Chloramphenicol residues in chicken liver, kidney and muscle: A comparison among the antibacterial residues monitoring methods of Four Plate Test, ELISA and HPLC


a Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, P.O. Box 1177, Urmia University, Urmia, Iran
b Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, P.O. Box 1177, Urmia University, Urmia, Iran

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ABSTRACT

There are many concerns about safety of food contaminated with antibacterial residues. This study was designed to investigate the occurrence of chloramphenicol (CAP) residue in broiler chickens tissues, namely liver, kidney and muscle. One hundred and sixty broiler chickens carcasses were collected from three provinces of Iran. Four Plate Test (FTP), ELISA and HPLC were used to qualify and quantify the contamination of the samples with CAP. The results of FTP revealed that up to 17.5% of the samples were contaminated with the antibiotic. The ELISA assay showed that out of 28 positive samples in FTP, 22 liver, 21 kidney and 14 muscle samples were positive for CAP. ELISA analyses demonstrated that the minimum and maximum levels of 0.54 and 155.2 ng/g were detected in the kidney and liver, respectively. HPLC analyses confirmed the ELISA findings although the level of contamination was lower than that of ELISA. These data showed that despite the prohibition of CAP application in food animals including poultry, the CAP residue was detectable indicating an illegal use of this antibiotic. Our findings also demonstrated the application of sensitive and more specific analytical assays in screening and quantitation of CAP residues in food products.

1. Introduction

Chloramphenicol (CAP) as a broad spectrum antimicrobial agent is used against many Gram negative and Gram positive bacteria (D’Aoust, 1994). CAP is rapidly absorbed and distributed in organs and edible tissues after oral and parenteral administration (Anadon et al., 1994). There are accumulating reports indicating undesirable effects of CAP in humans such as aplastic anemia, which has resulted to its limited usage. Even many countries including USA, Canada, Australia and EU member states have banned its use in food animals (JECFA, 1994). There are, however, great concerns regarding the human consumption of food products contaminated with drug residues especially in developing countries. They may contribute in the development of resistant bacteria strains and consequently lead to more serious health problems (Ferguson et al., 2005). Recently, it has been reported the presence of CAP residues detected in imported products to EU including poultry, shrimps and honey causing also a huge economical impact.

As the administration of CAP in food animals has been legally banned the maximum residual level (MRL) of zero should be anticipated. Thus, using the sensitive approaches to monitor and enforce the implementation of zero-tolerance level of CAP seems very crucial. During the last decade, different microbiological and chemical methods have been developed and validated for CAP detection and quantitation. The microbiological assay is traditionally used for detecting antibiotic residues in food products (Okerman et al., 1998a). There are two types of analytical methods including screening methods, normally utilizing immunoassay, and confirmatory methods performed based on gas-chromatography or liquid chromatography (De Wash et al., 1998; Fedeniuk and Shand, 1998; Gaudin and Maris, 2001; Neuhaus et al., 2002). Moreover, an alternative method of corona discharge ion mobility spectrometry has also been introduced for determining the residues of veterinary drugs in chicken meat (Jafari et al., 2007). In this study, we aimed to survey the occurrence of CAP residues in broiler chickens’ tissues including the liver, kidney and muscle from three different provinces in northwest of Iran. To achieve these goals we used the microbiological test as a screening method and immunological and chromatographical methods as the confirmatory tests.
2. Materials and methods

2.1. Chemicals

Muller Hinton Agar (Merck, 10664) were prepared and autoclaved. RIDASCREEN Chloramphenicol ELISA kit (R 1501) was purchased from r-biopharm, Landwehrstr, Germany. Chloramphenicol standard was obtained from Sigma-Aldrich (Germany). Chloramphenicol standard disc was purchased from Himedia LABORATORIES Pvt. Ltd., Mumbai 400 086, India.

2.2. Sample collection

One hundred and sixty poultry carcasses (160) were collected from five different poultry meat providing centers (I, II, III, IV, and V) of three provinces in north-west of Iran. The liver, kidney and muscle samples obtained from each carcass were kept at –20 °C for further analysis.

