ISSN 2706-6819 Vol.3, Issue 3: 40-46, DOI: 10.25236/IJFM.2021.030308

Study on Complete Antigen Synthesis of Chlorpromazine

Jiayan Zheng[1,2,a], Xiaoli Han[1,2,b], Yiqing Li[1,2,c], Tingxin Wang[1,2,d*]

[1]College of Quality and Technical Supervision, Hebei University, Baoding, China
[2]Institute of Food Safety and Standardization, Hebei University, Baoding, China
[a]574166857@qq.com, [b]1445706593@qq.com, [c]535789837@qq.com, [d]tingxinwang@126.com
**Corresponding Author*

Abstract: *Chlorpromazine (CPZ) is a small molecule, which cannot cause an immune response in the body and does not have active groups such as the amino carboxyl group. Therefore, the molecular structure of CPZ needs to be modified to introduce active groups, so that it can be conjugated with a carrier protein to form a complete antigen to induce an immune response and produce antibodies. In this paper, the molecular structure of chlorpromazine is modified by the chemical synthesis method. The diazotization of p-aminobenzoic acid into diazo salt by sodium nitrite is reacted with chlorpromazine. The aromatic side ring containing the carboxyl group is introduced into the counterposition of chlorpromazine. Artificial complete antigen CPZ-BSA is synthesized by mixed anhydride method catalyzed by triethylamine and isobutyl chloroformate and bovine serum albumin (BSA). The complete antigen is identified by ultraviolet spectroscopy. Results The characteristic absorption peaks of the synthetic complete antigen are not in the same position as those of chlorpromazine and bovine serum albumin, and the conjugation of chlorpromazine and bovine serum albumin is confirmed to be successful. The complete antigen of chlorpromazine is synthesized, which lays a foundation for the further determination of chlorpromazine residues, the establishment of immunological detection methods and the preparation of antibodies.*

Keywords: *Chlorpromazine, Mixed anhydride method, Complete antigen, Ultraviolet spectroscopic identification*

1. Introduction

Chlorpromazine is a phenothiazine antipsychotic, a central dopamine receptor blocker, mainly used to enhance hypnosis, anesthesia, sedation, etc[1,2]. The addition of chlorpromazine in animal feed can reduce the activity of livestock and poultry and make animals gain weight. At the same time, a large dose of chlorpromazine can be injected in the process of animal transportation to reduce the weightlessness and mortality of animals on the way[3,4]. In addition, there are also unreasonable situations in the breeding industry, such as increasing the dosage of chlorpromazine, prolonging the medication time of chlorpromazine, and not following the withdrawal period of chlorpromazine before slaughter [5]. This, therefore, inevitably leads to chlorpromazine residues in animal-based foods, and thus poses a potential hazard to public health. The elimination rate of chlorpromazine drugs in the human body is slow, and it is easy to produce drug residues in the body, especially the chlorpromazine sedative residues in food animals entering the human body through the food chain, which can cause adverse effects on the human endocrine, motor and circulatory

Systems as well as liver organs. It causes leukopenia, liver dysfunction, agranulocytosis, contact dermatitis and rash and other toxic and side effects, which bring great risks to human health[6,7]. In view of the potential harm to consumers, the European Union prohibits the use of chlorpromazines in any form in edible animals and cannot be detected in their tissues or products[8]. According to Announcement No. 235 of the Ministry of Agriculture of China, chlorpromazine tranquilizers are prohibited as feed additives and cannot be detected in usable tissues of animals[9]. Instrumental analysis methods are commonly used for the determination of chlorpromazine residues in animal-derived foods, while immunological detection methods are rarely used. However, compared with instrumental analysis methods, immunological detection methods have lower costs and shorter detection times, and are suitable for on-site detection of large quantities of samples. Complete antigen as the basis of the immunological detection method is very important. In order to enrich the

ISSN 2706-6819 Vol.3, Issue 3: 40-46, DOI: 10.25236/IJFM.2021.030308

immunological detection method for chlorpromazine residues in food, the complete antigen of chlorpromazine was successfully prepared, which laid a foundation for the establishment of relevant immunological detection methods later are shown in Figures 1.

