https://doi.org/10.31925/farmacia.2021.3.14 **ORIGINAL ARTICLE**

ESTIMATION OF QUINOLONES, CEFTIOFUR AND THIAMPHENICOL RESIDUES LEVELS IN HONEY

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Abstract

Quinolones, cephalosporins and amphenicols are antimicrobial agents effective in treating many infectious diseases caused by bacteria in both human and veterinary medicine. However, its residues in food products leads to increased bacterial resistance. Therefore, the quantification and limitation of such medicines should be verified through fast and reliable methods. The aim of the research was the simultaneous quantitative determination of quinolones, ceftiofur and thiamphenicol using biochip technology. The biochip method had excellent accuracy. The decision limit obtained for the analysed drugs was between 1.25 and 4.5 µg/kg. The detection capacity recorded values in the range of 2.05 - 8.51 µg/kg for the analysed drugs. The recovery coefficient obtained was in the range of 77 - 123%. The results of the analysis of Romanian honey samples using the biochip method were confirmed using a validated LC-MS/MS method.

Rezumat

Chinonolele, cefalosporinele și amfenicolii sunt agenți antimicrobieni eficienți în tratarea a numeroase boli infecțioase cauzate de bacterii atât în medicina umană, cât și în cea veterinară. Totuși, reziduurile acestor compuși în alimente conduce în timp la o rezistență bacetriană crescută. Prin urmare, cuantificarea și limitarea acestor medicamente ar trebui verificate prin metode rapide și fiabile. Scopul cercetărilor a fost determinarea cantitativă simultană a chinolonelor, ceftiofurului și tiamfenicolului folosind tehnologia *biochip*. Metoda *biochip* a avut o precizie excelentă. Limita de decizie obținută pentru substanțele analizate s-a încadrat între valorile 1,25 și 4,5 µg/kg. Capacitatea de detecție a înregistrat valori pentru chimioterapicele determinate în intervalul 2,05 - 8,51 µg/kg. Coeficientul de recuperare obținut s-a situat în domeniul 77 - 123%. Rezultatele analizei probelor de miere românească folosind metoda *biochip* au fost confirmate printr-o metodă LC-MS/MS validată.

Keywords: honey, quinolones, ceftiofur, thiamphenicol, biochip method

Introduction

The use of antibiotics in beekeeping is illegal in most EU countries. The maximum permitted limits for the antibiotic residues in honey have not been established in the European Community, which means that honey containing antibiotic residues cannot be marketed [11, 28].

Quinolones, cephalosporins and amphenicols are compounds commonly used for treating bacterial bee diseases [1, 3, 23, 29]. The widespread use of such drugs in veterinary medicine represents a potential danger to human health. They can be present as residues in food [25, 27], which may cause allergic reactions or antibiotic resistance phenomena in humans [4, 22].

The quinolones mostly used as veterinary antimicrobial drugs are the fluoroquinolones [6, 7, 8, 20]. Maximum residue limits (MRLs) have been established in different animal feed matrices for eight quinolones: marbofloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, flumequine and oxolinic acid [2, 5, 11, 30].

Ceftiofur is a third-generation cephalosporin, developed exclusively for veterinary use. It is bactericidal *in vitro* by inhibiting germ cell wall synthesis. Recently, ceftiofur has been used in beekeeping to treat bacterial infections [9, 16, 17]. Although it is not recommended during honey production, it has been detected in honey [14, 23].

Thiamphenicol is a broad-spectrum antibiotic, similar in structure and action mechanism to chloramphenicol [24]. Thiamphenicol is a broad-spectrum synthetic antibiotic that inhibits protein synthesis in the bacterial cell [21]. Thiamphenicol is used in beekeeping based

on the bacteriostatic effect on various Gram-positive and Gram-negative bacteria [2, 18, 27].

Biochip Array Technology allows the simultaneous testing of multiple analytes from a single sample. It is one of the most rapid and cost-effective analysis methods [2, 12, 13, 19].

The research included the validation of a method for the simultaneous quantitative determination of some quinolones, cephalosporins (ceftiofur), amphenicols (thiamphenicol) using the biochip technology and analysis of quinolones, ceftiofur and thiamphenicol in various honey samples purchased from the Romanian market.

