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Determination of albendazole-2-aminosulfone in bovine milk using high-performance liquid chromatography with fluorometric detection

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ABSTRACT

An analytical method for the determination of albendazole (ABZ) residues in bovine milk was developed using one of its major metabolites, albendazole-2-aminosulfone (ABZ2NH2) as the marker. The method involved acid hydrolysis of milk followed by liquid–liquid extraction and solid–liquid phase clean-up of the extract. A reversed-phase HPLC with fluorometric detection was used to quantitate the marker residue. The method exhibited a high degree of precision and good accuracy as demonstrated by a relative standard deviation (R.S.D.) $<5\%$ for the replicate analyses and 91.8 to 104.1% recovery of the fortification level (25–200 ng/ml), respectively. The ratio of the concentrations of the marker and total residues in milk over a 36–120 h withdrawal period was found to be steady at 43.1 indicating a definite relationship between the marker and the total residues of ABZ. The analytical method was used successfully to determine total residues in milk of cattle treated with ABZ.

INTRODUCTION

Albendazole (ABZ) is a broad spectrum anthelmintic which has been widely used for treatments of parasitic infections caused by gastrointestinal roundworms, lung worms, tapeworm and liver flukes in domestic animals [1]. The pharmacological activity of albendazole is believed to be attributable to its bioactivation of albendazole-sulfoxide [2,3]. With the growing concern about drug residues in foods of animal origin, analytical methods for the quantification of drug residues in edible tissues and milk have played an important role in determining the safe use of drugs.

A number of analytical methods are available for the determination of albendazole residues in edible tissues and milk [4,5]. Two analytical methods, determinative and confirmatory, had been developed by SmithKline Beecham Animal Health (SB Animal Health) for the quantification of the ABZ marker metabolite, albendazole-2-aminosulfone (ABZ2NH2), in liver tissue of cattle. In the determinative assay, HPLC with fluorometric detection was used in the quantification of the marker metabolite. The confirmatory portion involved detection of *tert.*-butyldimethyl (*t*-BDMS) derivatives of the marker by GC–MS with multiple ion detection. The methods have been approved by the U.S. Food and Drug Administration for the detection of ABZ2NH2 at or above 200 ng/g levels in liver tissues of cattle [6,7]. The determinative portion of the method has been applied successfully at SB Animal Health to determine ABZ2NH2 residues in muscle and kidney tissues at 100 and 300 ng/g levels,

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respectively. A multiresidue method for determination of a number of benzimidazoles (62.5–2000 ng/ml) in milk has been reported in the literature [6]. The method has been applied to quantitate albendazole residues in milk at 250 ng/ml.

The main objective of this study was to develop and validate an analytical method for the determination of an albendazole marker metabolite in milk of treated cattle at or around the expected maximum residue level at the time of withdrawal. The total residue level of milk three days after treatment with ABZ (10 mg/kg body weight) is below 100 ng/ml. Accordingly, the sensitivity of the method must be adequate (< 100 ng/ml) to detect marker residues in milk at withdrawal periods longer than three days.

EXPERIMENTAL

Apparatus

A modular HPLC system (Model 1050, Hewlett-Packard, Palo Alto, CA, USA) consisting of a solvent delivery system and an autosampler was used. A reversed-phase ODS-Econosphere column, 5 μm, 250 mm × 4.6 mm I.D. (Alltech Associates, Deerfield, IL, USA) and a RP 18 guard column, 10 μm, 40 mm × 4.6 mm I.D. (Brownlee Labs, Santa Clara, CA, USA) were used for chromatography. A fluorescence detector (Model LS-4, Perkin-Elmer, Norwalk, CT, USA) with excitation and emission wavelengths set at 300 and 320 nm, respectively, was used to detect the signal response. The detector was linked via an interface box (PE Nelson 1200, PE Nelson, Cupertino, CA, USA) to a personal computer for data transfer. The data acquisition and processing were performed by TurboChrom 3 software (PE Nelson, Cupertino, CA, USA). The HPLC mobile phase consisted of an isocratic solvent system: aqueous 0.02 *M* KH_2PO_4/0.01 *M* diethanolamine–methanol–acetonitrile (6:3:1, v/v). A mobile phase flow-rate of 1.1 ml/min was used for all the analyses unless specified otherwise. Typical injection volume was 20 μl. A liquid scintillation analyzer (Models 2200 CA or 2500 TR, United Technologies Tri-Carb, Meriden, CT, USA) was used for radio assay of the samples. Typically, a 5-ml milk sample was assayed in 10 ml of Insta-gel after dark adaptation. An IEC centrifuge (IEC Model K with a rotor #250, Internation Equipment Company, Needham Heights, MA, USA) was used in sample preparation.

