

## COMPARISON AND EVALUATION OF ANTIBIOTIC RESIDUES IN VARIOUS CHICKEN MEAT SAMPLES BY RP-HPLC METHOD

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### ABSTRACT

Antibiotics can be used in chicken meat as growth promoters and also to increase poultry production, but the antibiotics usage in chicken meat results into a major risk for humans due to antibiotic resistance. In the present study ten chicken meat samples were collected from different regions of Hyderabad. Liquid-liquid extraction process was used for the extraction of Sulphamethoxazole, Acetonitrile, Acetone and Dichloromethane were used as organic solvents. Microbiological method was done for the determination of Sulphonamides in chicken meat samples. Thin layer chromatography was done to determine sulphamethoxazole and other sulphonamides using Dichloromethane-methanol-water in the ratio of 69:35:6 as mobile phase. Retention factor (Rf) was calculated after chromatograms detected by spraying the plate with Fluorescamine and observed under Ultraviolet chamber. Quantification was done using RP-HPLC using KROMOSIL 100 C18 Column, 5 $\mu$ , Mobile phase: di sodium hydrogen phosphate:methanol (75:25), Flow rate: 1 ml/min, Pump mode: isocratic, Run time: 10min, Column temperature: 25 $^{\circ}$ C Injection volume: 20 $\mu$ l, Detection: UV at 266nm. Out of ten areas selected in Hyderabad region it was found that sulphonamide (sulphamethoxazole) concentration was more in Yakutpura and Mehdiapatnam areas which are above MRLs (0.1 $\mu$ g/ml) (Maximum Residue Limit (MRL) recommended limit according to the European Union (EC). All the results showed that the developed method was accurate, simple and can be used for the laboratory purpose for the analysis of antibiotic residues in chicken meat.

**Keywords:** Antibiotics, Chicken meat, poultry and sulphamethoxazole.

### 1. INTRODUCTION

Poultry industry is one of the biggest industries in India and globally in terms of production it is ranked 17th position. Accidentally, it was found that by-products of antibiotic production (dried streptomyces aureofaciens broth) which contain a high level of vitamin B12, when feed to poultry animals resulted in higher growth. Eventually, it was discovered that the trace amount of antibiotics remaining in these by-products accounted for high growth. Since

then the antibiotics have been used on poultry in large quantities to enhance production in poultry. However, the use of antibiotics in food animals poses a major risk for humans due to antibiotic resistance.

Antibiotic use is related to emergence of resistant bacteria in the animal which later transmits to human through food, environment and direct contact with the affected meat. Residues of antimicrobial compounds are also found in foods of animal's origin as a

result of inappropriate or excessive usage of these compounds. These residues are also known to transfer to humans through food and environment. To prevent any residues of antibiotics in food and food products of animal origin, withdrawal periods are set by regulatory agencies.

Withdrawal period is a time between the last dose of antibiotic given to food animals and consumption of food animals or food derived from it. It needs to be mentioned on the antibiotics that are used for animals. Antibiotics in poultry farming can be used as therapeutic agents, Prophylactic agents, growthpromoters.

Literature survey revealed that various analytical methods reported for the estimation of antibiotic residues. No method was developed for the estimation of antibiotic residues in various chicken meat samples in the area of Hyderabad by RP-HPLC. Hence RP-HPLC method can be developed for the estimation of antibiotic residues in various chicken meat samples from Hyderabad.

In the present study an attempt is made to know the concentrations of antibiotic residues in different areas by chromatographic techniques.

## 2. MATERIALS AND METHODS

### Sample collection

For determination of sulfonamide concentration, ten meat samples were collected from five different zonal areas of Hyderabad. Like Secundrabad in east side, YakutPura in west side, Mehdipatnam in north side, Amberpet in south side and khairthabad in center, these samples were taken and analyzed.

### INSTRUMENTS

Weighing machine of SARTORIUS, Incubator of CINTEX, Autoclave, Hot air oven of SISCO, UV chamber of SECOR, HPLC of YOUNG YL Instrument using UV detector, Sonicator of LALCO Scientific Instruments, Centrifuge of REMI.