2.3. Microbiological assay – Four Plate Test

2.3.1. Medium preparation

The culture medium of Muller Hinton Agar (MHA) with three different pH of 6.0, 7.2, and 8.0 (V381547, Merck, Germany) were prepared and autoclaved. Following cooling to 45–50 °C, the bacterial suspensions were added and plates were poured. Four different inoculated media were used for the antibiotic detection: medium I: MHA, pH 6.0; medium II: MHA, pH 7.2, and medium III: MHA, pH 8.0, and were seeded with Bacillus subtilis (ATCC 6633, 10^6–10^7 CFU/ml); medium IV: MHA, pH 8.0, seeded with Staphylococcus aureus (ATCC 6538, 10^6 CFU/ml).

2.3.2. Sample preparation

The chicken frozen samples including the liver, kidney and muscle were thawed at 4 °C overnight. The samples were homogenized using Ultra Turrax T25. Ten grams of homogenate was centrifuged at 10,000 g for 15 min. Ten microliters of supernatant for each sample was applied to paper discs (Mast Diagnostics, BD638W) and the discs, after drying at 40 °C for 10–15 min, were put on the earlier prepared and seeded agar plates. Plates containing media with various pH (I, II and III) previously inoculated with mentioned bacteria were incubated overnight at 37 °C, while plates with medium IV were incubated for 24 h at 30 °C. Following overnight incubation, evaluation of antibacterial potency of the samples was performed according to previously reported methods (Obaobegbulem and Fidels, 1996). In short, the mean width of the inhibition zone was calculated. The criterion for positive samples was the inhibition zone of 2 or more than 2 mm in width.

2.4. ELISA assay

In order to measure the amount of CAP in samples which were positive in FPT assay, the ELISA technique was performed according to manufacture’s instruction (RIDASCREEN Chloramphenicol ELISA kit (R 1501), r-biopharm, Germany). In brief, 4 g of each sample was weighed and homogenized with mixer, then the homogenized samples were mixed with 3 ml of distilled water and 6 ml of ethyl acetate and were centrifuged at 10,000 g for 10 min at room temperature. A 4 ml of ethyl acetate supernatant (corresponding to 2 g of sample) was transferred into a fresh tube and dried at 60 °C under a weak stream of N2. The residue was re-dissolved in 1 ml isooctane/chloroform (2:3) mixture. A half milliliter of the CAP buffer was added to this solution and vortexed intensively for approximately 1 min. The solution was centrifuged at 3000 g for 10 min at room temperature. A 50 μl of the aqueous (upper) layer was used per well of the ELISA plate. One hundred and sixty five wells containing a CAP buffer were added to each well followed by 50 μl of CAP standard sera (each spiked at 2 ng/ml) and CAP positive sera of different dilutions followed by 50 μl of antiserum, and incubated at room temperature for 2 h. A half milliliter of the CAP buffer was added to this solution and vortexed intensively for approximately 1 min. The solution was centrifuged at 3000 g for 10 min and the supernatants were transferred to fresh tubes and 3 ml trichloroacetic acid (15%) was added to each tube. Following 2 min vortexing the samples were centrifuged at 1400g for 10 min and the supernatant was subjected to solid phase extraction.

2.5. HPLC analysis

2.5.1. Sample clean-up

To confirm and determine the accurate level of CAP contamination in FPT and ELISA positive samples, the high-performance liquid chromatography (HPLC) measurement was conducted according to previously described method with minor modification (Xizhi et al., 2007). Briefly, 10 g from individual selected tissues including the liver, kidney and muscle was weighted and placed in 50 ml falcon tube. Then 20 ml phosphate buffer saline PBS (pH 7.2) was added and samples were cut into small pieces, followed by homogenization using Ultra Turrax T25. The extraction procedure was persuaded by addition of 20 ml PBS, vortexing for 2 min and sonicingating for 15 min. Then, the tubes were centrifuged at 1400g for 10 min and the supernatants were transferred to fresh tubes and 3 ml trichloroacetic acid (15%) was added to each tube. Following 2 min vortexing the samples were centrifuged at 1400g for 10 min and the supernatant was subjected to solid phase extraction.

