

**Validation of a competitive chloramphenicol enzyme linked immunosorbent assay for determination of residues in Ovine tissues**

**G. A. MURILLA<sup>1</sup>, J.O. WESONGAH\*<sup>2</sup>, T. FODDEY<sup>3</sup>, S. CROOKS<sup>3</sup>, A. N. GUANTAI<sup>4</sup>, W. M. KARANJA<sup>1</sup> AND T. E. MAITHO<sup>5</sup>**

<sup>1</sup> TRC, Residue Analysis Section, Kenya Agriculture Research Institute, P.O.Box 362, Kikuyu, Kenya.

<sup>2</sup> Medical Laboratory Sciences Department, Jomo Kenyatta University of Agriculture and Technology, P.O. Box 62000-00200, Nairobi, Kenya.

<sup>3</sup> Veterinary Sciences Division, Agri-Food Biosciences Institute, Stoney Road, Belfast BT4 3SD, UK

<sup>4</sup> Department of Pharmacology and Pharmacognosy, School of Pharmacy, College of Health Sciences, University of Nairobi, P.O. Box 19676-00202, Nairobi, Kenya.

<sup>5</sup> Department of Pharmacology and Toxicology Section, University of Nairobi, P.O.Box 30197-00100 Nairobi, Kenya.

**Chloramphenicol is a broad-spectrum antibiotic, which has been used for treatment of animals. However, in humans it leads to hematotoxic side effects particularly aplastic anaemia for which a dosage-effect relationship has not yet been established. The objective of this study was to validate a developed chloramphenicol enzyme linked immunosorbent assay for the determination of chloramphenicol residues in ovine tissues. Two groups (n=5) of sheep were injected with chloramphenicol sodium succinate at 25-mg/kg bodyweight and slaughtered one and four weeks post drug administration. Overall, the mean percentage recoveries in muscle, liver and kidney were 92 %, 70% and 78% respectively. The limits of detection were 1.2 ng/g, 0.6 ng/g and 0.8 ng/g while the detection capability was 2.5 ng/g, 1 ng/g and 1 ng/g in muscle, kidney and liver respectively. This enables the method to be used effectively as a screening tool for chloramphenicol residues in livestock products especially in the liver, muscle and kidney.**

**Keywords:** Chloramphenicol, enzyme linked immunosorbent assay, ovine, muscle, liver, kidney

### INTRODUCTION

The use of chloramphenicol (CAP) in veterinary medicine has been restricted to non-food producing animals because it possesses toxic properties [1]. Despite this restriction, residues have been found in samples taken from domestically produced animals in national monitoring programmes and in samples moving in international trade. For example Voedse en Waren analysed 20 samples of honey from Netherlands and reported an average mass concentration of 1.9 mg/kg (range 0.06-5.9

mg/kg) of CAP residues. In order to monitor and reduce the potential incidence of CAP residues in the food chain, a sensitive and specific method to detect CAP is of primary importance. Microbiological methods show a limited sensitivity and lack of specificity. For this reason various methods have been developed, such as gas chromatography [3] and high performance liquid chromatography (HPLC) [4]. However, these methods are very expensive and are mainly suitable for confirmatory analysis. The aim of the present study was to validate

\*Author to whom correspondence may be addressed

the CAP ELISA method developed by the authors for use in serum [5] for tissues from treated sheep. The validated method may be used as a screening tool to monitor CAP residues in livestock products destined for both local and international trade.

## MATERIALS AND METHODS

### Experimental animals

Twelve adult Red Maasai sheep aged between 9 to 12 months and weighing 20 kg to 28 kg were purchased and kept in a barn house. They were fed on hay plus water *ad libitum*. The animals were allowed to acclimatize for two weeks before the start of the experiments. During that time they were treated for ectoparasites by spraying with 12.5% w/v amitraz (Coopers Ltd., Nairobi, Kenya) diluted at the rate of 2 ml/l. They were drenched for endoparasites with albendazole (Norbrook Ltd, Newry, United Kingdom) at the recommended dose.

