Research Article

CONFIRMATION AND ESTIMATION OF CHLORAMPHENICOL RESIDUES IN GALDA (*MACROBRACHIUM ROSENBERGII*) BY ULTRA PERFORMANCE LC[®] (UPLC) AND MS/MS (TQD) SYSTEM

Alam Md. Ashraful^{1*}, Islam Md. Serajul¹, Talukdar Muhammad Waliullah², Akter Mst Yeasmin², Parvez Md. Anwar¹

¹FIQC Laboratory, Department of Fisheries, Matshya Bhaban, Ramna, Dhaka, Bangladesh

²Molecular and Cell Biology Laboratory, Bioscience Department, GSST, Shizuoka University, Suruga-ku, Shizuoka, Japan

*Corresponding Author Email: ashrafbemb@yahoo.com

Article Received on: 18/12/13 Revised on: 07/01/14 Approved for publication: 20/01/14

DOI: 10.7897/2230-8407.050219

ABSTRACT

An Ultra performance liquid chromatography – tandem quadrupole mass spectrometry (UPLC–MS/MS) method was developed for the determination of chloramphenicol in Galda shrimp (*Macrobrachium rosenbergii*). The UPLC separation was achieved on an AQUITY UPLC BEH C₁₈ Column (2.1 x 50 mm; 1.7 μm) with gradient elution using a mobile phase of methanol and water only. Mass spectral acquisition was done using electro spray ionization in the negative ion mode applying multiple reaction monitoring (MRM) of two diagnostic transition reactions for CAP (m/z 321 → 152 and m/z 321 → 257) and for d5-CAP (m/z 326.03 → 157). Shrimp samples were finally extracted with ethylacetate and evaporated to dryness, followed by a clean-up step using the liquid–liquid extraction with acetonitrile / hexane (1:1 v/v) mixture and reconstituted with 50 % methanol and water. The method validation was carried out according to the criteria of Commission decision 2002/657/EC. The calibration curve showed a good linearity in the concentration range from 0.05 to 1.0 ng/g with the correlation coefficient above 0.9995. The decision limits (CCα) and a detection capability (CCβ) were 0.041 and 0.070 ng/g respectively. The mean recoveries were in the range of 85–110 %.

Keywords: Ultra-performance liquid chromatography (UPLC), TQD mass spectrometry, chloramphenicol, Galda (*Macrobrachium rosenbergii*).

INTRODUCTION

Chloramphenicol (CAP) is a broad-spectrum antibiotic drug which interferes with protein synthesis of many gram-negative and gram-positive bacteria¹. In Asia, chloramphenicol has been widely used as a veterinarian antibiotic in aquaculture despite regal controls in many countries². The wide range antibiotic CAP has toxic effects on humans³. Miscellaneous toxic effects are due to the dichloride carbon alpha to the carbonyl group (Figure 1); this carbon readily undergoes substitution with nucleophiles such as those found on proteins⁴. Because of many serious side-effects (e.g. fatal aplastic anemia, grey syndrome, severe bone marrow depression)^{5–6}, use of CAP to treat food-producing animals was banned by the European Community in 1994⁷.

Figure 1: Structural formula of chloramphenicol

The minimum required performance limit (MRPL) for the detection of CAP residues in food of animal origin has been fixed at 0.3 μg/kg⁸. Thus, a sensitive and reliable method for the determination of CAP at residual levels is very important. In the past decade, several analytical methods have been developed for the screening and quantitation of CAP in foods and biological samples. For screening purposes, immunochemical tests^{9–11} and chromatographic techniques such as high-performance liquid chromatography (HPLC)

with diode array detection and gas chromatography (GC) with electron capture detection have been used^{12–13}. However, the confirmation of suspect positive samples must be carried out by mass spectrometry (MS) coupled to an adequate chromatographic separation. For this purpose, GC–MS methods using either electron impact (EI) or chemical ionization (CI) have been reported^{14–16}. But these procedures still require a tedious derivatization step prior to final analysis. Liquid chromatography methods, however, do not require a derivatization step and HPLC–MS sensitivity approaches that of GC–MS. The HPLC with tandem mass spectrometric detection (HPLC–MS–MS) has already been demonstrated for analysis of complex food matrices such as meat, seafood, eggs, honey, and milk and particularly for analysis of antibiotic residues^{17–18}. However, Ultra Performance LC (UPLC) and tandem quadrupole MS/MS (TQD) is a more sophisticated technique allowing a very effective isolation of analyte ions from the noise-producing matrix. This paper focuses on the optimization of UPLC–MS/MS technique for the conformation and estimation of CAP in Galda shrimp (*Macrobrachium rosenbergii*) with a very easy mobile phase which is better for long life of LC column. Validation of the confirmatory methods was performed according to EU decision 2002/657/EC.

