Determination of Antibiotic Residues in Chicken Liver by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

In this study, 0,5 kg of chicken liver sample was taken from 34 different markets in Antakya. The samples were analysed by LC-MS/MS in terms of 38 antibiotic residues from 7 groups. Only in one sample, Trimethoprim (298.5 µg/kg) and Sulfametoxazole (312.8 µg/kg) residue was detected. Both antibiotic residue amounts are above the limits announced in Turkey and EU regulations. Therefore antibiotic residue analyses should be performed within a plan and efficiently in terms of public health.

Keywords: Chicken liver, antibiotic, liquid chromatography, tandem mass spectrometry.

Likit Kromatografi Tandem Mass Spektrometri ile Tavuk Ciğerinde Antibiyotik Kalıntılarının Belirlenmesi

Özet

Bu çalışmada, Antakya'da 34 farklı tavuk marketinden 0,5 kg alınan tavuk ciğeri numunesinde antibiyotik kalıntısı araştırılmıştır. Numuneler, 7 grupta toplam 38 antibiyotik kalıntısı bakımından LC-MS/MS ile analiz edilmiştir. Sadece bir numunede Trimetoprim (298.5 µg/kg) ve Sulfametoxazole (312.8 µg/kg) kalıntısı tespit edilmiştir. Her iki antibiyotik kalıntı miktarı da Türkiye ve Avrupa Birliği mevzuatında bildirilen yasal limitlerin üzerinde

olduğu fark edilmiştir. Bu nedenle halk sağlığı açısından hayvansal ürünlerde antibiyotik kalıntı analizleri bir plan dahilinde ve etkin bir şekilde yapılmalıdır.

Anahtar Kelimeler: Tavuk ciğeri, antibiyotik, likit kromatografi, tandem mass spektrometri.

Introduction

Veterinary drugs have become an integral part of livestock production and play an important role in the maintenance of animal welfare, mainly for the prevention of disease, the curing of infection, controlling the risk of disease transmission to man and also increasing the productive capacity of animals [1].

The antibiotics are used at concentrations lower than those used for treatment; a potentially dangerous practice since it can encourage the production of antibiotic resistant strains of bacteria, potential allergic reactions and technological problems of fermented meat products. Some antibiotics are directly toxic, e.g. chloramphenicol, which causes fatal aplastic anemia, while allergic reactions and toxic side effects may have fatal consequences [2]. In addition to immediate adverse effects, there are also long-term effects to the exposure of low levels of residues that are still unknown [3].

Over recent decades, the predominant way of monitoring antibiotics has been by dividing the analysis into several steps, i.e. screening, post-screening and confirmation. Most commonly, screening is performed by microbiological plate tests and quantification and confirmation by class-specific liquid chromatographic methods. When screening is performed by plate tests a post-screening step by, for example, Charm is necessary to reveal the antibiotic class.

The described scheme for the analysis of antibiotics is time consuming, and requires several days from sampling to a confirmed result. If the analysis could be carried out in one step it would accelerate the process. One solution to this problem would be a rapid and simple multi-class liquid chromatographic–tandem mass spectrometric (LC–MS/MS) method [4].

Food safety is an important issue in the EU and a legal framework, which covers the whole food chain, has been established. The central goal is to guarantee a high level of protection of human health in relation to food. Regarding residues of veterinary drugs in foodstuffs of animal origin, the EU has set maximum residue limits (MRLs) for authorized drugs. An efficient control of residues is essential, and the member states implement national

residue monitoring plans with the aim of ensuring that MRLs are not exceeded and that forbidden substances are not present in food products [5].

In Turkey poultry sector, beta lactams, quinolones, macrolids, tetracyclines, trimethoprim, sulfonamides, amphenicols group antibiotics are widely used illegally.

According to Commission Regulation (EU) 37/2010 (EC 2010), quinolones range between 100-1900 µg/kg, Beta lactams range between 50-2000 µg/kg, Macrolids range between 400-1000 µg/kg, Tetracyclines 300 µg/kg, Trimethoprim 50 µg/kg, Sulfonamides 100 µg/kg, Florfenicol 2500 µg/kg, and Chloramphenicol no MRL in chicken liver [6]. Turkish Food Codex Regulation has also established the same levels as those of the EU [7].

