J. Fac. Pharm. Ankara / Ankara Ecz. Fak. Derg., 40(3): 29-40, 2016
Doi: 10.1501/Eczfak_0000000586

ORIGINAL ARTICLE / ÖZGÜN MAKALE

DEVELOPMENT AND VALIDATION OF A LC-ESI-MS METHOD FOR DETECTION OF PIPERIDIN-1-IUM {[5-(2-FURYL)-4-PHENYL-4*H*-1,2,4-TRIAZOL-3-YL] THIO}ACETATE RESIDUES IN POULTRY EGGS

KÜMES HAYVANLARI YUMURTALARINDAKİ PİPERİDİN-1-YUM {[5-(2-FURİL)-4-FENİL-4*H*-1,2,4-TRİAZOL-3-İL]TİYO}ASETAT ARTIKLARININ BELİRLENMESİ İÇİN BİR LC-ESI-MS YÖNTEMİNİN GELİŞTİRİLMESİ VE VALİDASYONU

Borys VARYNSKYI[1*], Vladymir PARCHENKO[2], Andriy KAPLAUSHENKO[1], Olexandr PANASENKO[2], Yeugeniy KNYSH[3]

[1]Zaporozhye State Medical University, Pharmaceutical Faculty, Physical and Colloidal Chemistry Department, 69035 Zaporozhye, Ukraine
[2]Zaporozhye State Medical University, Pharmaceutical Faculty, Toxicological and Inorganic Chemistry Department, 69035 Zaporozhye, Ukraine
[3]Zaporozhye State Medical University, Pharmaceutical Faculty, Department of Management and Pharmacy Economics, 69035 Zaporozhye, Ukraine

SUMMARY

A simple, rapid and reproducible isocratic reversed-phase LC-ESI-MS method has been elaborated for determination of the piperidin-1-ium {[5-(2-furyl)-4-phenyl-4H-1,2,4-triazol-3-yl] thio}acetate, active pharmaceutical ingredient (API) of the antiviral veterinary drug "Tryfuzol" in the poultry eggs. The samples were extracted by dimethyl sulfoxide at ultrasonic bath, centrifuged at 15000 g, filtrated through ultrafilter and injected into HPLC system. A mobile phase was used 0.1% (v/v) formic acid in water (A) and acetonitrile containing 0.1% (v/v) formic acid (B) at isocratic elution. The composition of the mobile phase was A/B (70%:30%, v/v) at flow rate of 0.4 ml min-1. Total running time was 5 minutes. Mass spectrometric conditions were SIM mode (m/z 302.1), positive polarity. The mean concentration were 0.2887-7.204% of the nominal values for the QC samples. The within-run CV values were 1.564-5.636% for the QC samples. The method is applicable for determination of the residual quantities of API of the "Tryfuzol" in the poultry eggs.

Keywords: *ESI mass spectrometry; high pressure liquid chromatography; poultry eggs; triazoles; tryfuzol*

ÖZET

Kümes hayvanları yumurtalarında bir antiviral veteriner ilaç Trifuzol (piperidin-1-ium {[5-(2-furil)-4-fenil-4H-1,2,4-triazol-3-il] tio}asetat)'un tayini için basit, hızlı ve tekrarlanabilir bir izokratik ters - faz LC-

* **Corresponding Author / Sorumlu Yazar:** Borys VARYNSKYI
e-mail: varinsky@zsmu.zp.ua

Submitted/Gönderilme: 21.02.2017 **Accepted/Kabul:** 07.04.2017

ESI-MS yöntemi tasarlanmıştır. Numuneler ultrasonik banyoda dimetil sülfoksit ile ekstre edilmiş, 15.000 g'da santrifüjlenmiş, ultrafiltre ile süzülmüş ve HPLC sistemine enjekte edilmiştir. Suda %0,1 (h/h) formik asit (A) ve asetonitrilde %0,1 (h/h) formik asit (B) içeren mobil faz ile izokratik elüsyon yapılmıştır. Mobil fazın bileşimi, 0,4 mL. dak^{-1} akış hızında, A/B (%70: %30, h/h)dır. Toplam çalışma süresi 5 dakikadır. Kütle spektrometrik koşullar, SIM modu (m/z 302,1), pozitif polaritedir. Doğruluk %0,2887-7,204 dır. Kesinlik, %1,564-5,636 dır. Yöntem, kümes hayvanları yumurtalarındaki Trifuzol kalıntısı kantitatif tayininde kullanılabilir.