MATERIAL AND METHODS

Negative Shrimp (Galda) Collection

Samples were cultured and collected in a standard condition without adding any kind of chloramphenicol contamination.

Apparatus and Chemicals

Micro pipette: eppendorf, analytical balance (4 dp), Shimadzu AUY 220; syringe filter: 13 mm PTFE 0.2 μm filter (Waters, USA); CAP and d5-CAP standards, assay > 99 %, Sigma Fluka, Germani; ethylacetate and n-Hexane (HPLC

grade), acetonitrile and methanol (HPLC and MS grade), Sigma Aldrich, Germani; NaCl: RANKEM, S0160; Phosphate buffered saline (PBS); Purified Water (de ionized) from Milli-Q apparatus (Millipore, Bedford, USA)

Samples

Shrimp samples were pasted with blender after removing shell and eight samples [reagent blank, matrix blank, matrix with internal standard (IS) blank and quality control (QC)] were taken as quality samples and another seven were taken to make calibration curve (0.05 to 1 ppb). All these samples and analytes were weighed out (3.00 ± 0.01 g) into 50 ml screw capped polypropylene centrifuge tube separately.

Extraction Procedure

300 μ l of D5-CAP solution (10 ng/ml) was added to each of matrix_IS, calibration curve, QC and analytes for equivalent concentration of 1.00 ppb and required volume of CAP standard (10 ng/ml) was added to the standard curve tubes to make equivalent conc. 0.05 to 1 ppb and that of QC samples 0.3 ppb and allowed to stand for 10 minutes. Then 4 ml PBS solution, 1 ml of 16 % NaCl solution and 4 ml of acetonitrile were added. The samples were vortexed for 1 minute and centrifuged at 4500 rpm for 20 minutes. Supernatant was transferred to 50 ml centrifuge tube and 10 ml de-ionized water and 10 ml hexane were added and shaken gently for 30 sec and again centrifuged at 4500 rpm for 20 minutes. After that the upper n-hexane layer was discarded and 8 ml of ethyl acetate was added and mixed by vortex for 1 minute and finally centrifuged at 4500 rpm for 20 minutes. Ultimately upper layer (ethylacetate) was transferred to glass tubes and taken to dryness at 55°C under N₂ gas. Residue was reconstituted in 1 ml of 50 % methanol and transferred to vial by filtering with syringe filter (0.2 μ m) for analysis with UPLC/MS/MS.

UPLC-MS/MS Analysis

Software: Mass lynx v4.1

LC Identity: ACQUITY UPLC® Waters, USA

Column: AQUITY UPLC BEH C18, 1.7 μ m, 2.1 x 50 mm (Waters, USA),

Solvent A: Water, Solvent B: Methanol,

Injection Volume (μ l): 10, Column temp: 35°C, Sample Temp: 10°C

LC Separation Method: Gradient

MS Method Parameters

Instrument Identity: ACQ_TQD#QBB933, Waters, USA.

Capillary (V): 30, Cone (V): 30, Extractor (V): 4, RF Lens: 0.20, Source Temp.: 120°C, Dessolvation Temp: 280°C, Cone Gas Flow(L/Hr): 50, Desolvation Gas Flow (L/H): 900, Collision Gas Flow (L/H): 0.10, LM1 Resolution: 14, HM1 Resolution: 15, Ion Energy 1: 0.80, MS mode entrance: 50, exit: 50. MSMS mode: entrance: 1, exit: 1, LM2 Resolution: 15, HM2 Resolution: 15, Ion Energy 2: 0.80, Ion mode: ESI (-Ve), Run time (Min): 5, Function type: MRM (multiple reaction monitoring)

Calculation

Ion Ratio, R = Peak area of primary ion (PAPI)

Peak area of secondary ion (PASI)

Response factor (RF)

RF = PAPI of interested substance x ISC

Peak area of internal standard ion

CAP concentration, X = (RF-b)/a

Where, ISC= Internal Standard Concentration, a=Slope of calibration curve, b= intercept of calibration curve.

