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Study on Complete Antigen Synthesis of Promazine Hydrochloride

Yiqing Li[1,2,a], Jiayan Zheng[1,2,b], Xiaoli Han[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]535789837@qq.com, [b]574166857@qq.com, [c]1445706593@qq.com, [d]tingxinwang@126.com
**Corresponding Author*

Abstract: *To explore the method of complete antigen synthesis of promazine hydrochloride. Based on the analysis of the molecular structure and immunogenicity of promazine hydrochloride, complete antigen was synthesized by mixed anhydride method,under the condition of excess acid, the diazotization reaction of nitrite and p-aminobenzoic acid produces diazosalt, diazonium salt reacts with promazine hydrochloride; Triethylamine and isobutyl chloroformate form a mixed anhydride, promazine hydrochloride complete antigen was synthesized by amino ligation of mixed anhydride and carrier protein bovine serum albumin (BSA), complete antigen synthesis was identified by ultraviolet spectroscopy, the characteristic absorption peaks of promazine hydrochloride, bovine serum albumin and synthetic promazine hydrochloride complete antigen were analyzed. The peak wavelength of the synthesized complete antigen, promazine hydrochloride and bovine serum albumin is different, the complete antigen synthesis of promazine hydrochloride was proved successful. The results of this study provided a basis for the rapid detection of promazine hydrochloride residues in animal-derived food by immunoassay.*

Keywords: *Promazine hydrochloride, Complete antigen synthesis, Mixed anhydride method, Ultraviolet spectroscopic method, Veterinary drug residue*

1. Introduction

Sedative is a class of drugs that have a sedative effect by inhibiting the central nervous system by reducing the activity of organs or tissues. Sedatives can reduce anxiety and anxiety, sedatives are used to treat stress without affecting normal brain activity. In animal husbandry, the use of sedatives has improved the quality of life of animals and reduced morbidity and mortality. But in the treatment of disease at the same time, sedatives also bring a lot of adverse reactions, and there are certain harmful side effects to people.

Promazine hydrochloride (N,N-dimethyl-10 H-phenothiazine-10-propylamine hydrochloride) belongs to the phenothiazine class of tranquilizers, which is a tranquilizer isomer with promethazine hydrochloride. Promazine hydrochloride blocks postsynaptic dopamine receptors D1 and D2 in the midbrain limbic and medullary chemoreceptor triggering area (CTZ), thereby reducing stimulation of the brain's vomiting center and psychotic effects, such as hallucinations and delusions. In addition, the drug blocks alpha-adrenergic receptors and exhibits strong anticholinergic activity. In the process of animal breeding, in order to fatten the animals and shorten the time of animal breeding, some farmers add sedatives such as promazine hydrochloride into the feed, resulted in the residue of promazine hydrochloride in animal-derived products, that affects food quality and safety seriously, and it will cause residue in the animal's body and accumulate as food in humans, causing neurological damage, it can lead to a rapid heartbeat, palpitation, liver damage, even cancer. So, sedative drugs are banned in many countries and there are strict limits on the amount of residues in processed meat products. Although the starting time of the veterinary drug residue monitoring work in China later than developed countries, but the relevant departments have been pushed to the job of a leading position, formulated the relevant laws and regulations, and revised the "veterinary drug residues in animal foods the highest standard of set limit to", and started across the country to establish drug residue monitoring system[1].The Ministry of Agriculture, in Announs 176 and 235, allows the use of phenothiazines in food animals, but prohibits their use in feed and drinking water for animals and does not allow the detection of drug residues[2].

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At present, the most widely used method for detecting tranquilizers is chromatographic analysis. Chromatographic analysis can generally be divided into high performance liquid chromatography, gas chromatography, and liquid chromatography-mass spectrometry[3].These methods are feasible for small batches of samples, but they also have disadvantages such as high cost, skilled technicians, expensive instruments and long time consuming. Therefore, it is very urgent to develop a simple and rapid new detection method that can meet the actual demand.

Immunoassay is an analytical method based on the specific and reversible binding reaction of antigen and antibody. It can be divided into radioimmunoassay, fluorescence immunoassay, luminescence immunoassay, enzyme immunoassay and electrochemical immunoassay. Compared with other physical and chemical methods, immunoassay has the advantages of higher sensitivity, lower cost and less impact on experimental results.

