Journal of Chromatography B, 878 (2010) 2013–2017

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Development and validation of an HPLC–FLD method for milbemectin quantification in dog plasma

Qianqian Xu [a], Wensheng Xiang [a], Jichang Li [a,*], Yong Liu [b], Xiaolei Yu [a], Yaoteng Zhang [a], Mingli Qu [a]

[a] College of Veterinary, Northeast Agricultural University, 59 Mucai Street, Xiangfang District, 150030 Harbin, China
[b] Heilongjiang Administration for Entry-Exit Inspection and Quarantine, 150030, Harbin, China

ARTICLE INFO

Article history:
Received 3 February 2010
Accepted 24 May 2010
Available online 31 May 2010

Keywords:
Milbemectin
HPLC–FLD
Dog plasma
Derivative

ABSTRACT

Milbemectin is a widely used veterinary antiparasitic agent. A high-performance liquid chromatography with fluorescent detection (HPLC–FLD) method is described for the determination of milbemectin in dog plasma. The derivative procedure included mixing 1-methylimizole [MI, MI-ACN (1:1, v/v), 100 μL], trifluoroacetic anhydride [TFAA, TFAA-ACN (1:2, v/v), 150 μL] with a subsequent incubation for 3 s at the room temperature to obtain a fluorescent derivative, which is reproducible in different blood samples and the derivatives proved to be stable for at least 80 h at room temperature. HPLC method was developed on C18 column with FLD detection at an excitation wavelength of 365 nm and emission wavelength of 475 nm, with the mobile phase consisting of methanol and water in the ratio of 98:2 (v/v). The assay lower limit of quantification was 1 ng/mL. The calibration curve was linear over concentration range of 1–200 ng/mL. The intra- and inter-day accuracy was >94% and precision expressed as % coefficient of variation was <5%. This method is specific, simple, accurate, precise and easily adaptable to measure milbemycin in blood of other animals.

1. Introduction

Among canine intestinal parasites, *Toxocara canis*, *Echinococcus granulosus*, *Ancylostoma* spp., *Giardia* spp. and *Cryptosporidium* spp., have received great attention due to their zoonotic potential [1]. Anthelmintics to those parasites had developed from metals or plant extracts to phenothiazine, piperazine, thiabendazole and levamisole, the benzimidazoles and finally the macrocyclic lactones (include avermectins and milbemycins) [2]. The macrocyclic lactones have selective toxicity to nematodicidal activity and no to their mammalian hosts.

Milbemectin (Fig. 1a and b) is a minor member of a group of analogues containing a 25-isopropyl substituent was isolated from *Streptomyces hygroscopicus* subspecies *aureolacrimosus*, which consists of a mixture of milbemycin A_3 and milbemycin A_4 at a 30:70 ratio [3,4], and the closely related chemical structure toavermectins [5–7] leads to its insecticidal activities against important pests, as has been found with the avermectins abamectin and emamectin [8–11], it was mainly used as an acaricide/insecticide for plant protection [12]. Milbemycin oxime A_3 and A_4, a related molecule, launched as a parasiticide for the control of *Dirofilaria immitis* [13] and other nematodes and arthropods. Members of pharmacology lab (NEAU, Harbin China) tested the activity of milbemectin to *Toxocara canis*, *Ancylostoma caninum* and *Dirofilaria immitis*, and it was effective both in vitro and in vivo. It is necessary to develop a detection method to study pharmacokinetics of milbemectin in animals.

The most suitable methods at present for determination of ML residues would appear to be LC fluorescence or LC–MS/MS [14,15]. Although recently a number of LC–MS/MS methods have been described for the detection of avermectins and milbemycins, the limit of detection (LOD) and quantification is not more satisfactory than the fluorescence detection which is still the most commonly applied detection technique [16–19]. Chou et al. determined milbemectin A_3 and milbemectin A_4 in bovine muscle [20] and Yoshii and coworkers developed a simultaneous analytical method for determining milbemectin in crops [21] using HPLC–FLD. To the best of our knowledge, the method has not been used in the plasma samples. The objective of the present work was to develop a HPLC–FLD method, considering the pre-column derivatization in blood and fluorescence detection, for the HPLC analysis of milbemycin in dog plasma.