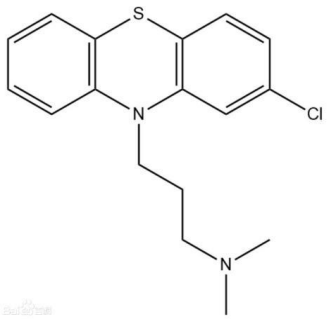

Figure 1: Chemical structure of chlorpromazine.

2. Manuscript Preparation

2.1. Materials and Instruments

2.1.1. The Instrument

Magnetic heating agitator (Jintan Zhongda Instrument Factory); High-speed centrifuge (TGL-10C, Shanghai Anting Scientific Instrument Factory); UV-Vis Spectrophotometer (Jingpu General Instrument Co., Ltd.)

2.1.2. Materials and Solutions

Chlorpromazine (purchased from Shanghai Yuanye Biotechnology Co., Ltd.); Bovine Serum Albumin (BSA, Roche)

0.6mg/mL Bovine Serum Albumin Solution: 12.0mg Bovine Serum Albumin was added to 0.02g/mL sodium tetraborate buffer and the solution was volume constant with ultrapure water to 20mL.

2.2. Methods

2.2.1. Molecular Modification of Chlorpromazine

Chlorpromazine was modified by diazotization and the carboxyl group was introduced. At low temperature, the pH value of 0.1mol/L p-aminobenzoic acid solution was adjusted to 1-2 with 6 mol/L HCl solution, and the magnetic stirring was performed for 10 min. The precooled 1 mL 0.1 mol/L sodium nitrite solutions was added to the p-aminobenzoic acid solution, and the color of the solution immediately changed from colorless to light yellow, and the reaction was carried out at 4℃ for 30minutes. 0.0355g of chlorpromazine was accurately weighed and dissolved in 1 mL of ultra-pure water, and 1.1mL of diazotized yellow solution was slowly added to the dissolved chlorpromazine solution under the condition of low-temperature stirring. The solution eventually turns dark red, and the centrifuge tube was placed in a thermostatic oscillator, where the oscillating reaction takes place overnight. After oscillation, the centrifugation was carried out at 6000 r/min for 6 min, and the lower precipitate was sterile and blown dry until it was dry reddish-brown are shown in Figures 2.

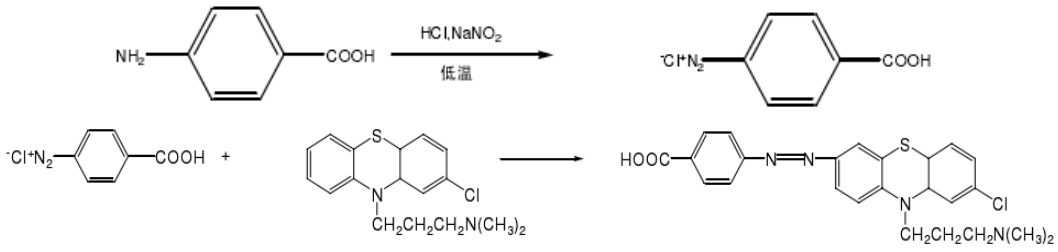

Figure 2: Hapen-modified pathways

ISSN 2706-6819 Vol.3, Issue 3: 40-46, DOI: 10.25236/IJFM.2021.030308

2.2.2. Complete Antigen Synthesis

1mL of 1, 4-dioxane and 1 mL of dimethylformamide were respectively taken to dissolve the reddish-brown precipitate, and the solution became clear after the precipitate was completely dissolved. After 10 min, add 27 μL triethylamine to the ice bath, react for 10min, then add 16μL isobutyl chloroformate, remove the ice bath, and oscillate for 30 min. 2mL 0.6mg/mL bovine serum albumin solution was added and reacted at 4℃ for 24h.

2.2.3. Complete Antigen Purification

The dialysis bag was boiled in 0.01mol/L $NaHCO_3$ solution under boiling state for 10min, then moved to 0.013mol/L EDTA solution under boiling state and continued to boil for 10min. After cleaning the dialysis bag with ultra-pure water 3 times, the synthesized complete antigen was put into the dialysis bag and dialysis was performed in PBS buffer solution. The dialysate was replaced every 8 h and dialysis was performed for 72h are shown in Figures 3.