Three New Zealand adult male rabbits were sacrificed and used as controls for the negative tissue samples used in the selection of the CAP extraction buffer

### Experimental design

Twelve experimental sheep were divided into 3 groups. Group 1 and 2 consisted of 5 sheep each while group 3 consisted of 2 animals. Groups 1 and 2 were treated intramuscularly with Chloramphenicol sodium succinate at a dose of 25-mg/kg body weight while group 3 remained untreated and was used as the control. Group 1 sheep were sacrificed after one week while groups 2 and 3 were sacrificed after four weeks.

### Drug preparation

A freshly prepared 10% (w/v) aqueous solution of CAP sodium succinate (Nabros Pharma Pvt. Ltd., Kheda, India) was administered to ten sheep (groups 1 and 2) at

a dose of 25 mg/kg-body weight, by deep intramuscular injection into the right hind limb muscles of each of the experimental sheep.

### Preparation of sheep tissue extract

Tissue extracts from control and the test animals were prepared from liver, kidney and muscle. Five gram tissues were weighed in 50 ml plastic tubes and cut into small pieces using a pair of scissors. The tissue samples were macerated using an Ultra-turax tissue homogenizer. To these were added 15 ml of phosphate buffered saline containing Tween 20 (PBST). The homogenized tissue was centrifuged at 4,000 rpm for 10 minutes and the supernatant stored at - 20°C. The control tissue extracts were used in the preparation of standards and determination of the limit of detection (LOD) and detection capability (CC $\beta$ ).

### CAP Enzyme-Linked Immunosorbent Assay (CAP ELISA)

The CAP ELISA developed for serum has been reported previously by the authors[5]. This method was validated using tissue extracts from sheep.

### Preparation of CAP standards in sheep tissues

Optical densities (ODs) of tissue samples (n=12) collected from two sheep were determined individually and those with ODs within two standard deviations from the mean were pooled separately. The tissue samples used consisted of muscles of Left Hind Limb (LHL), Left Fore Limb (LFL), Right Fore Limb (RFL), Right Hind Limb (RHL), liver and kidney. The pooled tissue samples were used in the preparation of standard solutions. The CAP standards used in developing the calibration curve were prepared by spiking the negative control tissues with CAP sodium succinate at 250, 500 and 1000 ng/ml and serially diluting the

solutions to obtain a range of concentrations. The calibration standard curve was fitted using the four-parameter logistic regression.

### **Limit of Detection (LOD)**

The limit of detection (LOD) was determined by analyzing 20 negative ovine tissues obtained from different animals for each matrix (liver, kidney and muscle) purchased from supermarkets in Belfast, Northern Ireland. These tissues were screened for CAP residues to ensure that they were negative for the residues. The mean OD values (n=20) and the standard deviation (SD) were determined for muscle, liver and kidney. The LOD was calculated as the concentration equivalent to the mean minus three standard deviations read from a matrix calibration curve.

### **Detection capability (CC $\beta$ )**

The 20 negative ovine tissues used in the determination of LOD were fortified at the level of interest determined by the LOD and used for determination of CC $\beta$ .

### **Cross-reactivity**

The cross-reactivity (CR) of CAP antibody with other commonly used antibiotics, such as penicillin, streptomycin and sulfamethazine and with the closely related antibiotics thiamphenicol and florfenicol as well as the structurally related compounds such as CAP sodium succinate and its major metabolite, CAP glucuronide was determined. The CR of CAP antibody with the CAP related compounds was carried out by analyzing negative control tissue spiked with these compounds at concentrations varying from 0.3 ng/g to 2000 ng/g. The percentage CR was calculated using the equation:

$$\% \text{ CR} = \left( \frac{\text{IC}_{50} \text{ of CAP}}{\text{IC}_{50} \text{ of the cross-reacting compound}} \right) \times 100$$

### **Parallelism**

The PBST was spiked with CAP at 1000 ng/ml and diluted serially at 100, 200, 400 and 800 times in sheep muscle PBST extract. The concentration of each dilution was determined by CAP ELISA, multiplied by the dilution factor and plotted against dilution. A horizontal line parallel to the ordinate indicated parallelism.