Anahtar Kelimeler: *kümes hayvanları yumurtası; kütle spektrometrisi; triazoller; trifuzol; yüksek basınçlı sıvı kromatografisi*

INTRODUCTION

1,2,4-triazole derivatives display different types of biological activity. At the present time there are developed drugs and veterinary medicines which contain 1,2,4-triazole derivatives. These derivatives are active pharmaceutical ingredients (APIs). The piperidin-1-ium {[5-(2-furyl)-4-phenyl-4*H*-1,2,4-triazol-3-yl] thio}acetate is the active pharmaceutical ingredient of the antiviral veterinary drug of a new generation that has hepatoprotective, cardioprotective, antioxidative, immunomodulating, interferonogenic, anti-inflammatory, detoxicative, wound-healing effect. It is designed for all types of productive and unproductive animals and birds. It is effective against corona-, rota-, reo-, paramyxo-, picorna-, parvo-, circo-, herpeso- and other viruses. The drug is used for preventive care and treatment of various diseases, such as an independent drug, and in the treatment with other drugs, such as antibiotics or vitamins.

A control of residual amounts of veterinary drugs' API is a rule for maintaining food safety and quality for aviculture and animal husbandry.

An article devoted to determination of this API in poultry meat was published before [1].

The presence of the proteins and lipids in eggs is cause of HPLC determination difficulties. Column efficiency may be decreased at its presence in the samples. The HPLC systems pressure dramatically increases due to protein clogging. Exist few ways to avoid it: deproteinization, liquid and solid phase extraction, dialysis.

There are a lot of articles which studied determination of different APIs in the poultry eggs.

Different classes of veterinary drugs may be occurred in the poultry eggs. The nitrofurans, fluoroquinolones, tetracyclines, sulfanilamides can be detected.

Draiscia *et al.* [2] determined nitrofuran residues, nitrofurazone, furazolidone and furaltadone in chicken eggs by the isocratic reverse phase high-performance liquid chromatography UV–Vis photodiode-array detection (HPLC–DAD) method at 362 nm. Identification of the substances was performed by high-performance liquid chromatography–mass spectrometry (HPLC–MS) using an atmospheric pressure ionization source and an ion spray interface. The minimal weight of added

substances was 5 μg kg^{-1} in a model mixture. They were extracted from eggs by acetonitrile and purified by liquid–liquid extraction. Finzi *et al.* [3] proposed RP LC–MS–MS analysis method of the metabolites of four commonly used nitrofuran drugs, furazolidone, furaltadone, nitrofurazone and nitrofurantoin in the poultry muscle and eggs. The multiple reactions monitoring (MRM) was used for confirmation and quantification. Quantification Limits were 0.5 ng g^{-1} and the total analysis time was 5 min. Minimal final concentration of calibration standard was 0.3 μg kg^{-1}.

An article about determination of the enrofloxacin (EFX) and ciprofloxacin (CFX) by HPLC in the eggs of laying hens was published by Gorla *et al.* [4]. Withdrawal treatment periods in hens are six days for EFX and five days for CFX to avoid violative levels of egg residues. During oral administration, minimal quantity of EFX 0.54 μg/g and CFX 0.14 μg/g and maximal in post-oral administration EFX 1.98 μg/g and CFX 0.28 μg/g were found. The ciprofloxacin, enrofloxacin and sarafloxacin (SFX) was determined by Chu *et al.* [5] in the egg yolk and egg albumen of laying hens by HPLC. Sample prepared by acidification of with 1 M phosphoric acid followed by deproteinization with acetonitrile. CFX, EFX and SFX were determined in eggs treated by drugs. Total concentrations for CFX, EFX and SFX were 0.229, 0.160, 0.211 μg/g respectively. Christodoulou *et al.* [6] presented two different RP diode array HPLC methods at 275 and 255 nm were for the determination of 10 quinolones (enoxacin, ofloxacin, norfloxacin, ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin, oxolinic acid, nalidixic acid and flumequine) in chicken muscle and egg yolk. 0.1% TFA in CH_3OH was used as extragent. The LODs for egg yolk was 8.0 g/kg. Ten of egg yolks were studied. There were not of quinolones residues found.