Chemicals

Uniformly ring labeled [^{14}C]albendazole, albendazole-2-aminosulfone [5-(propylsulfonyl)-1H-benzimidazol-2-amine] (SK&F 81038), the internal standard [(5-(butylsulfonyl)-1H-benzimidazol-2-amine] (the butyl congener of albendazole-2-aminosulfone) (SK&F 101437), albendazole sulfoxide (SK&F 77664) [Methyl (5-(propylsulfinyl)-1H-benzimidazol-2-yl)carbamate] and albendazole sulfone (SK&F 63896) [methyl (5-(propylsulfonyl)-1H-benzimidazol-2-yl) carmabate] were obtained from SB Animal Health repository (SB Animal Health, 1600, Paoli Pike, West Chester, PA, USA). The chemical structures of the compounds are given in Fig. 1.

Ethyl acetate, acetonitrile and methanol were of high purity grade solvents obtained from Baxter Burdick & Jackson, Muskegon, MI, USA. Mega Bond Elut SCX (strong cation exchange) SPE (solid phase extraction) cartridges were obtained from Varian Associates, Harbor City, CA, USA. Methyl sulfoxide, *ortho*-phosphoric acid (85%) and potassium phosphate monobasic were HPLC grade chemicals obtained from Aldrich, Milwaukee, WI, USA. Ammonia and sodium hydroxide were reagent grade chemicals purchased from Mallinckrodt, St. Louis, MO, USA. Milli-Q water was generated by the Milli-Q water purification system. All glassware was pre-silanized using 1% Prosil-28 obtained from PCR, Gainesville, FL, USA. The KH_2PO_4 buffer (pH 3.5) was prepared by adjusting the pH of a 10 m*M* solution of the phosphate with 5 *M* H_3PO_4.

Animal husbandry – dosing procedure – sample collection

Three Holstein cows in mid-lactation were used for the study after three weeks of acclimatization. Two of the three animals received a single oral dose (10 mg/kg body weight) of [^{14}C]alben-

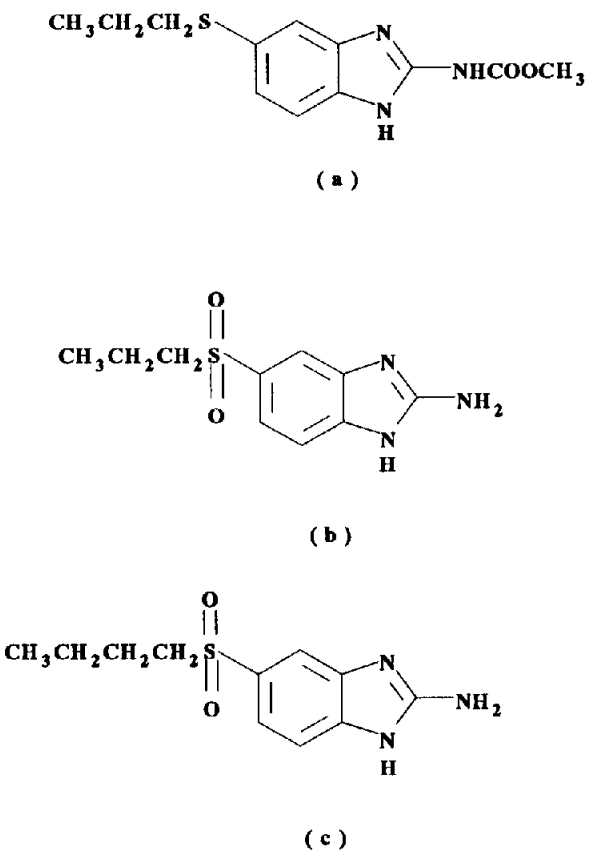

Fig. 1. Structures of (a) albendazole (ABZ), (b) albendazole-2-aminosulfone (ABZ2NH2), and (c) the butyl congener of ABZ2NH2 (internal standard).

dazole (specific activity = 1.79 μCi/mg) contained in a gelatin capsule. The third animal was used as the control. Animals were allowed access to food and water in sufficient quantities as determined by the established procedures at SB Animal Health. The animals were milked at 12 h intervals before and after the administration of the drug until sacrifice. Milk collected prior to drug administration was used as the blank during method development. A representative sample (1 liter) from each collection period was kept frozen (-20°C) until analysis.

