Chloramphenicol in Malaysia Waste Water and Its Residues in Animal Husbandaries Products

Marni S.[a], Malintan NT., Faridah I., Mustafa AM.[b]
[a]*Veterinary Public Health Laboratory, Department of Veterinary Services, Bandar Baru Salak Tinggi, Sepang, Selangor*
[a]*Faculty of Medicine, University of Malaya, Kuala Lumpur*

ABSTRACT: A simple and rapid liquid chromatography-mass spectrometry with electrospray ionisation (LC-(ESI)-MS) confirmatory method for the analysis of chloramphenicol (CAP) in poultry tissues has been developed. The method was applied to investigate the occurrence of CAP in poultry samples. A total of 448 samples from 12 poultry processing plants were analysed, covering eight states in Malaysia. Waste water samples (influent and effluent) from animal husbandaries collected from rivers in the state of Perak, Malacca and Selangor were analysed for chloramphenicol. The detectable levels of chloramphenicol in the water were between 10 to 90 ng/litres. Samples were extracted by using liquid-liquid extraction steps with ethyl acetate. LC separation was achieved by a C-18 column and methanol-water as a mobile phase. The mass spectrometer was operated in multiple reactions monitoring mode (MRM) with negative electrospray interface. Three transition ions monitored were *m/z* 321 > 257, *m/z* 321 >152 and *m/z* 326>157 (IS). For quantification, *m/z* 321 >152 was chosen. Validation parameter includes specification, linearity, precision, accuracy, decision limit and detection capability. Samples were fortified at CAP levels of 0.1, 0.2 and 0.3 μgkg^{-1} with D_5-CAP as internal standard. The limit of decision and detection capability was calculated at 0.032 and 0.054 μgkg^{-1} respectively.

Keywords: chloramphenicol, antibiotic, chicken, poultry, health

Introduction

Chloramphenicol is a highly effective antibiotic, used widely to treat food-producing animals. However, chloramphenicol were banned in the European Union (European Commission 1994) because of evidence that it may cause serious adverse health effects in humans (World Health Organization 1988).

In veterinary practices, runoff and leaching from feedlots or from the facilities themselves could carry these substances into the aquatic environment. Transformed and unmetabolised antibiotics are released into the aquatic system through manure or urine in waste water. In the environment, their conjugates are easily cleaved, thus they are released almost unchanged. Furthermore, as reported by Ternes; 1998 (1), Daughton and Ternes; 1999 (2) and Zwiener *et al;* 2000, (3) pharmaceuticals are not completely eliminated during waste water treatment and also not biodegraded in the environment. Thus, antibiotics for veterinary use or as growth promoters are excreted by the animals and end up in the waste.

Corresponding Author:
Marni S.
Veterinary Public Health Laboratory
Department of Veterinary Services
Bandar Baru Salak Tinggi, Sepang, Selangor

Published 12 January 2010

In Malaysia, where integrated animal and fish/vegetable farming is a common practice, manure readily available low cost fertilizer of good quality. This practice of manure disposal on land or incorporation into soil allows antibiotics to seep through the land system hence reaching the water source.

Materials and Method

Reagents and chemicals

Chloramphenicol (CAP) was supplied by Sigma-Aldrich, USA. Deuterated chloramphenicol (D_5-CAP), 99.6% isotopic purity was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). All chemical and chromatographic reagents used were of either HPLC or analytical grade. Ultrapure water was filtered through a Millipore Rios system followed by a Milli-Q Biocell system (18.2 $M\Omega\ cm^{-1}$ resistivity) (Millipore, Bedford, MA, USA). Nitrogen (99.999%) as desolvation and nebuliser gas was generated through a nitrogen generator from Whatman (Haverhill, MA, USA). Argon (99.999%) (MS–MS collision gas) was obtained from Malaysian Oxygen (MOX).

Sample collection and preparation for water sample

A total of 50 waste water samples were collected from three selected swine production facilities from

Malacca, Selangor and Perak, respectively. The samples were collected in pre-cleaned 1 L polypropylene bottles. The waste water was collected at the influent and effluent points of each farm.

The waste water samples were filtered using cotton wool to remove the suspended particles and then filtered with the 0.2 µm membrane filter (Whatman, UK). The samples were stored at 4 ºC until analysis.