Materials and Methods

Biochip technology was used for the simultaneous, quantitative determination of of three representatives classes of antibiotics. The biochips contain three Discrete Test Regions, each of those regions corresponding to an antibiotic class. Each biochip incorporated a reference spot and a correction spot on its surface. In the image processing stage, the reference spot was located by the analyser software on a predefined *x* and *y* coordinates to validate the respective biochip. The correction spot was then used to define and locate the surface of each discrete test region.

Antimicrobial II panel was used to determine 3 types of antibiotics, namely, ceftiofur (CEFT), thiamphenicol (TAF) and generic quinolones (QNL).

All reagents were of analytical grade and the device manufacturer supplied it's in a compact kit that included Anti-Microbial Array II (EV3524) and Multianalit Control (AMC5004), both produced by *Randox Laboratories* from The United Kingdom.

Compared to conventional test systems, in which each separate test requires different reagents, for all determinations included in the Antimicrobial II panel, the Evidence Investigator system requires only two types of reagents. The volume of the used reagent for a test was lower than in any other system. The total required reaction volume was 350 µL. All reagents used had barcodes and applications that were downloaded to the analyser software automatically, from a CD included in the kit of each reagent batch, making the system easy to use.

The method of simultaneous quantitative determination using the biochip technology was validated following a protocol that simultaneously met the requirements of decision 2002/657/EC and the possibilities of any laboratory processing a large number of test samples [10].

The validation parameters evaluated were linearity, sensitivity, specificity, selectivity, accuracy, intermediate accuracy and reproducibility, the limit of detection and recovery.

To confirm the linearity of the method, a 9-point calibration was performed for each analyte using the standards included in the Antimicrobial Array II kit. The results obtained were automatically processed using the analyser software [15, 26].

The standards were supplied in a compact kit and the calibration curves were automatically generated by the dedicated analyser software. However, given the complex nature of the honey sample matrix, the calibration curves were also obtained using standards from the same matrix, which was a honey sample proven negative for drug residues using an LC-MS/MS method, then spiked with the appropriate concentrations of the standards.

A standard multi-analyte solution was prepared. It contained 10 µg/kg of each analyte dissolved in methanol. Calibration curves were obtained by spiking the negative honey samples at 9 levels of concentration of the analytes: 0, 0.001 µg/kg, 0.01 µg/kg, 0.05 µg/kg, 0.1 µg/kg, 1 µg/kg, 4 µg/kg, 10 µg/kg and 50 µg/kg.

To evaluate the sensitivity of the method, IC50 was calculated for each analyte. IC50 represents 50% of the relative light unit (RLU) value corresponding to the zero concentration standards and extrapolating that RLU value from the x-axis of the calibration curve on which the units of concentration were expressed as µg/kg. That concentration corresponded to the concentration, which produced 50% inhibition.

To determine the specificity and selectivity, known concentrations of each analyte were used in serial dilutions. Three replicates were analysed for each drug level in serial dilution to assign the percentage of cross-reactivity.

According to the validation guides, the percentage of cross-reactivity for drug residue determination in honey should not exceed 25% for concentrating the analytes at the minimum limit of quantification [13].

According to the current legislation and validation guides for the methods of determining the drug residues in honey, the accuracy and precision are evaluated for concentrations representing 50 %, 100% and 150 % respectively of the minimum required performance limit (MRPL) required for antibiotic residues in honey bees: MRPL = 1 µg/kg [15, 26].

The accuracy within the same analytical series was determined by analysing 20 replicates of negative samples of honey that were spiked to achieve those 3 concentration levels of antibiotics.

The accuracy of different analytical series was determined by analysing 2 replicates of negative samples of honey that were spiked to achieve 3 concentration levels of antibiotics that were analysed during 10 working days. Accuracy and precision were acceptable if the coefficient of variation of the concentration in the control samples did not exceed ± 15% for all determinations executed during the same day.

To determine the decision limit (CCα) and the detection capacity (CCβ), 20 negative honey samples (blank samples) were selected. Aliquots of those samples were

spiked with drugs at the target-screening concentration of 0.5 µg/kg for CEFT and TAF, and 1 µg/kg for QNL. The blank and spiked samples were tested. CCα was calculated as the average of the analyte concentration in the 20 samples spiked with the analyte concentration at MRPL level plus 1.64 × standard deviation (SD) of repeatability at α = 5%. CCβ was calculated as the arithmetic average of the analyte concentration at CCα plus 1.64 × SD of repeatability at α = 5%.