2. Experimental

2.1. Chemicals and reagents

Milbemectin A_3 [10 ng/μL in acetonitrile (ACN)] and A_4 (10 ng/μL in ACN) were purchased from Wako Chemicals (Rich-

* Corresponding author. Tel.: +86 045155190674.
E-mail address: lijichang828@sina.com (J. Li).

1570-0232/$ – see front matter.
doi:10.1016/j.jchromb.2010.05.037

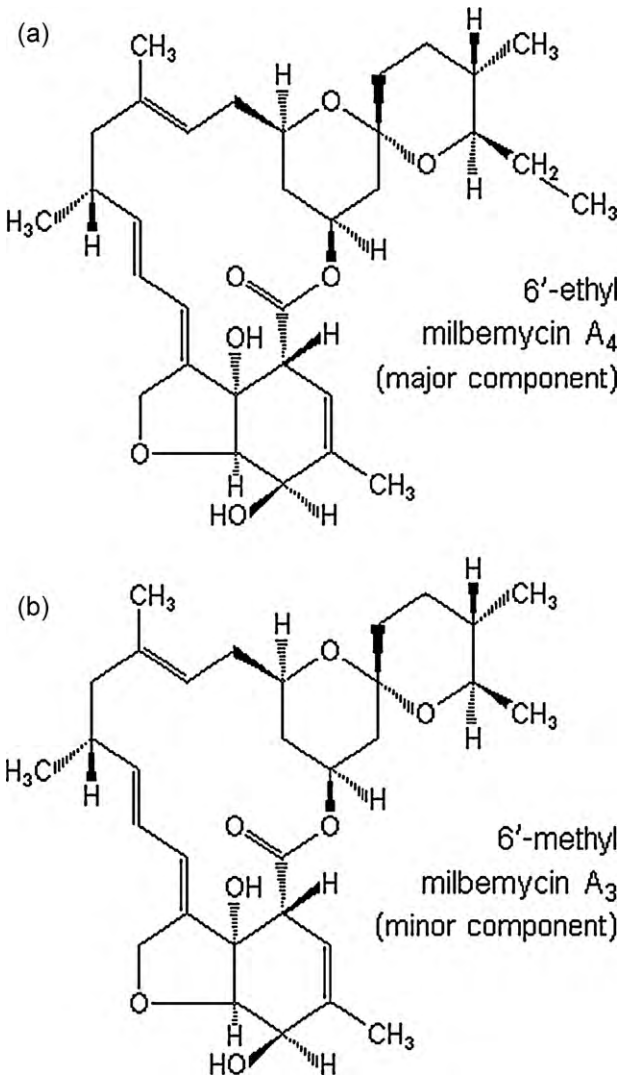

Fig. 1. Structures of milbemectin [milbemycin A_4 (a) and milbemycin A_3 (b)].

mond, VA, USA), trifluoroacetic anhydride (TFAA) (GC-grade), and N-methylimidazole of analytical-reagent grade, used in the derivatization process, were purchased from Aldrich (Sigma-Chimie, St. Quentin Fallavier, France). HPLC grade ACN and methanol were from Merk (Darmstadt, Germany). Deionized water for HPLC was prepared using Milli Q50 (Millipore, Bedford, MA) water purification system. Prepacked cartridges (Supelclean LC_{18}, 200 mg, 3 mL) for solid phase extraction were supplied by Sigma–Aldrich. For the preparation of in-house quality control and calibration samples, dog plasma was collected from normal dogs.