HPLC with ion-pairing chromatography and diode array detection at 355 nm proposed by Ruick *et al.* [7] for determination tetracycline antibiotics in eggs and broiler meat. The within-day precision ranged from 8.0 to 11.8% for oxytetracycline (OTC) in eggs. The limit of detection and the limit of quantitation for oxytetracycline in eggs were 2.2 ng/g. OTC was found in eggs during and after oral administration of 840 mg/kg feed. Minimal and maximal concentrations of OTC were 0.026 μg/g and 0.21 μg/g respectively.

The aim of the study was to develop a method based on HPLC-MS and to conduct rigorous tests of validation these methods under well-known requirements of European Medicines Agency [8]. This method can be applied for control of the residual amount of the API piperidine 2-{[5-(2-furyl)-4-phenyl-1,2,4-triazole-3-yl] thio}acetate in poultry eggs.

MATERIALS AND METHODS

Substances and Reagents

The substance piperidin-1-ium {[5-(2-furyl)-4-phenyl-4*H*-1,2,4-triazol-3-yl] thio}acetate corresponds to the chemical formula of "Tryfuzol" drug's active pharmaceutical ingredient (*TFZ API*). The molecular structure of the analyte is shown at Fig. 1. The substance was obtained from Toxicological and Inorganic Chemistry Department of Zaporozhye State Medical University (Professor Panasenko O.I. is a head of the department). Parchenko confirmed structure by ^{1}H-NMR, LC-MS [9].

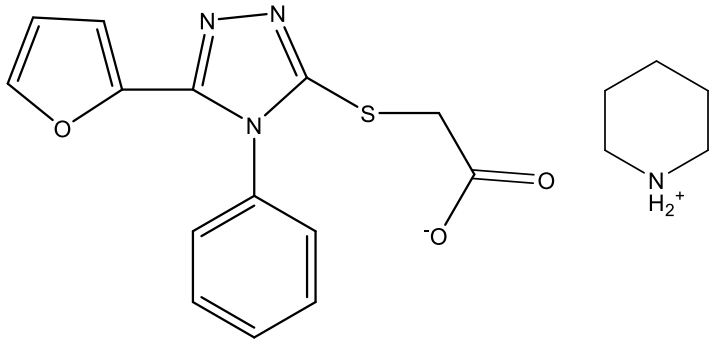

Figure 1. *TFZ API* structure

Acetonitrile graded for HPLC, Lab-Scan (Gliwice, Poland). Ultra-high pure water (18 MΩ at 25 °C) was obtained by using the system for water Direct Q 3UV Millipore (Molsheim, France). Formic acid (100%), Merck KGaA (Darmstadt, Germany).

Instruments

DMD HPLC-MS system: Agilent 1260 Infinity (degasser, binary pump, autosampler, single quadrupole mass spectrometry detector Agilent 6120 ionization in electrospray); software package OpenLAB CDS.

Chromatography conditions

Analytical column Agilent ZORBAX SB-C18 (30 mm x 4.6 mm; 1.8 μm, Agilent Corporation) with the corresponding guard column was used. The column temperature was 40°C, the injection volume was 1 mL. The mobile phase rate equals 0.4 mL min^{-1} in isocratic conditions. The composition of mobile phase is: water (formic acid 0.1%, v)/acetonitrile (formic acid 0.1%, v) (70:30, v/v). Total time was 5 min.

