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VALIDATION METHOD FOR EXTRACTION AND DETERMINATION OF MARBOFLOXACIN IN PLASMA AND EDIBLE CHICKEN TISSUES

(Kaedah Validasi bagi Pengesktrakan dan Penentuan Marbofloxacin di dalam Plasma dan Tisu Ayam yang Boleh Di Makan)

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Abstract

Marbofloxacin (MBX) is an antimicrobial for exclusive veterinary use, relatively new and of which there are few analytical methods for the extraction, identification and quantification in plasma and edible tissues (muscle, liver, kidney, lung, skin) in broiler chickens. The objective of developing a simple, sensitive and efficient extraction and detection method through high-performance liquid chromatography (HPLC) with fluorescence detection. For the preparation of the samples, known concentrations of MBX, enrofloxacin (ENX) as internal standard, methanol, water and perchloric acid, were added to plasma or tissues. Chromatographic conditions were: fluorescence detector at λex 295 and λem 490 nm, mobile phase composed of deionized water, acetonitrile and triethylamine adjusted to pH 3.0. For all matrices, the method was linear (r^2= 0.99) for MAR concentrations (0.039 to 2.5 µg/ml or gr), with an average retention time for MBX in the different matrices of 3.97 ± 0.144 minutes, and 4.99 ± 0.24 minutes for the internal standard (enrofloxacin). Detection limit in all matrices was less than 0.006 µg/ml, while recoverability percentage was 89 ± 4.65% and variation coefficients in intraday-interday assay ≤ 1.4%, determining the accuracy of the study. The analytical method applied is efficient, sensitive and reliable for future residue determinations or pharmacokinetic studies of marbofloxacin in plasma, muscle, liver, kidney, skin and lung in broiler chickens.

Keywords: marbofloxacin, high-performance liquid chromatography, chicken

Abstrak

Marbofloxacin (MBX) ialah antimikrob yang digunakan secara eksklusif bagi veterinar, secara relatifnya adalah baru dan hanya beberapa kaedah analisis bagi pengekstrakan, pengenalpastian dan kuantifikasi di dalam plasma dan tisu yang boleh di makan (otot, hati, buah pinggang, paru-paru dan kulit) di dalam ayam daging telah di perkenal. Objektif kajian adalah pembangunan kaedah pengesanan yang mudah, sensitif dan berkesan menggunakan kromatografi cecair berprestasi tinggi (HPLC) dengan pengesan pendaflour. Bagi penyediaan sampel, kepekatan MBX, enrofloxacin (ENX) sebagai piawai dalam, metanol, air dan asid perklorik ditambah ke dalam tisu plasma. Tetapan kromatografi adalah: pengesan pendaflour pada λex 295 dan λem 490 nm, fasa bergerak terdiri daripada air ternyahion, asetonitril, dan trietilamina yang diselaraskan pada pH 3.0. Bagi semua matriks sampel, kaedah adalah bersifat linear (r^2= 0.99) bagi kepekatan MBX (0.039 hingga 2.5 µg/ml atau µg/g), dengan purata masa tahanan di dalam matriks berbeza ialah 3.97 ± 0.144 minit, dan 4.99 ± 0.24 minit bagi piawai (enrofloxacin). Had pengesan di dalam semua matriks adalah lebih rendah dari nilai 0.006 µg/ml, manakala peratus perolehan semula ialah 89 ± 4.65% dan pekali variasi bagi ujian intra-hari dan inter-hari ≤ 1.4%, menjelaskan kejituan kaedah. Kaedah analisis yang digunapakai adalah

efisien, sensitif, dan dipercayai bagi tujuan penentuan sisa atau kajian farmakokinetik marbofloxacin di dalam plasma, otot, hati, buah pinggang, kulit dan paru-paru ayam daging.

Kata kunci: marbofloxacin, kromatografi cecair berprestasi tinggi, ayam

Introduction

Currently, the search of simple, reliable, efficient and selective analytical methods for determination and quantification of antimicrobial residues in animals for human consumption is important to provide safe foods [1]. Liquid chromatography is the most widely used, specific and selective method for fluoroquinolones determination, antimicrobial agents widely used in infections in animals and humans, but their improper use can lead to development of bacterial resistance, or even, cause allergies in antibiotics sensitive individuals [2-4].