Extraction and cleanup for chicken tissue

An amount of 2.0 ± 0.02 g of finely minced tissue was weighed into a 50 ml Falcon polypropylene tube. The sample was fortified with 200 µl of 20 ng/ml internal standard D_5-CAP (equivalent to 2 µg kg^{-1}). The sample was mixed with 800 µl HPLC grade water and homogenized for 15 s. CAP was extracted with 6 ml ethyl acetate by firstly mixing with end-over-end rotator for 10 min followed by centrifugation at 3000 rpm for 5 min (+4°C). After centrifugation, approximately 5 ml of the ethyl acetate layer from the upper phase was transferred to a clean test tube. The sample was evaporated to dryness under stream of nitrogen on a sample concentrator at 45-50°C. The residue was reconstituted in 0.5 ml iso-octane and mixed by vortexing for 25 s. 0.5 ml of water was added and was mixed gently by shaking. The sample was centrifuged at 3000 rpm for 5 min (+4°C). Approximate 0.2 ml of the aqueous phase (lower layer) was transferred into clean glass tube using a pasteur pipette or micropipette. The lipid layer that was located on top of the solution was avoided to ensure getting a clean sample. The aqueous phase was filtered through a 0.22 µm nylon filter directly into a HPLC vial.

Instrumentation

The LC system consisted of a 2695 Alliance Separations Module (Waters, Manchester, UK). Separations of the compounds were performed using HPLC Column: Luna C_{18} (150 mm × 2 mm; 3 µm particle diameter) from Phenomenex, Torrance, CA.

The LC separation of the CAP was achieved using a gradient elution mixture of water and methanol. The ESI-MS/MS detection of the CAP was carried out with a Quattro Ultima Pt triple-quadrupole mass spectrometer from Micromass Co. Inc. (Manchester, UK). The MS detector was operated with an electrospray ionisation (ESI) interface in the negative ion mode. The following tune parameters were used: capillary, 3.50 kV; cone, 35 Volts; source temperature, 120°C; desolvation temperature, 350°C; cone gas flow, 63 L h^{-1}; desolvation gas flow, 595 L h^{-1}; resolution (LM1, HM1) 13.0; resolution (LM2, HM2), 14.0; ion energy 1, 0.5; ion energy 2, 1.0; entrance, -1; exit, 0; photomultiplier, 650V; collision gas: argon (Pirani pressure, 2.52×10^{-3} mbar).

Results and Discussion

LC-MS/MS

Analytes were detected by negative multiple reaction monitoring, after transition of the precursor ion into two product ions. MS-scan of CAP showed that the most abundant molecule for CAP is 321 and the most intense fragment ions from this molecular ion are 152 and 257 where 152 is the major fragment ion. The transitions monitored for D_5-CAP was 325 > 157.

Calibration, linearity and quantification

CAP was quantified by an internal standard procedure based on matrix calibration curves. The calibration curves were constructed from five points spanning the concentration range from 0 to 0.5 µg kg^{-1}. The calibration curve was based on the transition *m/z* 321 > 157 i.e. the daughter ion with higher intensity. The concentration of the deuterated internal standard was fixed at 2 µg kg^{-1}. The calibration curve obtained for the chromatographic peak areas ratio of CAP/IS versus known concentrations of CAP was found to be linear in the range of 0.1 to 0.5 µg kg^{1}. The correlation coefficients, R^2 for the curve was 0.9996. The mean slope was 1.0453 with a 3.58% variation coefficient and the mean intercept was 0.0247.

Specificity

The specificity of the method was evaluated from the 20 blank matrix samples. The samples were collected from different sources to prove if other substances can interfere with the retention time of CAP. HPLC-chromatograms of blank and spiked sample of poultry muscle are shown in **FIG. 1** and **FIG. 2**. The result shows that there was no interferences at the same retention time as chloramphenicol were found in all the blank matrix samples at all transition ion. This confirms the good specificity of the method.

Precision and accuracy

The precision of the method was tested by repeatedly analysing the spiked chicken samples. The accuracy and "within-day" precision of the method were determined in chicken muscle using seven determinations *(n* = 7) at three concentration levels: at 1/3 x MRPL, 2/3 × MRPL and 1 × MRPL. The "within-day" precision analysis was done on the same day. The "between-day" precision was determined by repeating the study for two consecutive days. The precision of the entire method was expressed by the

RSD of multiple analyses at different concentration level.

The accuracy of the method was expressed as the mean recoveries of spiked analytes in chicken muscle at three concentration levels. **TABLE 1** showed the recovery data that ranged from 94 % to 104%. The recovery values obtained in this study exceeded the acceptable Decision No. 2002/657/CE (4) for the analysis of residues at ppb-level indicating good accuracy of the method. Data in **TABLE 2** showed a narrow spread of the RSD values of "within-day" and "between-day" precision. All R.S.D.s were below 7%.

