 samples (10.6%) were found containing FEB at concentrations exceeding the current MRL. ABZ-SO and ABZ-SO₂ were not detected in concentrations exceeding the MRL (Table 3).

As it can be seen from Table 2, the presence of anthelmintic drugs was not related to milk type ($p=0.548$, Fisher's exact test).

There was a noticeable geographical variation among the nine prefectures regarding the occurrence

of these substances in milk samples. For example, 78% of milk samples from Messinia were contaminated, but only 10% of milk samples from Attica contained these substances. The corresponding percentages were 16.7% for Argolida, 17.6% for Arcadia, 19.1% for Achaia, 58.3% for Ilia, 28.6% for Korinthia, 20% for Lakonia and 50% for Fokida.

The occurrence of positive samples is not affected by the season. Actually, 25 of 90 (27.7%) samples were positive during spring and 9 of 33 (27.2%) during autumn ($p=0.956$, Pearson χ^2 test).

Milk consumption data, as they were drawn from a previous study concerning dietary habits of children aged 10-12 years, are presented in Table 4. By combining the data given in Table 4 with the mean anthelmintic concentrations in the contaminated milk samples, it is possible to calculate the Estimated Daily Intakes (EDIs) of these compounds through milk consumption, and compare these with the corresponding

Table 4. Weekly and Daily Intake of milk in children (10-12 years old) according to the frequency questionnaire.

Glasses of milk	Population sample, %	Mean n of glasses consumed weekly	^a Quantity of milk consumed weekly (mL)	Mean n of glasses consumed daily	Quantity of milk consumed daily (mL)
0	2.9	0	0	0	0
1-5	5.5	4.23	1057	0.60	150
6-10	42.5	7.83	1957	1.12	280
>11	49.1	14.82	3705	2.12	530
Total	100.0	10.83	2707	1.55	387

^a A glass of milk is equal to 250 mL.

Table 5. ADIs and EDIs expressed as $\mu\text{g} \cdot \text{kg}^{-1}$ body weight/day and EDIs expressed as percentage of the ADIs for the detected compounds.

Body weight (kg) mean <i>sd</i>	Quantity of milk consumed daily (kg)	ABZ milk mean concentration ($\mu\text{g} \cdot \text{kg}^{-1}$)	ABZ-EDI ($\mu\text{g} \cdot \text{kg}^{-1}$ b.w.)	ABZ-EDI (as % of ^a ADI)	FEB milk mean concentration ($\mu\text{g} \cdot \text{kg}^{-1}$)	FEB-EDI ($\mu\text{g} \cdot \text{kg}^{-1}$ b.w.)	FEB-EDI (as % of ^b ADI)
50.8 13.3	0		0	0		0	0
47.1 9.8	0.154	18.8	0.061	1.23	20.1	0.066	0.94
44.4 10.8	0.288		0.122	2.44		0.130	1.86
41.8 10.4	0.546		0.245	4.91		0.262	3.75

^a ABZ-ADI = $5 \mu\text{g} \cdot \text{kg}^{-1}$ b.w. (EMEA 2004c)

^b FEB-ADI = $7 \mu\text{g} \cdot \text{kg}^{-1}$ b.w. (EMEA 2004a)

ADIs (Table 5). The EDI of each compound is calculated by multiplying the mean residue level with the daily milk consumption and dividing by the mean body weight per each class of milk consumption.

Discussion

The present study deals with the occurrence and the concentration levels of seven anthelmintic residues in milk destined for human consumption and an assessment of the children's daily intake of these drugs through milk and milk products' consumption.

ABZ was not found in any sample, indicating that its administration is employed properly and the necessary time between the drug administration and the milk withdrawal, is followed. Contrary, its metabolites were found, but in concentrations varying from nearly the limit of detection ($10 \mu\text{g} \cdot \text{L}^{-1}$) up to $70.5 \mu\text{g} \cdot \text{L}^{-1}$. Fletouris et al. (1997a) have shown that ABZ-SO₂ needs 156 h for elimination. Considering that the statutory withdrawal time is 3 d (72 h), it is not surprising that ABZ-SO₂ has been found in high frequency in milk. Nevertheless, no violation of the established MRL was reported, suggesting that in the

case of ABZ administration, the good veterinary practice is followed in the study area.