Sample preparation

A DMSO solution of the internal standard (100 ng/ml) was added to milk (10 ml) in a culture tube (15 mm × 200 mm), followed by the addition of 5 *M* H_3PO_4 (10 ml). The sample was capped, vortex-mixed for 30 s, and then hydrolyzed at 110 ± 4°C in an oven for 1 h. After cooling the tube to room temperature, the pH of the hydrolyzed milk was adjusted to 5–7 by adding NaOH pellets (4 g) slowly. To avoid foaming and thermal decomposition of the metabolites, the tube was immersed in an ice–water bath during the addition of NaOH. Acetonitrile (20 ml) was then added and the contents were vortex-mixed for 30 s and centrifuged at 213 *g* for 10 min. The top acetonitrile layer was transferred via a pipette to a conical/round bottom flask (200–300 ml). This step was repeated twice, and the acetonitrile extracts were combined and evaporated *in vacuo* to approximately 5 ml using a rotary evaporator. The concentrated extract was then transferred quantitatively to a centrifuge tube (50 ml) by rinsing the flask with 0.006 *M* H_3PO_4 acid (3 × 4 ml). The pH of the solution was adjusted to 1.5–3 using 5 *M* H_3PO_4 (*ca.* 200 μl) followed by centrifugation at 213 *g* for 10 min.

Solid phase clean-up

The supernatant after the centrifugation step was transferred to a Mega Bond Elut SCX cartridge (1 g or 6 ml) which had been prewashed with methanol (5 ml) and KH_2PO_4 buffer, pH 3.5 (10 ml) in succession. To ensure quantitative transfer of the residues, the centrifuge tube (50 ml) was rinsed with KH_2PO_4 buffer (5 ml) and the rinsate was transferred to the SPE cartridge. The cartridge was washed under gentle vacuum (138–173 kPa) with acetonitrile (5 ml), ethyl acetate (5 ml) and methanol (5 ml), successively. Finally, ABZ2NH2 was eluted with ammonia–water (20:80, v/v; 15 ml) under gentle vacuum. Ethyl acetate (15 ml) was added to the eluate and the liquid phases were vortex-mixed to facilitate partitioning of ABZ2NH2 into the organic layer. The ethyl acetate layer was carefully removed via a pipette and transferred into a fresh centrifuge tube (50 ml). This procedure was repeated twice and the combined ethyl acetate extract was evaporated to dryness under dry nitrogen gas using a N-Evap analytical evaporator. The residue was reconstituted in the HPLC mobile phase solution

(500 μl) and filtered directly into the HPLC autosampler vials using Bioanalytical System filter units fitted with 0.2 μm regenerated cellulose filters.

Marker metabolite identification

Milk samples collected at 24, 36, 48, 72, 96 and 120 h after treatment were analyzed by HPLC following extraction and clean-up to determine the metabolic profiles. The metabolism of albendazole in cattle, sheep, rats and mice is described elsewhere [8]. The major metabolites are the sulfoxide (ABZSO) and sulfone (ABZSO2) of albendazole and albendazole-2-aminosulfone. Authentic standard solutions of these major metabolites were also analyzed for the purpose of establishing their retention times. The HPLC conditions were similar to those already described except that a 300 mm × 3.9 mm I.D. μBondapak C_{18} column (Waters, Milford, MA, USA) and a flow-rate of 1.5 ml/min were used. After injecting the extracts of milk from various collection periods, the HPLC effluent was fractionated at 15 s intervals and assayed by liquid scintillation counting (LSC). The metabolic profiles at different collection periods were obtained by plottong LSC data (dpm) *versus* retention time (min). The ratio, concentration of a metabolite/total residues, for each collection period (24–120 h) was determined. The total residues were determined as [^{14}C]albendazole and/or its equivalents by assaying milk collected at various sampling periods by LSC. The metabolite with a steady ratio of concentrations at different collection periods was designated as the marker metabolite.

