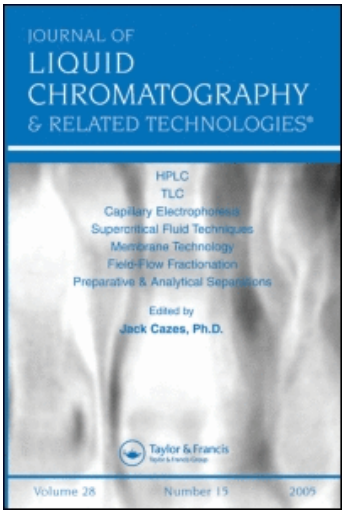

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Separation of Macromolecular Impurities in Penicillin G Sodium by Gel Filtration Chromatography

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Abstract: Macromolecular impurities in penicillin G sodium (PGS) may cause allergic reactions. Separation of these impurities and PGS by gel filtration chromatography was investigated in this study. The effects of gel, mobile phase, and temperature on separation were examined. The penamaldate method was applied to detect penicilloylated impurities separated on gel columns. Three gels, Sephadex G-10, G-25, and Toyopearl HW-40F were examined with water, acetate, phosphate, citrate buffers as mobile phases. Separation on HW-40F was better than on G-10 and G-25. Mobile phase significantly affected both the separation of macromolecular impurities and the chromatographic behavior of PGS. PGS was over excluded in water. The distribution coefficient of PGS was largest in citrate buffer, and smallest in acetate buffer. Ion strength influenced the chromatographic behavior of PGS greatly. Higher ion strength resulted in a greater distribution coefficient. Temperature hardly affected the chromatographic behavior of macromolecular impurities and PGS, but it greatly influenced the retention of small impurities. The suitable conditions for separation were using gel HW-40F as stationary phase, 5 mM citrate buffer as mobile phase, and temperature below 15°C.

Keywords: Gels, Interaction, Macromolecular impurities, Penicillin G sodium, Processing, Separation science, Sorption

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INTRODUCTION

β-Lactam antibiotics, such as penicillins, are widely used for the treatment of various infections on account of their bactericidal activity, broad spectrum, low toxicity, and excellent distribution throughout the body.[1] However, penicillin is still the most common cause of allergic drug reactions and the leading cause of anaphylaxis. Cross allergenicity exists between different penicillins and between penicillins and cephalosporins due to their similar molecular structures.[2,3]

Haptens, such as drugs and other low molecular weight chemicals, become immunogenic only upon binding to proteins. Allergic reactions are found to be elicited by macromolecular impurities such as penicilloylated proteins, penicilloylated peptides, or polymers which have at least two determinants per molecule.[4,5] Removal of these impurities is helpful to reduce the incidence of allergic reactions.[6,7] The macromolecular impurities may be carried over from the biosynthetic process or may be formed in solution during incubation by the degradation of its primary ring structure. The property and allergenicity of the macromolecular impurities have been studied by several authors.[8,9] Different methods had been used to separate the macromolecular impurities. Stewart[10] and Munro[11] used the dialysis method to separate proteinaceous contaminant and polymers in penicillins, and proved their allergic ability. Roets[12] separated and purified the oligomers of ampicillin and amoxicillin by ion exchange chromatography and studied the structure of oligomers. Gel filtration chromatography was the most frequently used method to separate these macromolecular impurities because of its high efficiency. Separation was implemented on the basis of their pore dimensions. Different gels had been utilized in previous studies. Kristofferson used Sephadex G-50 as the stationary phase to separate penicilloylated protein contaminants in penicillin.[13] Dewdney[14] and Ahlstedt[15] used Sephadex G-25 to separate polymeric materials, which were easily formed in aqueous solution of β-lactam antibiotics and proved their allergenicity by passive cutaneous anaphylaxis. The chemical structure of polymers separated by Sephadex G-25 was also studied.[16] Hu[17,18] used gel filtration chromatography for the quality control of cephalosporin and amoxicillin preparations. Iwata[19] applied TSK-G2000SW, a hydrophilic porous silica based gel, to analyze β-lactam antibiotic polymers, and found it offered high resolution.

