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ELISA validation and determination of cut-off level for chloramphenicol residues in honey

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Abstract

An analytical validation of a screening ELISA for detection of chloramphenicol (CAP) in honey was conducted according to the Commission Decision 2002/657/EC and Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines. The analyte was extracted from honey with a water and ethyl acetate mixture, and CAP concentrations were measured photometrically at 450 nm. The recovery rate of the analyte from spiked samples was 79%. The cut-off level of CAP in honey as the minimum recovery (0.17 units) was established. Detection capability (CCβ) was fixed at 0.25 μg kg^{-1}. No relevant interferences between matrix effects and structurally related substances including florfenicol and thiamphenicol were observed. The ELISA method should be useful for determination of CAP residues in honey monitoring.

Keywords: honey, chloramphenicol, ELISA, validation.

Introduction

Chloramphenicol is an antibiotic that was first isolated from the soil bacterium *Streptomyces venezuelae* in 1947. At present, this antibiotic is produced synthetically. Chloramphenicol is bacteriostatic and at high concentrations may be bactericidal when used against highly susceptible organisms. It blocks protein synthesis in bacteria by binding to the 50S subunit of the 70S ribosome. Teratogenicity studies in rats and rabbits showed that chloramphenicol failed to be teratogenic although it caused a high incidence of foetal deaths. The tests for carcinogenicity of chloramphenicol in experimental animals were inadequate and found only that the compound increased the incidence of lymphomas in two mouse strains and hepatocellular carcinomas in one mouse strain when administered in the drinking water. Epidemiological data evidence suggested that the treatment of humans with chloramphenicol induced blood dyscrasias, and particularly aplastic anaemia. (4, 10, 11).

The EU has totally banned the use of chloramphenicol in food-producing animals, including honey bees, since 1994. A zero tolerance level for chloramphenicol residues has been established. The acceptable daily intake (ADI) has never been fixed, because chloramphenicol induces aplastic anaemia in humans and consequently the maximum residue limit (MRL) has not been assigned. However, the Commission Decision 2002/657/EC introduced the idea of the minimum required performance limit (MRPL) meaning the minimum concentration of residues of a banned substance in a sample determinable and confirmable by the analytical method used with specified degrees of accuracy and precision. The MRPL for detection of chloramphenicol residue in honey has been fixed at 0.3 μg kg^{-1} (1, 2, 3). It should be stressed that the sensitivity and precision of the method used play a key role for correct detection of chloramphenicol in honey. Furthermore, the method should be as cost-effective and reliable as possible. In practice, it is difficult to develop one method that combines all these characteristics. Therefore, the analytical strategies used for the monitoring of residues involve a screening method followed by a confirmatory analysis of the samples that gave positive results with that screening method. A variety of analytical techniques have been used for the confirmation of chloramphenicol in honey and other biological matrices. CAP residue methods include mass spectrometry in combination with gas chromatography

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(GC/MS) and high performance liquid chromatography (LC/MS) (12, 13, 14). In this paper, a rapid screening enzyme-linked immunosorbent assay (ELISA) for chloramphenicol is developed and validated by the determined specific cut-off level.

Material and Methods

ELISA kit. An ELISA Ridascreen Chloramphenicol kit was purchased from R-Biopharm (Germany). The test kit contained: a microtitre plate with 96 wells coated with capture antibodies against chloramphenicol-antibodies; six chloramphenicol standards at concentrations of 0, 25, 50, 100, 250, and 750 ng L^{-1}; a bottle of peroxidase conjugated chloramphenicol concentrate; a bottle of substrate/chromogen solution; a bottle of stop solution (1 N (mol L^{-1}) sulphuric acid; buffer for conjugate and sample dilution; and washing buffer to prepare a 10 mM phosphate buffer containing 0.05% Tween 20.

Standard solutions. Chloramphenicol, florfenicol, and thiamphenicol standards were used to prepare stock solutions, sourced as pure drugs with a certificate of analysis (Sigma-Aldrich). Stock solutions of 1000 ng mL^{-1} were prepared from the three standards using methanol as a solvent. A working standard solution (10 ng mL^{-1}) was prepared before each analysis from the chloramphenicol stock solution diluted with the buffer provided with the test kit.

Spiked samples. For the fortified sample, 20 residue-free honey samples of various botanical origins (black and light) were used. The samples were obtained from a local apiary involved in the National Residue Programme. They were spiked with 10 ng mL^{-1} of working chloramphenicol standard solution at the screening target concentration (STC) of 0.25 µg kg^{-1}. The fortified samples were left for 30 min before the analysis.