Calibration

Two types of calibration curves, a standard curve and an analytical curve, were made during the study for quantification. To generate a standard curve, mobile phase solutions of ABZ2NH2 concentrations (encompassing 25–200 ng/ml) and the internal standard (100 ng/ml) were analyzed by HPLC. To obtain an analytical curve, blank milk samples were fortified with ABZ2NH2 (encompassing 25–200 ng/ml) and the internal standard (100 ng/ml). The samples were then taken through the whole analytical procedure as described above prior to HPLC analysis. Each standard solution injected contained a mixture of the marker and the internal standard. The purpose of generating two calibration curves during the study was to evaluate the use of a standard curve in a situation where blank milk samples are not readily available to construct the analytical curve.

Quantification by HPLC

An internal standard ratio method was used for quantification of ABZ2NH2 in milk samples. The calibration curves were constructed by plotting, on linear axes, the peak area ratio *versus* the respective ratio of concentration (ng/ml) for each standard solution. For example, a linear calibration curve was generated by plotting peak area ratios ($\text{marker}_{\text{area}}$/$\text{internal standard}_{\text{area}}$) *versus* the concentration ratios ($\text{marker}_{\text{concentration}}$/$\text{internal standard}_{\text{concentration}}$). At least five reference points encompassing the expected concentration of the marker were analyzed prior to the analysis of unknown milk extracts. The concentration of the marker in the unknown sample was calculated by multiplying its concentration ratio ($\text{marker}_{\text{concentration}}$/$\text{internal standard}_{\text{concentration}}$) corresponding to the area ratio ($\text{marker}_{\text{area}}$/$\text{internal standardf}_{\text{area}}$) from the curve with the fortification level of the internal standard.

Validation of the method

The analytical method described above was validated by fortifying control milk samples with ABZ2NH2 at 25, 50, 100 and 200 ng/ml and with the internal standard (100 ng/ml). Five replicate samples at each fortification level were assayed to evaluate the accuracy and precision of the method. Duplicate HPLC injections were made for each sample. A standard curve, used for quantification, was constructed by analyzing HPLC mobile phase fortified with ABZ2NH2 (25 to 200 ng/ml) and the internal standard (100 ng/ml). The fortifications were made immediately prior to analysis.