Fluoroquinolones are synthetic antimicrobial agents widely used for their efficacy against pathogens with importance in commercial poultry [5], with their activity being the inhibitor the enzyme gyrase, preventing bacterial replication [6]. Marbofloxacin (MBX) belongs to second-generation fluoroquinolones with Gram-negative antimicrobial activity, some Gram-positive and mycoplasmas [7], including bird pathogens such as *Mycoplasma gallisepticum*, *Escherichia coli* [8, 9] and *Pasteurella multocida* [10].

For MBX identification and quantification, some chromatographic analytical methods have been developed for plasma and edible tissues of chickens, including high performance liquid chromatography (HPLC) with UV detector [6, 9, 11-13]. HPLC with fluorescence detector [14], HPLC with photodiode-array detector [15] and HPLC with MS/MS tandem detector [16, 17]. The method has an extensive extraction and sample preparation processes, especially in complex matrices (skin, liver or lung). The aim of this work was to validate a new analytical method for the extraction, identification, and quantification of MBX by HPLC with fluorescence detector in plasma and edible broiler chickens tissues. This is because MBX is one of the last quinolones approved for use in food animals such as cows and pigs [18-19], due to its high efficiency in bacterial respiratory infections, which determines MBX as a potential therapeutic tool in poultry farming.

The importance of this research is to propose an optimization of the analytical method for the determination of MBX, promoting food safety, avoiding the presence of harmful pharmaceutical residues in food, one of the causes of bacterial resistance.

Materials and Methods

Reagents and instrument

Antimicrobials namely MBX (Guobang Pharma 99.9%) and enrofloxacin (Laboratory Chile 99.38%) were used as internal standard. Acetonitrile (Applichem Panreac 99.9%), triethylamine (Sintorgan 99%), deionized water, methanol (Applichem Panreac 99.9%), 70% perchloric acid (Lab. Cicarelli) and 85% orthophosphoric acid, were used to carry out the study.

A Hewlett-Packard 1050 liquid chromatography, equipped with quaternary pump, in-line mobile phase degasser, manual injector, was achieved on an Agilent octadecylsilane C18 column (25cm x 4.6 mm, 5 μm) (Agilent Technologies, Wald-bronn, Germany), Phenomenex precolumn, Hamilton injection syringes 100 μl, Hewlett-Packard fluorescence detector series 1046-A adjustable wavelength and personal computer with plate and program for equipment control, acquisition, data processing and printing of chromatograms.

HPLC analysis

The mobile phase was composed of deionized water, acetonitrile and triethylamine (790: 200: 10 v/v/v) adjusted to pH 3.0 with orthophosphoric acid, filtered with vacuum pump an 0.22 μm nylon membrane. The determination was performed at room temperature by reverse phase isocratic elution at flow rate of 0.8 ml/min, with a fluorescence detector set at 295 nm excitation and 490 nm emission [17] and an injection volume of 50 μl.

Sample pre-treatment and extraction procedure
Samples were processed according to Böttcher et al. method with modification [20]. For plasma, a liquid-liquid extraction was carried out by incorporating 150 µl of chicken plasma, 150 µl of a known MAR concentration, 600 µl of methanol and 40 µl of the internal standard (enrofloxacin 2.5 µg/ml) into an Eppendorf tube of 1.5 ml. For the tissue samples, 150 µg of the tissue, 150 µl of known MBX concentration, 600 µL of a homogenization solution, composed of deionized water, methanol and 70% perchloric acid (50: 48: 2) and 40 µl of the internal standard was added to an Eppendorf, proceeding to homogenize the whole for one minute with a homogenizer (Dremel 3000®). Then, samples were stirred in vortex for 30 seconds, held for 1 hours at room temperature 22 °C and then 12 hours at 4 °C; at the end of this time, were centrifuged at 13,500 rpm at 4 °C for 25 minutes. The supernatant was filtered through a 0.22 µm nylon membrane, obtaining the sample to be eluted.