The chromatographic behavior of penicillin G sodium could greatly be affected by the ion strength of the mobile phase and the gel chosen. It was of significance to study all these factors on the chromatographic behavior of penicillin G sodium in order to optimize the separation of macromolecular impurities. However, there was no report concerning this. The present work is intended to systematically study the separation

of macromolecular impurities. The mechanism of interaction between gel and solute was discussed. Except for the dextran gels mentioned above, another hydrophilic gel Toyopearl HW-40F was also explored for separation, and it was found more effective than dextran gels.

EXPERIMENTAL

Chemicals and Reagents

Penicillin G sodium (PGS) was purchased from the North China Pharmaceutical Group Co., Shijiazhuang, China. Toyopearl HW-40F was made by Tosoh, Tokyo, Japan. Sephadex G-10, G-25, and blue dextran 2000 (M.W. 2×10^6 Da) was made by Amersham Pharmacia Biotech, Uppsala, Sweden. Penicilloic acids were prepared as described by Schwartz.[20] All other reagents were of AR grade. Caution: $HgCl_2$ is hazardous and should be handled carefully.

Gel Filtration Chromatography

A Waters system which consisted of a 1525 binary pump, a 717 plus autosampler, and a 2487 dual λ absorbance detector was used in the experiment. Column temperature was controlled by a thermostat water bath with the precision of $\pm 0.1^{\circ}C$. A glass column with diameter of 1.6 cm was slurry packed with gels. The length of columns was 32, 33, and 33.7 cm for G-25, G-10, and HW-40F, respectively. Prepared buffers were used as mobile phases and all the experiments were run at a mobile phase flow rate of $1.0\,mL{\cdot}min^{-1}$. Considering the instability of PGS in acidic and basic solutions due to the labile β-lactam ring at the 7-position,[21] all the buffers were adjusted to pH 7.0 with corresponding acids. Wavelength of 254, 322, and 280 nm was used for detection of PGS and impurities.

An aliquot of PGS solution was applied to the column which had previously been equilibrated with mobile phase. PGS solution was prepared immediately before use. The distribution coefficient of PGS, K_{av}, on the column was calculated by the equation:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0} \tag{1}$$

where V_e was the elution volume of PGS obtained corresponding to the maximum concentration of PGS with a maximum UV absorption, V_0 was the void volume of column, V_t was the column volume. The void

fraction of the column was determined using blue dextran 2000. Because of its high molecular weight, blue dextran 2000 was totally excluded from the gel pores. The elution volume for blue dextran was taken as the void volume of the column.

Effect of Gel

The separation of macromolecular impurities was carried out on the gels of G-25, G-10, and HW-40F using 5 mM citrate buffer as mobile phase. The temperature of the column was maintained at 5°C in order to ensure that no additional PGS degradation product was produced in the process of experiments.

Effect of Mobile Phase

The effect of mobile phase with and without electrolytes on the separation of macromolecular impurities and the chromatographic behavior of PGS was studied at 5°C. Water and three buffers, acetate, phosphate, and citrate buffers, were used as mobile phases. The ion strength of buffer I was calculated by:

$$I = \frac{1}{2}\sum c_i z_i^2 \tag{2}$$

where c_i was the molar concentration of ion, z_i was the charge number of ion. Furthermore, the effect of citrate buffer concentration on the distribution coefficient of PGS was investigated in the buffer concentration range of 5 to 50 mM.

Effect of Temperature

Experiments were implemented at four temperatures of 5, 15, 25, 35°C on the columns of G-25 and HW-40F. The mobile phases used for the gels of G-25 and HW-40F were 20 and 5 mM of citrate buffer, respectively.