In only one sample MBZ was found in concentration between the LOD and the LOQ. Even if its quantitation is doubtful, the presence of MBZ is surprising since the specific compound is not for use in animals from which milk is produced for human consumption, but it is authorized as a constituent of medicated feed (EMEA 2001). This is the reason why there is no established MRL for MBZ. A species not authorized to receive MBZ, is possible to ingest it through cross-contamination in feed mills. Remaining quantities of medicated feed containing MBZ could be kept at various points along the production line, contaminating subsequent batches of feed processed (De Ruyck 2003).

The parent compound FEB was identified in 21 samples and with concentrations from the limit of detection through $71.8 \mu\text{g} \cdot \text{L}^{-1}$, that is seven times above the current MRL. This finding along with the absence of its two metabolites, FBZ and FBZ-SO₂, could be attributed to a short time span between the drug administration and the milk withdrawal, since the withdrawal time, which is the minimum time needed between the last recommended drug administration and

the time of collection of milk, allows the veterinary drug and its residues to decrease to levels below the established MRL (De Ruyck 2003). The finding becomes even more important considering that according to Delatour et al. (1983) the parent FEB and three of its metabolites need 45 to 65 h post-treatment to reach non detectable levels ($<5 \mu\text{g} \cdot \text{L}^{-1}$), while in 12 h post-treatment FEB and the three metabolites are present. At this time point, FEB is the compound with the higher concentration. All the violations reported in the present study concern FEB residues, a fact revealing a repeated negligence in compliance with the statutory withdrawal periods before putting the milk on the market.

An important issue that arises is the absence of cooperation between stock-farmers and veterinary doctors. The present study indicates that areas that are located at distance from the urban centers and with deficiently staffed food and veterinary agencies, such as Messinia and Ilia, have a relatively raised percentage of violative samples. This finding suggests that the farmers are not informed about the possible toxic significance of the drugs and have not access to the veterinary experts to take advice about the recommended drug application and the proper withdrawal time. According to De Ruyck (2003), violations of MRLs are the result of improper use of veterinary drugs or illegal use of unlicensed substances. Poor treatment records, failure to identify treated animals and uppermost inconsistency between the product label and the drug application are factors contributing to the residue problem. The stock-farmers should be notified about the drugs they use and their possible effects and should be trained in order to comply with the legislation.

The present study indicates that the EDIs, expressed as percentage of the corresponding ADIs, are relatively low (up to 3.74% for FEB and 4.90% for ABZ residues). However, what is of greater concern is the total dietary intakes of anthelmintics taking into account not only milk consumption but also dairy products and meat. Several studies indicate that the concentration levels of ABZ (De Liguoro et al. 1996, Fletouris et al. 1997b, 1998) and TCB residues (Imperiale et al. 2011) in a dairy product, e.g. cheese, are higher than those in the contaminated milk used for its production. In sequence, dairy products originated from contaminated milk are expected to contain increased concentrations of the investigated substances. Furthermore, there are not established MRLs for anthelmintics in the EU concerning cheese, butter or any other milk products (EU 2010), which is a legislation gap that results in the lack of the corresponding controls.

Additionally, the current status of MRLs and

ADIs, for anthelmintic residues, has been established on the basis of observations in laboratory animals (EMEA 1999, 2001, 2004a,b,c). In the light of studies which refer to adverse effects and high toxicity of anthelmintics to humans (Capleton et al. 2006, Nuray et al. 2007, Oztas et al. 2007), there is a possibility of MRLs and ADIs re-evaluation. Food safety assessments are based on scientific data, which clearly has its limitations (Botsoglou and Fletouris 2000). Rules for setting international food standards must respect consumer needs and consumer protection should be their main goal. It is consumers who eat food, who pay for it, and who face the impact of the risks related to evaluation procedures (Botsoglou and Fletouris 2000). Thus food legislation gaps should be minimized in order to assure the production of dairy products with no residues of such substances or at least at "safe" levels.

In conclusion, taking into account the increased number of positive milk samples reported in the present study, we suggest firstly that organized campaigns must take place to inform the stock-farmers about the anthelmintics drugs and educate them about the good veterinary practice and secondly the food authorities should inform consumers with detailed lists of the residues found in milk, violative or not.

Although the consumption of contaminated milk does not lead in exceedance of the ADIs, in view of the number of positive findings and taking into account a) the variety of benzimidazoles residues food sources, b) their tendency to be increased during cheesemaking and c) the lack of further toxicological studies and detailed studies for products such as butter, we suggest a stricter frame for dairy products. Education of the farmers and access to advisory veterinary agencies together with intensified and updated monitoring programs for these veterinary drugs should be implemented to ensure the safest residue levels not only in dairy foods, but also in all foods of animal origin, so as to avoid a possible public health concern.

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