Method validation
The validation of the chromatographic methodology was performed according to the *Codex Committee on Residues of Veterinary Drugs in Foods* (CCRVDF), proposed by the European Medicines Agency (EMEA) [21].

Calibration curve
The calibration curve was performed with different concentrations of MBX in the range of 0.0097 - 2.5 µg/ml (plasma), 0.0048 - 1.25 µg/g (muscle) and 0.0195 - 2.5 µg/g (skin, lung, liver and kidney); concentrations were incorporated into the sample preparation as described previously dependent on the tissue used. Determination was performed by linear regression, linearity, regression coefficient, slope, intercept, detection limit (LOD) and quantification limit (LOQ). The intercept and slope values of the calibration curve were used for a linear function to obtain the ratio of the peak of marbofloxacin and internal standard (enrofloxacin), determining the final concentration of the analyte.

Precision and recoverability assays
For precision, intra-day tests were employed, which refers to the dilution of calibration standards (0.039 to 1.250 µg/ml or µg/g) of plasma and muscle; meanwhile in tissues, concentrations from 0.078 to 2.5 µg/g were used, by six-fold. Test was considered acceptable when the coefficient of variation (CV%) between elution, as the area index of the chromatograms was achieved ≤ 1.5%.

Finally, inter-day test consisted in the dilution of the calibration standards from 0.039 to 1.25 µg/ml or µg/g of plasma and muscle and 0.078 to 2.5 µg/g in tissues performed in 6 different days. It was considered acceptable when the CV% of the area indices of the chromatograms was ≤ 3%. For recoverability (plasma and muscle) MBX calibration standards were used in the same concentration of 0.039 to 1.250 µg/ml or µg/g, whereas in the other tissues (liver, kidney, lung, skin) it was 0.078 to 2.5 µg/g with fortified samples, observing differences between standard versus fortified samples calculating the recoverability percentage.

Results and Discussion

Table 1 shows results of the intercept, slope and the adjustment coefficient for each matrix studied. Table 2 shows variation coefficients of intraday and inter-day assays and the percentages of relative recovery of marbofloxacin in all matrices studied. Table 3 indicates the different detection and quantification limits for marbofloxacin in the different biological matrices studied. Retention time for MBX in the different matrices studied was 3.97±0.144 minutes, and 4.99±0.24 minutes for internal standard (enrofloxacin) without interferences in the chromatogram.

The coefficient of adjustment of linearity (Table 1) in the range of concentrations studied, shows that this analytical method is efficient and sensitive for the determination of MBX in different tissues of chickens, for pharmacokinetic behavior studies and for residues monitoring of this drug in broiler chickens (plasma, muscle, liver, lung, kidney, skin) [21].

Table 1. Linearity of marbofloxacin in plasma and chicken tissues

Matrix	Ranges of Concentrations (*)	A	B	r^2
Plasma	0.0097-2.50	0.008128	0.4154	0.9928
Muscle	0.0048-1.25	0.01456	0.7084	0.9999
Liver	0.0097-2.50	0.1087	0.8059	0.9908
Kidney	0.0097-2.50	-0.01264	0.4973	0.9993
Lung	0.0097-2.50	0.01509	0.4398	0.9986
Skin	0.0097-2.50	0.01264	0.5294	0.9992

(*) µg/ml o µg/g. **A**: intercept **B**: slope r^2: coefficient of adjustment

Intra-day and inter-day precision tests (Table 2), coefficients variation (%CV) obtained in plasma and tissues tested were ≤1.73 and ≤ 2.14%, respectively; being less than those obtained by other authors ranging from 5.7 to 15.5%, [6, 8, 11-17] after the application of more complex extraction methods, with amount of reagents and a large procedures such as evaporation with nitrogen at 30 °C, confirm the accuracy of the analytical method implemented. The results obtained in the validation tests confirm the sufficiency of the procedures destined to extract the analyte from the plasma, using in the extraction methanol as a polar solvent followed by centrifugation [22].

