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Institute for Reference Materials and Measurements
Community Reference Laboratory for Feed Additives

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CRL Evaluation Report on the Analytical Methods submitted in connection with the application for modification of authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q- 2007-180 FAD-2007- 0035
Product name:	Cycostat 66G
Active Substance(s):	robenidine hydrochloride
Rapporteur Laboratory:	Istituto Superiore di Sanità (ISS) Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare - Rome, Italy
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Report approved by:	Christoph von Holst (CRL-FA)
Date:	18/07/2008

EXECUTIVE SUMMARY

Cycostat 66G is a coccidiostat already authorised as feed additive for use in rabbits for breeding by Commission Regulation (EC) No 2430/1999 and for use in rabbits for fattening, chickens for fattening and turkeys by Commission Regulation (EC) No 1800/2004.

The active agent of *Cycostat 66G* is robenidine hydrochloride. The authorised inclusion level is ranging from 30 to 66 mg active substance/kg complete feedingstuffs, depending on the species or category of animal.

In the current application a modification of the conditions of authorisation is sought for *Cycostat 66G* according to Article 13(3) of Regulation (EC) No 1831/2003. Specifically Maximum Residue Limits (MRLs) in chickens for fattening and turkeys are proposed. The provisional MRLs proposed for chickens for fattening are (1) 200 μg kg^{-1} in muscle, (2) 2000 μg kg^{-1} in skin/fat, (3) 1250 μg kg^{-1} in liver and (4) 750 μg kg^{-1} in kidney. For turkeys the MRLs proposed are (1) 200 μg kg^{-1} in muscle, (2) 1000 μg kg^{-1} in skin/fat, (3) 1000 μg kg^{-1} in liver and (4) 200 μg kg^{-1} in kidney.

Since robenidine hydrochloride belongs to group B of Annex I to Council Directive 96/23/EC[1], the confirmatory methods for the detection of residues in target matrices that are suitable for official control have to comply with the criteria specified in Commission Decision 2002/657/EC[2].

For the determination of the residues of robenidine in tissues of all target species the applicant proposed a High Pressure Liquid Chromatography (HPLC) method with *single* wavelength Ultraviolet (UV) detection adjusted at 317 nm. A limit of quantification (LOQ) of 100 μg kg^{-1} has been established for all tissues and animal species which is well below the proposed MRLs. Also acceptable values for the precision and accuracy have been obtained and therefore the method is considered suitable for *quantification* of robenidine in target tissues at concentrations below or at the proposed MRLs. However, the method does not comply with the required criteria for the *confirmation* of the presence of robenidine in the case of exceeding the proposed MRLs, since Commission Decision 2002/657/EC requires that for LC/UV, *two* different chromatographic systems or a *second, independent detection method* are used.

[1] Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC, OJ 125, 23.05.1996, p. 10

[2] 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, OJ L 221, 17.08.2002, p. 8

For confirmatory purposes a method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has been developed and is available at the Community Reference Laboratory for Residues of Veterinary Drugs at the German Federal Office of Consumer Protection and Food Safety, Berlin[3]. This method was successfully in–house validated in accordance with the requirements of Commission Decision 2002/657/EC. The decision limit (CC_α) for robenidine was 2.79 μg kg^{-1} and the detection capability (CC_β) was 4.64 μg kg^{-1}.

Another confirmatory method for detection of robenidine in muscle validated according Commission Decision 2002/657/EC is available and published in literature (*Dubois M. et al., (2004). Journal of Chromatography B, 813: 181-189*). Also this method is based on liquid chromatography coupled to low resolution tandem mass spectrometry (LC-MS/MS). The CC_α was 0.2 μg kg^{-1} and the CC_β was 0.5 μg kg^{-1}.

The CRL concludes that both LC-MS/MS method can be applied for confirmatory purposes of robenidine in animal tissue.

Further testing or validation is not considered necessary.

KEYWORDS

Cycostat 66G, robenidine hydrochloride, chickens for fattening, turkeys, Maximum Residue Limits.

[3] Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL), Berlin, Germany

BACKGROUND

Cycostat 66G is a coccidiostat already authorised as feed additive for rabbits for breeding [1] and for fattening, and for chickens for fattening and turkeys [2].

