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ORIGINAL ARTICLE

IMMUNOCHEMICAL ASSAY OF CHLORAMPHENICOL IN HONEY

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Abstract

The veterinary use of chloramphenicol in food-producing animals is forbidden in the European Union. As it is still used for the treatment of infections in animals not bred for consumption, traces of the drug may be found in honey. Developing accessible methods to detect traces of chloramphenicol is of high interest to food residue monitoring and regulation programs. We propose an immunochemical method as an alternative to detect chloramphenicol in honey using Biochip Technology. The sensitivity of the new quantitative determination method for chloramphenicol was high (IC50 = 0.74 µg/kg). The method presented very good accuracy both within the same analytical series and in different analytical series with typical values lower than 15% for concentration levels of 0.5, 1 and 1.5 µg/kg. The decision limit (CCα) of chloramphenicol was 0.37 µg/kg, while the detection capability (CCβ) was 0.42 µg/kg. The method was applied to the determination of chloramphenicol residues in Romanian honey samples.

Rezumat

Utilizarea cloramfenicolului în medicina veterinară la animalele crescute pentru consum este interzisă în Uniunea Europeană. Se găsesc însă urme de substanţă medicamentoasă în miere, deoarece este folosită pentru tratamentul infecţiilor la animalele care nu sunt crescute pentru consum. Dezvoltarea unei metode accesibile pentru detecţia urmelor de cloramfenicol este de mare interes pentru programele de monitorizare şi control a reziduurilor din alimente. Propunem o metodă imunochimică folosind tehnologia Biochip ca o alternativă pentru analiza cloramfenicolului în miere. Sensibilitatea noii metode de determinare cantitativă a cloramfenicolului este mare (IC50 = 0,74 µg/kg). Metoda prezintă o precizie foarte bună atât în cadrul aceleiaşi serii analitice, cât şi în serii analitice diferite, cu valori mai mici de 15% pentru nivelurile de concentraţie de 0,5, 1 şi 1,5 µg/kg. Limita de detecţie (CCα) a fost 0,37 µg cloramfenicol/kg, în timp ce capacitatea de detecţie (CCβ) a fost 0,42 µg/kg. Metoda a fost aplicată pentru determinarea reziduurilor de cloramfenicol în probe de miere din România.

Keywords: chloramphenicol, honey, Biochip

Introduction

Honey from across the world is contaminated with potent pesticides [8, 10, 14, 15]. When analysed almost half the samples contained a cocktail of pesticides [1, 5, 6, 9, 17-22, 24-26].

Chloramphenicol is an aminoglycoside obtained for the first time in 1947 from *Streptomyces venezuela* [3, 7], but since 1950 it is obtained through synthesis. Chloramphenicol has bacteriostatic activity and it is effective in treating infectious diseases [23]. The mechanism of action of chloramphenicol is based on the inhibition of the transport of activated amino acids to the site of protein synthesis (ribosomes), i.e. inhibiting the synthesis of bacterial proteins. Resistance installs very slowly and it is due to the development of enzymes that break down chloramphenicol such as hydrolases, reductases or acetyltransferases [7].

Because of the side effects that chloramphenicol has on human health, its use in veterinary medicine has been banned by the FDA in the US, by the Canadian Health Protection Branch and by the European Union in those animals whose products and by-products are used for human consumption. However, chloramphenicol has been shown to be one of the most commonly found drug residues found in honey [1-2, 5, 11-13, 27, 29-34].

The present study aimed to achieve validation of a quantitative determination method for chloramphenicol, using Biochip technology and to validate the new method for the determination of chloramphenicol residues in Romanian honey samples.

Materials and Methods

All reagents were supplied in a compact kit that included Antimicrobial Array III - kit EV3695 and Antimicrobial Array III Control - kit AMC5036, produced by Randox Laboratories, UK.