To estimate the recovery percentage, negative honey samples were used, spiked with the analytes at 3 different levels of concentration.

The validated biochip method was applied to 43 honey samples of different assortments for the determination of antibiotic residues. Honey samples analysed using an already validated LC-MS/MS method and identified as being drug-free were used as negative samples in the validation protocol [27].

Confirmation of the results obtained through the biochip method on the tested honey samples was done by the LC-MS/MS method [27]. The determinations were made using the Agilent 1100 LC system (Agilent Technologies, USA) coupled with the 4000 Q TRAP mass spectrometer (Applied Biosystems, USA).

The following parameters were followed to confirm the results: the signal/noise ratio selected for the characteristic ions was > 3; differentiation of analyte retention time and the corresponding standard were ± 2.5%; the deviation of the relative abundance of the characteristic ions of the target analyte and the deviation of the characteristic ions of the corresponding standard were between ± 20% and ± 50%.

Results and Discussion

Nine biochips were used for calibration. The remaining 45 biochips were used for the control samples, to check the accuracy and precision within the same analytical series.

The calibration intervals were 0 - 7 µg/kg for QNL, 0 - 11.5 µg/kg for CEFT and 0 - 5 µg/kg TAF. The correlation coefficients (r) obtained for the drugs were in the range 0.982 - 0.998, and the lowest coefficient was obtained for TAF.

The calculated IC50 were for each analyte 0.52 µg/kg for QNL, 0.25 µg/kg for CEFT and 0.5 µg/kg TAF.

Specificity and selectivity were studies based on the cross-reactivity percentage that was determined against the parent compound and the corresponding chain of related compounds (Table I).

Table I

Cross-reactivity study results

Cross-Reactant	Cross-reactivity (%)			Cross-Reactant	Cross-reactivity (%)		
	QNL	CEFT	TAF		QNL	CEFT	TAF
Amoxicillin		< 1		Levofloxacin	13		
Ampicillin		< 1		Marbofloxacin	16		
Cefadroxil		< 1		Nadifloxacin	14		
Cefazolin		< 1		Nafcillin		< 1	
CEFT	< 1	100	< 1	Nalidixic acid	< 1		
Chlortetracycline		< 1	< 1	Norfloxacin	100	< 1	< 1
Cinoxacin	< 1			Ofloxacin	21		
Ciprofloxacin	19			Orbifloxacin	11		
Cloxacillin		< 1		Oxacillin		< 1	
Danofloxacin	10			Oxolinic acid	12		
Dicloxacillin		< 1		Pazufloxacin	3		
Difloxacin	3			Pefloxacin	24		
Enoxacin	5			Penicillin G		< 1	
Enrofloxacin	8			Pipemidic acid	9		
Fleroxacin	12			Sarafloxacin	6		
Florfenicol			23	Streptomycin		< 1	< 1
Florfenicol amine			< 1	TAF	< 1	< 1	100
Flumequine	< 1			Ticarcillin		< 1	
Gatifloxacin	< 1			Tylosin		< 1	< 1

Multianalyte Control (AMC 5004) produced by Randox Laboratories, UK was used to evaluate the accuracy and precision, and the average concentration and CV% was calculated. The method presented excellent accuracy both within the same analytical series and in different analytical series (Table II), with typical values less than 15% for the determined concentrations.

The decision limit obtained for the classes of antibiotics determined was between 1.25 µg/kg and 5.5 µg/kg. Also, the detection capacity obtained for the same drugs was between 2.05 µg/kg and 8.81 µg/kg. The obtained values are presented in Table III.

Table II

Precision and accuracy data

Concentration	Concentration (µg/kg)			CV (%)		
	CEFT	QNL	TAF	CEFT	QNL	TAF
The same series						
0.5 µg/kg	1.25	0.63	0.31	6.8	4.30	3.40
1 µg/kg	2.50	1.25	0.63	5.80	6.60	4.50
2 µg/kg	5.00	2.50	1.25	5.00	6.80	6.80
Different series						
0.5 µg/kg	0.49	0.93	0.38	6.80	4.30	3.40
1 µg/kg	1.05	1.80	0.59	5.80	6.60	4.50
2 µg/kg	1.97	3.29	1.17	5.00	6.80	6.80

Table III

Decision limit and detection capacity

Validation parameters	CEFT	QNL	TAF
Average concentration (µg/kg)	0.49	0.93	0.38
Standard deviation (SD)	2.44	1.52	0.53
1.64 × DS	4.01	2.50	0.87
CCα (µg/kg)	4.50	3.43	1.25
CCβ (µg/kg)	8.51	5.93	2.05

Each spiked sample was then analysed and the recovery percentage was calculated using the formula: Recovery (%) = ((A-B)/C) × 100, where: A was the average concentration determined for the analyte, B was the average analyte concentration in the sample and C was the analyte concentration in the spiked sample.