2.5.2. Solid phase extraction (SPE)

To perform the SPE, prior to extraction, the cartridge C18 (3 ml, 5 mg, J.T. Baker, The Netherlands) was conditioned with distilled water and methanol subsequently. The samples from chemical extraction were passed through the cartridge. The columns were washed with distilled water and dried under N2 stream. Ultimately, the bound compound to the cartridge was eluted with methanol. The eluted samples were dried under gentle stream of N2 and dissolved in mobile phase for HPLC analyses.

2.5.3. HPLC

CAP level in extracted samples was determined using HPLC, according to previously described method (Xizhi et al., 2007). Shortly, the chromatographic system consisted of an auto sampler (Autosampler Triathlon type 900, Germany) and dual pumps (Wellchrom HPLC pump, K-1001, KNAVEG Germany). Twenty microliters of the extracted sample was injected onto an ODS C18 (250 × 4.60 mm, 5 μm, Phenomenex) column. The mobile phase consisted of a mixture of water–methanol (60:40, v/v) eluted at a flow-rate of 1.0 ml/min. CAP was detected by means of an UV detector (RF-10A XL KNAUER, Germany), set at wavelength of 276 nm. CAP levels were quantified by measuring the areas under the peaks and comparing them to the relevant peaks generated by the standard CAP.

2.6. Evaluation of data

To quantify the levels of CAP in HPLC method an external standard was used. The limit of detection (LOD) for CAP was established by determining the signal-to-noise ratio at 3 and was found 2.5 ng/g. To obtain calibration curve for CAP, we subjected a concentration range from 0 to 100 ng/ml (r^2 = 0.9992). The mean recovery and RSD for each individual sample type were obtained with three times spiking of 5 ng/g. The Student t-test was used to analyze the difference between samples subjected to both ELISA and HPLC. Statistical differences taken at P < 0.05 were considered significant.

3. Results

The FPT test showed that out of 160 samples, 28 (17.5%) were positive as demonstrated the diameter of inhibition zone higher or equal than 2 mm. This result was confirmed for all three types of tissues including the liver, kidney and muscle. It came clear that the obtained results included all media with various chosen pH and both selected bacteria. The minimum and maximum diameter of inhibition zone observed from muscle (5.3 ± 1.7 mm for B. subtilis and 3.8 ± 0.4 mm for S. aureus) and the liver (18.7 ± 1.2 for B. subtilis and 14.3 ± 1.9 mm for S. aureus) samples, respectively. The results of FPT are presented in Table 1. Using 1 μg CAP as standard antibacterial agent at pH 8.0, which considered as optimal growth pH for either bacteria resulted in the diameter of inhibition zone of 28 ± 3.2 and 25 ± 2.6 mm for B. subtilis and S. aureus, respectively.

To determine the level of CAP in positive samples, the quantitative method of ELISA was conducted and the results showed that out of 28 positive samples from former assay (FPT), 22 liver, 21 kidney and 14 muscle samples were positive. The levels of contamination with CAP are depicted in Fig. 1A–C demonstrating the minimum measured level of 0.54 ng/g in the kidney and the maximum measured level of CAP (155.2 ng/g) in the liver samples. Interestingly, we found the maximum level of the CAP in the tissues of the same carcasses, namely carcasses number 14, 15, 16, 20, and 25. The second part of this study was devoted to confirm the previous assays and compare the accuracy of two routinely used analytical methods of ELISA and HPLC. To this end, we selected the five carcasses, which based on former method had shown the highest level of CAP in the examined organs. The HPLC analyses revealed that all selected samples and examined organs were contaminated with CAP. Interestingly, the level of CAP which detected with HPLC method was remarkably lower than that of ELISA (Fig. 2A–C). At the same time, the HPLC results confirmed the entire data obtained with former assay as the exactly same samples had also shown the minimum and maximum levels in ELISA. We found that the CAP residue was at the highest level (153 ± 15 ng/g) in the liver and at the lowest level in the muscle (2.64 ± 0.2 ng/g). The mean recovery percentages and RSD for each sample type following three times spiking of 5 ng/g of CAP were calculated as it follows:
87.5 ± 9.3%, 79.3 ± 6.8%, and 63.2 ± 6.4% for the liver, kidney and muscle samples, respectively (n = 5).