The validation method and the honey analysis procedure were performed in accordance with 2002/657/EC Decision, FDA approved validation guidelines and validation guidelines for screening methods for veterinary drug residues. The validation parameters evaluated were: linearity,

sensitivity (IC50), specificity and selectivity, precision (intermediate and reproducibility), accuracy, detection limit and recovery [17, 18, 20].

The linearity of the method was evaluated by performing a 9-point calibration using the calibrators included in the Anti Microbial Array III kit. Considering the complex honey composition as a sample matrix, the linearity of the method was also checked by spiking blank honey samples in order to obtain nine concentration levels: 0, 0.001, 0.01, 0.05, 0.1, 1, 4, 10 and 50 µg chloramphenicol/kg.

The analysis software used a specific calibration equation [4, 16]:

$$y = D + [(A-D)/1+(x/C)^B], \text{ where:}$$

x = analyte concentration (µg/kg); y = the intensity of the chemiluminescent signal expressed as relative light units (RLU); A, B, C, D = parameters of the competitive method, predefined in the analyser software as A = the intensity of the chemiluminescent signal of the blank, B = slope factor, C = the inflection point of the calibration curve and D = the intensity of the luminous response signal at an infinite theoretical concentration of the analyte [17].

An initial estimate was made for each parameter, and then it was optimized by minimizing the sum of the squares of the residuals using Microsoft Excel Solver.

The procedure used in order to obtain the calibration curves included the following steps: adding 50 µL of each calibrator to the surface of each Biochip followed by the addition of 150 µL of reaction buffer (AM III DIL ASY); incubating the Biochips at 25°C while stirring at 370 rpm for 30 minutes; adding 100 µL of enzyme conjugate solution to each Biochip; incubating for 30 minutes the Biochips at 25°C while stirring at 370 rpm; removing the reaction mixture by washing the reaction surface 6 times, in order to remove the components that did not bind to the polyclonal antibodies present on the solid substrate on surface of the Biochip; after complete removal of the reaction mixture, 250 µL of reagent (luminol: peroxide 1:1 v/v) had been added; the Biochips were left to rest in the dark for the development of the reaction and exactly 2 minutes after, they were placed in the image capture chamber for processing and interpretation of the signal.

The sensitivity of the method expressed as the Inhibitory Concentration (IC50) was calculated based on 50% of the value of the RLU signal generated by the zero-concentration calibrator and extrapolating the RLU value thus obtained on the X-axis of the calibration curve where the concentration units were expressed as µg/kg. The concentration thus obtained corresponded to the inhibitory concentration that produced 50% inhibition [18].

The specificity and selectivity of the method were analysed by adding the analyte separately in known concentrations of (10 and 100 µg/kg) to the zero-concentration calibrator in serial dilutions. To assign the cross-reactivity percentage, three replicates were assessed for each analyte level in the serial dilution.

Cross-reactivity was calculated using the formula:

$$\% \text{ CR} = [IC50_{analyte}/IC50_{cross\text{-}reactant}] \times 100.$$

Antimicrobial Array III Control - code AMC5036, manufactured by Randox Laboratories, UK, was used to evaluate the accuracy and precision. Mean concentration, standard deviation, and coefficient of variation (%CV) were also calculated.

Because the residues of chloramphenicol have a 1 µg/kg reference point for action, that was the reason the target concentration for screening was set at 0.5 µg/kg (50% of reference point for action).

According to current legislation and validation guidelines for methods of drug determination in honey, precision and accuracy must be assessed for the minimum required performance limit (MRPL) which was 1 µg/kg, and then for 50% and 150% MRPL.

The precision in the same analytical series was determined by analysing 20 replicates of negative honey samples, spiked in order to obtain 3 different concentration levels: 0.5, 1 and 1.5 µg/kg.

The precision in different analytical series was determined by analysing two replicates of blank honey samples spiked with chloramphenicol at three different concentration levels (0.5, 1 and 1.5 µg/kg) in 10 different days of analysis.