### CHEMICALS

- ✓ Different antibiotics like Sulphamethoxazole and sulphamethazine were obtained from yellow Chem product.
- ✓ Methanol, Acetonitrile of analytical grade and Glacial Acetic Acid were obtained from MERCK laboratories.
- ✓ Water of HPLC grade from Milli-Q, di-Natrium Hydrogen Phosphate.

### Determination of Antibiotic Residues

#### Microbiological assay for the determination of Sulphonamides using Agar Plate Method

Take the sterile nutrient agar tubes, at 40-45°C transfer a loop of culture of 24hrs old *Bacillus subtilis* and mix thoroughly. Pour the solution into sterile Petri plates under aseptic conditions. Allow the plates to solidify, after complete solidification, make the wells using sterile borer and label the cups. Fill the cups using disposable syringe with the respective dilution. Do not disturb the plates after addition of antibiotic solution and let the solution diffuse. Then place all the plates in the incubator at 37°C for 24hrs. After 24hrs, measure the diameter of zone of inhibition.

#### Thin Layer Chromatographic method for the determination of Sulphonamides

##### TLC Conditions

- ✓ TLC plates: Plate coated with silica gel
- ✓ Mobile phase: ethyl acetate: n-butanol: methanol: aqueous ammonia (35:45:15:2 v/v/v/v)
- ✓ Spraying reagent: fluorescamine
- ✓ Detection: ultraviolet light

#### Preparation of TLC plate

TLC 20×10 plate coated 0.25mm thickness with silica gel were used for the determination of Sulphamethaxazole in chicken sample.

##### Preparation of mobile phase:

Mobile phase was prepared by mixing of ethyl acetate, n-butanol, methanol and aqueous ammonia in the ratio of 35:45:15:2 v/v/v/v

#### Preparation of Standard Solution

Standard solution of Sulphamethaxazole and Sulphamethazine was prepared by dissolving 0.05g of Sulphamethaxazole and Sulphamethazine powder in 100ml of mobile phase each.

#### Sample preparation

A volume of 30 ml acetonitrile was added to 10 g of minced and homogenized muscle tissue weighed in a glass centrifuge tube. The sample was homogenized for 1min in a vortex and centrifuged at 3500rpm for 10min. Then the supernatant was transferred into a pear-shaped flask, add 20ml of acetone to the sediment before the mixture was sonicated for 10min. The solution was centrifuged once again and the supernatant was added to same pear-shaped flask. The mixed solution was evaporated 40°C until near dryness. Afterwards, 5ml of dichloromethane was added, homogenized by vortexing and transferred into a test tube. The step of addition of dichloromethane was repeated three times and the combined

dichloromethane was dried at 40°C. The residue was reconstituted with 1ml of 50% methanol in di-sodium hydrogen phosphate solution (6g/1000ml) and mixed properly by vortexing. N-hexane (2 ml) was added into the test tube for defeating and vortexing before being removed. 10µl of standard and sample solutions were placed on TLC plate. Treated plate were transferred to TLC tank containing mobile phase. After reaching the solvent front to end of plates, chromatograms detected by spraying the plate with a fluorescamine and observed under ultraviolet light.

### **RP-HPLC Method for the determination of Sulphamethaxazole**

#### **Preparation of di sodium hydrogen phosphate**

Weigh 6g of di sodium hydrogen phosphate in 1000ml volumetric flask and make up the volume with distilled water.

#### **Preparation of mobile phase**

Prepared a required volume of degassed mixture of di sodium hydrogen phosphate and methanol in the ratio of 75:25 v/v.

#### **Preparation of standard solution**

Weighed and transferred 100mg of sulphamethaxazole working standard into a 100ml clean, dry volumetric flask. Make up the volume with mobile phase (1000µg/ml). Diluted 1ml standard stock solution to a 100ml volumetric flask with mobile phase as diluents and mixed well (10µg/ml). Further the solution is diluted to get 0.2, 0.4, 0.6, 0.8, 1, 1.2µg/ml concentrations respectively.