The purpose of this study is to successfully synthesize the complete antigen of promazine hydrochloride, and lay the foundation for exploring more methods of complete antigen synthesis of promazine hydrochloride, establishment of immunological detection methods and preparation of antibodies in the future.

1.1. Current Status of Complete Antigen Synthesis

A complete antigen is a substance that can both induce and stimulate the body's immune system to produce antibodies (immunogenicity) and bind specifically to the corresponding antibodies (immunoreactivity).Only an antigen that has both of these properties is a complete antigen. Any substance that can induce and stimulate the body's immune system to produce antibodies should have a molecular weight of more than 10,000, small molecules to be tested need to be conjugated with macromolecules in order to become complete antigens. In order to achieve conjugation with macromolecules, it is necessary to analyze the structure of small molecules to determine whether they have active groups that bind to carrier proteins. The following steps are used to synthesize complete antigens from small molecules without active groups: the small molecule to be tested was introduced into the active group through chemical reaction, and the modified molecule to be tested reacted with the large molecule carrier protein under the corresponding conditions to form complete antigen.

1.2. The Synthesis of Haptens

The most important determinant of antibody specific recognition is haptens, which have only immunoreactivity but no immunogenicity. Usually, farming veterinary drug residues in food molecular weight is less than 10000, have no immunogenicity, so if you want to stimulate the body's immune system to produce antibodies, need to combine with macromolecular protein carrier form the complete antigen, but many of these small molecules residue without active group, therefore cannot be directly connected to the carrier protein.

1.3. Modification of Hapten Molecules

Like general veterinary drug residue molecules, promazine hydrochloride does not have immunogenicity, and there is no active group in the molecular structure. It needs to modify its structure through chemical reaction before it can be coupled with carrier protein to form complete antigen, promazine hydrochloride was modified by referring to the modification method of chlorpromazine molecule [4]. Under the condition of excess acid, diazotization reaction was carried out between nitrite and p-aminobenzoic acid to generate diazosalt, the hapten molecule is modified by the reaction of diazonium salt with promazine hydrochloride and the introduction of benzene ring with carboxyl group.

The level of immunogenicity of haptens is related to its shape and structure. The more complex the structure of the hapten molecule, the more characteristic it is, the lower the probability of having the same epitope with other substances, and the higher the immunogenicity of hapten. In other words, the level of immunogenicity is related to the number of rings in the structure, the number of heteroatoms, the heterogeneity of the structure and the number of branching structures. In general, the more complex the haptens are, the easier it is to induce the corresponding antibodies in experimental animals. On the contrary, the haptens with too simple a structure are less likely to induce the corresponding antibodies in experimental animals.

Selection of active sites. Generally speaking, more than one active site can be selected in the

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molecular structure of the hapten to be tested, and the selection of appropriate active site will be of great help to the synthesis of high-quality small molecule haptens, which will have a direct impact on the immune effect and affinity of antibody generation[5].The molecular structure of each active site is different, and the antigen determinants exposed are also different, so the specificity of antibodies will be affected; In addition, in order to maintain the characteristic structure of the object to be tested to the maximum extent, the active site should be far away from this part to avoid the destruction of the characteristic structure and the shielding of the macromolecular carrier, so that the characteristic structure part can be fully exposed.

After the selection of the active site, the next step is the introduction of the active group. Generally, the introduction of the active group can be done in the following ways. First, the molecular structure of the object to be tested already contains the active group that can be connected to the carrier protein, they are generally amino (-NH2), carboxyl (-COOH), hydroxyl (-OH), etc. Different active groups have different ways of connecting with carrier protein[6];Or some of the substances to be tested have inactive groups on the molecular structure, and these inactive groups can be oxidized or reduced to the corresponding active functional groups through chemical reactions, for example, in the design of phenobarbital hapten, the para-nitro group on phenobarbital molecule is reduced to para-amino group, and then the corresponding antibody is prepared through coupling reaction[7];Third, the substances or metabolites of the upper stage of small molecule substances can also be used as haptens; Fourthly, according to the position of the active site on the structure of the substance to be measured, the active functional groups are introduced through chemical reactions under appropriate reaction conditions.