Honey sample preparation. Two grams of honey sample were placed into 25 mL glass centrifuge tubes. Distilled water and ethyl acetate were added to the centrifuge tube in 4 mL volume and shaken upside-down vigorously for 10 min using a mechanical shaker. The tube was then centrifuged for 10 min at 2900 rpm at room temperature. Next, 1 mL of ethyl acetate supernatant was transferred into a new vial; the extract was then dried with a stream of nitrogen on a hot plate at 60°C. The dried residue was dissolved in 0.5 mL of the buffer provided with the ELISA kit and the samples were mixed for about 1 min. A volume of 50 µL/well was used in the assay.

ELISA procedure. Each standard solution at the supplied concentrations mentioned above, and prepared blank and fortified samples were added to separate duplicate pre-coated wells in a 50 µL volume. The same volume of a diluted enzyme conjugate was added to the bottom of each well. After gentle manual mixing, the solutions were incubated for 60 min at 20°C to 22°C. The non-bound enzyme-conjugate reagent was removed and washed three times by a washing buffer. The CAP enzyme conjugate amount was visualised by adding 100 µL of substrate/chromogen and incubating for 15 min at room temperature in darkness to transform it into a product coloured by the bound enzyme conjugate. Stopping the substrate reaction was achieved by addition of 1N of sulphuric acid followed by a colour change from blue to yellow. The absorbance was measured photometrically at 450 nm.

Validation. The procedure was performed according to the Commission Decision 2002/657/EC and Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines 20/1/2010. The performance characteristics including the specificity/selectivity, detection capabilities of CCβ, and cut-off level were determined. The selectivity and specificity were evaluated by analysing blank honey samples fortified with thiamphenicol and florfenicol (chemically related to CAP) at the concentration corresponding to the MRL.

Results

The enzyme immunoassay for the quantitative analysis of chloramphenicol was validated by the determination of the specificity/selectivity, detection capabilities of CCβ, and cut-off level. The cut-off level is the response or signal in a screening test which indicates that a sample contains a substance at a concentration similar to or higher than the STC and specifies the concentration at which a screening test categorises the samples as potentially non-compliant and needing confirmatory analysis. During the validation process, the cut-off level was established using the matrix blank sample analysis and replicates of the same samples spiked (fortified) at the STC. For chloramphenicol, the STC was less than the MRPL and fixed at a concentration of 0.25 µg kg^{-1}. The cut-off level for honey was calculated at a concentration of 0.17 µg kg^{-1}/units (Table 1). Twenty different blank honey samples were selected. Blank and spiked samples were analysed in various periods. The analysis also included the range of response in the blank samples. The highest response of the blank samples was noted as a 0.082 unit and the lowest response of the spiked samples was noted as a 0.17 unit. In this case, none of the fortified samples overlapped with the response ranges of the blank samples. For this reason, the CCβ was established to be 0.25 µg kg^{-1}. Consequently, the CCβ of this assay and the β error were 0.25 µg kg^{-1} and 0 respectively. This meets the condition laid down in Commission Decision 2002/657/EC which permits less than 5% of false compliant results (β error).

Table 1. The cut off level for CAP in honey

Sample number	Blank samples	Spiked samples 0.25 µg kg^{-1}
1	0.050	0.169
2	0.051	0.174
3	0.052	0.181
4	0.056	0.180
5	0.049	0.184
6	0.043	0.220
7	0.052	0.232
8	0.057	0.231
9	0.061	0.186
10	0.052	0.194
11	0.053	0.196
12	0.056	0.187
13	0.070	0.166
14	0.027	0.192
15	0.035	0.200
16	0.037	0.206
17	0.082	0.299
18	0.075	0.233
19	0.077	0.168
20	0.036	0.167