Detection of Penicilloylated Impurities by Penamaldate Method

Penicilloic acids and functional derivatives of their α-carboxyl group converted to penamaldic acids and penamaldic acid derivatives,[20,22] respectively, when treated with $HgCl_2$ solution (Figure 1). The penamaldates exhibited an intense UV-absorption band with a characteristic absorption at 282 nm. It was found that the absorbance of penamaldate

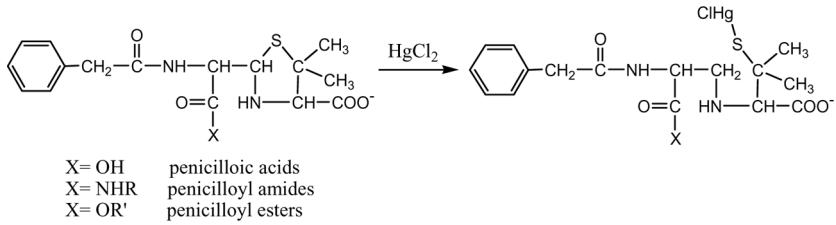

Figure 1. Formation of the penamaldate structure from penicilloyl derivatives.

formed from penicilloic acid was much less stable than that formed from an ester or amide derivative. Using this property, penicilloic acids could be differentiated from penicilloylated impurities, like penicilloylated proteins, penicilloylated peptides, and polymers.

An amount of 0.2 g PGS was dissolved in mobile phase and applied to a G-10 or HW-40F column. The eluent was collected in 2 mL fractions. Penicilloylated impurities were detected at 285 nm after treated with 0.001 and/or 0.002 M $HgCl_2$ solutions, according to the impurities concentrations. To 2 mL of sample solution, $HgCl_2$ solution was added in small increments of 10 μL or 20 μL, rapidly mixed, until the ultraviolet absorbance was not increased anymore. Penamaldate value (*P* value) was defined as the difference of ultraviolet absorbance before and after the addition of $HgCl_2$ solution. Penamaldate stability PS_{10} was a comparison of the absorbance at 10 and 0 min after the addition of $HgCl_2$ solution:

$$PS_{10} = \frac{Absorbance_{10\,\mathrm{min}}}{Absorbance_{0\,\mathrm{min}}} \times 100\% \tag{3}$$

RESULTS AND DISCUSSION

Effect of Gel

The chromatograms of PGS samples on the columns of G-25, G-10, and HW-40F were shown in Figure 2. Elution volumes (*EV*) of solutes in the chromatograms were normalized by dividing by colomn volume. Absorbance at 254, 280, and 322 nm were signed as *A254*, *A280*, *A322* for short. On the column of G-25, macromolecular impurities could not be baseline separated from PGS. An impurity eluted after PGS was detected at 322 nm. It exhibited a maximal UV-absorption at 320 nm, which was considered to be the characteristic absorption of penicillenic acid, a degradation product of PGS that could be esaily formed in