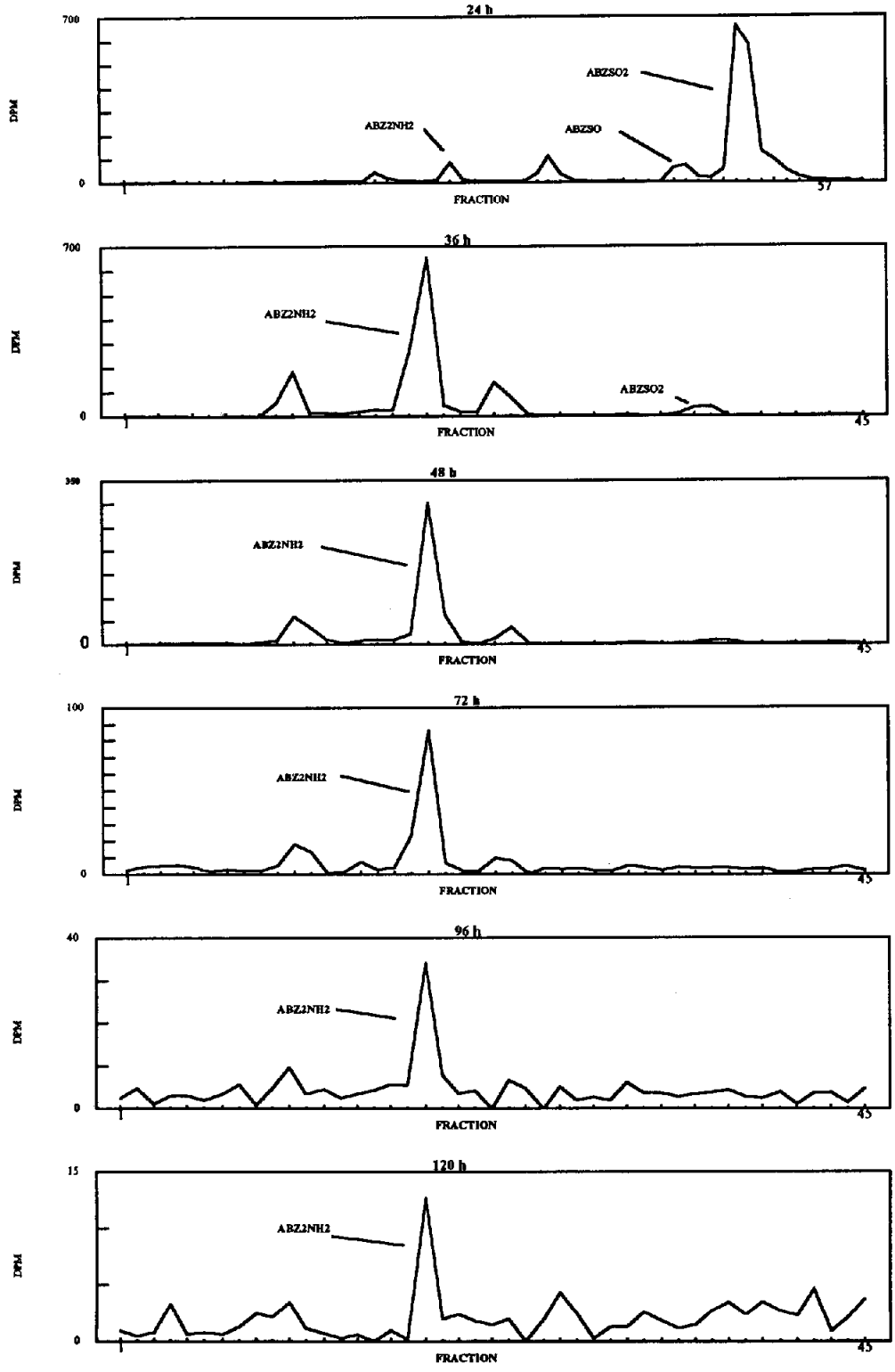

Fig. 2. Metabolic profiles of milk at various sampling periods.

Application of the method to milk from treated cattle

The method was also applied to quantify the marker metabolite in milk samples collected from cattle at 36 and 48 h after treatment with albendazole. Five replicates at each time point were analyzed. Duplicate HPLC injections were made for each replicate. The ABZ2NH2 in sample extracts was quantified using both standard and analytical curves.

RESULTS AND DISCUSSION

The metabolic profiles of ABZ in milk over a period of 120 h indicated that ABZ2NH2 is the predominant metabolite after 36 h following treatment (Fig. 2). The concentration ratio, concentration of ABZ2NH2/total residues, was relatively uniform with an average of 43.1 (σ = 9.4, n = 6) over the 36- to 120-hour sampling period after drug treatment (Table I). Accordingly, ABZ2NH2 was designated as the marker metabolite.

The results of the method validation indicated that the milk samples fortified at levels 25 to 200 ng/ml with ABZ2NH2 were detected with an average relative error of −3.7% when the standard curve was used for quantification (Table II). Good repeatability of the analysis at all levels (five replicates at each level) was demonstrated by the average relative standard deviation (R.S.D.) of 3.4%. A representative chromatogram of a milk extract fortified with ABZ2NH2 and the internal standard is given in Fig. 3. The retention times of ABZ2NH2 and the internal standard were 6.5 and 10.8 min, respectively. The average recoveries of ABZ2NH2 and the internal standard were 75.8 and 78.7%, respectively. The limit of detection for ABZ2NH2 was estimated to be 8 ng/ml which corresponds to a signal re-

TABLE I

RELATIONSHIP BETWEEN MARKER METABOLITE AND TOTAL RESIDUES

Collection period (h)	Total residues (ng/ml)	Marker concentration (ng/ml)	Ratio
120	10.2	3.0	29.4
96	17.3	9.6	55.5
72	47.3	21.3	45.0
48	164.0	67.3	41.0
36	462.5	206.0	44.5
24	2687.2	576.2	21.4
Average (excluding the 24 h collection period)			43.1
S.D.			9.4

TABLE II

SUMMARY OF RESULTS FOR THE VALIDATION OF THE REGULATORY METHOD FOR MILK

Fortification level[a] (ng/ml)	Average concentration found (ng/ml)	Relative standard deviation (%)	Relative error (%)
25	26.0	4.6	4.1
50	46.9	4.6	−6.2
100	91.8	3.1	−8.2
200	191.1	1.5	−4.4
Average		3.4	−3.7

[a] Five replicates were fortified with ABZ2NH2 and the internal standard at each level.