Mass spectrometry conditions for the TFZ API quantitative analyzes

Mass spectrometric conditions were optimized for maximum response. Selective ion monitoring mode (SIM) with m/z 302.1 (*TFZ API*); positive polarity; the rate of drying gas (nitrogen) is 10 L/min; capillary voltage at 4000 V; drying gas temperature was 247 ^{o}C; the fragmentor voltage was 149 B; nebulizer pressure was 46 psi.

Preparation of standard solutions, calibration solutions and quality control solutions (QC samples)

Standard stock solution of *TFZ API (*0.1 mg mL^{-1}) (solution A) was prepared in 100 mL capacity measuring flask by dissolution of 0.01000 g of this substance in water and bringing the solution to line.

The final standard solution of *TFZ API* (0.01 mg mL^{-1}) (solution B) was prepared by transfer of 10.00 mL of solution A in 100 mL capacity measuring flask and bringing the solution to line with water.

The model mix was made by preparing eggs homogenate with mixer.

Seven model solutions for testing the linearity were made. The egg homogenates mass of 0.2000 g were spiked with variable micropipets by 0 µL, 10 µL, 20 µL, 40 µL, 50 µL, 80 µL, 100 µL of solution B and weighed. Then to these mix solutions were added 100 µL, 90 µL, 80 µL, 60 µL, 50 µL, 20 µL, 0 µL of water accordingly. Afterwards, acted in accordance to the procedure in the scheme starting from paragraph 3 (Fig. 2).

Twenty model solutions videlicet five solutions of four levels of concentration to check precision and accuracy were made. Various amounts of the solution were added to the 200 mg egg homogenate, namely at LLOQ was 10 µL of solution B. Within three times LLOQ (low QC (quality control) sample) was 20 µL of solution B. About 50% of the calibration curve range (medium QC) was 50 µL and not less than 75% of the calibration curve upper range (high QC) was 80 µL of solution B, and weighed. Then, added 90, 80, 50 and 20 µL of water. Then, acted according to the procedure in the scheme, starting from the paragraph 3 (Fig. 2).

Non-extracted solutions of the *TFZ API* at four levels of concentration were prepared. This was gained by mixing 10 µL, 20 µL, 50 µL and 80 µL of solution B and 90 µL, 80 µL, 50 µL and 20 µL of water respectively and 1mL of dimethyl sulfoxide for testing of the recovery.

All standard solutions were stored at 5^{o}C. They were stable during the validation experiment.

Sample Liquid Extraction

Eggs samples were homogenized with using a mixer. Hundred µL of water was added to the 0.2000 grams (exact weights) of homogenized eggs. It was sonicated for 15 minutes at ultrasonic bath. Then, 1.00 mL of dimethyl sulfoxide was added. It was sonicated for 15 minutes at the ultrasonic bath, centrifuged 15000 g for 10 minutes and filtered through a nylon syringe filter (13 mm internal diameter, pore size 0.2 µm). Samples preparation procedure is shown at the diagram (Fig. 2)