The purpose of the present study is to determine the levels of the seven aforementioned groups of antibiotics in chicken liver samples by LC MS/MS and to compare the obtained results with antibiotic tolerance limits accepted by the EC and Turkey.

Material and Methods

Material

The samples used in this study were of chicken liver. About 0.5 kg chicken liver samples (10 pieces) were purchased from 34 different local poulterers in Antakya. Upon arrival at the laboratory, the samples were homogenized and stored at -18 ºC and thawed before analysis.

Reagents

Amoxicillin trihydrate (AMX), ampicillin trihydrate (AMP), chloramphenicol (CLP), nafcillin sodium salt (NAF), oxytetracyclin hydrochloride (OXT), spiramycin (SPR), sulfadiazine (SDZ), and tylosin phosphate (TYL), were obtained from Sigma – Aldrich (Seelze, Germany). Cefapirin sodium (CEP) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Cefalexin monohydrate (CEL), ceftiofur (CET), chlorotetracycline hydrochloride (CLT), ciprofloxacin hydrochloride (CPF), cloxacillin sodium salt hydrate (CLX), danofloxacin mesylate (DNF), dicloxacillin sodium hydrate (DLO), difloxacin hydrochloride (DFO), doxycycline hyclate (DXC), enrofloxacin (ENO), florfenicol (FLF), flumequine (FLQ), marbofloxacin (MAF), nalidixic acid (NAL), norfloxacin (NOR), oxacillin sodium salt hydrate (OXC), oxolinic acid (OXL), penicilinle G potassium salt (PEN), sarafloxacin hydrochloride (SRF), sulfachinoxalin (SUC), sulfachloropyridazine (SCP), sulfadimethoxine (SDM), sulfamerazine (SMR), sulfamethazine (SMT), sulfamethoxazole (SMX), sulfathiazole

(STH), tetracycline hydrochloride (TEC), tilmicosin (TİL), and trimethoprim (TRM) were obtained from Dr. Ehrenstorfer (Augsburg, Germany).

Acetonitrile, formic acid and methanol were HPLC of gradient grade and purchased from Merck (Darmstadt, Germany). Solid reagents used were all analytical grade; oxalic acid 2-hydrate, sodium hydroxide and ethylenediaminetetraacetic acid disodium salt were purchased from Merck (Darmstadt, Germany). Double-deionized water (Sartorius Arium 611 Goettingen, Germany) of 18.2 MΩ.cm resistivity was used.

Stock standard solutions of 50 μg/mL were prepared in 40 mM methanolic sodium hydroxide for Quinolones, in water for AMP, DLO, CLX, CLP, NAF, OXC, PEN, CEL, CEP; in 50% acetonitrile for AMX, CET; in acetone for SDZ; in acetonitrile for SDM, SUC, SMR, SMT, SMX, STH, and in methanol for the remaining 10 analytes. All stock standard solutions were stored at +4 ºC. Antibiotics dissolving in same solvent were mixed among each other and used in validation studies.

Mixed working standard solutions were prepared daily in water. The working standard solutions contained the analytes in concentrations appropriate to achieve MRL-level in the samples by spiking 3 g of sample with 100μL of working standard. Matrix matched standards were prepared in exactly the same way as the other samples.

Instrumentation

A Shimadzu Prominence LC system interfaced to an AB SCIEX API 3200 LC-MS/MS system equipped with Turbo V source and Electrospray Ionization (ESI) probe was used. The column used was C_{18} Synergi (50 mm × 2 mm; 2.5 μm particle diameter) from Phenomenex. Instrument control and data processing were carried out by means of Analyst 1.6.2 software. A high-performance dispersing machine from Wisd (Korea) and a Hettich refrigerated centrifuge (Tuttlingen, Germany) were used in the extraction process.

