ECRONICON OPEN ACCESS

EC VETERINARY SCIENCE
Research Article

Enrofloxacin and Ciprofloxacin Residues in Broiler Chicken Feathers after Enrofloxacin Oral Administration

María Laura Marchetti, Andrea Buchamer, Daniel Buldain and Nora Mestorino*

Laboratory of Pharmacological and Toxicological Studies (LEFyT), Faculty of Veterinary Science, Universidad Nacional de La Plata, La Plata, Argentina

***Corresponding Author**: Nora Mestorino, Laboratory of Pharmacological and Toxicological Studies (LEFyT) Veterinary Faculty, Universidad Nacional de La Plata, La Plata, Argentina.

Received: March 22, 2019; **Published:** April 30, 2019

Abstract

Antibiotics in animal feed are a public health concern. Drug residues could eventually be detected in animal food products intended for human consumption. Our aim was to study the residue depletion of enrofloxacin and its metabolite ciprofloxacin in broiler chicken feathers. A validated HPLC-fluorescence method was used to quantify both compounds in feather samples. Broiler chickens were treated through drinking water with 10 mg Kg^{-1} d of enrofloxacin for 5 consecutive days. Feather samples were taken from 10 random birds per day until 9 days. Extraction was performed by a liquid/liquid technique. Both fluoroquinolones concentrations were determined by liquid chromatography with fluorescence detection. High levels of enrofloxacin and ciprofloxacin were found in feathers after oral administration and these compounds were detected for 9 days. Feather meal is a potential source of drug residues that can pass through the food chain when contaminated meal is fed to food-producing animals. In the present study enrofloxacin and ciprofloxacin concentrations were 0.20 to 5.46 μg g^{-1} between 1 to 9 days after final treatment, which means that withdrawal time fit for edible tissues is not adequate to reduce antimicrobial residues in chicken feathers. Further studies to establish a withdrawal time may be useful to avoid that drug residues could result in adverse health consequences like increase in antibiotic resistance.

Keywords: *Antimicrobials; Enrofloxacin; Ciprofloxacin; Feathers; Poultry; Residues*

Abbreviations

ENR: Enrofloxacin; CIP: Ciprofloxacin; HPLC: High-Performance Liquid Chromatography; PVDF: Polyvinylidene Fluoride; LOQ: Limit of Quantitation; LOD: Limit of Detection; SD: Standard Deviation; CV: Coefficient of Variation

Introduction

Enrofloxacin is an antimicrobial agent from the group of fluorinated quinolones with broad antimicrobial spectrum and high bactericidal activity. The antimicrobial properties of enrofloxacin indicate that it has advantages for use in poultry. A major use of this product is for the treatment of mycoplasmal infection, colibacillosis and pasteurellosis in birds [1-4]. Antibiotics in animal feed are a public health concern. Drug residues could eventually be detected in animal food products intended for human consumption [5]. For decades, antibiotics have been added to livestock feeds in low doses to serve as growth promoters [6]. Antibiotics have recently been shown to accumulate in poultry feathers, which is significant because poultry feathers serve as a high protein ingredient in animal feed, such as poultry feed [6]. The continued use of these compounds as feed additives has inadvertently created antibiotic-resistant microorganisms, which has caused human health concerns. Antimicrobials used in poultry production have the potential to bioaccumulate in poultry feathers but available pharmacokinetics and tissue depletion studies are very scarce [6]. Following poultry slaughter, feathers are converted in feather meal [7]

and sold as fertilizer [8]. Feather meal is often incorporated as a protein source into the diets of other food animals, such as cattle, swine, rainbow trout, shrimp and salmon [9,10], thereby providing a potential pathway for reentry of antimicrobials into the human food supply. Although feathers after been processed are introduced in the food chain as a protein source in animal feed, withdrawal periods are not established yet. In view of the pharmacokinetic characteristics of enrofloxacin (ENR) and its metabolite ciprofloxacin (CIP), good absorption after parenteral and oral applications, large volume of distribution suggesting wide tissue penetration, and a long terminal half-life [1,2,4,11,12], drug accumulation in non-edible tissues such as feathers is highly probable. During the last 3 decades, fluoroquinolones in the poultry industry have become a matter of concern because the common use of these compounds to control bacterial infections has led the emergence and dissemination of resistance in *Salmonella enterica* serovars Enteritidis and Typhimurium [13,14], *Campylobacter* spp. [15] and *Escherichia coli* [16]. This resistance has led to lowered efficacy of these compounds as therapy for human infections [3]. Hence, the present study has been conducted to evaluate the residue profile of enrofloxacin and its primary metabolite ciprofloxacin in feather of broiler chicken, after ENR (Carval®) administration at 10 mg kg^{-1}d for 5 consecutive days with drinking water.