4. Discussion

This study reports certainly for the first time that the residue of CAP in edible tissues of poultry including the liver, kidney and muscles in northwest of Iran. Additionally, the efficiency of three methods was compared. One of the commonly used assays for antibacterial residues screening in food and feed materials is microbiological assay (Okerman et al., 1998a). In microbiological assay, the broad inhibition zone formation indicating the high concentration of antibacterial in examined foodstuff. In the present study FPT assay was conducted to screen the wide range of poultry

Table 1
The results of FPT presented as the width of inhibition zone (mm); data are representing the mean values ± SD of the diameter of inhibition at pH 8.0 from five various poultry meat suppliers.

<table>
<thead>
<tr>
<th>Collection site</th>
<th>Liver</th>
<th>Kidney</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B. subtilis</td>
<td>S. aureus</td>
<td>B. subtilis</td>
</tr>
<tr>
<td>I</td>
<td>12.3 ± 0.8</td>
<td>9.4 ± 1.6</td>
<td>11.7 ± 2.6</td>
</tr>
<tr>
<td>II</td>
<td>15.4 ± 1.7</td>
<td>12.0 ± 2.3</td>
<td>13.4 ± 1.8</td>
</tr>
<tr>
<td>III</td>
<td>18.7 ± 1.2</td>
<td>14.3 ± 1.9</td>
<td>11.8 ± 2.1</td>
</tr>
<tr>
<td>IV</td>
<td>12.5 ± 2.1</td>
<td>11.0 ± 1.8</td>
<td>9.5 ± 1.9</td>
</tr>
<tr>
<td>V</td>
<td>14.9 ± 1.8</td>
<td>9.3 ± 1.6</td>
<td>12.7 ± 1.4</td>
</tr>
</tbody>
</table>

Fig. 1. Chloramphenicol concentrations in the (A) liver; (B) kidneys and (C) muscles samples from broiler chickens analyzed by ELISA assay (n = 28).
showed that all five samples which showed the highest level of CAP in the examined tissues were also positive in HPLC analyses. One of the interesting findings of this study was significant differences between the detected level of CAP residue by ELISA and HPLC methods. The low concentrations of CAP, particularly in the kidney and muscles, in HPLC analyses indicates the possible cross reaction between CAP itself and its metabolites which could be detected in ELISA system but not in HPLC. Previous reports showed that using QFlex® Kit Chloramphenicol, which contains CAP antibody, led to a high cross-reactivity for CAP and chloramphenicol–glucuronide in milk, honey and poultry meat (Ferguson et al., 2005). The metabolism of CAP takes place in various organs including liver, kidneys and muscles by cytochrome P450 system and the produced metabolites are distributed in entire body (Akhtar et al., 1996). It has been shown that the glucuronidation is the major biotransformation route of CAP in humans and animals.

In summary, as CAP is a prohibited antibiotic in food animal industry, a zero tolerance must be applied for this antibacterial agent. Our data indicates that illegal uses of CAP in poultry industry should be taken into account seriously. At the same time to detect the low concentrations of the CAP, food authorizing centers should provide more sensitive analytical methods such as chromatography or biosensor systems. Moreover, as shown in this study, the high concentration of CAP can be accumulated in the liver and kidneys raising awareness for more cautious consumption of these organs where CAP is used inconsiderately in food animal industry.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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