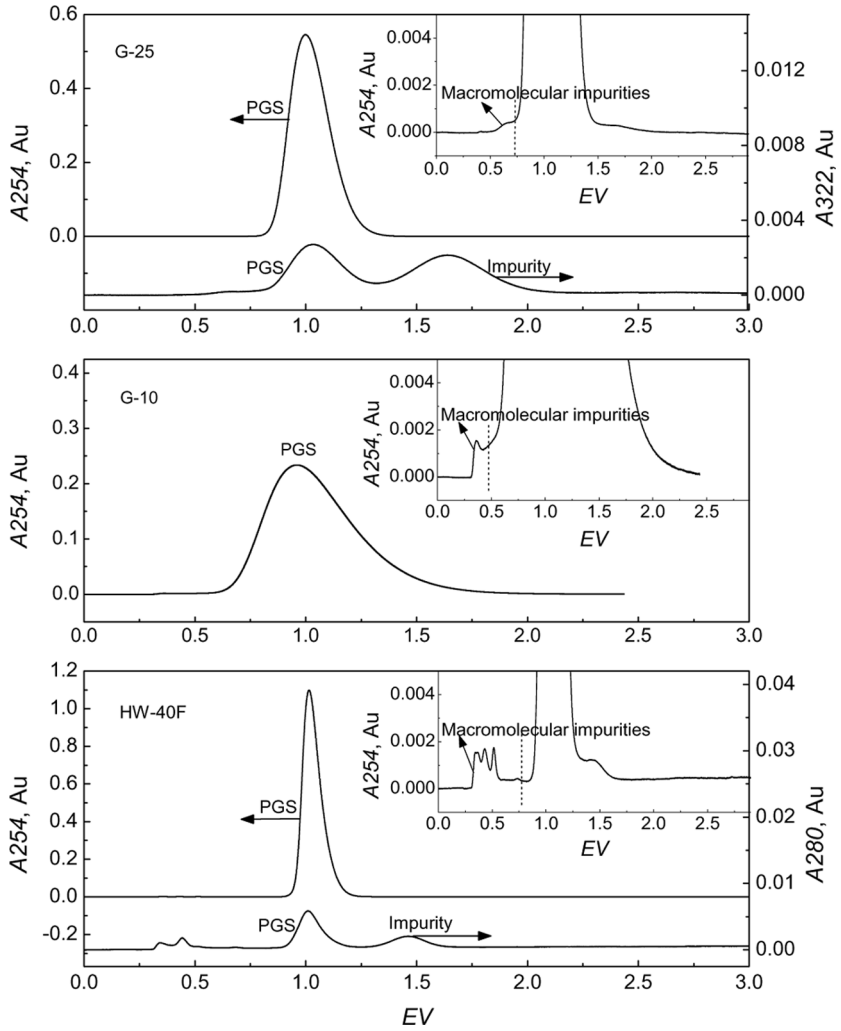

Figure 2. Chromatograms of PGS samples on the gels of G-25, G-10, and HW-40F using 5 mM citrate buffer as mobile phase. PGS sample concentration: 20 mg·mL^{-1}; injection volume: 0.5 mL.

aqueous solution. The elution volume of this impurity was 1.65 times of column volume, indicating the adsorption effects between molecule and gel. Compared to G-25, G-10 had smaller pore size with an exclution limit of 700 Da. The separation of macromolecular impurities was improved as seen in the amplified figures. Macromolecular impurities were eluted in a single peak because of the low exclution limit. Since the particle size of G-10 was larger (dry particle size: G-10, 40–120 μm;

G25, 20–80 μm), the peak of PGS was broadend. This made the macromolecular impurities not be baseline separated from PGS either. Among the gels examined, the separation was best on the column of HW-40F. Macromolecular impurities were not eluted in a single peak on HW-40F indicating the nonuniform molecular weights of impurities. Smaller particle size (30–60 μm) of HW-40F leads to a higher column efficiency, thus resulting in a narrower peak of PGS. An impurity eluted after PGS showed a maximal UV-absorption around 280 nm. This might be attributed to the degradation product of PGS, penamaldic acid. Similar to penicillenic acid, an adsorption effect existed between this impurity and HW-40F.

Effect of Mobile Phase

As shown in Figure 2, the separation of macromolecular impurities could adequately be processed when buffers were used as mobile phases. The elution volume of PGS was nearly equal to the bed volume of the column on all three gels. This indicated that the chromatographic behavior of the PGS sample on gel was not only dominated by molecular sieving mechanism, but also influenced by gel-solute interactions.[23] The interactions between the solutes and stationary phase made the chromatographic behavior differ from the molecular sieving mechanism. In the application of the preparation mode, this departure was welcome when it enhanced the resolution. On this account, the effect of mobile phase on the chromatographic behavior of PGS was studied.