#### **Preparation of sample solution (Liquid-liquid extraction method)**

A volume of 30 ml acetonitrile was added to 10 g of minced and homogenized muscle tissue weighed in a glass centrifuge tube. The sample was homogenized for 1min in a vortex and centrifuged at 3500rpm for 10min. Then the supernatant was transferred into a pear-shaped flask and added 20ml of acetone to the sediment before the mixture was sonicated for 10min. The solution was centrifuged once again and the supernatant was added to same pear-shaped flask. The mixed solution was evaporated 40°C until near dryness. Afterwards, 5ml of dichloromethane was added, homogenized by vortexing and transferred into a test tube. The step of addition of dichloromethane was repeated three times and the combined dichloromethane was dried at 40°C. The residue was reconstituted with 1ml of 50% methanol in di-sodium hydrogen phosphate solution (6g/1000ml) and mixed properly by

vortexing. N-hexane (2 ml) was added into the test tube for defeating and vortexing before being removed. The remaining solution was filtered through the filter of 0.2µm or not more than 0.45µm, and was ready for injection into HPLC system.

#### **Method Validation**

The method was validated in-house according to EMEA- notice to applicant and guideline- veterinary medical products (vol-8)-, and Commission Decision (EEC) No. 657/2002. For the criteria specified for quantitative method, and the validation parameters were determined by spiking blank chicken meat at three levels 0.5, 1, 1.5 MRL. The measured parameters were Calibration Curve, Specificity, Repeatability, Accuracy, Response Linearity, Limit of Detection and Limit of Quantification.

##### **A. Linearity and Range**

Linearity is the ability of a method to the analyte concentration within the given range.

Range is the interval between upper and lower levels of analyte that have been demonstrated to the determined with precision, accuracy & linearity using method as described.

##### **B. Accuracy (% Recovery)**

The accuracy of an analytical method measures the agreement between the value, which is either as a conventional true value or and accepted reference value and the value found (i.e accuracy is a measure of the exactness of & analytical method). Accuracy is measure as the percent of analyte recovered after spiking samples in a blank.

##### **C. Precision**

Precision is the measure of the degree of repeatability of an analyte method under normal operation and is normally expressed as relative standard deviation for a statistically significant number of samples.

There are two types of precisions

##### **1. Repeatability**

This is the closeness of agreement between mutually independent test results obtained with the same method on identical test materials in the same laboratory by the same operator using the same equipment within short intervals of time.

##### **2. Intermediate precision (ruggedness)**

Intermediate precision is also called as ruggedness. This method measures the repeatability of the results obtained with the same method, on the same sample, in the

same laboratory, but conducted by different operators in different days.

#### D. Specificity

The specificity of an assay is the capability to differentiate similar organisms or analytes or the other interferences from the matrix elements that could have a positive or negative effects on the assay value.

#### E. LOD & LOQ

LOD is the lowest concentration of an analyte in a sample that can be detected but not necessarily quantitated under the stated experimental conditions. It can be determined by preparing a solution that is expected to produce a response i.e about 3 to 10 times that base line noise.

The solution is injected three times, and the s/n ratio for each injection is recorded. The concentration of the solution is considered and LOD if the s/n ratio is between 3 to 10 LOQ can be determined in the same manner but with and s/n ratio of 10 to 20.

$$\text{LOD} = 3 \times \text{SD} / \text{slope}$$

$$\text{LOQ} = 10 \times \text{SD} / \text{slope}$$

### 3. RESULTS AND DISCUSSION

#### Antimicrobial assay

Zone of inhibition for standard and sample was calculated (See Table No. 1 & Figure. No. 1, Figure No:2). Out of twenty samples taken, the chicken meat samples in Kukatpally, Mehdipatnam, Yakatpura, Khairtabad, Secundrabad, Amberpet showed zone of inhibition when compared with the standard sulphamethoxazole. Hence it proved that the meat samples containing antibiotics (sulphonamides).

#### Thin Layer Chromatographic method For the determination of Sulphonamides

TLC was performed for the identification of the sulphamethazine and Sulphamethaxazole by comparing Rf value of samples with the standard but meat sample showing only Sulphamethaxazole (see Figure No.2).