RESULTS

MS-MS detection

The detector (TQD MS-MS) was firstly operated in negative ESI-MS mode to select characteristic ions as the precursors of CAP (m/z 321) and its deuterated internal standard d5-CAP (m/z 326) (Figure 2). Both CAP and d5-CAP were then analyzed by UPLC-ESI- MS-MS in a negative ionization product ion scan mode by selecting m/z 321 and m/z 326 ions as the precursor ion, respectively. The collision-induced dissociation (CID) experiments of these ions; giving rise to m/z 257 and m/z 152 ions for CAP and m/z 157 for d5-CAP (Figure 3)

Table 1: Transitions monitored for CAP determination and optimal MS-MS conditions

S. No	Parant (Da)	Daughter (Da)	Dwell(s)	Cone (V)	Collision (eV)	Compound
1	321.00	152.00	0.050	32	18.00	CAP
2	321.00	257.00	0.050	32	11.00	CAP
3	326.03	156.94	0.050	34	16.00	CAP_D5

Table 2: Gradient Table

S. No	Time (minute)	Flow Rate (ml)	% A	% B
1	Initial	0.200	80	20
2	0.5	0.200	80	20
3	1.60	0.200	10	90
4	1.70	0.200	10	90
5	2.00	0.200	80	20
6	5.00	0.200	80	20

Table 3: UPLC-MSMS performance tables for QC samples

Parameter	Fortification level ng/g		
	0.10	0.30	0.40
Overall mean $\pm$ SD	0.10 $\pm$ 0.007	0.32 $\pm$ 0.02	0.42 $\pm$ 0.02
Overall Recovery	104	105	104

Table 4: Analysis for the Quantitative detection of CAP at 0.3 ppb by UPLC-MSMS

S. No.	Date/File	Recovery (ppb)	Mean	Mean + 1SD	Mean + 2SD	Mean + 3SD	Mean - 1SD	Mean - 2SD	Mean - 3SD	Trend	Date
1	CAP-301210	0.31	0.312353	0.35932	0.40630	0.45327	0.26537	0.21840	0.17142	0.30	16032012
2	CAP-311210	0.35	0.312353	0.35932	0.40630	0.45327	0.26537	0.21840	0.17142	0.31	30012012
3	CAP-151210	0.3	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142	0.30	16112011
4	CAP-141210	0.3	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142	0.30	17102011
5	CAP-121210	0.27	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142	0.29	17082011
6	CAP-051210	0.42	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142	0.31	13072011
7	CAP-021210	0.3	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142	0.30	24052011
8	CAP-241110	0.33	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142	0.30	21042011
9	CAP-121110	0.22	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142	0.27	19032011
10	CAP-041110	0.39	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142	0.29	12022011
11	CAP-031110	0.28	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142		
12	CAP-281010	0.29	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142		
13	CAP-201010	0.3	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142		
14	CAP-191010	0.3	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142		
15	CAP-121010	0.31	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142		
16	CAP-121010	0.28	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142		
17	CAP-071010	0.36	0.312353	0.35932	0.406302	0.45327	0.26537	0.21840	0.17142

Figure 2: Mass Spectrum of CAP and CAP_D5

Figure 3: UPLC-MS-MS chromatograms of daughter ions of CAP and d5-CAP in matrix spiked at 0.3ng/g

Figure 4: Chromatogram of solvent blank

Figure 5: Chromatogram of matrix blank

Figure 6: Chromatogram of internal standard (IS) in matrix

Figure 7: Trend analysis of chloramphenicol in quality control chart

The selected transitions for CAP and d5-CAP for the optimal MS–MS conditions are given in Table 1. The developed gradient method with methanol and water is shown the Table 2. Concentrations were calculated by comparing the ratio of m/z 321→152 response of CAP with the ratio of m/z 326→157 response of d5-CAP. There were no peak in

solvent blank and matrix blank and a single peak in case of matrix_IS (Figure 4-6). The experiment was run whole over the year and trend analysis was done in quality control chart comparing with previous data (Table 4 and Figure 7) which not exceed even 1SD (standard deviation).