The recovery rates for honey samples can be found in Table IV. A recovery percentage > 70% required for each analyte was achieved with values in the range of 77 - 125%.

Table IV

Recovery data

Concentration	Analysed Concentration (µg/kg)			Recovery (%)		
	CEFT	QNL	TAF	CEFT	QNL	TAF
0.5 µg/kg	0.56	0.62	0.60	112	123	119
1 µg/kg	0.87	1.00	1.00	87	100	100
2 µg/kg	1.54	1.74	1.98	77	87	99

Table V

Comparison of the results obtained using the biochip method *versus* the LC-MS/MS method

Sample No.	Method	QNL (µg/kg)	CEFT (µg/kg)	TAF (µg/kg)
1	biochip	10.4	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 5 µg/kg Norfloxacin 7 µg/kg	< LOD	< LOD
2	biochip	54.9	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 41 µg/kg Norfloxacin 10 µg/kg	< LOD	< LOD
3	biochip	12.7	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 4 µg/kg Norfloxacin 2 µg/kg	< LOD	< LOD
4	biochip	11.4	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 7 µg/kg Norfloxacin 3 µg/kg	< LOD	< LOD
5	biochip	9.4	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 6 µg/kg Norfloxacin 4 µg/kg	< LOD	< LOD
6	biochip	24.6	< LOD	< LOD
	LC-MS/MS	Ciprofloxacin 3 µg/kg Norfloxacin 15 µg/kg	< LOD	< LOD

After testing 43 honey samples, only residues of QNL were quantified in 6 samples. Both positive and negative samples were confirmed by the LC-MS/MS method. As can be seen from the data presented in Table V, the concentration values obtained using the biochip method are comparable to those obtained from the LC-MS/MS method. Following the application of the LC-MS/MS method, the samples having drug concentration greater than 1 µg/kg were confirmed to be positive.

The results of the 6 positive samples identified by the biochip method and confirmed by the LC-MS/MS

method revealed the same QNL at concentration levels above MRPL, namely: ciprofloxacin and norfloxacin.
Private beekeepers from different geographical regions in Romania provided the 6 QNL-positive samples of honey. The results obtained in the study were comparable to those previously published by other research teams in the scientific literature [16, 23].
As a result of the current study, we were able to confirm that the biochip method was suitable for the proposed purpose. The performances of the biochip method were adequate, and the results obtained were similar to the concentration values determined using the confirmatory method, for both positive and negative samples.

Conclusions

In terms of analytical performance, the biochip method presented excellent accuracy both within the same analytical series and in different analytical series, with typical values less than 15% for concentrations of 0.5, 1 and 1.50 µg/kg. The decision limit was between 1.25 µg/kg and 4.5 µg/kg. The detection capacity recorded values in the range of 2.05 - 8.51 µg/kg. The recovery coefficient obtained was in the range of 77 - 125% compared to the initial concentration.
The method validation criteria (specificity, accuracy, linearity, limits of detection and quantification) complied with the recommendations of European Commission Decision 2002/657/EC and proved that the method can detect and quantify drug residues, without necessarily having to be tested by mass spectrometry or derivatization for fluorescence analysis of analytes.
The immunological method proposed in our study presented the advantage that it did not require extraction of antibiotics from the biological matrix with organic solvents, as well as the advantage of obtaining a large number of results in a short time.
Instrumental methods, such as LC/MS are sensitive and specific, are suitable for confirmation, but would be too laborious for screening a large number of samples.
The validation and application of the biochip method highlighted the fact that the Evidence Investigator System and the Antimicrobial Panel II represent an efficient system for the simultaneous detection and quantification of multi-analytes in honey samples. A comparison of the results obtained by the biochip method and the LC-MS/MS method revealed that the proposed biosensor method is suitable for the proposed purpose, namely, the determination of the concentration of each analyte during European food safety monitoring programs.

Conflict of interest

The authors declare no conflict of interest.

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