2.2. HPLC chromatographic conditions

Quantitative analysis of milbemectin in dog plasma was performed using an HPLC–FLD analytical system. The separation of compounds was carried out using an Agilent binary system consisting of an Agilent 1200 well plate auto-sampler fitted with a 20 μL sample loop, a quaternary pump, a column oven and a Model RF551 fluorescence detector. Chromatographic software HP ChemStation was used for data collection and processing. Separations were performed using elite C_{18} analytical column, 4 mm × 250 mm (elite, Dalian, China) packed with 5 μm particle size.

The fluorescence intensity was measured on a PerkinElmer luminescence spectrometer equipped with a xenon lamp and a Dell model 110 L, computer working with WinLab software. All the measurements were performed in a 10 mm pathlength quartz cell thermostated at 25 ± 0.5 °C, with 5 nm band-widths both emission and excitation monochromators.

Four mobile phases were tested for the elution step onto the analytical column based on acetic acid (0.2%)–methanol–ACN (8:30:62; v/v/v), 100% methanol, methanol–water (95:5, v/v), and methanol–water (98:2, v/v). The mobile phases were delivered at a constant 1.0 mL/min flow. Fluorescence detection was performed at an excitation wavelength of 365 nm and emission wavelength of 475 nm.

2.3. Extraction and cleanup

Blood samples were centrifuged for 15 min at 3000 rpm, and the supernatant was filtered through a Minisart plus syringe filter (0.2 μm pore size, Supelo) to remove remaining blood elements and high molecular weight proteins. 3 mL ACN was added to 1 mL of plasma and 1 mL of water, mixing for 20 min, and centrifuging at 2620 × *g* for 5 min, the supernatant was used.

The supernatant (≤5 mL) was manually transferred into a tube which was then placed on the appropriate rack of a Benchmate II (Hopkinton, MA, USA). Automatic sample preparation was performed as follows. Condition of the cartride: the column, positioned on the holder, was first conditioned with 3.0 mL of methanol and 3.0 mL of water (flow-rate 6 mL/min). Loading of the plasma sample: all of the supernatant was applied to the cartridge (flow-rate 3.0 mL/min). The cartridge was washed with 2 mL of water followed by 1 mL of water–methanol (75:25, v/v) at a flow-rate of 3.0 mL/min before elution, the cartridge was dried with nitrogen for 10 s (flow-rate 6.0 mL/min), then, 3.0 mL of methanol was applied to the cartridge at a flow-rate of 3.0 mL/min and the elute was collected.

2.4. Derivatization

The eluate was evaporated to dryness under a gentle stream of dry nitrogen at 50 °C in a water bath. The derivatization was started by adding 100 μL MI-ACN (1:1, v/v), then 150 μL TFAA-ACN (1:2, v/v) was added to the mixture, resulting in an exothermic reaction, coloring of the solution and the release of fumes. Exclusion of daylight for 30 s, the solution was evaporated for 10 min under a gentle stream of dry nitrogen at 50 °C in the water bath, 1 mL mobile phase was added, vortexed for 1 min, filtered through a Minisart plus syringe filter (0.2 μm pore size, Supelo) to remove the dopant, transferred into sample vials and analyzed using HPLC with fluorescence detection. The optimized procedure was tested in blood samples fortified with 1 ng/mL of milbemectin (milbemycin A_3: milbemycin A_4 = 30:70). Subsequently, the stability of the derivatised sample extracts was tested by storing for 80 h at room temperature with exclusion of daylight.

2.5. Calibration curve

Stock (1000 ng/mL) and substock (500 ng/mL) solutions of milbemectin (milbemycin A_3: milbemycin A_4 = 30:70) were prepared in ACN. A total of seven milbemectin concentrations (1, 2, 5, 10, 40, 80, 100, 150, and 200 ng/mL) in drug-free plasma were used as calibrators and three in-house quality control standards (QCs), containing 2, 10 and 150 ng/mL of milbemectin were used to estimate the accuracy and precision of the assay. All the stock and diluted stock solutions, calibrators and QC standards were stored at −80 °C until being used.