### **Intra- and inter-assay coefficient of variation**

Chloramphenicol standards of 2 ng/ml and 4 ng/ml were prepared in muscle extract. Each standard was replicated fifteen times on microtitre plates and analysed on three days consecutive days. The concentrations were determined and variations within the plate (intra-assay) and between assays (inter-assay) were calculated using the method described by Rodbard [6].

### **Spiking of samples and determination of percentage recovery**

Approximately one gram of respective tissues obtained from untreated sheep was spiked with 6 different CAP concentrations at 0.3 ng/g, 0.5 ng/g, 1 ng/g, 5 ng/g, 50 ng/g and 500 ng/g and the drug extracted using PBST at pH 7.4. CAP concentrations in the extracts were determined using the CAP ELISA developed for serum [5] and validated for tissues.

### **Statistical Analysis**

Chloramphenicol residue concentrations were compared between tissues of different animals and different tissues of the same animals using statview statistical programme (SAS Institute Inc., California, USA).

## RESULTS

### Optimal conjugate and antibody dilutions

The optimal conjugate and antibody dilutions in tissues were both 1/12000 as the dilutions that gave optical densities of approximately one and a competition of 85.4%.

### Limit of detection and Detection capability

The limits of detection (LOD) determined by assaying 20 of each negative ovine tissue were 1.2 ng/g, 0.6 ng/g and 0.8 ng/g for muscle, kidney and liver respectively. The detection capabilities (CC $\beta$ ) determined by assaying CAP fortified ovine tissues were 2.5 ng/g, 1.0 ng/g and 1.0 ng/g for muscle, liver and kidney respectively.

### Parallelism

Parallelism was demonstrated through a parallel line inferred from a 1000 ng/ml spiked tissue (muscle) sample diluted serially and analysed for CAP residues.

### Cross-reactivity

Cross-reactivity (CR) with the most commonly used antibiotics (penicillins, tetracycline, sulfamethazine and streptomycin) and closely related antibiotics such as thiamphenicol and florfenicol was not observed. Eighty percent (80%) CR was observed with CAP glucuronide and 100% CR was observed with CAP sodium succinate as shown in Table 1.

### Intra- and inter-assay coefficient of variation (Precision)

The within assay (CV<sub>w</sub>) and between assay (CV<sub>b</sub>) coefficients of variation for 2 ng/ml were 3.8% and 7.8%, while the CV<sub>w</sub> and CV<sub>b</sub> for 4 ng/ml were 4.5% and 8.9% respectively.

**Table 1: Cross-reactivity of the anti-CAP antibody with related chemical compounds and antimicrobial agents**

Compound	Cross-reactivity
Chloramphenicol (CAP)	100
CAP Sodium succinate	100
CAP Glucuronide	80
Penicillin G	NO
Tetracycline	NO
Sulfamethazine	NO
Streptomycin	NO
Florfenicol	NO
Thiamphenicol	NO