1.4. Complete Antigen Synthesis

Complete antigen is composed of hapten connected to carrier protein. The carrier is not only used to increase the molecular weight of hapten, but more importantly to induce the immune response of the body by the strong immunogenicity of the carrier. The relative molecular weight of promazine hydrochloride is 320.88, which has only immune reactivity but not have immunogenicity. However, only complete antigens with two characteristics can stimulate the body to produce promazine hydrochloride antibody, so the first thing to do is to modify the structure of promazine hydrochloride molecule, through chemical reaction, the active group is introduced, and then the carrier protein is linked to synthesize the complete antigen. Moreover, no matter which protein carrier is used, the groups involved in the reaction are the same, so it can be seen that the specific binding method is determined by the types and properties of the active groups of haptens.

When the hapten molecular structure has an amino group, the synthesis method is generally as follows: diazotization method [8], periodic acid oxidation method, glutaraldehyde method.

When the hapten molecular structure has carboxyl group, the synthesis method is generally as follows: mixed anhydride method, carbonic diimine (EDC) method, and active ester method.

1.5. Identification of complete antigens

After complete antigen synthesis, it is necessary to confirm whether the hapten binds to carrier protein, so the identification of antigen synthesis is a necessary work. At present, the identification methods are mainly as follows: ultraviolet spectroscopy, infrared spectroscopy method[9], thin layer chromatography[10], gel electrophoresis analysis[11].

After the complete antigen synthesis of promazine hydrochloride, the solution was dialysis purified with 0.01M PBS buffer. The obtained solution was centrifuged, and part of the supernatant was diluted several times, the success of the synthesis was determined by the ultraviolet spectrophotometer at 200-400nm. After the scanning, the corresponding spectral map will be obtained. Because each substance has its own different molecular spatial structure, each substance has its own unique and fixed absorption spectrum curve. After the synthesis of the complete antigen of promazine hydrochloride, the characteristic absorption peaks of promazine hydrochloride, bovine serum albumin and the complete antigen were observed to determine whether the complete antigen was synthesized successfully.

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2. Materials and Methods

2.1. Instrument and Equipment

Electronic balance: PTX-FA210S Fuzhou Huazhi Scientific Instrument Co., Ltd; TGL-10C Flying Pigeon Series Centrifuge: Shanghai Anting Scientific Instrument Factory; Ultraviolet Visible Spectrophotometer: Jingpu General Instrument Co., Ltd;

2.2. Materials and Reagents

Para-aminobenzoic acid, purchased from Shanghai Macklin Biochemical Company; Promazine hydrochloride 99.5% purity, purchased from LGC Ltd; Bovine serum albumin (BSA), from Roche; N, N-dimethylformamide, purchased from Tianjin Komil Chemical Reagent Co., Ltd; Isobutyl Chloroformate, purchased from Shanghai Macklin Biochemical Company; Triethylamine, purchased from Tianjin Damao Chemical Reagent Factory; Wet ready-to-use dialysis bag (the maximum allowable transmissible molecular weight is less than 6000), reduced iron powder: purchased from Baoding Huaxin Reagent Co., Ltd.

2.3. Methods

2.1.1. Hapen Synthesis

Mixed anhydride method

(1) Take 3.45g sodium nitrite, add 35 mL water, constant volume to 50mL, and put it into the freezer for use after constant volume is completed. (2) Take 0.137g para-aminobenzoic acid, add 10mL water and a few drops of 6mol/L hydrochloric acid after the solution is completely dissolved, the solution was stirred on a magnetic stirrer for 10 minutes in ice bath conditions. Remove 1mL of the prepared sodium nitrite solution and add it to the completely dissolved p-aminobenzoic acid solution. Place the mixture in the freezer for half an hour. Take 0.0119g promazine hydrochloride and add 0.35mL water to dissolve. Under the condition of stirring in the ice bath, 0.37mL solution was dropped into promazine hydrochloride solution and left to rest overnight in the freezer. The reaction was oscillated at 14℃ for 6 hours on the second day. After the oscillation, the supernatant was centrifuged at 6000r/min for 6 min, then discard supernatant and blow dry.

Diazotization

(1) Take 0.005g promazine hydrochloride into 50μL fuming nitric acid and 50μL concentrated sulfuric acid, the reaction was sealed at 55℃for 1 hour, add 4 times the volume of water, then centrifuge at 5000 r/min for 5 min, then discard supernatant and blow dry. (2) Dissolve the reactant in 1mL 1mol/L hydrochloric acid, add 0.03g of reduced iron powder, constant temperature sealed reaction for 40minutes at 65℃,after the reaction, wash with water for 3 times, then centrifuge at 5000r/min for 5min, the precipitate was dissolved in 1.5mL dimethylformamide, add 1.5mL water, centrifuge, and leave the precipitate.