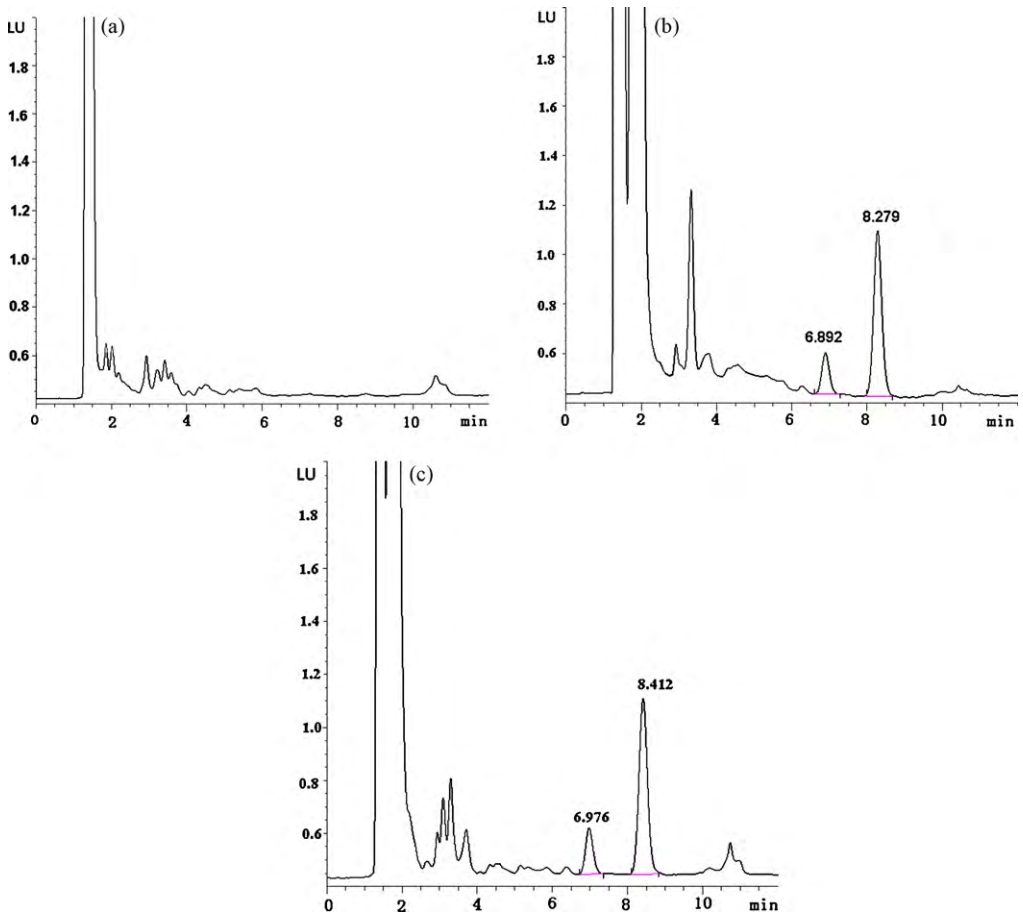

Fig. 2. Typical chromatograms: (a) blank plasma; (b) standard of milbemectin (milbemycin A_3: milbemycin A_4 = 30:70) (5 ng); (c) plasma sample fortified with 5 ng/mL of milbemectin(milbemycin A_3: milbemycin A_4 = 30:70).

2.6. Method validation

For selectivity, analysis of blank samples of the biological matrix (blank perfusion) was obtained from six different animals and each was tested for interference and selectivity was ensured at LOQ level for the drug.

Quality control samples were prepared by spiking of substock solution in the blank perfusion fluid. Accuracy was determined at three different concentrations of QC samples (2, 40, 150 ng/mL) each in five replicates. Similarly, precision was measured using five determinations per concentration for all QC samples. Intra- and inter-assay precision was measured by determinations at a particular day and also at three consecutives days.