A gradient containing 0.2% formic acid containing 0.1mM oxalic acid (A) and 100% acetonitrile (B) was applied. The flow rate was set at 0.3 ml/min and the injection volume was 20 μl. The gradient went from 0% B to 75% B in 1 min, was kept at 75% B until 2.6 min and was back at 0% B after 2.6 min. The runtime for each injection was 7.2 min. The mass spectrometer was operated in the negative ion mode for amphenicols. Others were operated in the positive ion mode. The mass spectrometric parameters are shown in Table 1.

Samples

The samples were prepared exactly as described previously. In short, 200µL of 0.1 M EDTA (ethylenediaminetetraacetic acid) was added to 3 g of homogenized tissue. The samples were spiked as appropriate and then the antibiotics were extracted from the tissues using 15mL of 70% methanol. After extraction the samples were centrifuged at 3800×g for 5 min [4]. Finally, 500µL of the extract was diluted to 2mL with water, and filtered through 0.45-µm membrane filters. The samples were injected in the LC–MS/MS [5]. The samples were judged against a matrix-matched standard curve.

Table 1. MS/MS parameters for 38 analytes

ID	Q1 Mass (Da)	Q3 Mass (Da)	Ret. Time (min)	DP (Volts)	EP (Volts)	CEP (Volts)	CE (Volts)	CXP (Volts)
Amoxicillin	366.2	114	2.36	21	10.5	30	31	4
	366.1	349.2	2.36	26	7.5	20	15	6
Ampicillin	350.1	106.1	2.4	31	12	28	35	4
	350.1	160.3	2.4	31	12	28	17	6
Danofloxacin	358.1	340.2	2.42	46	12	18	29	6
	358.1	314.2	2.42	46	12	18	25	6
Difloxacin	400.1	356.1	2.45	51	10.5	24	23	8
	400.1	299.1	2.45	51	10.5	24	35	6
Dicloxacillin	469.9	160.3	3.07	66	5	24.2	21	4
	469.9	311	3.07	70	10	24.2	50	4
Doxycycline	445.1	409.9	2.41	41	9	18	33	8
	445.1	428.3	2.41	46	7.5	20	17	34
Enrofloxacin	360.1	316.2	2.42	21	12	24	25	8
	360.1	245.3	2.42	21	12	24	37	4
Florfenicol	355.8	184.9	100*	-15	-12	-16	-26	-4
	355.8	335.9	100*	-15	-12	-16	-12	-6
Flumequine	262.1	244.2	2.8	36	10.5	14	23	8
	262.1	202.1	2.8	36	10.5	14	41	4
Cloxacillin	436.0	160.1	2.97	26	11	24	19	6
	436.0	277.1	2.97	26	11	24	21	8
Chloramphenicol	320.9	152	100*	-20	-12	-26	-22	-4
	320.9	256.8	100*	-20	-12	-26	-14	-8

Chlorotetracycline	479.1	444	2.43	36	8.5	24	29	14
	479.1	154	2.43	36	8.5	24	41	6
Marbofloxacin	363.1	72.1	2.41	36	12	18	37	4
	363.1	320	2.41	36	12	18	21	8
Nafcillin	415.0	199.2	2.97	21	8	22	21	6
	415.1	256.1	2.97	26	8.5	20	21	4
Nalidixic acid	233.1	215	2.84	26	8	14	17	6
	233.1	187.2	2.84	26	8	14	33	4
Norfloxacin	320.1	302.1	2.41	51	12	16	23	8
	320.1	276.3	2.41	51	12	16	19	8
Oxacillin	402.0	160	2.91	26	10.5	22	19	4
	402.0	243.3	2.91	26	10.5	22	19	4
Oxytetracycline	461.1	425.9	2.41	26	7	20	23	8
	461.1	444	2.41	26	7	20	23	6
Oxolinic acid	262.0	244.1	2.69	36	8.5	16	21	6
	262.0	216.2	2.69	36	8.5	16	37	4
Penicilline G	335.1	160	2.77	31	12	18	19	6
	335.1	176.2	2.77	31	12	18	19	6
Sarafloxacin	386.0	342.1	2.44	51	12	16	25	6
	386.0	299.2	2.44	51	12	16	33	8
Cefalexin	348.1	158.2	2.38	21	8	14	17	4
	348.1	173.9	2.38	21	8	14	19	6
Cefaprin	424.0	292.1	2.37	26	10	20	21	10
	424.0	152.1	2.37	26	10	20	31	4
Ceftiofur	524.0	241.2	2.6	16	12	26	25	4
	524.0	209.9	2.6	51	8.5	24	29	6
Ciprofloxacin	332.2	314.2	2.41	41	10.5	14	27	10
	332.2	288.1	2.41	41	10.5	14	23	8
Spyramicin	843.4	174.3	2.39	71	11	44	49	6
	843.4	540.2	2.39	71	11	44	41	6
Sulfadimethoxine	311.0	156.1	2.71	46	11	16	27	4
	311.0	92.1	2.71	46	11	16	43	4
Sulfadiazin	251.1	156	2.49	36	10.5	14	21	6
	251.1	92	2.49	36	10.5	14	35	4