Materials and Methods

Study design treatment and administration

The experiment was performed on three week old broilers (n=90). The birds were randomly divided into control (n = 20) and treatment group (n = 70). Treatment group was administered with enrofloxacin (Carval®) at recommended therapeutic dose 10 mg kg^{-1}, with drinking water for five consecutive days (0, 1, 2, 3 and 4 days), whereas control group received non-medicated water. The birds were kept in a special space designed for performing experiments on animals and before treatment chickens were deprived of water. Antibiotic free food was available *ad libitum*. The chickens treated with enrofloxacin were euthanized by cervical dislocation after desensitization by passage of an electric current through the head 1d, 2d, 3d, 5d, 7d and 9d after final drug administration (ten animals at each time point). Feather samples were collected, washed with saline solution and stored at -20°C until sample preparation and chromatographic analysis. Control chickens were sacrificed on day 9 post treatment. The protocol was according to the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science societies -FASS) and was approved by the Experimental Ethics Committee of the Faculty of Veterinary Science, UNLP, Argentina.

Enrofloxacin and ciprofloxacin analysis

Enrofloxacin (ENR) and ciprofloxacin (CIP) standards were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol, trifluoroacetic, triethanolamine, acetonitrile and acetone were of HPLC grade and purchased from J. T. Baker (Phillipsburg, NJ, USA). All other reagents were of analytical reagent grade.

Enrofloxacin and ciprofloxacin feathers analysis was performed by high-performance liquid chromatography (HPLC) with fluorescence detection. Extraction was performed following a technique described by San Martin., *et al.* (2007) and modified by us (SOP: ENR/CIP-VMA-04/02) [14]. Briefly, feather samples (0.5g) were thinly triturated; placed in centrifuge tubes and 5 mL of acetone was added. Samples were shaken and centrifuged at 2000g for 10 minutes at 4°C. The clear supernatant was transferred into drying tubes. The extraction procedure was repeated two times more. Supernatants were combined (S1 + S2 + S3) and evaporated to dryness in a vacuum concentrator (AVC 2-25CD Christ, Germany) at 40°C. Residues were dissolved in 75 µL methanol 0.1% trifluoroacetic. After 475 µL aqueous 0.1% trifluoroacetic were added. The total solution was vortexed for 2 minutes and then filtered by Millex-GV syringe filter Unit, 0.22 µm pore size polyvinylidene fluoride (PVDF) membrane. Finally, 100 µL of clear solution was injected into the chromatographic system.

Standard curve

Standards were prepared by adding 0.1, 0.25, 0.5, 1 and 2 µg mL^{-1} of ENR-CIP to test-tubes, evaporating to dryness at 60ºC and dissolving as described above.

Apparatus

The chromatographic system consisted of an isocratic pump (Gilson Inc. 307), an automatic injector (Gilson Inc. 234), a FluoroMonitor IM III Detector (excitation 278 nm and emission 446 nm) (Sp Thermo Separation products) and Eppendorf CH-30 Column Heater (set at a 30ºC). The system is controlled through the Unipoint® Gilson system. A C_{18}column (Luna, 5 µm, 4.6 mm x 150 mm; Phenomenex, Torrance,

CA, USA) was eluted with a mixture of water: acetonitrile: triethanolamine (80:19:1, v/v/v) pH3, at a flow rate of 1.2 mL min^{-1}. Identification of ENR-CIP in bird feathers was accomplished by comparison with the retention times of the reference standards.