The analytical recovery of milbemectin was assessed by comparing the peak area ratio of QCs extracted from plasma with the peak area ration of reference standards prepared in the same way at the stock solution.

Stability of milbemectin after freeze and thaw cycles was determined at all three QC concentrations in triplicate. The QC samples were frozen at −20 °C for 24 h and then thawed unassisted for next 24 h, this cycle was repeated three times before analysis. Bench-top stability of milbemectin in the blank matrix was determined by holding separate QC samples at above three concentrations and in triplicate at room temperature for 24 h. Samples were analyzed thereafter with the same method, and comparing the accuracy against freshly prepared stock solution. Stability of the stock solution stored at −20 °C for seven days and subsequently for 6 h at room temperature was also determined. Stock solution was spiked in blank perfusion fluid as mentioned above to produce three QC concentrations in triplicate and analyzed against the QC samples prepared from fresh stock solution.

2.7. Application of the method

Tablets of milbemectin (1 mg, tablets were made in pharmacology lab of NEAU, China) were used for oral administration in two healthy beagles (5.1 kg and 5.0 kg) with no clinical sighs of either acute or chronic illness. The trial was started after an overnight fast which continued for another 4 h after dosage. Blood samples were taken by jugular vein puncture 1 h, 2 h and 4 h after administration of a single dose of 1 mg table. Blood specimens were stored at −20 °C until analysis. After reconstitution, the samples were treated in the same procedure as for the plasma calibration line.

3. Results and discussion

3.1. Method development

The validation of avermectins [22–24] and milbemycins [25] in animal blood and tissues has been reported based on microbiology, immunoassay, HPLC and liquid chromatography–tandem mass spectrometry (LC–MS/MS) [26–31]. Only Chou et al. reported determination of milbemectin A_3, and milbemectin A_4 residues in bovine muscle using HPLC with fluorescence detection [20].

In the extraction portion, modified method of De Montigny et al. [32] was used, while the amounts of ACN, and water or NaCl were tested. That is protocol A: 1 mL ACN was added to 1 mL of plasma and 0.25 mL of water; protocol B: 3 mL ACN was added to

1 mL of plasma and 1 mL of water; protocol C: 3 mL ACN was added to 1 mL of plasma and 0.5 g NaCl, mixing for 20 min, and centrifuging at 2620 × *g* for 5 min. The recovery were protocol B > protocol A > protocol C, so protocol B was selected, 3 mL ACN was added to 1 mL of plasma and 1 mL of water, mixing for 20 min, and centrifuging at 2620 × *g* for 5 min, the supernatant was used.

The derivate of milbemectin was stable, the Alvinerie [33] method were used here. This method was easier compared with Chou et al. used for determination of milbemectin A_3 and A_4 in bovine muscles, which contained 60 °C sand bath for 30 min and 0 °C ice bath for 5 min [20].

Mobile phase composition used in the test was methanol–water (98:2, v/v), which was different from the reference [acetic acid (0.2% in water)–methanol–ACN (4:15:31, v/v/v)] [25], and the two peak separate perfectly.

3.2. Method validation

Fig. 2a–c shows the typical chromatograms of blank dog plasma and a plasma sample collected from a dog 0.5 h after oral administration of milbemectin (1 mg/kg). The derivatives of milbemectin was well resolved and had retention times of 10.03 min. There was no chromatographic interference to the derivatives of milbemectin from endogenous compounds or reaction by-products.

Calibration curve for the milbemectin was prepared in the range of 1–200 ng/mL. These concentration ranges were selected on the basis of drug concentration anticipated while analyzing the samples in the above conditions, The concentration–peak area relationships were described by simple regression analysis and analytical procedure was in a given range to obtain the test results which were directly proportional to the concentration (amount) of analyte in the defined range for the samples, i.e. correlation coefficient of $r^2 = 0.9992$ for the plot of concentration versus response (peak area). Standard curve was constructed and regression parameters, range, slop and intercept were determined as shown in Table 1.