Sulfachinoxalin	301.1	156.2	2.7	36	10.5	18	21	4
	301.1	107.9	2.7	36	10.5	18	33	4
Sulfachloropyridazine	284.9	155.9	2.63	31	10	16	21	6
	284.9	108.1	2.63	31	10	16	35	4
Sulfamerazine	265.0	155.8	2.52	36	12	14	21	4
	265.0	92.1	2.52	36	12	14	39	4
Sulfamethazine	279.1	186.1	2.55	41	10.5	18	21	6
	279.1	156.1	2.55	41	10.5	18	25	4
Sulfamethoxazole	254.0	91.9	2.65	21	12	12	41	4
	254.0	156.1	2.65	21	12	12	21	4
Sulfathiazole	256.0	156	2.48	41	9.5	20	21	6
	256.0	92	2.48	41	9.5	20	35	4
Tetracycline	445.1	410.1	2.39	31	8.5	18	27	8
	445.1	154.1	2.39	31	8.5	18	35	4
Tilmicosin	869.4	174.1	2.41	116	12	36	61	6
	869.4	156.1	2.41	116	12	36	61	4
Tylosin	916.4	174.2	2.46	91	12	38	49	6
	916.4	772	2.46	91	12	38	49	6
Trimethoprim	291.1	230.2	2.38	66	12	18	31	6
	291.1	123	2.38	66	12	18	33	4

* Dwel time (msec)

Method Validation

Calibration curves, precision (repeatability and within-laboratory reproducibility) were performed to validate the whole procedure. Linearity was evaluated using matrix-matched calibration, spiking blank extracts at five concentration levels (from 0.5 to 8 μg/kg). Precision of the method was studied by spiking blank samples. Repeatability (intraday precision) was performed by spiking blank liver at one concentration level (100 μg/kg), using six replicates in one day. To evaluate interday precision (reproducibility), two concentration levels (50 and 200 μg/kg) were studied, spiking blank liver during six consecutive days. Recovery was studied by analyzing a blank sample that was fortified before extraction at 100 μg/kg concentration level. For limit of detection (LOD) and limit of quantitation (LOQ), 20 different blank samples were spiked at 100 μg/kg level for each analyte. Spiked blank samples were analyzed at LC MS/MS. LOD and LOQ were calculated as described below.
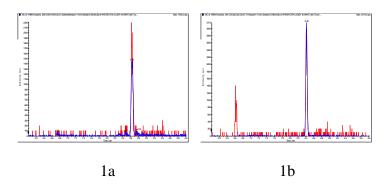
$$LOD=3*[C/(S/N)], LOQ=10*[C/(S/N)]$$

$$C= Concentration, \quad S= Signal, \quad N= Noise$$

Result

In this study totally 38 types of antibiotics were studied by injecting extracts obtained by single extraction to LC-MS/MS device. Amphenicol group antibiotics were studied in negative ion mode while other 36 antibiotics were studied in positive ion mode. Validation results of all antibiotics are shown in Table 2.