Precision and accuracy are acceptable if the coefficient of variation in the concentration of the control samples measured does not exceed ± 15% for determinations on the same day or on different days or analytical series.

In order to determine the decision limit (CCα) and the detection capability (CCβ) 20 blank honey samples were selected and spiked at the target concentration for screening - 0.5 µg/kg. CCα was calculated as the arithmetic mean of the concentration in 20 spiked samples. The concentration level of each analyte was MRPL plus 1.64 × standard deviation of repeatability at α = 5%. CCβ was calculated as the arithmetic mean of the concentration analyte at CCα plus 1.64 × standard deviation of repeatability at α = 5%.

The recovery percentage was calculated for three chloramphenicol concentration levels 0.5, 1, 1.5 µg/kg in spiked honey samples. Recovery percentages were calculated by plotting the ratio of the analyte concentration in the sample against the theoretical concentration of the analyte in the standard

solution. According to the validation guidelines, the requirement for the recovery percentage for the determination of drug residues in honey must be higher than 70%.

The validated method was used for the analysis of Romanian honey samples purchased from supermarkets or from private producers. The samples had been stored at room temperature and in the dark.

Sample processing included derivatization and extraction from honey samples. The procedure included the following steps: 1g of honey sample was mixed with 4 mL distilled water, incubated at 37°C for 30 minutes, and stirred 10 minutes until dissolved; 0.5 mL of 1M HCl and 100 µL of 10 mM 4-nitrobenzaldehyde solution were added to the sample solution; the mixture was stirred for 10 minutes, and then incubated for 16-24 hours at 37°C; after incubation, 5 mL dipotassium phosphate 0.1M solution was added to each sample, and the pH was adjusted to 7.4 with 1M NaOH solution; 15 mL ethyl acetate were added to 5 mL of derivatized honey sample, which was stirred on a Vortex shaker for 12 minutes; the sample was centrifuged for 10 minutes using 4500 relative centrifugal force; 6 mL of supernatant from each sample was transferred into microtubes, which were then evaporated at 60°C and 15 psi; the residue was mixed with 375 µL of sample diluent provided in the kit (AM III DIL SPE) and stirred for 2 minutes.

Confirmation of the results obtained using the Biochip method for the analysed honey samples was performed by using a validated LC-MS/MS method [15] on an Agilent 1100 LC (Agilent Technologies, USA) coupled with a 4000 Q TRAP mass spectrometer (Applied Biosystems, USA), and the acceptance rules were: signal/noise ratio > 3 ± 2.5% differentiation of analyte retention time and corresponding standard, ± 20% deviation of the relative abundance of the analyte and ± 50% deviation of the corresponding standard.

Results and Discussion

The calibration curve (Figure 1) showed a correlation coefficient (r) of 0.991 for the linearity range 0 - 5 µg/kg, thus fulfilling the admittance condition of the calibration curve (r > 0.949).

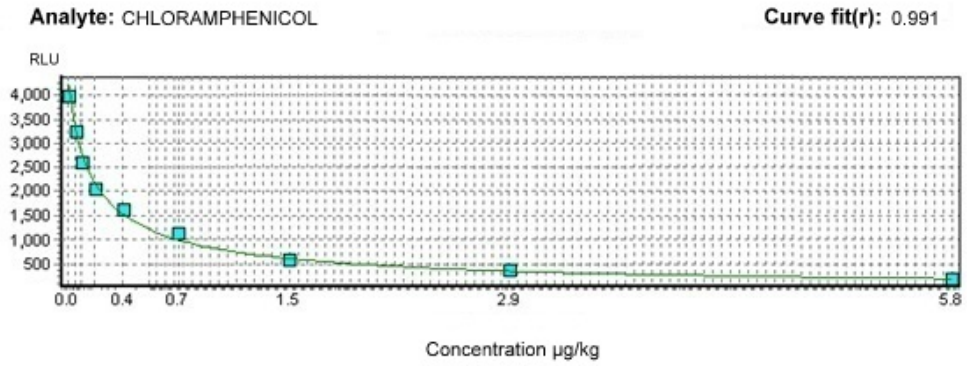

Figure 1.
Calibration curve for chloramphenicol

The sensitivity of the method expressed as IC50 for the simultaneous quantitative determination of chloramphenicol was 0.74 µg/kg.