Method validation

The validation procedure was performed following Commission Decision 2002/657/CE of the EU (2002) [17]. The following parameters were evaluated for the analysis of ENR and CIP in feathers: linearity (concentrations ranging between 0.1 - 2 μg mL^{-1} or μg g^{-1}), precision and accuracy, limit of quantitation (LOQ), limit of detection (LOD) and selectivity. Samples from untreated animals (blank feathers) were analyzed to confirm ENR and CIP absence and the specificity of the analytical method. The mean accuracy (% recovery) should be within the range 85 - 115% and the variation in precision should be ≤ 20%. The LOD was estimated through the analysis of 10 aliquots of control feathers (free of ENR-CIP).

The noise of the base-line was measured; the average and the standard deviation were calculated, the LOD corresponds to three of those SD (sign/noise ≥ 3/1). The LOQ is defined as the level where the reproducibility of the replicate analysis does not exceed a variation coefficient of 20% and the accuracy is from 85 - 115% after the analysis of 12 replicates of fortified sample matrix with the smallest concentration.

Results

The development and validation were successfully accomplished. This method performed accurately and reproducibly over a range of 0.1 to 2 μg mL^{-1} for ENR-CIP.

Precision of the system

One standard solution was prepared containing 1 μg mL^{-1} of ENR and CIP, respectively and the precision of the system was evaluated after the placement of twenty (20) injections in the chromatographic system. In this manner the efficiency of the column and of the system were evaluated. After twenty injections a coefficient of variation (CV) of 6.85% and 13.02% for ENR and CIP respectively, were determined.

Assay linearity

This assay exhibited a linear dynamic range between 0.1 and 2 μg mL^{-1}. A linear relationship was obtained across one dynamic range with r values ranged from 0.997407 to 0.998399 and 0.997442 to 0.998605 for ENR and CIP respectively (Figures 1A and 1B).

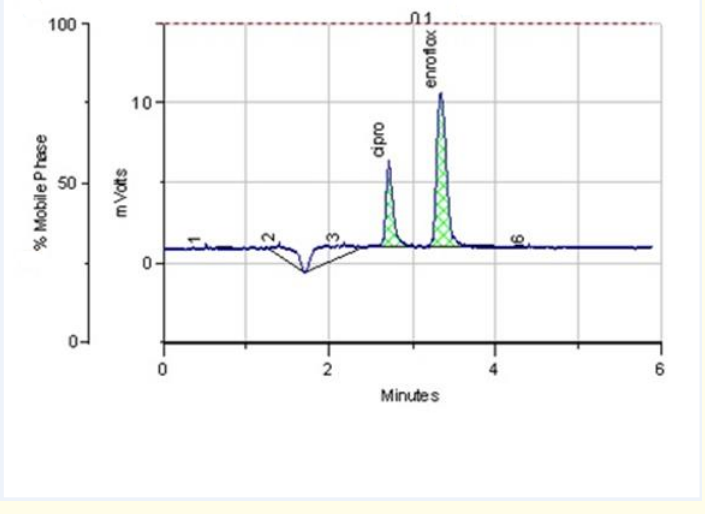

Figure 1A: *HPLC Chromatograms of ENR and CIP standard solution (0.1 μg mL^{-1}).*

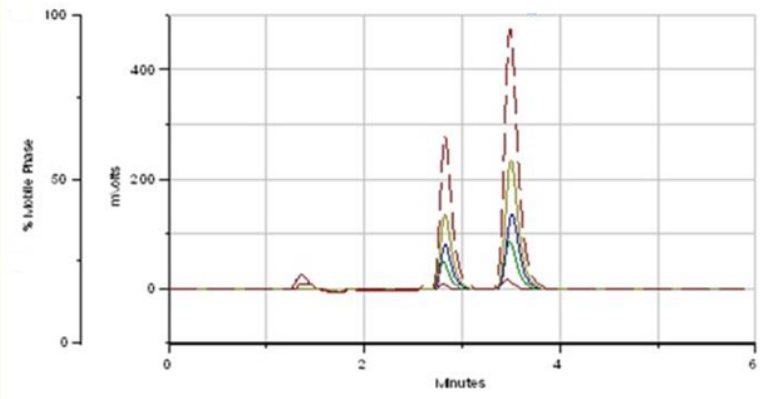

Figure 1B: *HPLC Chromatograms of ENR and CIP standards calibration curve (0.1, 0.25, 0.5, 1 and 2 µg mL^{-1}).*

Specificity

Six different samples from control feathers (free of ENR-CIP) and 6 feather samples fortified with ENR and CIP were analyzed by HPLC and the corresponding chromatograms were compared. No matrix interferences were observed on the chromatograms of the samples with the same retention time as ENR-CIP (Figure 2). The chromatographic analysis time was short and ENR and CIP were presented in 3.4 and 2.8 minutes respectively, as a sharp and symmetrical peak with no interfering peaks.