Table 2. Recovery and precision assay of marbofloxacin in plasma and chicken tissues

Matrix	Ranges of Concentrations (µg/ml or µg/gr)	Recovery (%)	Precision (%CV)	
			Intra-day	Inter-day
Plasma	0.039 – 1.25	86.2 ± 3.75	1.39 ± 0.61	1.73 ± 0.65
Muscle	0.039 – 1.25	87.6 ± 6.91	1.47 ± 0.34	1.54 ± 0.50
Liver	0.078 – 2.50	86.5 ± 5.17	0.91 ± 0.11	2.16 ± 0.62
Kidney	0.078 – 2.50	93.7 ± 7.17	1.02 ± 0.42	1.21± 0.13
Lung	0.0078 – 2.50	92.8 ±4.44	0.74 ± 0.07	1.54 ± 0.39
Skin	0.0078 – 2.50	89.0 ± 2.17	1.16 ± 0.22	2.14 ± 0.63

CV: Coefficient of variation

Moreover, recoverability percentages in this study were 89 ± 4.65% (Table 2) among all matrices studied, higher than 64% to 75% observed with methods developed by other authors ranging for plasma and different tissues [6, 11, 14]. Yu et al. [16] and Rocha et al. [17] obtained percentages of MBX recovery similar to this work, between 78 and 95% in the different tissues (muscle, kidney and liver), Rocha et al. [17] performed two different extraction methods and with an HPLC coupled to tandem MS/MS, their percentages in muscle were 88 and 89%, while in the kidney they were greater between 95 to 96%.

Zhao et al. [12] obtained between 90 to 95% in muscle while Yang et al. [13] in plasma obtained between 88 to 95% and in tissues (lung, muscle, kidney and liver) between 81 to 89%. These data corroborate that the results obtained are in the acceptable ranges, promoting the greater recovery of the analyte in the study sample, in complex matrices such as lung, kidney or liver, validating the extraction method.

On the other hand, LOQ for MBX matrices (Table 3) are lower than those observed in extraction methods performed by Anadón et al. [6] and Ding et al. [8] in plasma obtained at 0.01 µg/ml and 0.02 µg/ml, respectively. In tissues samples, where Ding et al. [8] determined to LOQ of 0.05 µg/g (liver, kidney and muscle). Yorke and Forc

[14] reported LOQ 0.75 µg/g for muscle and Anadón et al. [6] recorded 0.01 µg/g (muscle, liver and kidney). A study performed by Anadón et al. [6] is similar to the one obtained in our research, although the previous studies are much higher, but the high sensitivity of our analytical method still considerable for the drug studied, in the different chicken tissues.

Table 3. Limits of detection (LOD) and limits of quantification (LOQ) in chicken tissue (µg/g)

Matrix	**LOD**	**LOQ**
Plasma	0.002	0.006
Muscule	0.003	0.009
Liver	0.003	0.009
Kidney	0.006	0.018
Lung	0.005	0.017
Skin	0.006	0.018

Considering the complexity of determining fluoroquinolones for their amphoteric character [22], added to the complexity of the matrices worked (muscle, kidney, lung, liver and skin), the processes of sample preparation and analyte extraction are complicated, with great quantity of organic solvents, for dilution and cleaning of the sample, removing macromolecules that interfere with the correct determination of the analyte. The above shows that our study by optimizing the process of sample preparation and extraction of the analyte, in addition to the validation of the analytical method, demonstrates the reliability of routine studies for marbofloxacin in the edible tissues of broiler chickens.

Conclusion

The optimization method developed in this study for the determination and quantification of MBX in plasma and edible tissues of broiler chickens (muscle, lung, kidney, liver and skin) could be considered as reliable, efficient, fast, precise, and also economical procedure due to the low amount of solvents used, being a useful tool for the monitoring of the pharmacokinetics and/or residues of MBX in broiler chickens, since it has been determined in previous studies as a potential therapeutic tool in poultry farming.

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