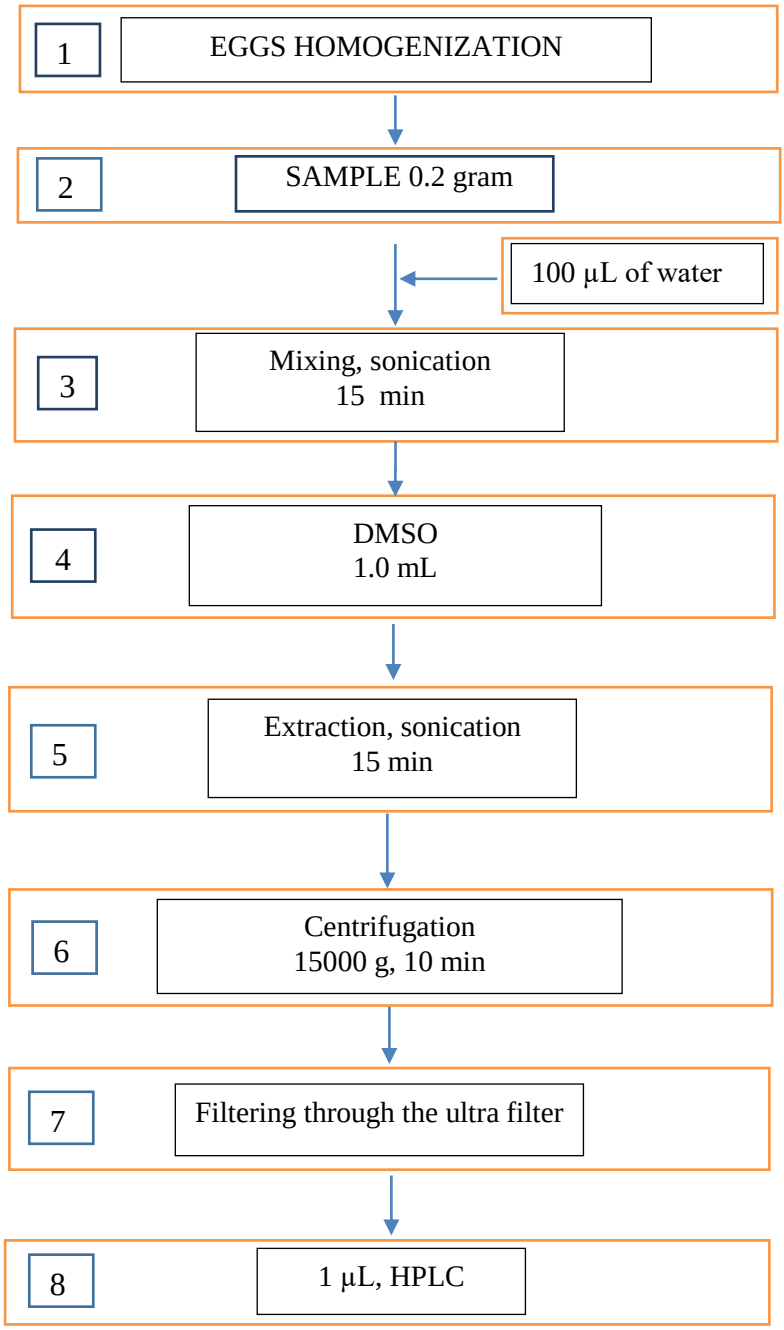

Figure 2. Procedure of sample preparation

The method validation

The selectivity was confirmed by analysis of blank samples without analytes to determine the absence of interference with analyte.

The lower limit of quantification (LLOQ) was determined by the model mix, which provides 5-fold signal-noise ratio. RSD should not exceed 20%. The calculated value should be within 20% of the actual analyte amount value, which was introduced in the model mix.

Lower calibration standard is set by lower limit of quantification (LLOQ) [8].

Precision and accuracy was defined by the quality control solutions study, which were made according to the *Preparation of standard solutions, calibration solutions and quality control solutions (QC samples)***.** The recovery was determined by comparing the extracted standard samples with four levels of concentration for samples that have been made without extraction step.

Application of analytical method for real egg samples

One mL of 1% solution of drug was added to the one L of water. Final concentration of drug 10 mg/L. During the feeding one chicken receive of drug about 5 mg/kg. Residual amounts determination of drug substance was conducted according to the procedure (Figure 2) comparing with calibration homogenate samples. The eggs have taken at random from different chickens.

Concentration calculation

It was held by the calibration curve equation, which should be checked every time in the terms of research.

RESULTS AND DISCUSSION

Sensitivity described by a lower limit of quantification (LLOQ). LLOQ was 0.12 μg in the weighed sample or 0.6 mg/g of homogenate.

TFZ API peak identified at the SIM 302 chromatogram the model mixture at LLOQ and had a retention time 3.4-3.5. Selectivity was confirmed by absence of interference with impurities (Figure 3). Total time of chromatography was five minutes.