While studying of specificity and selectivity, the cross-reactivity (%) for chloramphenicol was 100 and for cross-reactant - chloramphenicol glucuronide, it was 17. According to the validation guides, the cross-reactivity percentage (%CR) for the determination of drug residues in honey must not exceed 25% for the analyte concentration at the minimum limit of quantification. The assayed validation parameters are shown in Table I and Table II.

The validated Biochip method was applied to the analysis of 16 samples of honey from various geographic regions in Romania. The results were confirmed using the LC-MS/MS method. The performance of the Biochip method was very good, the values obtained were comparable to the results obtained for both positive samples and negative samples (Table III).

Table I
Precision of the used method

Series	Level	Concentration (µg/kg)	CV (%)
Identical	1	0.32	9.06
	2	0.75	6.97
	3	1.06	7.34
Different	1	0.44	8.92
	2	0.75	7.26
	3	1.06	9.84

Table II

Recovery, decision limit and detection capability

	Level 1	Level 2	Level 3
Concentration (µg/kg)	0.32	0.75	1.06
Recovery (%)	64	75	71
Average concentration (µg/kg)	0.32		
Standard deviation (SD)	0.03		
1.64 x SD	0.05		
CCα (µg/kg)	0.37		
CCβ (µg/kg)	0.42		

Table III

Comparison of the results determined through Biochip and LC-MS/MS methods

Sample No	Biochip (µg/kg)	LC-MS/MS (µg/kg)
1	0.79	0.88
2	0.84	0.33
3	3.07	2.34
4	0.23	0.07
5	0.76	0.61
6	0.49	0.29
7	0.77	0.43
8	0.44	0.94
9	0.11	0.32
10	0.11	0.32
11	0.11	0.32
12	0.85	0.12
13	0.44	0.14
14	0.11	0.02
15	0.11	0.53
16	0.11	0.26

For most of the samples the results determined through the Biochip method were close to those obtained through LC-MS/MS method. There were also some samples that produced different results, most likely because of the great complexity of honey sample composition, viscosity and sugar amount. But for screening purposes, the Biochip method proved to be a valuable monitoring instrument.

In terms of literature similar studies, there is more data that confirms the capacity and sensitivity of the Biochip method for identification of nitrofuran antibiotics in food [21, 22]. The limit of detection for the investigated Biochip technique was below 0.9 µg/kg for all metabolites, where the threshold limit was generally set at 1 µg/kg. Therefore, our data is in accordance to other published studies that sustain the use of Biochip assay for the fast screening of banned antibiotic residues in different food samples. Moreover, the detection costs, speediness and reliability of this method represent more arguments for its use in food safety domain.

Conclusions

The Biochip technology allowed the quantification of residues of chloramphenicol in honey at levels that were lower than the minimum required performance limits. The sensitivity of the new quantitative determination method for chloramphenicol was high and it was evaluated as IC50 (0.74 µg/kg). The method presented very good accuracy both within the same analytical series and in different analytical series with typical values lower than 15% for concentration levels of 0.5, 1 and 1.5 µg/kg. The decision limit (CCα) of chloramphenicol was 0.37 µg/kg, while the detection capability (CCβ) was 0.42 µg/kg.

The validated Biochip method was applied to the analysis of 16 samples of honey from various geographic regions in Romania and the results were confirmed using a validated LC-MS/MS method. Although honey is considered a healthy natural product, the incidence of honey samples contaminated with traces of chloramphenicol was high.

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