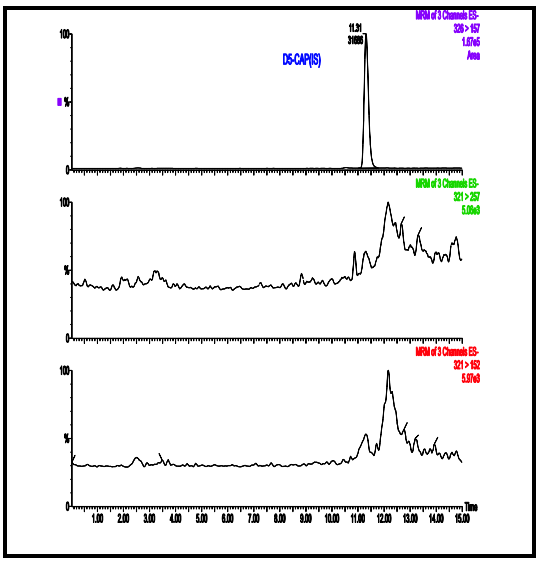

FIG.1- MRM chromatogram of blank chicken muscle

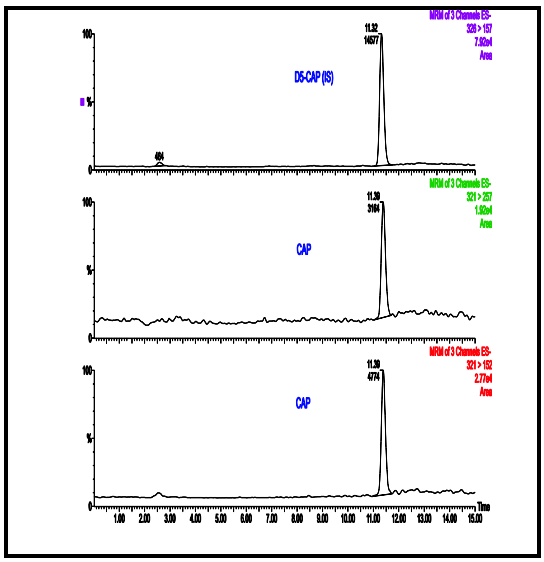

FIG. 2- MRM chromatogram of chicken muscle spiked with 0.1 µgkg^{-1} CAP

TABLE 1- Accuracy of the methodology expressed as recovery of spiked chicken samples

Performance criteria	Fortification level (µg kg^{-1})		
	0.1	0.2	0.3
Accuracy (%)			
Assay 1	100	99	97
Assay 2	104	103	99
Assay 3	101	99	94
Overall	102	100	97

TABLE 2- Method precision expressed as RSD of spiked chicken samples

Performance criteria	Fortification level (µg kg^{-1})		
	0.1	0.2	0.3
Precision CV (%)			
Assay 1	8.16	8.65	4.17
Assay 2	7.54	2.60	1.92
Assay 3	3.73	5.38	7.23
Intra-assay[a]	6.77	5.99	4.84
Inter-assay[b]	5.67	6.06	8.45

[a] Intra-assay precision of data analysed within the same day *(n* = 7)
[b] Intermediate precision of data analysed on different day *(n* = 3)

Limits of detection and limit of quantification

The limit of detection (LOD) was defined as the concentration giving a signal to noise ratio of 3. The limit of quantification (LOQ) was considered as the concentration for which S/N was 10 (5). An estimate of LOD and LOQ was performed by extrapolating the S/N ratio of the peak areas obtained from a chicken meat sample fortified at 0.1 µgkg^{-1}. The LOD and LOQ were estimated at 0.015 and 0.05 for m/z 321>257.

Application of the method

The Veterinary Public Health Laborotary Salak Tinggi (VPHL), Department of Veterinary Services Malaysia, provided chicken tissue samples used in this study. The samples were collected by the veterinary meat inspectors from the Meat Inspection and Food Safety Unit. Chicken samples were collected as the government approved and certified poultry processing plant. All the plants operated for the commercial poultry production, where the production levels ranged from 2,000 to 90,000 birds per day. The sample source was from 12 processing plants located in Perak, Pulau Pinang, Kuala Lumpur, Selangor, Negeri Sembilan, Melaka, Terengganu and Sarawak. Samples were transported under cooled or frozen conditions to the laboratory and stored at temperature of -20^{o}C prior to analysis.

Results of samples tested are shown in **TABLE 3**. From 448 samples analysed, 2 samples were detected to contain CAP at concentrations of 0.05 and 0.06 µg kg.^{1}. These concentrations exceeded LOQ value. The source of the positive samples was from Plant B.

For the waste water analysis, fifty bottles of waste water samples (influent and effluent) were collected from each state of Perak, Malacca and Selangor then analysed. In this study, following HPLC analysis, LCMS was used to reconfirm the results. The results of HPLC analysis were summarised in **TABLE 4**. All the samples were then quantified using LCMS.