Analytical performance

Method validation was carried out according to criteria described in Decision 2002/657/EC. The parameters taken into account were: response linearity, decision limit (CC α), detection capability (CC β), reliability and accuracy. The calibration curve showed a good linearity in the conc. range from 0.05 to 1.0 ng/g with the correlation coefficient above 0.9995. The decision limit (CC α) and the detection capability (CC β) were 0.041 ng/g and 0.07 ng/g, respectively. The trueness was expressed in terms of recovery rates. The results are presented in Table 3.

DISCUSSION

The precursor and daughter ions obtained in the result have a good agreement with previous findings¹⁹ which indicates the compound was identified accurately. The gradient method for compound separation was developed by methanol and water only with sufficient washing protocol without adding any salt, acid or base. As a result, liquid chromatographic column has been experiencing longer life. The trend analysis of the result of quality control samples of this method in quality control chart prepared by previous data explains the experiments of whole year were in good and homogeneous condition. The EU decision⁸ introduces the concepts of decision limit (CC α) and detection capability (CC β) for a chemical analytical method. These parameters are to be used instead of the more familiar limit of detection (LOD) and limit of quantification (LOQ). The definition of the CC α for a forbidden compound is: "The limit at and above which it can be concluded with an error probability of 1 % that a sample is noncompliant". The definition of the CC β for a forbidden compound is: "The lowest concentration at which a method is able to detect truly contaminated samples with an error probability of 5 %". This means that a signal responding to CC α or lower will be regarded as the background noise, while a signal corresponding to CC β or higher will be regarded as originating from a forbidden compound. The decision limit (CC α = 2.33 $\times$ standard error of the y intercept) and the detection capability [CC β = CC α + (1.64 $\times$ standard deviation of 21 spikes at CC α)] for CAP were 0.041 ng/g and 0.07 ng/g, respectively which are significantly below the MRPL of 0.3 ng/g and demonstrates that the method was sufficiently fit for the purpose. As shown in the Figure (4-6) there were no chromatogram of a solvent blank, matrix blank (negative shrimp sample) and a single chromatogram in case of matrix with internal standard (IS) which indicates no contamination was found in sample and in the extraction process. The sample spiked at MRPL level (0.3 ng/g) showed good chromatogram (Figure 3) for each transition. The performance characteristics of the method presented in this paper indicate that it may be preferably used in food control.

AKNOWLEDGEMENT

We would like to provide humble gratitude to the deputy director of Fish Inspection and Quality Control Laboratory. We express our sincere thanks to all officials and staffs of the laboratory for their support in works.

REFERENCES

1. Austin S, Weisberger MD. Inhibition of Protein Synthesis by Chloramphenicol, *Annual Review of Medicine* 1967; 18: 483-494. <http://dx.doi.org/10.1146/annurev.me.18.020167.002411>