2.1.2. Complete Antigen Synthesis

Mixed anhydride method

(1) Take 0.34mL 1, 4-dioxane and dimethylformamide into the precipitation to dissolve the precipitate, add 9μL triethylamine under ice bath and oscillating condition. After 15minutes of oscillating reaction, add 5.5 μL isobutyl chloroformate, oscillate at a constant temperature of 14℃ half an hour. Take 0.05g precipitate and dissolve it in 5mL water, add 0.03g bovine serum albumin,0.67mL was absorbed into the activated promazine hydrochloride solution under the condition of low temperature ice bath, and then put into the freezer for standing overnight. (2) Dialysis was soaked in 0.1M PBS buffer for 3days, and the dialysate was changed twice a day.

Diazotization

Dissolve the precipitate in 1.5mL 1 mol/L hydrochloric acid, add 31.25mg sodium nitrite, stir for 6hours under ice bath conditions, take pH to 7.0 with 1mol/L sodium hydroxide, add 28 mg 0.01mol/L precooled bovine serum albumin phosphate buffer (pH =7.4).Stir at 4℃overnight. The dialysis process is the same as the mixed anhydride process.

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3. Results and Discussion

3.1. Results

3.1.1. Result of Hapten Synthesis

The method of mixed anhydride

The pre-cooled sodium nitrite solution was slowly dropped into the p-aminobenzoic acid solution, and the fully reacted solution was slowly dropped into the dissolved promazine hydrochloride solution while stirring under the conditions of ice bath. The solution was left standing overnight in the freezer, then the oscillating reaction was centrifuged for 6 hours and the precipitation was taken out.

The method of diazotization

After dissolving promethazine hydrochloride with mixed acid, heating in 55 ℃ water bath, dissolving the reactant with 1 mol/L hydrochloric acid and adding reduced iron powder, heating the reaction in water bath at 65 ℃ for 40 minutes, there were bubbles and residual iron powder at the bottom of the reaction, but there was no precipitation, so the experiment cannot proceed further.

3.1.2. Result of Complete Antigen Synthesis

The precipitation was dissolved with 1-4-dioxane and dimethylformamide, then add the triethylamine and isobutyl chloroformate to activate promazine hydrochloride solution, and bovine serum albumin solution was added to react overnight to obtain complete antigen.

3.1.3. Identification Result of Complete Antigen Synthesis

The composite was scanned by ultraviolet spectrophotometer. The scanning results are shown in Figure1, 2, 3,4, 5.