According to the applicant, *Cycostat 66G* is a greyish product containing robenidine hydrochloride, a chemical synthesised substance, with a minimum purity of 97%. The percentage of incorporation of robenidine hydrochloride in *Cycostat 66G* is 6.6% w/w. The authorised inclusion level is ranging from 30 to 66 mg active substance/kg complete feedingstuffs, depending on the target animal species [3].

In the current application a modification of the terms of authorisation according to Article 13 (3) of Regulation (EC) No 1831/2003 is sought for *Cycostat 66G*. Specifically, a set of Maximum Residue Limits is proposed by the applicant.

TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and the tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority for each application. For this particular dossier, the methods of analysis for detection and quantification of robenidine residues in tissues of target animals, submitted in connection with *Cycostat 66G* (EFSA-Q-2007-180), and their suitability to be used for official controls in the frame of the authorisation, were evaluated.

EVALUATION

Description of the qualitative and quantitative methods for determining the marker residue(s) of the active substance in target tissues and animal product

Cycostat 66G is currently authorised as feed additive for rabbits for breeding and for fattening, for chickens for fattening and turkeys. The Applicant proposes the following MRLs for chickens for fattening and for turkeys [3]:

Maximum Residue Limit (MRL)			
Marker residue	Species or category of animal	Target tissue(s) or food products	Maximum content in tissues
Robenidine	Chickens for fattening	Muscle Skin/fat Liver Kiney	200 µg/kg 2000 µg/kg 1250 µg/kg 750 µg/kg
Robenidine	Turkeys	Muscle Skin/fat Liver Kiney	200 µg/kg 1000 µg/kg 1000 µg/kg 200 µg/kg

The parent molecule, unchanged robenidine, has been identified as a suitable marker residue in chicken and turkeys [4].

The applicant suggests a High Pressure Liquid Chromatography (HPLC) method with Ultraviolet (UV) detection (single wavelength – detector wavelength 317 nm) [5] that has been developed and validated to determine robenidine residues in the target tissue from chicken [6], turkey [7] and rabbit [8]. Suitable extraction and cleaning procedures have been established for muscle, liver, kidney, and skin/fat for all target species. The method was characterised by most of the criteria laid down in Annex III to Regulation (EC) 882/2004 [9]. The obtained values for the limit of detection (LOD) of the method were different between the three species and among the different tissues. In detail, in poultry the LOD was 3.9 µg kg^{-1} for muscle, 5.12 µg kg^{-1}, for liver, 3.78 µg kg^{-1} for kidney, 9.15 µg kg^{-1} for fat and 9.13 µg kg^{-1} for skin with fat. In turkey the LOD was 8.11 µg kg^{-1} for muscle, 23.0 µg kg^{-1} for liver, 8.80 µg kg^{-1} for kidney, 5.12 µg kg^{-1} for fat and 7.14 µg kg^{-1} for skin with fat. In rabbit the LOD was 4.6 µg kg^{-1} for muscle, 4.5 µg kg^{-1} for liver, 4.1 µg kg^{-1} for kidney and 15.9 µg kg^{-1} for fat. A limit of quantification (LOQ) of 100 µg kg^{-1} has been established for all tissues and animal species. In addition the methods showed sufficient values for the accuracy and precision, since the percentage recovery rate ranged between 70-110% and the within laboratory percentage reproducibility standard deviation was below 20%.

Based on the acceptable performance characteristics the CRL considers this method suitable for the quantification of robenidine in the target tissues.

Since robenidine belongs to group B of Annex I to Council Directive 96/23/EC, the analytical methods for the confirmation of its residues in target matrices need to fulfil the criteria specified in Commission Decision 2002/657/EC. However, the HPLC method proposed by the applicant employs a *single* chromatographic system measuring at a *single* wavelength. In

consequence the proposed method does not comply with these criteria, since *two* different chromatographic systems or a *second, independent detection method* are required to fulfil these criteria.