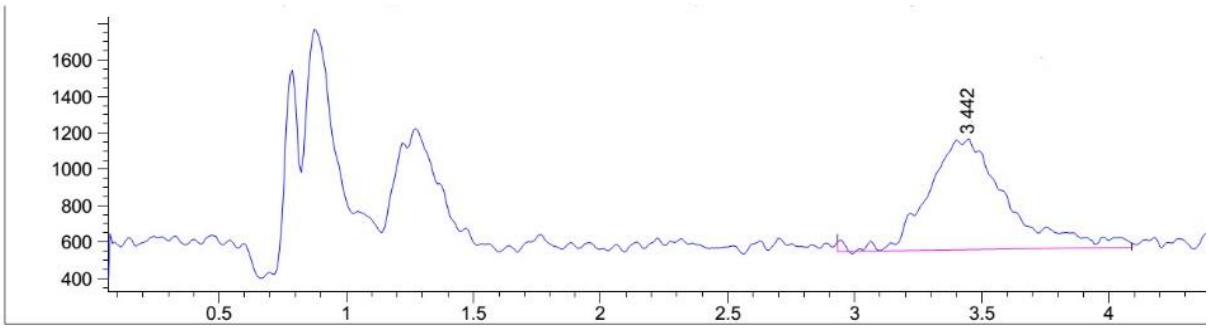

Figure 3. Homogenate extract chromatogram spiked with TFZ *API* at LLOQ (lower limit of quantification) (*lowest calibration standard).*

Calibration curve was built on the basis of mass spectrometric detector response at m/z 302.1 depending on content of the substance at the homogenate. The calibration was performed by external standard. Calibration curve was linear within 0.12-1.07 µg in the weighed sample homogenate or 0.61-3.6 µg/g of homogenate. Linearity curve was described by the equation:

$y = 93332x + 3205.4$; $R^2 = 0.9988$, $R = 0.9994$.

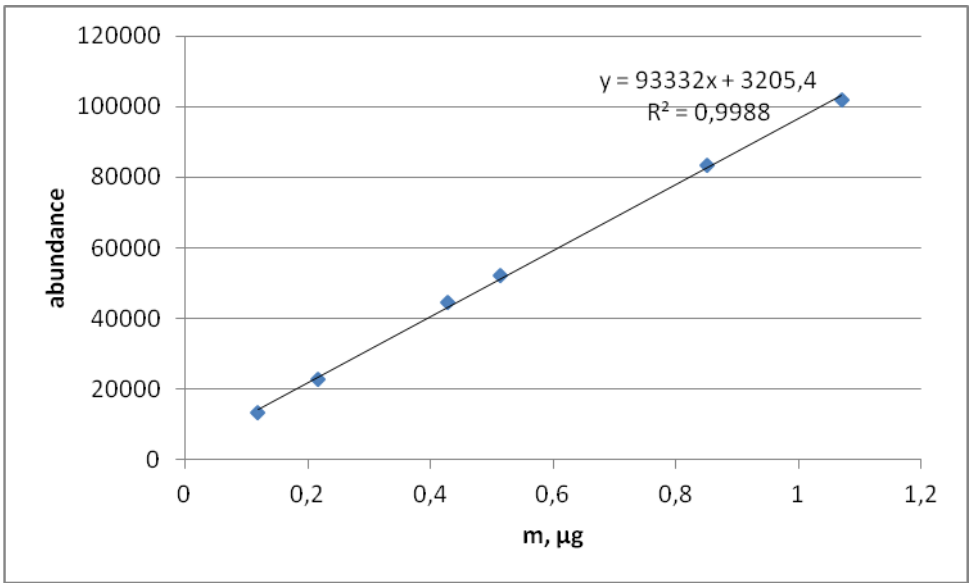

Figure 4. The calibration curve of TFZ *API* in the sample of egg homogenate

TFZ API content in the QC samples was determined using the calibration curve equation. Accuracy and precision were determined for the quality control solutions (QC) of the API. The chromatograms of QC solutions at LLOQ and high (at least at 75% of the upper calibration curve range) concentrations are shown at Fig. 3, 5.