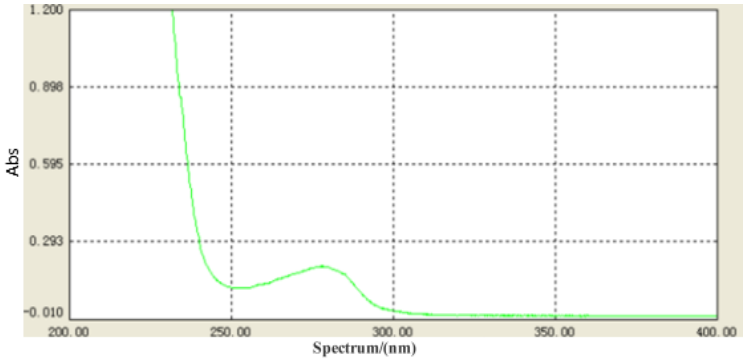

Figure 1: UV spectrogram of bovine serum albumin

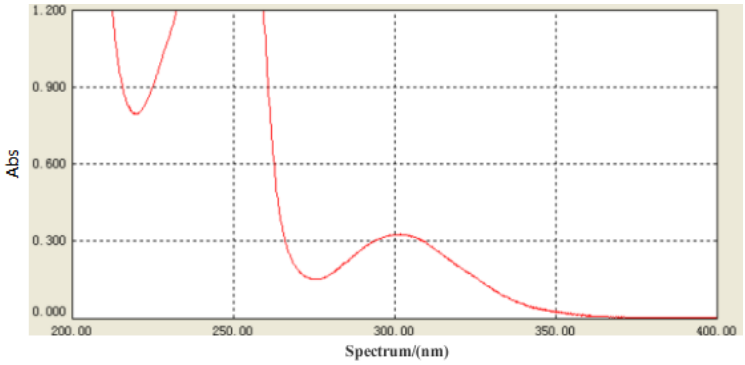

Figure 2: Ultraviolet spectra of promazine hydrochloride

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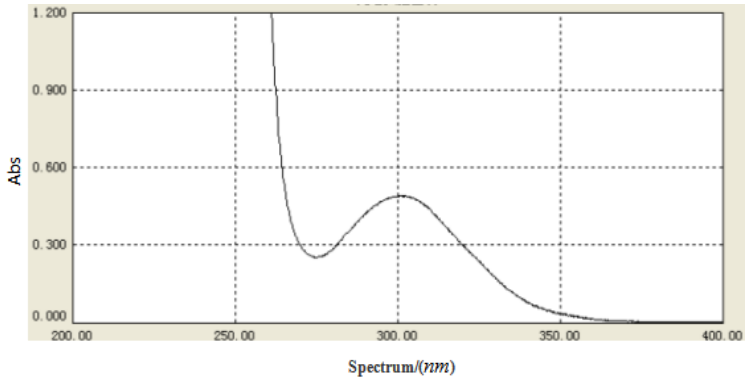

Figure 3: Ultraviolet spectrum of promazine hydrochloride -BSA mixture

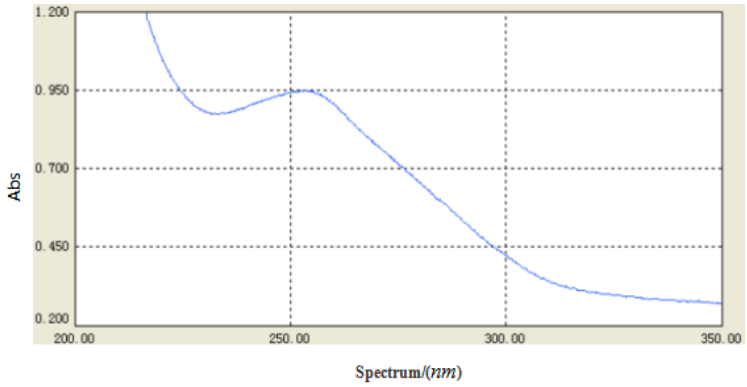

Figure 4: Complete antigen ultraviolet spectrogram

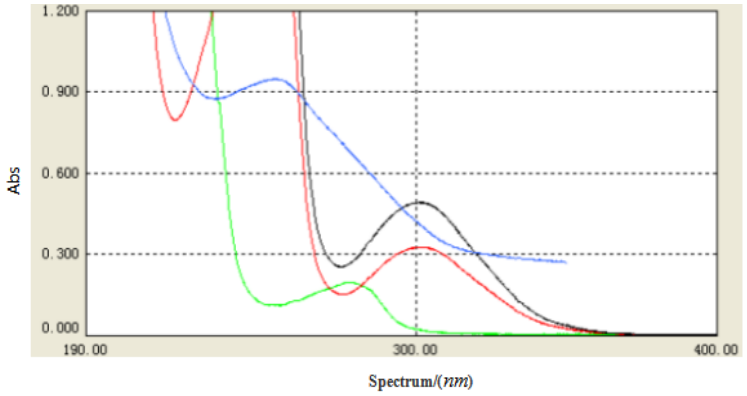

Figure 5: Identification of ultraviolet spectra

From the figure above we can see, the characteristic absorption peaks of promazine hydrochloride and promazine-BSA mixture are both about 300 nm, the characteristic absorption peaks of bovis serum albumin are about 280 nm, and the characteristic absorption peaks of complete antigen are about 260 nm. The peak locations and shapes of the characteristic absorption peaks of complete antigen are different from those of the other three substances. It can be preliminarily judged that complete antigen synthesis is successful.

3.2. Discussion

3.2.1. Selection of Carrier Proteins

The carriers used in complete antigen synthesis mainly fall into the following categories :(1) protein carriers, (2) peptide polymers, (3) macromolecular organic compounds. In the selection of carrier proteins, the following considerations should be taken into account: the carrier should have chemically active groups that can react with hapgens; the carrier should have a certain capacity and can conjugate enough molecules. The carrier should be inert and not affect the function of the conjugate. The carrier

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should be sufficiently stable and cheap and easily available [12]. In the immunoanalysis, the carrier is usually protein. No matter which protein has strong immunogenicity, it is easy to bind to haptens, the protein solubility is relatively large, and the extraction and purification operation is simple and easy. Human serum albumin (HSA), bovine serum albumin (BSA) and ovalbumin (OA) are the most commonly used carrier proteins in normal conditions during the experiment. Considering the kinds of agents available in the laboratory, bovine serum albumin was selected as carrier protein in this experiment.