TABLE 3- Results of CAP Detection in Chicken Samples

Processing Plant ID	Number of samples analysed	Number of samples >LOD	LOD <Number of samples <LOQ	Number of samples ≥ LOQ
A	52	0	0	0
B	104	0	0	2 (0.05 & 0.06 µgkg-1)
C	130	0	0	0
D	32	0	0	0
E	12	0	0	0
F	7	0	0	0
G	62	0	0	0
H	8	0	0	0
I	7	0	0	0
J	7	0	0	0
K	5	0	0	0
L	22	0	0	0

TABLE 4- The detection frequency in the influent and effluent collected from the swine facilities in selected states in Malaysia. Forty-five were found to be positive from the total of 150 samples analysed.

State	Influent	Effluent	Concentration (ngL^{-1})
Perak	1	0	10.02
Malacca	9	6	90.31 / 47.43
Selangor	2	1	12.75/10/58

[a]The concentrations displayed in the table were the highest concentrations detected in the samples. The levels detected in the influent precede the levels detected in the effluent.

From the results, 12 influent samples and 7 effluent samples were found positive for CAP out of the total 150 waste water samples. The concentration ranged from 9.04 ng/L to 90.31 ng/L in the influent samples and 8.81 ng/L to 47.43 ng/L in the effluent samples. Based on the results, detection of CAP in the influent samples were higher than in the effluent samples (**FIG. 3**). The levels of CAP detected in each positive sample were summed to assist interpretation. This was expected as CAP would be in its most concentrated at the influent point as this is the entry point of the waste water from the farm into the waste lagoons. The distance between the influent and effluent points could have affect the concentration of CAP in the effluent samples. Thus, it could have been diluted over the distance and the concentration becomes lesser until it is too low to be detected. Furthermore, many environmental factors could have affected the antibiotic while it travelled from the entry (influent) to the exit (effluent) point such as rainfall, pH, temperature and organisms content. We are convinced at some point in the waste lagoons, CAP could be degraded or adsorbed into soil although this could not be proven unless point measurements at certain distance or analysis on the soil for CAP are done.

Since chloramphenicol and also other antibiotics biodegradation varies according to temperature, pH, bacterial activity, chemical composition, water flow and/or distance between facility and sedimentation area, it is difficult to predict the bioaccumulation fate of antibiotics. This complicates the estimation of environmental exposure and also risk assessment.

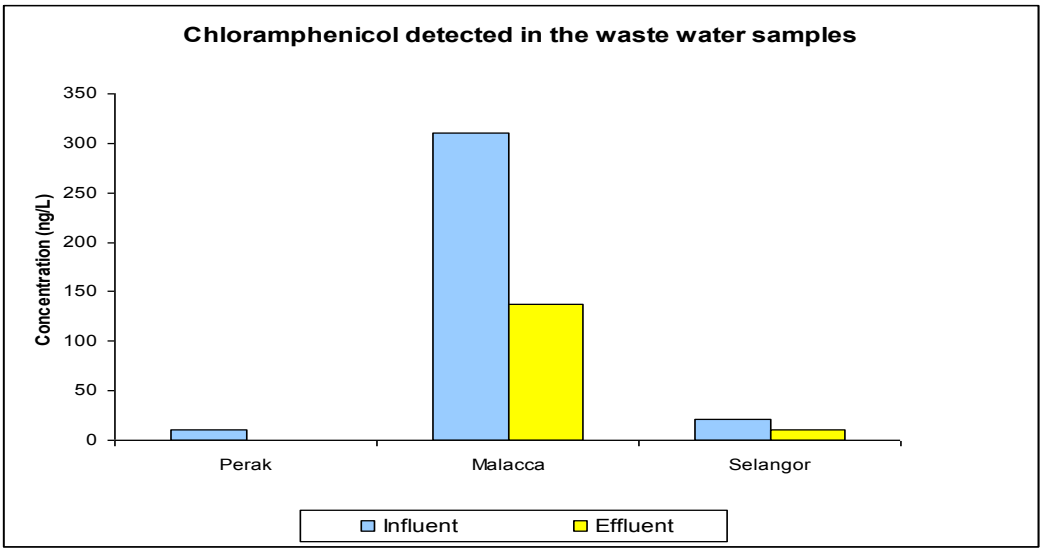

FIG. 3- Chloramphenicol detection in the waste water samples collected from the swine facilities in Perak, Malacca and Selangor

Conclusion

The method applied for determination of chloramphenicol in chicken is very simple and fulfils required sensitivity limit. It allows analysis of 20–30 samples per day. The performance of the method is in accordance with the requirements for the analysis of veterinary drug residues. Studies on waste water from animal husbandries showed that there is certain extent of contamination of these antibiotics in the Malaysian aquatic system.

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