2. Amornchai Somjetlercharoen Chloramphenicol in Aquaculture AAHRI Newsletter Article Vol. 11 No. 1; 2002. http://www.fisheries.go.th/dof_thai/Division/Health_new/Health_new/AAHRI/AAHRI/Topics/Newsletter/art54.htm
3. Allen EH. Review of chromatographic methods for chloramphenicol residues in milk, eggs, and tissues from food-producing animals. *J Assoc off Anal Chem* 1985; 68(5): 990-9.
4. BR Baker. Design of Active-Site-Directed Irreversible Enzyme Inhibitors. *The Organic Chemistry of the Enzymatic Active-Site*, John Wiley and Sons, New York 1967; 159: 3819.1091-a
5. Joan Ramon Laporte, Xavier Vidal, Elena Ballarín and Luisa Ibáñez. Possible association between ocular chloramphenicol and aplastic anaemia—the absolute risk is very low. *Br J Clin Pharmacol* 1998; 46(2): 181–184. <http://dx.doi.org/10.1046/j.1365-2125.1998.00773.x>
6. Chloramphenicol Injection Side Effects. Drug.com <http://www.drugs.com /sfx/chloramphenicol-injection-side-effects.html>
7. Commission Regulation (EC) 1430/94, Off. J. Eur. Commun., L156/6; 1994.
8. 2003/181/EC: Commission Decision of amending Decision 2002/657/EC as regards the setting of minimum required performance limits (MRPLs) for certain residues in food of animal origin, Official J, L71/17; 2003.
9. KH Hsieh, CP Lin, CC Cheng. Screening Procedures for Chloramphenicol Residue Determination in Serum, Milk and Feedstuff Using Immuno chromatographic Assay 2013; 27(4): 328-341.
10. Yogeswari S, Ching AL, Chiew KT and Chua SB. Rapid screening method for chloramphenicol in tissues using enzyme-linked immune sorbent assay. *Singapore J Pri Ind* 2005; 32: 46-51.
11. G Scortichini, L Annunziata, MN Haouet, F Benedetti, I Krusteva, R Galarini. ELISA qualitative screening of chloramphenicol in muscle, eggs, honey and milk: method validation according to the Commission Decision 2002/657/EC criteria. *Analytica Chimica Acta* 2005; 535: 43–48. <http://dx.doi.org/10.1016/j.aca.2004.12.004>
12. Mónica Cecilia Vargas Mamani, Felix Guillermo Reyes Reyes, Susanne Rath. Multi residue determination of tetracyclines, sulphonamides and chloramphenicol in bovine milk using HPLC-DAD. *Food Chemistry* 2009; 117.3: 545–552. <http://dx.doi.org/10.1016 /j.foodchem.2009.04.032>
13. Mamani MCV, Reyes FGR, Rath S. Multi residue determination of tetracyclines, sulphonamides and chloramphenicol in bovine milk using HPLC-DAD. *Food Chemistry* 2009; 117.3: 45-552.
14. Yang X, Zhou R, Zhao YF, Wu YN. Analysis of chloramphenicol in shrimps by gas chromatography tandem mass spectrometry. *Wei Sheng Yan Jiu* 2005; 34(5): 584-7.
15. Boerner, S, Fry H, Balizs G, Kroker R. Confirmation of chloramphenicol residues in egg by gas chromatography/high-resolution mass spectrometry and comparison of quantitation with gas chromatography-electron capture detection. *J AOAC Int* 1995; 78(5): 1153-60.
16. Hao Yu Shen, Hai Liang Jiang. Screening, determination and confirmation of chloramphenicol in seafood, meat and honey using ELISA, HPLC–UVD, GC–ECD, GC–MS–EI–SIM and GCMS–NCI–SIM methods. *Analytica Chimica Acta* 2005; 535(1–2): 33–41. <http://dx.doi.org/10.1016/j.aca.2004.12.027>
17. Huang JF, Zhang HJ, Feng YQ. Chloramphenicol extraction from honey, milk, and eggs using polymer monolith micro extraction followed by liquid chromatography-mass spectrometry determination. *J Agric Food Chem* 2006; 54(25): 9279-86. <http://dx.doi.org/10.1021/jf062246e>
18. Jing Fang Huang, Hui Juan Zhang, Yu Qi Feng. Chloramphenicol extraction from honey, milk, and eggs using polymer monolith micro extraction followed by liquid chromatography-mass spectrometry determination. *Journal of Agricultural and Food Chemistry* 2007; 54(25): 9279-86. <http://dx.doi.org/10.1021/jf062246e>
19. Kevin J, Mc Hale. Trace Level Quantitation of Chloramphenicol Using the TSQ Quantum Discovery. Application note: 337. Thermo Fisher Scientific, Somerset, NJ, USA. http://www.thermo.com/eThermo /CMA/PDFs/Articles/articlesFile_4253.pdf

Cite this article as:

Alam Md. Ashraful, Islam Md. Serajul, Talukdar Muhammad Waliullah, Akter Mst Yeasmin, Parvez Md. Anwar. Confirmation and estimation of chloramphenicol residues in Galda (*Macrobrachium rosenbergii*) by ultra performance LC® (UPLC) and MS/MS (TQD) system. *Int. Res. J. Pharm.* 2014; 5(2):92-96 <http://dx.doi.org/10.7897/2230-8407.050219>