Figure 3 showed the chromatograms of PGS on the column of G-25, G-10, and HW-40F when water was used as mobile phase. Macromolecular impurities could not be separated with water. PGS was eluted earlier than would be expected from the value of its molecular weight alone. It began to be eluted at the position of void volume corresponding to the elution volume of blue dextran 2000. It had been reported that the non-size-related behavior of the solute was mainly attributed to hydrophobic interactions, ion exchange, ion exclusion, and electrostatic repulsive interactions.[24] Hydrophobic interactions and electrostatic interactions were two opposing effects. Suppression of electrostatic effects enhanced hydrophobic interactions and vice versa.[25] The chromatographic behavior of PGS was in accordance with this rule. Because Sephadex gels G-25 and G-10 were both prepared by crosslinking dextran with epichlorohydrin, and Toyopearl HW-40F was ethylene glycol and methacrylate copolymer, the gels of G-25, G-10, and HW-40F were all weakly negatively charged in aqueous media due to the presence of ether bonds and hydroxyl groups in the gel skeleton. When water was used as mobile phase, PGS was also negatively charged caused by the carboxylic group in the

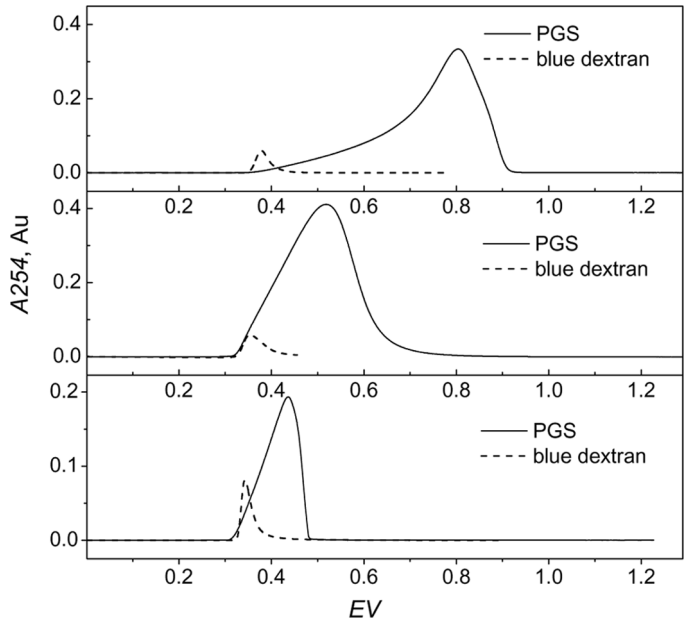

Figure 3. Chromatograms of PGS sample on the gels of G-25, G-10, and HW-40F using water as mobile phase.

thiazolidine ring. Electrostatic repulsive interactions dominated the gel filtration process and PGS was excluded from entering the gel matrix.

When buffer was used as mobile phase, separation was improved. The distribution coefficients of PGS, K_{av}, on the columns of G-25, G-10, and HW-40F were calculated at low PGS sample concentration, when acetate, phosphate, and citrate buffers with concentration of 20 mM were used as mobile phases. As shown in Figure 4, the K_{av} values of PGS were affected by both stationary phases and mobile phases. The cross linked structure of gels was likely to create a hydrophobic site on its surface. When buffers were used as mobile phases, the electrostatic interactions between gels and solutes were partially suppressed, and the hydrophobic interactions worked in the process. Among the gels examined, the adsorption effect of PGS increased in the sequence of G-25, G-10, and HW-40F. Hydroxylated methacrylic polymer HW-40F had the most potential hydrophobic sites. This might be induced by several aspects, such as the composition of the gel skeleton, the surface area of particle, and the degree of cross linking. The distribution coefficients of PGS on the gels of G-25 and G-10, which had similar skeleton structures were different. This should be attributed to the different degree of dextran cross linking. A smaller pore size of G-10 resulted from a high degree