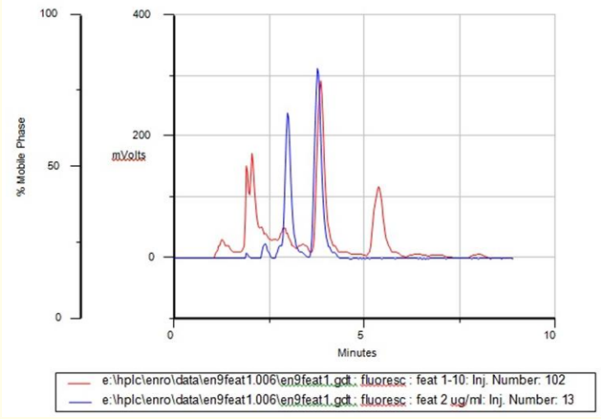

Figure 2: *HPLC Chromatograms of feather spiked with ENR and CIP at 2 µg mL-1 and problem feather (1 - 10 = 1 day post treatment/animal N°10).*

Limits of detection (LOD) and quantitation (LOQ)

The LODs were 0.040 and 0.062 µg g^{-1} and LOQs were 0.050 and 0.080 µg g^{-1} for CIP and ENR respectively in chicken feathers.

Intra-day and inter-day accuracy and precision

The method for the analysis of feather samples was thoroughly validated and the results are presented in Table 1. To assess the inter-day (over 3 days) assay accuracy and precision, 6 sets of feather samples were prepared containing ENR and CIP at 0.1, 0.25 and 2 µg g^{-1}.

The inter-day variation in accuracy (recovery) and precision were assessed. The mean accuracy (recovery) should be within the range 85 - 115% and the variation in precision should be ≤ 20%.

To determine the intra-day accuracy and precision, 6 replicates of each 3 concentrations were analyzed along with duplicate standard calibration curves prepared from 2 separate stock solutions (Table 1).

			Intra-day		Inter-day (over 3 days)	
Compound	**r**	**µg g-1**	**Recovery (%) n = 6**	**Precision (%) n = 6**	**Recovery (%)**	**Precision (%)**
ENR	0.99953 (0.1 - 2 µg g^{-1})	0.1	110.00	4.76	100.00	10.00
		0.25	96.00	3.38	94.67	2.44
		2	98.00	5.57	97.33	0.59
CIP	0.99863 (0.1 - 2 µg g^{-1})	0.1	110.00	5.34	100.00	10.00
		0.25	96.00	5.54	93.33	2.47
		2	100.00	3.94	96.67	2.99

Table 1: *ENR and CIP recovery and precision intra-day and inter-day from feather samples spiked with ENR and CIP.*

ENR and CIP feather concentrations

High levels of ENR and CIP were found in feathers after ENR oral administration (10 mg kg^{-1}) for consecutive 5 days with drinking water and both compounds persisted throughout the study period. Mean ± SD values of ENR, CIP, ENR + CIP and CIP to ENR ratio are presented in Table 2. Figure 3 shows concentration evolution of ENR, CIP and ENR + CIP in function of time.

Days post-treatment	CIP ± SD	ENR ± SD	ENR+CIP ± SD	R CIP/ENR
1	0.40 ± 0.16	4.66 ± 0.78	5.07 ± 0.81	0.09 ± 0.04
2	0.29 ± 0.11	5.17 ± 1.21	5.46 ± 1.19	0.06 ± 0.03
3	0.54 ± 0.20	2.48 ± 0.95	3,02 ± 0.91	0.28 ± 0.20
4	0.48 ± 0.38	1.42 ± 0.71	1.90 ± 0.92	0.40 ± 0.26
5	0.39 ± 0.34	1.22 ± 0.41	1.60 ± 0.46	0.38 ± 0.41
7	0.23 ± 0.13	0.65 ± 0.31	0.88 ± 0.34	0.43 ± 0.29
9	0.09 ± 0.03	0.11 ± 0.03	0.20 ± 0.05	0.85 ± 0.42