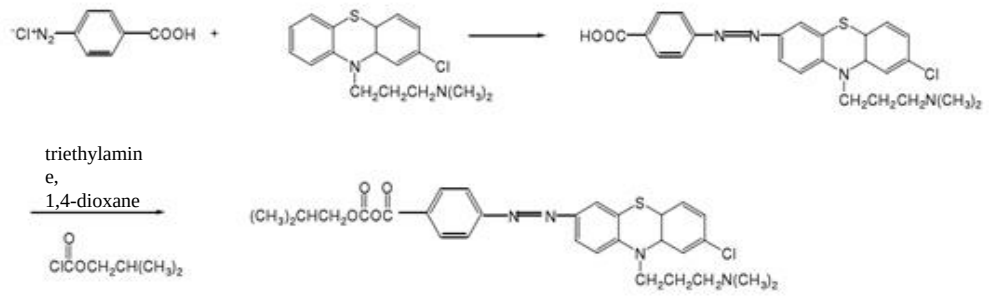

Figure 3: Complete antigen synthesis pathway

2.2.4. Complete Antigen Identification

UV scanning identification is the basic method to detect whether the artificial antigen is synthesized successfully. Different substances will have their characteristic absorption peaks. The maximum absorption peak of the synthesized complete antigen was measured by ultraviolet spectrophotometer and compared with the maximum absorption peak of carrier protein BSA and hapten chlorpromazine to observe the difference and evaluate whether the synthesized complete antigen was successful[10].

After dialysis, the complete antigen solution was centrifuged at 6000r/min for 6min, and the supernatant was taken. Complete antigen identification was carried out after dilution of certain times. Ultrapure water was calibrated at baseline, BSA and complete antigen solution were scanned at the wavelength of 200~400nm by UV, while methanol was calibrated at baseline, a chlorpromazine-methanol solution was scanned at the wavelength of 200~400nm by UV, and the waveform was recorded.

2.3. Results and Discussion

2.3.1. The Results

2.3.1.1. Molecular Modification Results of Chlorpromazine

The precooled 1mol/L sodium nitrite solution was slowly added to the dissolved p-aminobenzoic acid solution, and the color of the solution immediately turned pale yellow from colorless. The complete yellow solution was slowly added to the dissolved chlorpromazine solution under low-temperature agitation. The solution eventually turned a deep red. The red solution was oscillated overnight and centrifuged in a high-speed centrifuge to produce reddish-brown precipitation.

2.3.1.2. Complete Antigen Synthesis Results

After 1, 4-dioxane, N, N-dimethylformamide was used to dissolve the reddish-brown precipitate, the precipitate became red clear after being completely dissolved. The complete antigen of chlorpromazine reacted overnight with triethylamine and isobutyl chloroformate at low temperatures.

2.3.1.3. Complete Antigen Identification Results

The diluted complete antigen is scanned by an ultraviolet spectrophotometer. The identification results are shown in Figures 4, 5, 6 and 7.