For the determination of robenidine residues in poultry tissues, a confirmatory method based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS), which was validated in accordance with the requirements of Commission Decision 2002/657/EC, is available at the Community Reference Laboratory for Residues of Veterinary Drugs at the German Federal Office of Consumer Protection and Food Safety [10]. The target analyte was extracted from the matrix using acetonitrile and the extract was subjected to a clean up with solid-phase extraction. For quantification purposes a deuterated hydroxylated metabolite of ipronidazole (IPZOH-d3) was applied. The method was validated for the simultaneous determination of several coccidiostats, including robenidine, in muscle and liver of poultry, bovine, ovine and sheep in a concentration range of 2.0 to 10.0 μg kg^{-1}. Validation was carried out with underlying α- and β- error probabilities of 1% and 5% respectively, and showed sufficient sensitivity expressed in terms of the decision limit (CC_α) for robenidine of 2.79 μg kg^{-1}. The detection capability (CC_β) was 4.64 μg kg^{-1}.

Alternatively another confirmatory method for the determination of robenidine in poultry muscle is available in the literature [11]. The method was validated according to Commission Decision 2002/657/EC, for the simultaneous detection and quantification of nine coccidiostats in eggs and muscle. This method is based on liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Dinitrocarbanilide-d8, nigericin and diclazuril-bis were used as internal standards. The extraction was executed in acetonitrile followed by a clean-up on an silica solid-phase extraction column. High-performance liquid chromatography separation was performed on a C18 column. For unequivocal identification of each analyte, two ions were detected and chosen for multiple reaction monitoring (MRM). Validation was carried out on spiked muscle and egg samples. The percentage recovery after extraction of muscle sample spiked with robenidine at 2 μg kg^{-1} was 56%. Validation results are presented with the measured decision limit (CC_α) and the detection capability CC_β values. The CC_α for robenidine was 0.2 μg kg^{-1} and the CC_β was 0.5 μg kg^{-1}.

CONCLUSIONS AND RECOMMENDATIONS

For the determination of the robenidine, marker residue of robenidine hydrochloride, in chicken and turkey tissues various methods have been evaluated. The applicant's method is suitable for the *quantification* of the target analyte in the target tissue at concentration *below*

the proposed MRL. However this method does not comply with the criteria for confirmatory methods required by Commission Decision 2002/657/EC, thus in cases where the analytical result indicates an exceeding of the MRL, a confirmatory method must be used.

Two LC-MS/MS methods, namely a method developed at the Community Reference Laboratory for Residues of Veterinary Drugs [10] and another method published in the literature [11], which have been validated in accordance with the requirements laid down by Commission Decision 2002/657/EC are available and are recommended by the CRL for Feed Additives for confirmatory purposes.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Method for quantification of residues in target tissues: Liquid chromatography coupled with UV detection (LC-UV)

Method for confirmatory purposes: Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS).

DOCUMENTATION AND SAMPLES PROVIDED TO CRL

Reference samples of Cycostat 66G have been sent to the Community Reference Laboratory for Feed Additives. The dossier has been made available to the CRL by EFSA.

REFERENCES

[1] Regulation (EC) No 2430/1999 (O.J. No L296/ 17.11.1999, p. 3).
[2] Regulation (EC) No 1800/2004 (O.J. No L317/37 16.10.2004, p. 37).
[3] Annex III. Proposal of Registry entry.
[4] Technical Dossier, Section I – p. 22.
[5] Technical Dossier, Section II – p. 35.
[6] Technical Dossier, Section II – Annex II.6.8.
[7] Technical Dossier, Section II – Annex II.6.9.
[8] Technical Dossier, Section II – Annex II.6.10.
[9] Regulation (EC) No 882/2004 of 29 April 2004 (O.J. L 191, 28.5.2004, p. 1).

[10] BVL in-house method: Coccidiostats in liver and muscle (COCC_004-sd_Ver.2-22.03.07.doc).

[11] Dubois M., et al., (2004). Journal of Chromatography B, vol. 813: 181-189.

RAPPORTEUR LABORATORY & NATIONAL REFERENCE LABORATORIES

The Rapporteur Laboratory for this evaluation was Istituto Superiore di Sanità – Dipartimento di Sanità Pubblica Veterinaria e Sicurezza Alimentare - Rome, Italy.

This report is in accordance with the opinion of the consortium of National Reference Laboratories as referred to in Article 6(2) of Commission Regulation (EC) No 378/2005.

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- Central Institute for Supervising and Testing in Agriculture, Praha, Czech Republic
- Saxonian National Institute for Agriculture, Leipzig, Germany

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