In the present study, 34 chicken liver samples were subjected to LC-MS/MS for confirmatory analysis of the antibiotic residues. A single liver sample was found to contain TRM (298.5 µg/kg) and SMX (312.8 µg/kg). The levels of residues were higher than the international levels set by the European Union and limits allowed in Turkey (100 µg/kg for SMX and 50 µg/kg for TRM). Antibiotic residues were not detected in the other 33 chicken liver samples.

LC-MS/MS chromatograms of chicken liver sample positive for SMX and TRM are shown in Figures 1a and 1b, respectively.

1a   1b

Figure 1. Typical chromatograms of chicken liver samples positive for sulfamethoxazole (1a) and trimethoprim (1b)

Table 2. Results of method validation

	Analyte	Linearity	Recovery (%)	Repeatability RSD %	Within-laboratory reproducibility RSD %		LOD (µg/kg)	LOQ (µg/kg)
				100	50	200		
1	Amoxicillin	0.9940	65	5.8	9.4	9.8	0.28	0.92
2	Ampicillin	0.9978	83	8.7	14.1	7.6	0.29	0.96

3	Cefalexin	0.9990	67	13.3	9.5	9.8	0.35	1.16
4	Cefaprin	0.9945	76	7.8	9.1	8.4	0.37	1.22
5	Ceftiofur	0.9992	62	8.3	17.1	14.0	0.04	0.13
6	Chloramphenicol	0.9938	77	9.8	8.7	11.6	1.32	4.36
7	Chlorotetracycline	0.9965	55	11.3	8.4	10.2	1.22	4.03
8	Ciprofloxacin	0.9958	61	15.5	12.2	8.8	1.49	4.92
9	Cloxacillin	0.9972	80	5.8	10.8	15.0	0.38	1.25
10	Danofloxacin	0.9899	74	15.0	9.2	9.7	1.11	3.66
11	Dicloxacillin	0.9952	79	14.2	14.8	12.9	1.44	4.75
12	Difloxacin	0.9932	102	16.8	13.6	8.2	1.57	5.18
13	Doxycycline	0.9954	69	11.5	10.7	7.9	4.81	15.87
14	Enrofloxacin	0.9988	88	16.8	10.1	8.5	1.12	3.70
15	Florfenicol	0.9897	94	11.9	6.8	5.5	0.69	2.28
16	Flumequine	0.9945	107	7.1	4.5	6.4	0.08	0.26
17	Marbofloxacin	0.9988	93	11.2	18.6	12.8	0.58	1.91
18	Nafcillin	0.9985	73	5.8	6.0	5.5	0.68	2.24
19	Nalidixic acid	0.9967	112	11.9	8.4	4.1	0.12	0.40
20	Norfloxacin	0.9858	66	13.8	10.5	8.6	3.51	11.58
21	Oxacillin	0.9977	76	11.2	11.4	6.2	0.19	0.63
22	Oxolinic acid	0.9944	108	14.9	9.3	4.8	0.07	0.23
23	Oxytetracycline	0.9931	54	12.2	8.9	3.3	0.17	0.56
24	Penicilline G	0.9959	65	11.3	14.2	7.5	0.61	2.01
25	Sarafloxacin	0.9974	78	12.2	10.3	8.3	2.41	7.95
26	Spyramicin	0.9922	90	17.9	8.9	11.1	0.78	2.57
27	Sulfachinoxalin	0.9877	60	10.1	11.8	12.4	0.04	0.13
28	Sulfachloropyridazine	0.9975	70	4.8	14.1	5.8	0.58	1.91
29	Sulfadiazin	0.9914	72	10.1	9.8	8.1	0.24	0.79
30	Sulfadimethoxine	0.9929	56	5.8	14.5	3.8	0.03	0.10
31	Sulfamerazine	0.9900	69	7.8	6.1	3.7	0.19	0.63
32	Sulfamethazine	0.9985	88	9.8	11.7	6.3	0.08	0.26
33	Sulfamethoxazole	0.9958	74	7.2	14.9	9.5	0.07	0.23
34	Sulfathiazole	0.9899	101	2.9	8.3	6.9	0.11	0.36
35	Tetracycline	0.9921	45	8.4	11.6	7.4	0.42	1.39
36	Tilmicosin	0.9982	44	6.1	9.1	7.8	0.32	1.06

| 37 | Trimethoprim | 0.9991 | 83 | 5.7 | 7.9 | 5.9 | 0.85 | 2.81 |
| 38 | Tylosin | 0.9988 | 86 | 3.8 | 12.8 | 12.7 | 0.89 | 2.94 |