ISSN 2706-6819 Vol.3, Issue 3: 40-46, DOI: 10.25236/IJFM.2021.030308

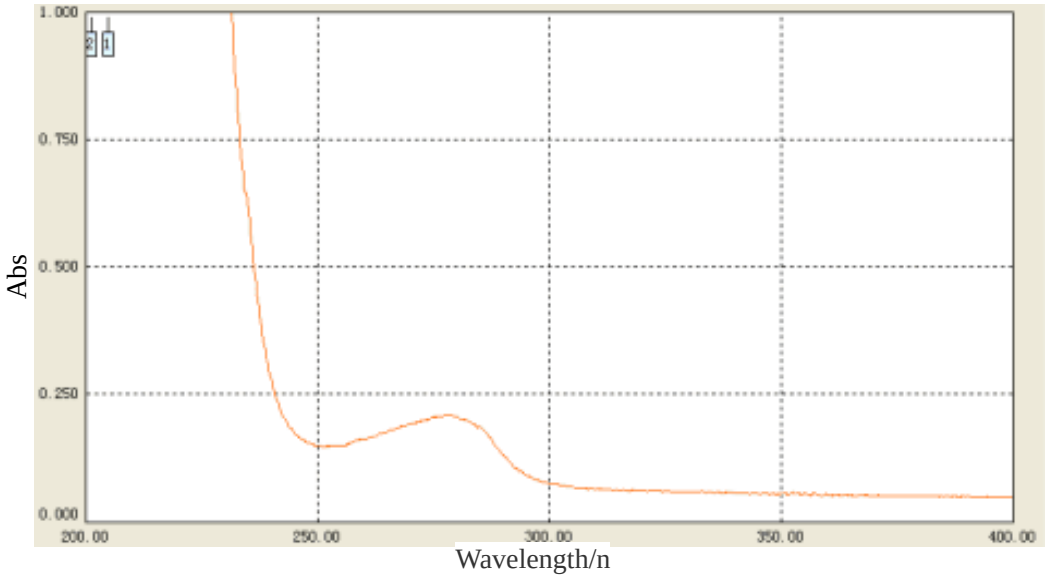

Figure 4: UV absorption spectra of bovine serum albumin

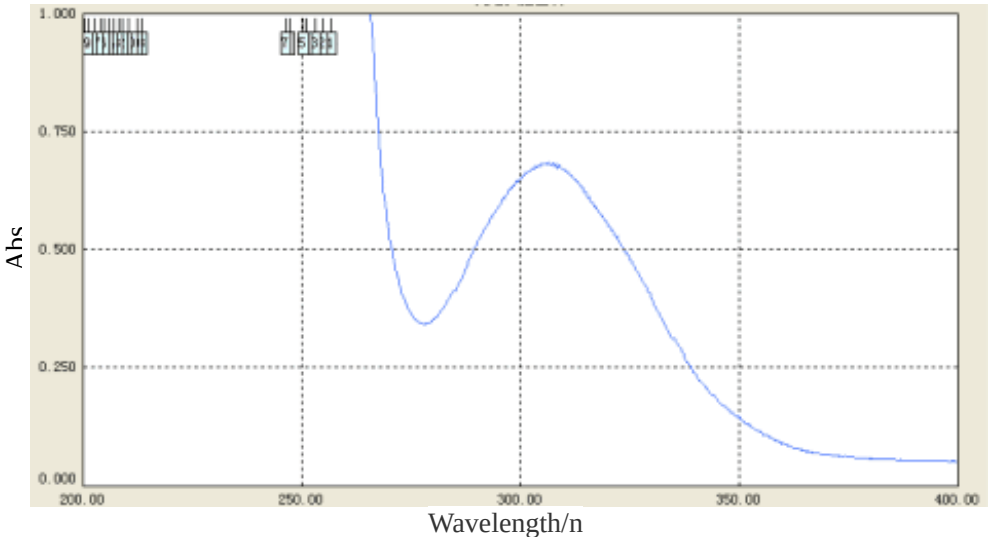

Figure 5: Ultraviolet absorption spectra of chlorpromazine

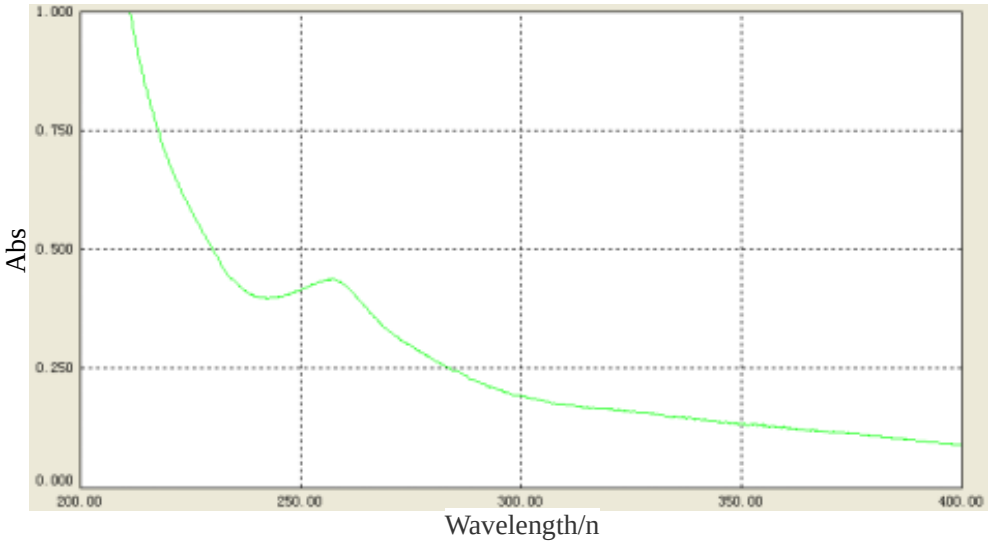

Figure 6: Ultraviolet absorption spectra of complete antigen

ISSN 2706-6819 Vol.3, Issue 3: 40-46, DOI: 10.25236/IJFM.2021.030308

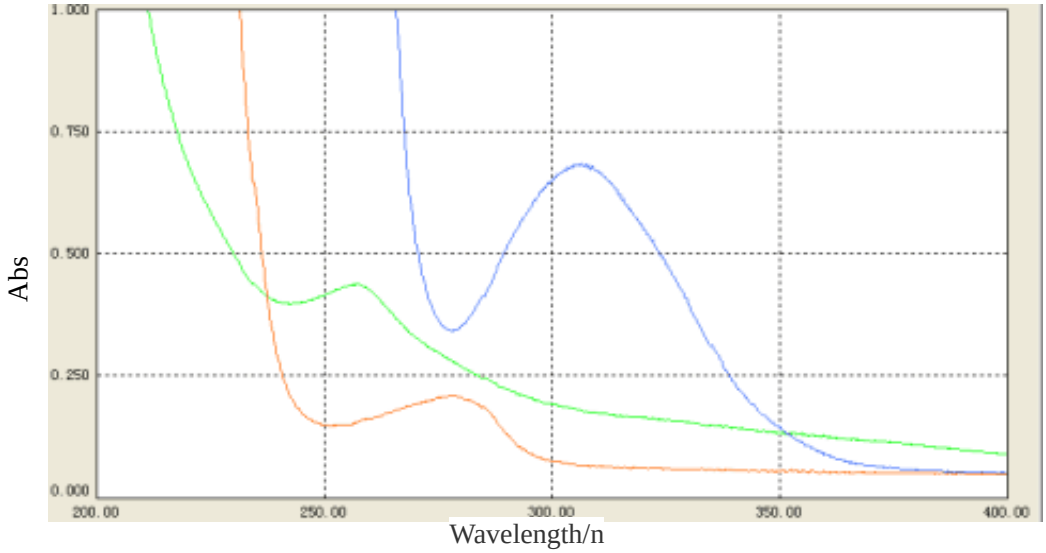

Figure 7: UV analysis and identification

Chlorpromazine has a strong UV absorption capacity. From the UV scanning results, it can be seen that the characteristic absorption peak of BSA is about 280 nm, that of chlorpromazine is about 310 nm, and that of complete antigen is about 210 nm. The characteristic absorption peak of the synthesized complete antigen moves to the left, and it is preliminarily judged that the synthesis of complete antigen is successful. After the modified chlorpromazine molecule is coupled to bovine serum albumin, the characteristic absorption curve of the complete antigen formed is not in the same position as the characteristic absorption peak of the ultraviolet absorption characteristic curve of chlorpromazine and bovine serum albumin, so it can be preliminarily confirmed that chlorpromazine and bovine serum albumin are successfully coupled.

2.3.2. Discussion

2.3.2.1. Selection of Carrier Proteins

Appropriate carrier proteins play an important role in the synthesis of complete antigens. A suitable carrier protein must first have the active group. Only carrier proteins with active groups can be conjugated to haptens. Selecting the appropriate carrier protein cannot only increase the yield of the antibody but also simplify the experimental process. The proteins commonly used as artificial antigen carriers are bovine serum protein (BSA), ovalbumin (OVA), keyhole hemocyanin (KLH), human serum protein (HSA), etc. At present, BSA is most widely used because of its physical and chemical stability, non-deniability, low price and easy availability, high lysine content, many free amino groups, and high solubility at different pH and ionic strength. It can be conjugated with hapten in the presence of organic solvents and remains soluble after conjugation. Therefore, bovine serum albumin is used as a carrier protein in this experiment.