#### RP-HPLC Method For the determination of Sulphamethoxazole

Several trails were done and the optimized conditions was achieved on KROMOSIL 100 C18, 5 $\mu$  column, di sodium hydrogen phosphate: methanol (75:25) mobile phase in isocratic mode and the Run time was 10min, the Column temperature was 25°C, Injection volume was 20 $\mu$ l, peak observed at 4.5min using flow rate of 1 ml/min with UV detection at 266nm. The developed method

was validated according to ICH guidelines. (See Figure No:3&4)

#### METHOD VALIDATION

##### A. Linearity and range

Established by plotting the peak area of each active component against its associated concentrations. The concentration used for sulphamethoxazole ranged from 30 $\mu$ g/kg to 150 $\mu$ g/kg (0.5MRL – 1.5MRL), at five levels, according to the recommendation made by the Commission Decision No. 657/2002.

A statistic linear regression was performed. Acceptance criteria:  $r^2 > 0.999$  (coefficient of correlation). correlation coefficient was found to be 0.9993 (see Table No.2, Figure No:6).

##### B. Accuracy (% Recovery)

It was determined by recovery experiments using blank matrices. 18 aliquots of a blank material were spiked (six aliquots) at each of 0.5, 1 and 1.5 x MRL the samples were analyzed and the sulfonamides concentration present in each sample was Calculated; using the equation. The percentage recovery of Sulphamethaxazole is from 71.05 -74.24% these values are within the criteria % Recovery (R) = 100 x measured content / spiked level. (See Table No:3)

##### C. PRECISION

###### 1. REPEATABILITY

Three test samples were spiked at analyte levels, which encompass 0.5x and 2x the MRL. Then, 6 test portions of each level were taken, analyzed and the residue concentration of each test portion was determined. The relative standard deviations (RSDs) of the sulfonamides for six measurements at 50 $\mu$ g kg<sup>-1</sup>, 100 $\mu$ g kg<sup>-1</sup> and 150 $\mu$ g kg<sup>-1</sup> were from 0.3-1.46%. These values are within the criteria stipulated for residue analysis by the Commission Decision No. 657/2002: RSD < 20% (see table No.4) RSD = SD/MEAN x 100 (See Table No:4).

##### D. SPECIFICITY

Specificity was study by analyzing blank samples and spiked samples at relevant concentration (0.5, 1, 1.5 x MRL) and checking any interferences in the region of interest.

Percent peak width was found to be 103.6%. So, there were no interfering peak in blank, sample and standard at retention time of these analyte in this mode and the peak width of analyte is within 90-110% of standard width (see figure No.7&8).

##### E. LOD & LOQ

LOD of Sulphamethaxazole was found to be 1.5443 $\mu$ g/kg.

LOQ of sulphamethoxazole was found to be 4.6798 $\mu$ g/kg.

#### Comparative Studies

Out of ten samples, Sulphamethoxazole concentration was detected in meat samples of six different areas from Hyderabad. Yakatpura and Mehdhipatnam samples are having concentration of Sulphamethoxazole more than MRLs established by INDIA (0.1 $\mu$ g/g). The samples from the Amberpet, Secunderabad and Kukatpally are having Sulphamethoxazole concentration within the maximum residual limits (see table No.5).

#### 4. CONCLUSION

An attempt was made for the analysis of antibiotic residues in various chicken meat samples of different regions in Hyderabad by RP-HPLC method. The screening of 20 chicken meat samples showed occurrence of antibiotic residues like sulphamethoxazole in six different areas of Hyderabad, whereas Mehdipatnam and Yakutpura regions showed antibiotic residues above MRL which causes serious health concerns in humans who consumes chicken meat in that regions. The level of antibiotic consumption in these areas should be evaluated and this study provides a basis for the need of an investigation on antibiotic residues in various chicken meat samples. Hence various educational and awareness programs should be conducted on the proper use of antibiotics in poultry industry.