Discussion

Antibiotics are normally used for therapeutic, prophylactic and growth-promoting purposes. Antibiotic residues may have direct toxic effects on consumers, e.g., allergic reactions in sensitive individuals, or may indirectly cause the growth of antibiotic-resistant bacteria in humans.

Çetinkaya et al. [8] analyzed chicken meat samples available in Bursa (Turkey) for the antibiotics of class tetracycline (Oxytetracycline, Chlortetracycline, Doxycycline and Tetracycline) using LC-MS/MS technique. Doxycycline was found in four of the 60 samples in the range of 19.9 to 35.6 µg/kg. Tetracycline was detected in only one sample (17.2 µg/kg). Chlortetracycline and Oxytetracycline were not detected in any of the samples tested.

Er et al. [9] randomly collected 127 chicken meat samples from markets of Ankara (Turkey) and determined quinolones using ELISA technique. Of the 127 chicken meat samples tested 58 samples (45.7%) were positive for quinolones. The mean levels of quinolones were found to be 30.81 ± 0.45 µg/kg in chicken meat samples.

Cheong et al. [10] analyzed four common Sulfonamides (SAs), Sulfadiazine, Sulfamethazine, Sulfamethoxazole and Sulfaquinoxaline in chicken breast and liver samples using reverse phase HPLC equipped with UV detector at 266 nm. The concentration of SAs detected in samples from 11 states in Peninsular Malaysia ranged from 0.004 to 0.152 µg/g in liver samples. Except for the sample from Johor, concentrations of SAs in all the samples were lower than MRLs established by Malaysia (0.1 µg/g).

In Korea, Kim et al. [11] analyzed a total of 65 chicken meat samples purchased from local Korean markets. No residues of narasin or lincomycin were detected in any of the samples.

Lopez et al. [12] obtained 11 chicken meat samples from local supermarkets (Almeria, Spain) and analyzed them for Tylosin, Sulfadiazin, and Trimethoprim by LC MS/MS. No residues of antibiotics were detected in any of the samples.

Al-Ghamdi et al. [13] screened 110 raw chicken liver samples for oxytetracycline (OXT), tetracycline (TET), chlortetracycline (CHT) and doxycycline (DXC) residues using microbiological methods. OXT, TET, CHT and DXC were detectable in 77.3%, 46.4%, 53.6%, and 33.6% of the samples, respectively.

Nizamlıoğlu and Aydın [14] examined 50 chicken liver samples for quinolone antibiotics in Konya. The samples were analyzed by an enzyme-linked immunosorbent assay (ELISA) screening method. Of the 50 chicken liver samples analyzed for residues of quinolone, 17 (34%) were positive and in one of them the value (147.88 µg/kg) was above the maximum residue limits (MRLs).

Shareef et al. [2] purchased 25 chicken livers from different markets in Mosul, Iraq. Samples were analyzed for gentamycin, neomycin, sulfadiazine and oxytetracycline by TLC. From 25 liver samples tested, seven (28%) were positive for oxytetracycline and sulfadiazine. No neomycin or gentamycin residues were detected on TLC plates in all samples tested.

Although display methods are widely used in antibiotic residue analyses the results can be false positive or negative at a high ratio. Therefore for exact determination samples should be confirmed by chromatographic methods such as LC-MS/MS.

Due to hazard posed on human health, European Union and Turkey banned antibiotics that to be used for long time as growth promoters. Antibiotics should only be used for treatment of animal diseases.

In animal production audits should be performed efficiently in order to prevent uncontrolled and unconscious usage of antibiotics. Also the meat and interior organs should be analysed by accurate and confirming methods such as LC-MS/MS from the aspect of antibiotic residue after slaughtering.

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