Accuracy and precision are the closeness between experimental and true value, and closeness amongst experimental value with multiple monitoring of same sample, respectively. Both are the prerequisite for determining the concentration of any unknown sample concentration. Three concentration level quality control (QC) samples ($n = 5$) were used to measure accuracy and precision of the method. The percent recovery with the drug, which is an indication of accuracy, did not deviate more than 5% from true value at all three concentrations with %R.S.D. of 3.25%. All the values are within the limits prescribed by FDA-CDER guidelines for bioanalytical method validation. %R.S.D. of inter-day and intra-day of slopes for each standard curve obtained within day (intra-assay) and between three consecutive days (inter-assay) proved intermediate precision of inter-day and intra-day validation (Table 1). In conclusion, the method was judged to be accurate and precise for intended purpose and minor variation like time and day did not affected the analysis.

Determinations at LOQ were found accurate (105.22%) and precise (3.85%) for milbemectin. All the values complied with the specified limits.

Freeze–thaw, storage at room temperature of samples and stock solution did not affect the stability of the samples. The accuracy and %R.S.D. values for all stability samples are given in Table 1 and all of them are under the required limits.

3.3. Application of the method to real samples

The method was applied to determination of mean plasma concentration of milbemectin 1, 2, 4 h after oral administration of 1.0 mg of tablets to four healthy dogs. The results of analysis were given in Table 2.

Table 1
HPLC method validation parameters for determination of milbemectin with excitation at 365 nm and emission at 475 nm.

Parameter			
Regression equation			
Range (ng/mL)		1–200	
Slope (±S.D.)		0.12 ± 0.00	
Intercept (±S.D.)		0.76 ± 0.33	
Regression coefficient		0.9999	
Validation	Concentration (ng/mL)	Accuracy (%)	%R.S.D. (CV)
LOQ	1	105.22	3.85
Precision			
Intra-assay	2	98.58	1.28
	40	99.24	1.52
	150	99.67	1.25
Inter-assay	2	94.44	1.42
	40	96.42	3.25
	150	99.59	2.76
Stability			
Freeze–thaw	2	95.06	3.26
	40	95.43	1.86
	150	97.84	2.33
Bench-top	2	98.96	2.99
	40	96.63	1.24
	150	95.12	2.25
Stock solution	2	101.15	1.58
	40	95.42	4.65
	150	97.89	3.32

Table 2
Results of dog plasma samples after administration of tablets. Concentrations are expressed in ng/mL ± S.D.

Time (h)	Concentration (ng/mL ± S.D.)
1	5.7 ± 0.2
2	53.8 ± 2.3
4	93.6 ± 3.4

4. Conclusions

In summary, a simple HPLC–FLD method for the assay of milbemectin in dog plasma has been developed and validated. To our knowledge, this is the first report of an HPLC method for the analysis of milbemectin in animal plasma. The proposed HPLC–FLD method is reliable, free of interference, precise and with a broad linear range.