3.2.2. Selection of Coupling Methods

The method of coupling should be selected according to the molecular structure characteristics of small molecule compounds. The coupling reaction has three basic conditions :(1) The hapten structure contains at least one of the free amino and carboxyl groups. (2) The haptens with hydroxyl, ketone or aldehyde groups cannot directly bind to the carrier protein. It is necessary to chemically bind the carboxyl group on the haptens and then connect to the carrier. (3) If aromatic haptens have carboxyl groups on the ring, the ortho hydrogen is very active and can be easily replaced. The coupling effect between hapgens and carrier proteins is influenced by many factors, including temperature, pH value, reagent concentration, etc. In this experiment, the method of modification of promazine hydrochloride molecule is that under the condition of excess acid, diazotization reaction of nitrite and p-aminobenzoic acid is carried out to generate diazosalt, which reacts with promazine hydrochloride and introduces benzene ring with carboxyl group. The modified promazine hydrochloride molecule contains carboxyl group. The complete antigen was synthesized by mixed anhydride method according to the current conditions.

3.2.3. Selection of Identification Methods

At present, the main methods for the determination of complete antigen include ultraviolet spectroscopy, nuclear magnetic resonance, isotope tracer, infrared spectroscopy, gel electrophoresis, high performance capillary electrophoresis, LC-ESI-MS and so on. The most common method for detecting antigen coupling is ultraviolet spectroscopy. Other methods require a certain level of chromatography-analysis ability and operating experience and are expensive [13]. Considering the existing equipment in the laboratory, the complete antigen was preliminarily judged by ultraviolet spectrophotometry in this experiment, and the immunogenicity of the synthesized complete antigen was identified by immunoassay in the later stage.

3.2.4. Diazo Analysis

Method first through nitration in hydrochloric acid c oxazine molecules on the nitro, again through the reduction reaction nitro reduction into amino, reduced iron powder in the reaction as a reductant reduction of nitro, but may be reduced iron powder reducing weak cannot nitro reduction into amino, through the communication with the reagent company that zinc powder, magnesium powder and aluminum powder for tube products, Therefore, under the current laboratory conditions, it is impossible to replace the metal element with stronger reducibility as the reducing agent.

4. Conclusion

The complete antigen of promazine hydrochloride was synthesized by diazotization and mixed anhydride method, and the synthesis was successful. In the process of molecular modification, the solution of p-aminobenzoic acid dissolved in water should be maintained in a strong acidic environment, because the diazonium salt is easy to decompose, and can remain relatively stable only in an excessive acidic environment. Nitrite is unstable and easy to decompose, so the reaction should be carried out at low temperature and should be prepared on the spot. In the process of diazotization, p-aminobenzoic acid was dissolved in water and acidified with 6 mol/L hydrochloric acid, and the complete antigen was successfully synthesized. However, different results were obtained by directly dissolving p-aminobenzoic acid in 0.2mol/L hydrochloric acid. Due to the limitation of the quantity of the drug, the trial dose in the experimental process is less, and the stirring reaction is needed, so the stirring process is replaced by the oscillation in the thermostatic oscillator. The configuration of dialysate should be carried out in strict accordance with the ratio of agents; otherwise the acid and alkalinity deviation of the solution prepared will be great, which will affect the dialysis results. After the preparation, 1 mol/L sodium hydroxide solution was used to adjust the pH to 7.4. After dialysis, the dialysate should be sterilized at high temperature. It should be noted that the whole dialysis process should be carried out in the freezer and sealed with plastic wrap to prevent bacteria from affecting the

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dialysis results.

Two experimental methods of complete antigen synthesis of promazine hydrochloride were tried, the first method successfully synthesized complete antigen, which proved that this method was successful, and laid a foundation for exploring more methods of complete antigen synthesis of promazine hydrochloride, residue detection of promazine hydrochloride and preparation of specific antibodies in the future. Although the second method failed to synthesize the complete antigen, if the metal with stronger reducing ability can be used as the reducing agent, it can be determined whether the nitro group is too weak to be reduced to the amino group.

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