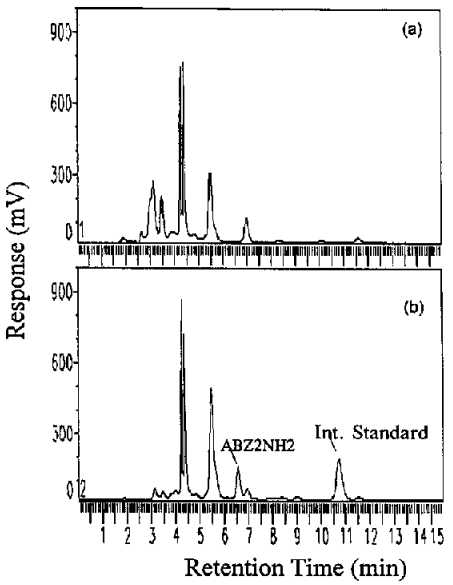

Fig. 3. Representative chromatograms of extracts: (a) control milk and (b) milk fortified with ABZ2NH2 (100 ng/ml) and the internal standard (100 ng/ml).

TABLE III

SUMMARY OF RESULTS DEMONSTRATING THE UTILITY OF THE REGULATORY METHOD

Sample I.D.[a]	Total ^{14}C residues[b]	ABZ2NH2 found (ng/ml)		Total residues calculated[c] (ng/ml)	
		Analytical curve	Standard curve	Analytical curve[d]	Standard curve[e]
392, 48 h	171.4	68.7 ± 4.2	63.1 ± 3.8	159.4	146.4
393, 48 h	164.0	64.7 ± 2.4	59.4 ± 2.2	150.1	137.8
393, 36 h	462.5	184.0 ± 3.5	167.9 ± 3.5	426.9	389.6
392, 36 h	468.0	192.2 ± 14.3	175.3 ± 13.0	445.9	406.7

[a] 392 and 393 are animal I.D.s.

[b] Total ^{14}C residues in milk were determined by radio assay methods.

[c] Total residues in milk were calculated using the ABZ2NH2/total residue ratio of 43.1.

[d] The average under estimation of the total residues was 7%.

[e] The average under estimation of the total residue was 15%.

sponse of three standard deviations over the mean of the blank.

The analysis of milk samples collected from animals at 36 and 48 h after treatment with ABZ demonstrated the practicality of the method. Both standard and analytical curves were used for quantification. Results of the quantification using these two curves are shown in Table III. Once again, good analytical repeatability was evident for all the analyses (R.S.D. <8%). The results obtained from the standard curve were slightly lower than those derived from the analytical curve. A similar trend was observed in the method validation study where results obtained for ABZ2NH2 using the standard curve were 4.4 to 8.2% lower than the nominal fortification levels between 50 and 200 ng/ml. The deviation is more apparent when the ABZ2NH2 values were converted to total residues in milk using the ratio of 43.1 (ABZ2NH2/total residues). The total residues are underestimated on the average by 15% when calibrated against the standard curve compared to 7% when an analytical curve was used. Thus it is apparent that the use of an analytical curve is the preferred method for quantification. However, if blank milk is not readily available, with an appropriate correction, a standard curve may be used to quantify ABZ2NH2 in milk.

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REFERENCES

1 V. J. Theodorides, R. J. Guyrik, W. D. Kingsbury and R. C. Parish, *Experientia*, 32 (1976) 702.
2 P. Delatour and R. Parish, in A. G. Rico (Editor), *Drug Residues in Animals*, Academic Press, New York, 1986, p. 175.
3 Q. A. McKeller and E. W. Scott, *J. Vet. Pharmacol. Therap.*, 13 (1990) 223.
4 R. T. Wilson, J. M. Groneck, A. C. Henry and L. D. Rowe, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 56.
5 A. R. Long, L. C. Hsieh, M. S. Malbrough, C. R. Short and S. A. Barker, *J. Assoc. Off. Anal. Chem.*, 72 (1989) 739.
6 J. Markus and J. Sherma, *J. AOAC Int.*, 75 (1992) 1129.
7 J. Markus and J. Sherma, *J. AOAC Int.*, 75 (1992) 1135.
8 R. J. Gyurik, A. W. Chow, B. Zaber, E. L. Brunner, J. A. Miller, A. J. Villani, L. A. Petka and R. G. Parish, *Drug Metab. Disp.*, 9 (1981) 503.