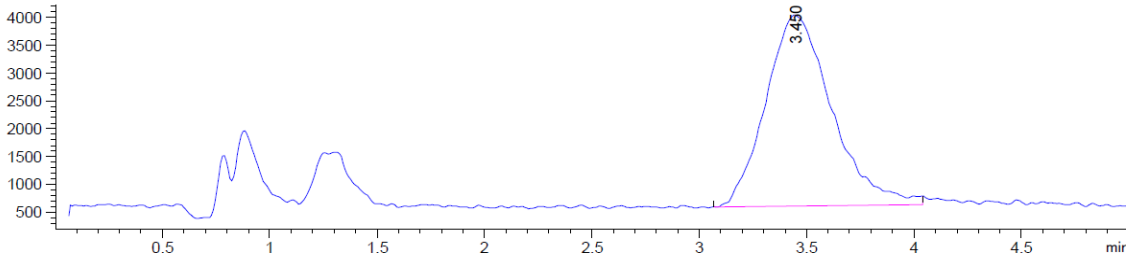

Figure 5. The high QC solution chromatogram of the egg homogenate spiked with TFZ API.

Accuracy and precision data are given in the Table 2.

Table 1. Accuracy and precision in the determining of *TFZ API* (n = 5) at 4 concentration levels

Levels	$\bar{x}$, µg Injected into the weighed sample	$\bar{x}$±SD, µg found	Precision RSD (%)	Accuracy RE* (%)	Recovery (%)
1	$1.095x10^{-1}$	$1.146x10^{-1} \pm 5.505x10^{-2}$	4.804	4.585	96.13
2	$2.112x10^{-1}$	$1.96x10^{-1} \pm 3.242x10^{-2}$	1.654	7.204	80.57
3	$5.312x10^{-1}$	$5.127x10^{-1} \pm 8.020x10^{-2}$	1.564	3.493	82.94
4	$8.456x10^{-1}$	$8.43x10^{-1} \pm 4.753x10^{-2}$	5.638	0.2887	97.52

*Accuracy was determined by the calculated values' deviations from nominal values of QC samples as a percentage to nominal values (relative error).

The recovery was determined for the quality control solutions. The recovery values are shown in the Table 1.

The method was applied for the determination of residues of TFZ API in egg homogenate samples after chicken feeding by drug. This method was reproducible, accurate and sensitive, and can be used for the determination of the residual amounts of the *TFZ API*. Every day at least six eggs were analyzed.

Table 2. *TFZ API* results determination in the eggs.

Day	Measured weight (mean) m, µg	Measured concentration (mean) C, µg /g
1	0.6872	3.227
2	0.3648	1.679
3	-	-

Content calculations performed using calibration curve equation. Results of content in µg and µg/g in Table 2.

ESI-MS conditions, such as drying gas temperature, the fragmentor voltage, nebulizer pressure were proposed earlier [10]. A protonated acidic form of analyte is eluted and detected. So, the value of m/z SIM mode was equal 302.1, which coincides to the molecular weight of protonated molecule of corresponding acid.

LC behavior of *TFZ API* was shown previously [11]. Addition of acid to the eluent need for formation of the positive ions for ESI ion source and for the suppression of silanole groups of stationary phase to avoid with triazole nitrogen interaction. Formation of acidic form also increases retention time. The formic acid was chosen as acidic agent due to volatility and compatibility with ESI-MS detector. The dependence of capacity factor *k* from concentration of the acetonitrile was studied in our previous article. The acetonitrile content 30% for *TFZ API* corresponds to the capacity factor 2.6 and run-time 5 min, which is satisfactory for separation from impurities.

Dimethyl sulfoxide was used as extraction and deproteinization agent in sample preparation.

Validation results meet the requirements European Medicines Agency [8]. Signal/noise ratio for QC sample was five times at LLOQ (must be ≥5).

Identification and selectivity was acceptable because detection was performed in the SIM mode at m/z = 302.1 and it corresponded to the value of specific monoizotopic mass of quazimolecular ion received by joining proton to corresponding acid of *TFZ API*.

Precision, accuracy was satisfied according to the requirements [8]. The RSD value was not exceeded 15% for the QC samples. The mean concentration was within 15% of the nominal values for the QC samples.

Application of the method to the real samples show that the TFZ API was not detected at third day after finishing of the feeding by drug (Table. 2).