References

[1] B.L. Blagburn, D.S. Lindsay, J.L. Vaugham, N.S. Rippey, J.C. Wright, R.C. Lynn, W.J. Kelch, G.C. Ritchie, D.I. Hepler, Comp. Cont. Educ. Pract. Vet. 18 (1996) 483.
[2] A. Quintin, McKellar, J. Frank, Trends Parasitol. 20 (2004) 456.
[3] A.G.M. Barrett, R.A. Carr, S.V. Attwood, M.A.W. Finch, G. Richardson (Eds.), Recent Advances in the Chemistry of Insect Control, Royal Society of Chemistry, London, UK, 1985, p. 257.
[4] J. Vercruysse, R.S. Rew, Macrocyclic Lactones in Antiparasitic Therapy, CABI Publishing, 2002, p. 432.
[5] R. Baker, C.J. Swain, J. Head, in: James (Ed.), Recent Advances in the Chemistry of Insect Control, Royal Society of Chemistry, London, UK, 1985, p. 245.
[6] M.H. Fisher, ACS Syrup. Ser. 658 (1997) 220.
[7] R.K. Jansson, R.A. Dybas, in: I. Ishaaya, D. Degheele (Eds.), Insecticides with novel modes of action: Mechanisms and application, Springer-Verlag, Heidelbery, Germany, 1998, p. 152.
[8] R.A. Dybas, in: W.C. Campbell (Ed.), Ivermectin and abamectin, Springer-Verlag, New York, 1989, p. 287.
[9] A.R. Horowitz, Z. Mendelson, I. Ishaaya, J. Econ. Entomol. 90 (1997) 349.
[10] J.A. Lasota, R.A. Dybas, Annu. Rev. Entomol. 36 (1991) 91.
[11] H. Mrozik, et al., Experientia 45 (1989) 315.
[12] K. Okuno, K. Tanaka, Agrochem. Jpn. 78 (2001) 13.
[13] J. Ide, T. Okazaki, A. Ono, Annu. Rep. Sankyo Res. Lab. 45 (1993) 1.

[14] M. Danaher, L. Howells, S.R.H. Crooks, V. Cerkvenik-Flajs, M. O'Keeffe, J. Chromatogr. B 844 (2006) 175.
[15] B. Kinsella, S.J. Lehotay, K. Mastovska, A.R. Lightfield, A. Furey, M. Danaher, Anal. Chim. Acta 637 (2009) 196.
[16] S.B. Turnipseed, J.E. Roybal, W.C. Andersen, Anal. Chim. Acta 529 (2005) 159.
[17] X. Hou, X. Li, S. Ding, Chromatographia 63 (2006) 543.
[18] R. Sheridan, L. Desjardins, J. AOAC Int. 89 (2006) 1088.
[19] X.L. Hou, H.Y. Jiang, S.Y. Ding, J. AOAC Int. 89 (2006) 1110.
[20] H.K. Chou, C.Y. Lai, T.H. Chen, G. Yen, J. Food Drug Anal. 12 (2004) 146.
[21] K. Yoshii, A. Kaihara, Y. Tsumura, J. Chromatogr. A 896 (2000) 75.
[22] J.W. Tolan, P. Eskola, D.W. Fink, J. Chromatogr. 190 (1980) 367.
[23] M. Pollmeier, S. Maier, K. Moriarty, J. Chromatogr. B 772 (2002) 99.
[24] L. Antonian, P. De Montigny, P.G. Wislocki, J. Pharm. Biomed. Anal. 16 (1998) 1363.
[25] M. Alvinerie, J.F. Sutra, M. Badri, J. Chromatogr. B 674 (1995) 119.
[26] M. Malaňıkovĭa, V. Malaňık, J. Chromatogr. 513 (1990) 401.
[27] T. Reuvers, R. Diaz, J. Gomez, Proceedings of EuroResidue II Conference on Residues of Veterinary Drugs in Food, Veldhoven, The Netherlands, University of Utrecht, Faculty of Veterinary Medicine, Utrecht, The Netherlands, 1993, p. 567.
[28] M.S. Ali, T. Sun, G.E. McLeroy, E.T. Phillippo, J. AOAC Int. 83 (2000) 39.
[29] M. Danaher, M. O'Keeffe, J.D. Glennon, Analyst 126 (2001) 576.
[30] H. Kitagawa, Y. Sasaki, K. Ishihara, K. Ishizako, Jpn. J. Vet. Sci. 50 (1988) 1184.
[31] T. Nagata, F. Miyamoto, Y. Hasegawa, E. Ashizawa, J. AOAC Int. 86 (2003) 490.
[32] P. De Montigny, J.S.K. Shim, J.V. Pivnichny, J. Pharm. Biomed. Anal. 8 (1990) 507.
[33] M. Alvinerie, J.F. Sutra, M. Badri, P. Galtier, J. Chromatogr. B 674 (1995) 119.