NO=Not Observed

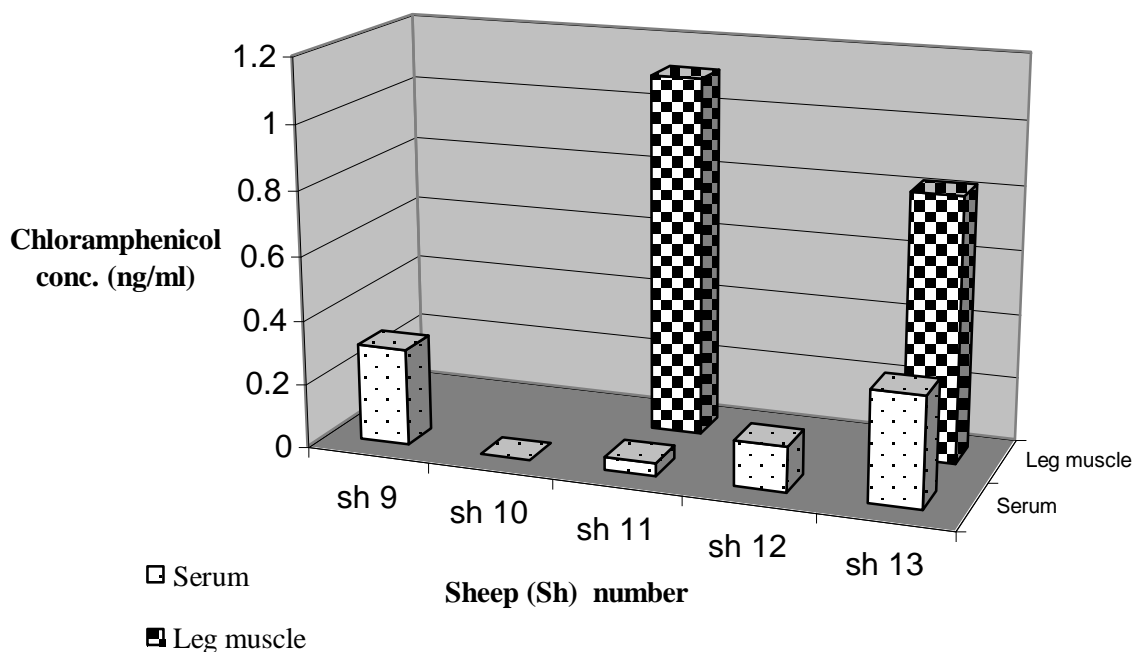
### CAP mean percentage recoveries in sheep tissues

The mean percentage recovery of CAP determined in the liver, kidney and muscle of sheep spiked with six different drug concentrations of 5 ng/g, 50 ng/g and 500 ng/g ranged from 52.4% to 95.6 %, 59% to 97.5% and 67.33% to 113% respectively. Overall mean percentage recoveries in muscles, liver and kidney were 92 %, 70% and 78% respectively.

### CAP residues in tissues from treated sheep

Figure 1 shows CAP residue levels in different muscles sampled from 5 sheep sacrificed at one-week post drug administration.

The percentage recovery was taken into account when calculating the residue levels in all the tissue samples. One-week post drug administration, CAP residue levels in the liver and the kidney were below the detection capability (CC $\beta$ ) of the assay in all the five sheep. There were significant differences ( $P < 0.05$ ) in CAP residues levels of the leg muscles obtained from the five sheep.



**Figure 1: CAP residue concentration in serum and different leg muscles sampled from 5 sheep one-week post drug administration**

In sheep number 9, 10 and 12 the residue levels were below the  $CC\beta$  in all the different parts of muscles sampled. The residue levels in the different leg muscles (RHL, LFL, RFL) ranged from 2.9 to 7.2 ng/g (mean  $4.5 \pm 2.3$  ng/g). The residue levels were 7.2 ng/g in the right hind leg (RHL) of sheep number 11 while the levels in LFL, LHL and RHL of the same sheep were below the  $CC\beta$  of the assay because this was the injection site. However, in the other four sheep the site of injection was not observed to have such high drug residue levels. During this period CAP serum levels had also declined rapidly to levels ranging from 0 to 0.34 ng/ml (mean  $0.16 \pm 0.15$  ng/ml) in the five sheep as shown in Figure 1.

## DISCUSSION

The competitive enzyme linked immunosorbent assay for detection and

monitoring of CAP in serum (CAP ELISA) validated for sheep tissues described in the present study had a  $CC\beta$  of 2.5ng/g, 1.0ng/g and 1.0ng/g for muscle, kidney, liver respectively and LOD of 1.2ng/g, 0.6ng/g and 0.8 ng/g for muscle, kidney and liver, respectively. The LOD determined in the present study were low but more work is required to further lower the values in order to achieve the recommended EU standards.

The antibody cross-reactivity obtained with CAP sodium succinate and CAP glucuronide in the present study was 100% and 80 % respectively. This shows that CAP ELISA developed for serum and validated for residue analysis in ovine tissues was very specific for CAP and it is major metabolite (CAP glucuronide). Thus the method is suitable for screening for CAP residues in animal products. The ELISA method validated for tissues in the present study had very good mean percentage recoveries for liver, muscle and kidney, of

70%, 92% and 78 % respectively. The overall recoveries were close to those obtained in bovine muscle tissue and raw cow's milk in Slovenia [7].