Conclusions

1. For the first time the method, which allows to control the residual amount of the *TFZ API* in the poultry eggs, was developed.
2. We have held the thorough research on the validation of this method. It was proven that the method is suitable for use, basing on the checking of validation, precision, selectivity and sensitivity results.
3. Method is available for research as well as control laboratories. It can be used in the toxicological research and also for the veterinary inspections.

ACKNOWLEDGEMENTS

We acknowledge complete support of the Zaporozhye State Medical University. We thank rector of Zaporozhye State Medical University Prof. Yu. M. Kolesnyk for his help and support.

There are no conflicts of interest have been declared.

REFERENCES

1. Varynskyi, B.O., Knysh, Ye.G., Parchenko, V.V., Panasenko, O.I., Kaplaushenko, A.G. (2015). Quantitative analysis of piperidin-1-ium {[5-(2-furyl)-4-phenyl-4*H*-1,2,4-triazol-3-yl]thio}acetate, substance of the veterinary drug «Tryfuzol», in poultry meat by LC–DAD–MS, Current issues of pharmaceutical and medical science and practice, 2, 25-31.
2. Draiscia, R., Giannettia, L., Lucentinia, L., Palleschia, L., Brambilla, G., Serpe, L., Gallo, P. (1997). Determination of nitrofuran residues in avian eggs by liquid chromatography–UV photodiode array detection and confirmation by liquid chromatography–ion spray mass spectrometry, Journal of Chromatography A, 777, 201–211.
3. Finzi, J.K., Donato, J.L., Sucupira, M., Nucci, G.De. (2005). Determination of nitrofuran metabolites in poultry muscle and eggs by liquid chromatography-tandem mass spectrometry, Journal of Chromatography B, 824, 30–35.
4. Gorla, N., Chiostri, E., Ugnia, L., Weyers, A, Giacomelli, N., Davicino, R., Ovando, H.G. (1997). HPLC residues of enrofloxacin and ciprofloxacin in eggs of laying hens, International Journal of Antimicrobial Agents, 8, 253–256.
5. Chu, P.S., Wang, R.Ch., Chu, H.V. (2002). Liquid Chromatographic Determination of Fluoroquinolones in Egg Albumen and Egg Yolk of Laying Hens Using Fluorometric Detection, Journal of Agricultural and Food Chemistry, 50, 4452-4455.
6. Christodoulou, E.A., Samanidou, V.F., Papadoyannis, I.N. (2007). Validation of an HPLC-UV method according to the European Union Decision 2002/657/EC for the simultaneous determination of 10 quinolones in chicken muscle and egg yolk, Journal of Chromatography B, 859, 246–255.
7. Ruyck, H.De., Ridder, H.De., Renterghem, R.Van, Wambeke F.Van. (1999). Validation of HPLC method of analysis of tetracycline residues in eggs and broiler meat and its application to a feeding trial, Food Additives & Contaminants, 16, 47-56.
8. European Medicines Agency (2011). Guideline on bioanalytical method validation. http://www.ema.europa.eu/ema/index.jsp?curl=pages/includes/document/document_detail.jsp?webContentId=WC500109686&murl=menus/document_library/document_library.jsp&mid=WC0b01ac058009a3dc.

9. Parchenko, V. (2006). Synthesis, physicochemical and biological properties of derivatives of 1,2,4-triazoles-3-thione containing core furan, Candidate of PharmSci thesis, Zaporozhye State Medical University, Zaporozhye, 208 p.
10. Varynskyi, B.O. (2015). Optimization of the detection conditions 1,2,4-triazol-3-yl-thioacetate acids series and their salts by HPLC-ESI-MS, The Odessa Medical Journal, 150, 17-21.
11. Varynskyi, B.O., Knysh, Ye.G., Parchenko, V.V., Panasenko, O.I., Kaplaushenko, A.G. (2015). The study of retention regularities for the potential drug substances of 1,2,4-triazol-3-ylthioacetic acids and their salts series by the method of HPLC/DAD-MS, Journal of Organic and Pharmaceutical Chemistry, 52, 68-75.