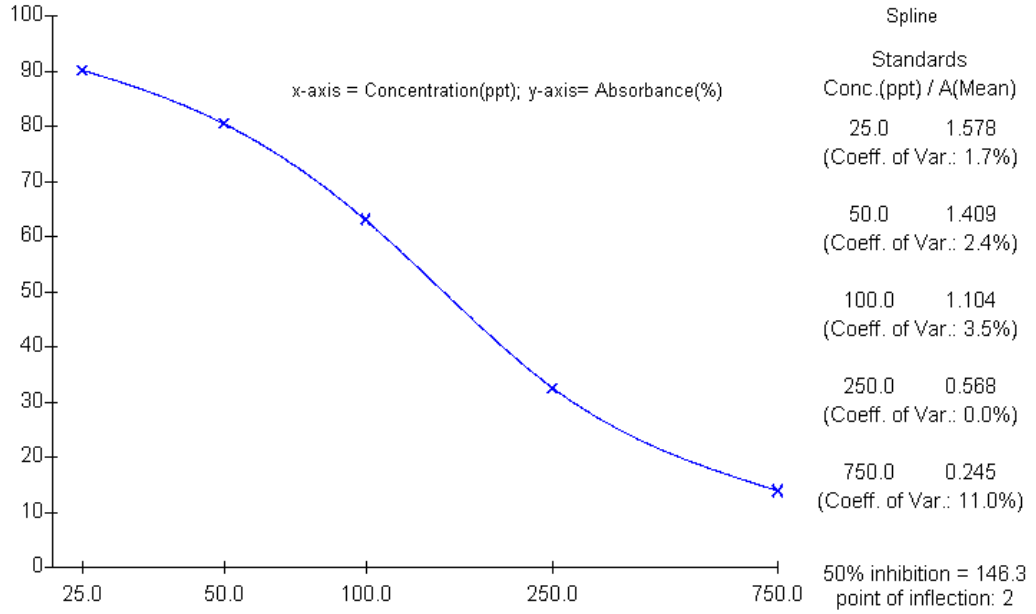


Fig. 1. Calibration curves of the competitive ELISA for chloramphenicol in honey. Each point presents the average of two well replicates

The selectivity/specificity data indicated that no relevant matrix interferences were observed during the validation. Moreover, no interference was found when the thiamphenicol and florfenicol were added to honey samples at the MRL concentration. The antibodies directed against chloramphenicol did not show cross-reactivity with other phenicols, indicating that the monoclonal antibodies used in the test were highly specific to CAP. Fig. 1 shows a representative standard curve for chloramphenicol that was prepared with standard solutions in water at final concentrations of 0, 25, 50, 100, 250, and 750 ng kg-1.

Discussion

Several methods have been used to detect residue of chloramphenicol in honey and other matrices (7, 12, 13, 14). However, an enzyme immunoassay is now considered to be the most sensitive and has been used

for the last few decades. The validation of methods is fundamental to the quality of the final results and the applicability of qualitative methods of residue analysis. For several years so far, method validation studies, guidelines, and procedures have mainly focused on the quantitative methods of analysis. As a result, the literature considering performance parameters, discussing procedures, and describing theoretical studies has been growing (5, 6, 8, 9). The validated method presented in this paper meets the performance criteria and the requirements set out in the regulations of the European Union for validation of screening methods to be used for regulatory purposes. Particularly, the current Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines are addressed to those analytical methods that provide binary results including YES/NO based on the determined cut-off level (3).

The validated ELISA shows optimal performance in terms of sensitivity and acceptable specificity. Consequently, the method is suitable for detecting chloramphenicol in honey and may be used for screening purposes connected with the National Residue Programme.

Conflict of Interests Statement: The author declares no conflict of interests regarding the publication of this article.

References

1. Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. O J 2002, L 221, pp. 8–36.
2. Commission Decision of 13 March 2003 amending Decision 2002/657/EC as regards the setting of minimum require performance limits (MRPLs) for certain residues in food animal origin 2003/18/EC. O J, 2003, L71.
3. Community Reference Laboratories Residues (CRLs) Guidelines for the Validation of Screening Methods for Residues of Veterinary Medicines (Initial Validation and Transfer) 20/1/2010.
4. EMEA Committee for veterinary medicinal products. Chloramphenicol. Summary report, http://www. ema.europa.eu.
5. Eurachem.: The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. Eurachem 1998, http://www.eurachem.org
6. International Union of Pure and Applied Chemistry.: Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis. Pure Appl Chem 2002, 74, 835.
7. Forti F., Campana G., Simonella N.: Determination of chloramphenicol by liquid chromatography–tandem mass spectrometry. Anal Chem Acta 2005, 259, 257–263.
8. Gaudin V., Hedou C., Verdon E.: Validation of a wide-spectrum microbiological tube test, the EXPLORER® test, for the detection of antimicrobials in muscle from different animal species. Food Add Contamin 2009, 26, 1162–1171.
9. Gowik P.: The validation of methods for regulatory purposes in the control of residue. J Chromatogr A 2009, 1216, 8051–8058.
10. Hazardous Substances Data Base.: HSDB, National Library of Medicine, http://toxnet.nlm.nih.gov/
11. Jimenez J.J., Jimenez J.G., Daghistani D., Yunis A.: Interaction of chloramphenicol and metabolites with colony stimulating factors: possible role in chloramphenicol-induced bone marrow injury. Am J Med Sci 1990, 300, 350–355.
12. Posyniak A., Żmudzki J., Niedzielska J.: Evaluation of sample preparation for control of chloramphenicol residues in porcine tissues by enzyme-linked immunosorbent assay and liquid chromatography. Anal Chim Acta 2003, 483, 307–311.
13. Ronning H.T., Einarsen K., Asp T.N.: Determination of chloramphenicol residues in meat, seafood, egg, honey, milk, plasma and urine with liquid chromatography–tandem mass spectrometry, and the validation of the method based on 2002/657/EC. J Chromatogr A 2006, 1118, 226–233.
14. Rodziewicz L., Zawadzka I.: Rapid determination of chloramphenicol residues in honey by liquid chromatography–tandem mass spectrometry and the validation of method based on 2002/657/EC. Apiacta 2007, 42, 25–30.