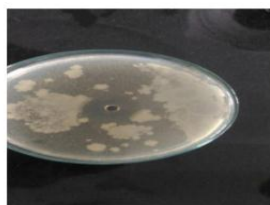


Fig. 1: Zone of inhibition of standard



Fig. 2: Zone of inhibition of sample

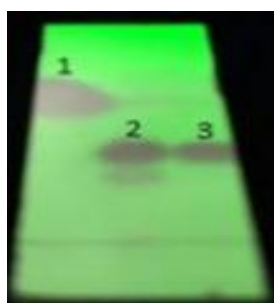
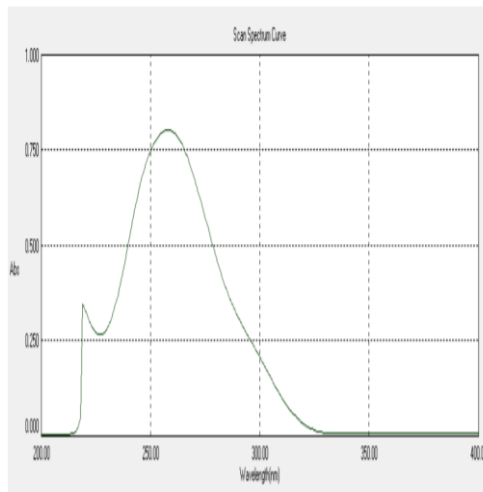
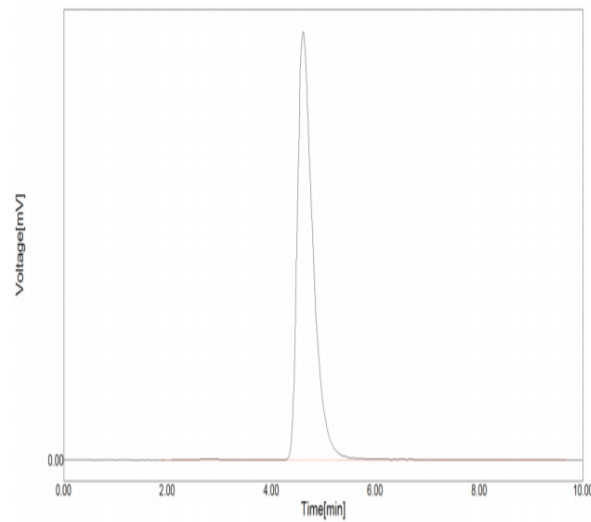