Table 2: *Enrofloxacin and its active metabolite, ciprofloxacin (µg g^{-1}) in broiler chicken feathers after enrofloxacin administration of 10 mg kg^{-1} during five days with drinking water (Mean ± SD, n = 10).*

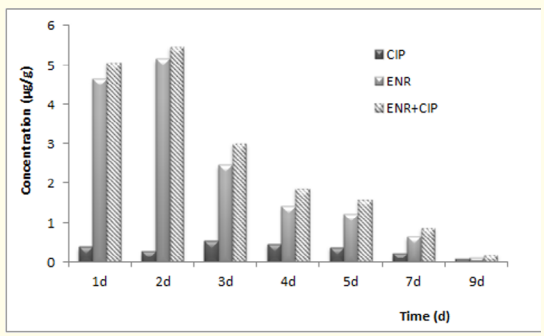

Figure 3: *Mean feather concentrations of ENR, CIP and ENR + CIP of chickens after ending the enrofloxacin (Carval®) administration by oral route with drinking water for 5 consecutive days.*

Discussion

The analytical method developed to determine ENR and CIP in broiler chicken feathers demonstrated linearity, precision and accuracy under the analytical conditions which include acetone extraction and quantitative analysis by liquid chromatography with fluorescent detector. This simple technique might have important applications in residues studies of ENR and CIP in feathers.

Feather meal is a potential source of drug residues that can pass through the food chain when contaminated meal is fed to food-producing animals. In the present study, feathers had high ENR and CIP concentrations, coincident with results of others authors who analyzing different compounds in chicken feathers [14,18]. Although after the feathers have been processed are introduced in the food chain as a protein source in animal feed, because poultry feather constitutes the most abundant keratinous material in nature, withdrawal periods are not established yet. Our results were coincident with San Martín., *et al.* (2007) and Love., *et al.* (2012) [6,14]. The characteristics of enrofloxacin include good absorption after parenteral and oral applications, large volume of distribution, suggesting wide tissue penetration, including peripheral tissues (feathers) as we observed in our study, and a long terminal half-life [1,2,12]. The high concentrations found in the feathers cannot be explained by blood contribution to this tissue because feather vasculature reaches only the lower portion of the calamus. One possible source of feather contamination is secretion from the uropygial gland, which may reach the feathers via grooming behavior [4,14]. However, such high concentrations of ENR and CIP found in feathers cannot be attributed only to external contamination of feathers. Feather generation and molting can play an important role in drug disposition kinetics in feathers of treated animals. As the birds grow from chicks to adult birds, they undergo a series of molts, in which four generations of feathers develop and grow from the same follicle. All these follicles are formed during embryo development; once the bird has hatched, the follicle number is fixed. Both the follicle and the emerging feathers are derived from the epidermis of the skin [19]. The slow elimination of enrofloxacin residues from feathers could be explained by the reabsorption of the vascularized pulp that fills the shaft of the feather throughout the maturation process. This process is discontinued and terminates in a pulp cap, in which the drug can be retained. Enrofloxacin and its metabolite ciprofloxacin, accumulate in higher and persistent concentrations in feathers than in edible tissues [6,20]. In the present study ENR + CIP concentrations were 0.20 to 5.46 μg g^{-1} between 1 to 9 days after final treatment, which means that withdrawal time fit for edible tissues is not adequate to reduce antimicrobial residues in chicken feathers. Further studies to establish a withdrawal time may be useful to avoid that drug residues could result in adverse health consequences like increases in antibiotic resistance.

Conclusion

According to our results, high ENR + CIP concentrations were reached in feathers, which were maintained up to 9 days post treatment. As feathers are used as a protein source to supplement food for different food producing species, they must be considered potential reservoirs of drug residues that can reach man through the food chain and could result in adverse health consequences such as selection and spreading of resistant microorganisms to antibiotics. Therefore, we conclude that it is very important to establish an withdrawal time similar to that of edible tissues.

Bibliography

1. Mestorino N., *et al*. "Estudio de depleción tisular de enrofloxacina, de su metabolito ciprofloxacina y de bromhexina en pollos parrilleros". XXI Congreso Latinoamericano de Avicultura, La Habana, Cuba (2009): 793-795.