2.3.2.2. Selection of Coupling Method

The common method of coupling hapten with the carrier is to link hapten with the carrier by using a coupling agent. Coupling agent binds hapantigen to the carrier at the group sites of -COOH, -NH2 or -SH. Carbodiimine and mixed anhydride are linked by carboxyl groups. The succinic anhydride method is linked by hydroxyl group, and Yu Wanxiang et al[11] synthesized Sudan red antigen artificially by succinic anhydride method; the glutaraldehyde method and the acrylic acid method are linked by amino group, and Song Jianwu et al[12] synthesized huperzine A artificial antigen by glutaraldehyde method; the diazotization method is linked by aromatic amino group and phenolic hydroxyl group. Sainter[13] etc, with the method of diazotization successful synthesis of chloramphenicol artificial antigen; amino acid method through a keto connection, etc[14]. This experiment with the method of diazotization modifies hapten, connects a hapten carboxyl, so as to choose mixed anhydride method and the carbon 2 imide method of hapten and the carrier protein coupling. This experiment chlorpromazine molecule itself does not contain active group, therefore require modification. Considering the laboratory conditions and experimental operation modification of this experiment adopts the method of diazotization, chlorpromazine molecules on connecting a carboxyl, Zhao Yingxia[15] used the diazo method to introduce carboxyl group in the construction of

ISSN 2706-6819 Vol.3, Issue 3: 40-46, DOI: 10.25236/IJFM.2021.030308

nonylphenol immunoassay. The coupling method was mixed anhydride method. Mixed anhydride method is also called isobutyl chloroformate method. When coupled, the carboxyl group in hapten molecule can form mixed anhydride with isobutyl chloroformate in organic solvent, and form peptide bond with amino group in protein molecule. Wang Xinzhou[16] prepared the complete antigen of Tymycin by the mixed anhydride method. Liu Xiyang[17] successfully synthesized cyproheptadine complete antigen by the isobutyl chloroformate method.

2.3.2.3. Selection of Coupling Conditions

The coupling process is actually a chemical reaction process of hapantigen, carrier protein and coupling agent under certain conditions. The temperature of the reaction process, the choice of the relative concentration of hapten, carrier protein and coupling agent are very important for the synthesis of complete antigen. Excessive temperature is easy to destroy the molecular structure, and the diazotization reaction requires to be carried out in a low-temperature environment, otherwise, it is easy to be dangerous. Secondly, the buffer composition should not react with the haptens and the solution pH should be moderate, otherwise, the protein will easily hydrolyze in an acid or alkaline environment. Also pay attention to the solubility of tranquilizers. Most tranquilizers are not soluble in water, but chlorpromazine is soluble in water, so water can be used as a reaction solvent.

2.3.2.4. Selection of Purification Methods

Due to the incompleteness of the reaction, the complete antigen solution formed after the modified hapten is coupled with the carrier protein, and there are unreacted haptens and other small molecules in the solution, which has to be purified before it can be used in the subsequent experiments. Complete antigens are usually purified by dialysis or gel chromatography. Dialysis is more thorough and simple than gel chromatography, but it takes longer to complete the purification. The time required for gel chromatography is short but the operation is complicated. In view of this, the dialysis method was used to purify the complete antigen.

2.3.2.5. Selection of Identification Methods

There are many methods for the identification of complete antigens, such as ultraviolet spectroscopy, infrared spectroscopy, fluorescence labeling, SDS-PAGE, non-denatures-agar gel electrophoresis, nuclear magnetic resonance, isotope tracer combined with thin layer chromatography, MALDI, high-performance capillary electrophoresis, LC-ESI-MS. Among them, the UV scanning method is widely used to identify antigen synthesis, while other methods require a certain level of chromatogram analysis ability and operating experience, and the cost is very high[18].

3. Conclusions

In the process of modifying the molecular structure of chlorpromazine, diazotization reaction is needed, and it is very important to control the pH value of solution in the diazotization reaction. The pH value of the solution should be strictly controlled between 1 and 2 after the solution is dissolved in water, because the diazonium salt is easy to decompose under normal circumstances, and it can remain relatively stable in the acidic environment. Nitrite is unstable, so the reaction should be controlled at a lower temperature. In the experiment, the reaction is first mixed and then used, and the required reaction is carried out under ice bath conditions. Due to the small amount of liquid, it is necessary to stir the reaction. Under the due conditions in the laboratory, the stirring process is changed to oscillating reaction overnight in a thermostatic oscillator. In the dissolution process of chlorpromazine, the acidity should be strictly controlled. A very small amount of acid or directly dissolved in ultra-pure water has little influence on the experimental results, and the amount of ultra-pure water has little influence on the results. In the experiment, chlorpromazine was dissolved with 1mL of ultra-pure water, 2mL of ultra-pure water, 1mL of 0.02mol/L of hydrochloric acid and 1mL of 0.06mol/L of hydrochloric acid, respectively, and relatively ideal results were obtained. Reddish-brown precipitates were produced overnight during the oscillating reaction. But in the more concentrated hydrochloric acid solution, the oscillating reaction overnight turns the solution green and produces no desired result. In the dialysis process, the pH value of the PBS buffer solution was accurate to 7.4 with a precision pH test paper, and the buffer solution was sterilized at a high temperature. The dialysis was carried out at a low temperature of 4℃ to prevent the contamination of the complete antigen during the dialysis process. After cooking the dialysis bag and then touching the dialysis bag, both hands should be operated on with rubber gloves. The successful development of artificial antigen provides practical technical support for further immunodetection, and also provides a reference method and approach for