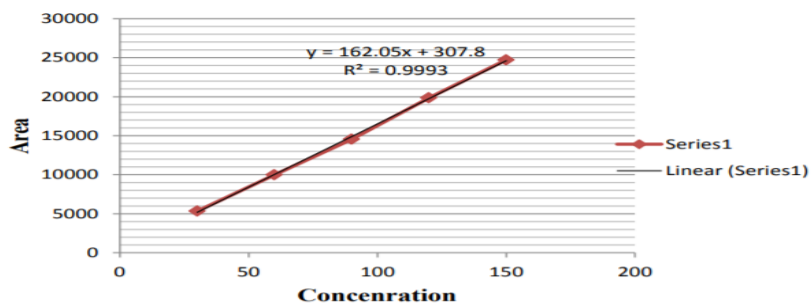
Fig. 3: TLC of standard and sample



**Fig. 4: UV spectra of sulphamethoxazole at 266nm**



**Fig. 5: Rt of sulphamethoxazole at 4.5 min**



**Fig. 6: Linearity of sulphamethoxazole**

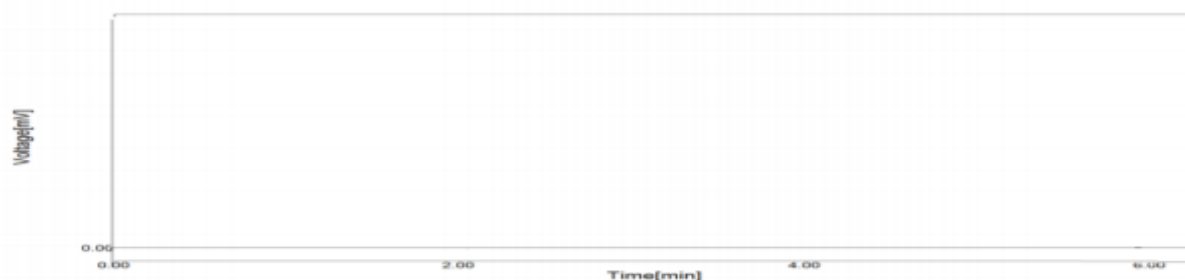


Fig. 7: chromatogram of blank

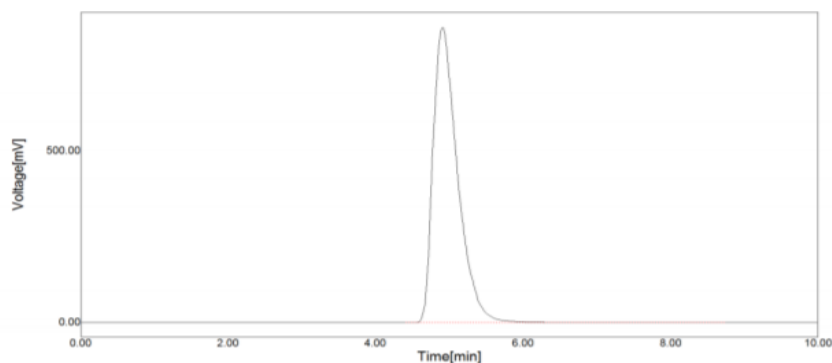


Fig. 8: Chromatogram of sample

Table 1: Microbial assay results

concentration	Zone of inhibition
2	1.92
4	2.11
6	2.29
8	2.48
10	2.66
sample	1.97

Table 2: linearity data of sulphamethoxazole

Concentration	Area
30	5346
60	9989
80	14550
120	19855
150	24720

Table 3: Accuracy data of sulphamethoxazole

50µg/ml			100µg/m			120µg/ml		
Area	Con. obtained	% recovery	Area	Con. obtained	% recovery	Area	Con. obtained	% recovery
6375.6	37.45	74.91111	11497.2	36.03	72.07037	18797.8	38.045	76.09053
6407.7	37.65	75.30741	11562	35.73	71.47037	19300	39.07	78.1572
6164.8	36.15	72.30864	11775.4	35.39	70.78765	18462	37.35	74.70864
6262	36.75	73.50864	11664.8	35.05	70.10494	17526.8	35.43	70.86008
6326.8	37.15	74.30864	11875.4	35.7	70.4049	17429.6	35.23	70.46008
6116.2	35.85	71.70864	11726.8	35.24	70.48765	18575.4	37.585	75.17531
%mean recovery = (36.8/50)×100 = 73.6%			% mean recovery = (71.054/100)×100 =71.05%			% mean recovery = (111.36/150)×100 =74.24%		

**Table 4: Repeatability data of sulphamethoxazole**

Con. At 50µg/ml		Con. At 100 µg/ml		Con. At 150 µg/ml	
Area	Con. Obtained	Area	Con. obtained	Area	Con. obtained
8375.6	49.8	16297.2	98.7	23797.8	148.8
8407.7	50	16362	99.1	24300	148.1
8164.8	48.5	16475.4	99.8	24462	149.1
8262	49.1	16264.8	98.5	24526.8	149.5
8326.8	49.5	16475.4	99.8	24429.6	148.9
8116.2	48.2	16426.8	99.5	24575.4	149.8
Avg = 49.1 STD = 0.719 RSD = 1.465		Avg = 99.23333 STD = 0.557 RSD = 0.561		AVG = 149.0 STD = 0.592 RSD = 0.397	

**Table 5: Comparative studies of meat sample concentrations in different areas**

S.No	Location	Area	Sulphamethaxazole (µg/g)	MRL (µg/g)
1	Amberpet	329.5	0.037	0.1
2	Khairthabad	-	0.042	0.1
3	Kukatpally	343.72	0.067	0.1
4	Mehdipatnam	355.96	0.202	0.1
5	Secunderabad	316.18	0.015	0.1
6	Yakutpura	398.8	0.182	0.1

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