2. Mestorino N., *et al*. "Depleción de enrofloxacina y su metabolito ciprofloxacina en tejidos comestibles y en plumas de pollos parrilleros". XXII Congreso Latinoamericano de Avicultura, Buenos Aires, Argentina (2011).

3. Gouvêa R., *et al*. "Fluoroquinolones in Industrial Poultry Production, Bacterial Resistance and Food Residues: a Review". *Brazilian Journal of Poultry Science* 17.1 (2015): 1-10.

4. López-Cadenas C., *et al*. "Enrofloxacin: Pharmacokinetics and metabolism in domestic animal species". *Current Drug Metabolism* 14.10 (2013): 1042-1058.

5. Sapkota AR., *et al*. "What do we feed to food production animals? A review of animal feed ingredients and their potential impacts on human health". *Environmental Health Perspectives* 115.5 (2007): 663-670.

6. Love DC., *et al*. "Feather Meal: A previously unrecognized route for reentry into the food supply of multiple pharmaceuticals and Personal Care Products (PPCPs)". *Environmental Science and Technology* 46.7 (2012): 3795-3802.

7. Nachman KE., *et al*. "Arsenic pieces in poultry feather meal". *Science of the Total Environment* 417-418 (2012): 183-188.

8. Hadas A and Kautsky L. "Feather meal, a semi slow-release nitrogen fertilizer for organic farming". *Fertilizer Research* 38.2 (1994): 165-170.

9. Cheng Z., *et al*. "Effect of feather meal on growth and body composition of juvenile Pacific white shrimp Litopenaeusvannamei". *Journal of Applied Aquaculture* 12.1 (2002): 57-69.

10. Divakala K., *et al*. "Amino acid supplementation of hydrolyzed feather meal diets for finisher pigs". *Journal of Animal Science* 87.4 (2009): 1270-1281.

11. Dimitrova DJ., *et al*. "Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin in male and female turkeys following intravenous and oral administration". *Veterinary Research Communications* 30.4 (2006): 415-422.

12. Otero JL., *et al*. "Pharmacokinetics of enrofloxacin after single intravenous administration in sheep". *Revue Scientifique et Technique (International Office of Epizootics)* 28.3 (2009): 1129-1138.

13. San Martín B., *et al*. "Isolation and molecular characterization of quinolone resistant Salmonella spp. from poultry farms". *Veterinary Microbiology* 110.3-4 (2005): 239-244.

14. San Martín B., *et al*. "Depletion study of enrofloxacin and its metabolite ciprofloxacin in edible tissues and feathers of white leghorn hens by liquid chromatography coupled with tandem mass spectrometry". *Journal of Food Protection* 70.8 (2007): 1952-1957.

15. Griggs DJ., *et al*. "Incidence and mechanism of ciprofloxacin resistance in Campylobacter spp. isolated from commercial poultry flocks in the United Kingdom before, during and after fluoroquinolones treatment". *Antimicrobial Agents and Chemotherapy* 49.2 (2005): 699-707.

16. Ponce-Rivas E., *et al*. "Identification and molecular characterization of Class 1 integrons in multiresistant Escherichia coli isolates from poultry litter". *Applied and Environmental Microbiology* 78.15 (2012): 5444-5447.

17. European Union. "Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of the results". Official Journal of the European Communities L221 (2002): 23-33.

18. Malucelli A., *et al*. "Tissue distribution and residues of clenbuterol, salbutamol, and terbutaline in tissues of treated broiler chickens". *Journal of Animal Science* 72.6 (1994): 1555-1560.

19. Kumar SV., *et al*. "Feather - a potential source for reentry of enrofloxacin and its metabolite ciprofloxacin residues in food chain". *Indian Journal of Veterinary and Animal Sciences Research* 43.2 (2014): 151-155.

20. Jansen L., *et al*. "The analysis of tetracyclines, quinolones, macrolides, lincosamides, pleuromutilins, and sulfonamides in chicken feathers using UHPLC-MS/MS in order to monitor antibiotic use in the poultry sector". *Analytical and Bioanalytical Chemistry* 409.21 (2017): 4927-4941.

Volume 4 Issue 3 May 2019