ISSN 2706-6819 Vol.3, Issue 3: 40-46, DOI: 10.25236/IJFM.2021.030308

immunology research.

Acknowledgements

This project is supported by Hebei Provincial Science and Technology Support Plan - Health and Medical Special Fund (18275502D)

References

[1] LIU X J. Causes and control of veterinary drug residues in animal products [J]. Veterinary Drugs and Feed Additives, 2004, 9(5): 1-3.
[2] MOTAHARIAN A, HOSSEINI MRM, NASERI K. Determination of psychotropic drug chlorpromazine using screen printed carbon electrodes modified with novel MIP-MWCNTs nano-composite prepared by suspension polymerization method [J]. Sensors Actuat B-Chem, 2019, 288: 356-362.
[3] Chinese Veterinary Pharmacopoeia Committee.Beijing: China Agriculture Press, 2005.
[4] Cui Yaowen, Xia Xi, Wang Yuanyuan, Shen Jianzhong.Research progress in instrumental analysis of phenothiazines and benzodiazepines residues in animal tissues [J].China Animal Husbandry and Veterinary Medicine, 2011,38 (2) : 136-141.
[5] Liu Jianjing, Yang Shuming.Research progress in determination of chlorpromazine residues [J].China Animal Husbandry and Veterinary Medicine, 2008,35 (10):142.
[6] Liu Yan, Su Ying, Lan Ruirong, Peng Zhangming.Determination of antipsychotic drugs chlorpromazine and haloperidol in pork by HPLC-MS/MS [J].Journal of Pharmaceutical Analysis, 2015,35 (4): 685-688.
[7] Committee for Veterinary Medicinal Products. Chlorpromazine-summary report, in EMEA MRL 111/96-FINAL, The European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit. 1996.
[8] EEC, Council Regulation (EEC) 2377/90 of 26 June, Off. J. Eur. Comm. 1990, L224:1-6.
[9] Announcement of the Ministry of Agriculture.Maximum residue limits of veterinary drugs in animal food (No. 235)[EB/OL]. [2002-12-24].
[10] Bao Xiaoyan, He Fuyuan, Zeng Jioli, et al.China Journal of Chinese Materia Medica, 2013, 38(03): 397-401.
[11] Yu Wanxiang, Tang Xia, Zhu Hongyi et al.Review on synthetic methods of Sudan red antigen [J]. Guangzhou Chemical Industry,2015,43(19):16-17.
[12] Song Jianwu, Chao Zhi, Dong Renren.Journal of Third Military Medical University, 2009, 31(21): 2128-2130.
[13] Li Hao, Han Le.Journal of Food Science and Technology,2013,13(05):177-184.
[14] Li Nan, Wang Xiaojie, Hou Lanmei et al.Journal of Food Safety and Quality, 2016, 7(04): 1535-1545. (in Chinese with English abstract)
[15] Zhao Yingxia.Guangzhou: Guangdong University of Technology,2019.
[16] Wang Xinzhou.Preparation of complete antigens and development of polyclonal antibodies against Tymyosin [D]. Zhengzhou: Zhengzhou University,2014. (in Chinese)
[17] Liu Xiyang.Study on synthetic method of artificial antigen of zaheptadine [D]. Zhengzhou: Henan Agricultural University, 2014.
[18] Liu Jianjing.Establishment of an enzyme-linked immunosorbent assay (ELISA) for the detection of chlorpromazine [D].Chinese Academy of Agricultural Sciences, 2009.