Following one week of withdrawal, the CAP residues detected in the muscles ( $4.5 \pm 2.3$  ng/g) was significantly higher ( $P < 0.05$ ) than the levels in serum ( $0.16 \pm 0.15$  ng/ml) while in the liver and kidney these levels had declined to below the LOD of the method. This shows that CAP persisted in the muscles for a longer period of time than in the liver, kidney and serum. This could be attributed to protein binding of CAP in the muscle. Chloramphenicol has been reported to have a 42-50% protein binding [8]. The injection site of one out of five sheep (number 11) had significantly ( $P < 0.05$ ) high residue levels of CAP suggesting individual differences in the metabolism of CAP in the sheep. Several studies [9-10] show that dehydrochloramphenicol, a CAP metabolite produced by intestinal bacteria may be responsible for DNA damage and carcinogenicity. This metabolite can undergo nitro-reduction in the bone marrow, where it causes DNA single-stranded breaks. This shows that the ban of CAP use is justified because the metabolites that cause aplastic anaemia may accumulate in the body if they are consumed frequently in animal products.

The present study shows that the sensitivity of CAP ELISA validated for tissues can be improved further by thorough cleaning of tissues during the extraction of CAP residues in order to reduce unspecific binding and lower the CC $\beta$  and the LOD. It may be necessary to carry out interlaboratory validation of the CAP ELISA method in order to harmonize the analytical performance of method.

#### ACKNOWLEDGEMENTS

The authors gratefully acknowledge the International Atomic Energy Agency (IAEA) and KAPP-KARI for funding this

project. All the members of staff of the Agri-Food Biosciences Institute (AFBI), Belfast, Northern Ireland and residue analysis laboratory of the Kenya Agriculture Research Institute - Trypanosomiasis Research Centre (KARI-TRC) are acknowledged. The authors would also like to thank Dr. M.C. Eisler for providing the ELISA software used for data analysis.

#### REFERENCES

- [1] Lancaster, M., (2006). Veterinary drug residues and MRLS, 2006 Edition, ISBN: SR251.
- [2] Voedsel en Waren Autoriteit. (2004) Communication of the Voedsel en Waren Autoriteit of the Netherlands to the FAO /JECFA Secretariat.
- [3] Ginkel, V.L.A, Van Rossum, H.J., Zoontjes, P.W., Van Blitterswijk, H., Ellen, G., Van der Heeft, E., de Jong, A.P.J.M. and Zomer G. (1990). *Anal. Chim. Acta* 237, 61-69.
- [4] Gard A. and Johansson J. (2004). Development of sample workup and analysis scheme for determination of CAP in shrimp feed using HPLC, Analytical Chemistry, University of Science in Ho Chi Minh City, Vietnam, Minor Field Studies.
- [5] Wesongah J. O., Murilla G. A., Guantai, A. N., Elliot C., Fodey T., and Cannavan A. (2007). *J. Vet. Pharmacol. Therapeut.* 30, 1-6.
- [6] Rodbard, D. (1974) , *Clin. Chem.* 20, No. 10, pg 1255-1270.
- [7] Cerkvenik, V. (2002). Analysis and monitoring of CAP residues in food of animal origin in Slovenia from 19991 to 2000, *Food Additives and Contaminants*, 19, 357-367.

- [8] Pilloud M.(1973). Res. Vet. Sc. 1973; 15: 231-238. (1990).Am. J. Med. Sci. 300 (6) 350-3.
- [9] Jimenez, J. J., Jimenez, J.G., Daghistani, D. and Yunis A .A.
- [10] Kimatura, T., Ando J., Ishihara R., Takano S., Iijima T., Nishimura, S., Yoshida M., Takahasi M., and Maekawa, A. (1997) Food Chem. Toxicon; 35(10-11): 1075-80.
-