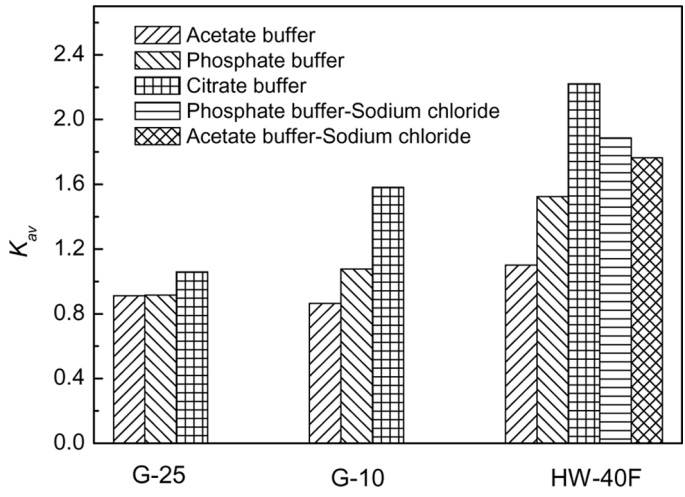

Figure 4. Distribution coefficients of PGS, K_{av}, on the gels of G-25, G-10, and HW-40F when different buffers were used as mobile phases.

of cross linking, which could provide increased numbers of hydrophobic sites in the gel.

Buffers had different effects on the interactions between PGS and gels. On three gels, the trend of the buffer effects was identical. PGS had the largest K_{av} value with the citrate buffer, while it had the smallest K_{av} value with the acetate buffer. The difference of K_{av} values was larger on the gel which had greater adsorption effect with PGS. The ion strength of buffers calculated by Equation 2 were 110, 36 and 20 mM for citrate, phosphate, and acetate buffer, respectively. This might be one of the reasons that caused the difference of PGS adsorption on gels. A buffer with higher ion strength suppressed the electrostatic repulsive interactions more effectively, thus resulting in a higher adsorption capacity. In order to check whether the type of electrolyte affected the interactions between PGS and gel, phosphate and acetate buffers (20 mM) were adjusted by adding sodium chloride to get the same ion strength with the citrate buffer (20 mM), then applied to the HW-40F column. It was found that the K_{av} values obviously increased with the adjusted buffers compared to that of the unadjusted. The difference of the K_{av} values between different kinds of buffers decreased.

Since the ion strength of the buffer could affect the chromatography process greatly, a further study was carried out concerning the effect of citrate buffer concentration on the K_{av} value of PGS. The results in Figure 5 show that the K_{av} values increased with buffer concentration. This means that the hydrophobic interactions were enhanced by higher

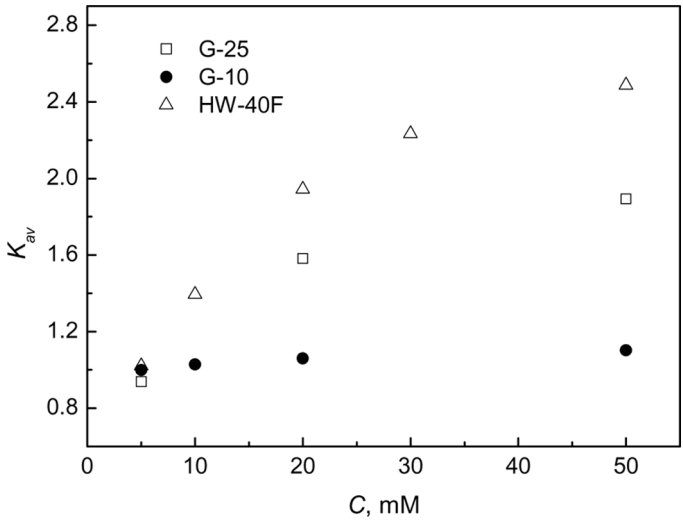

Figure 5. Effect of citrate buffer concentration on the distribution coefficient of PGS, K_{av}, in the concentration range of 5 to 50 mM. PGS sample concentration: 20 mg·mL^{-1}; injection volume: 0.5 mL.

ion strength of the buffers. It had been found that carboxylic, and aromatic functional groups had strong influence on the elution volume of the solute.[24,26] The electrostatic repulsive interactions between PGS and gels induced by carboxylic group could be inhibited by the electrolyte, so that the hydrophobic interactions induced by aromatic groups in PGS molecules were facilitated. On the G-25 gel the influence of buffer concentration was small, and the K_{av} values increased slightly with buffer concentration. This was caused by its lack of hydrophobic sites in the gel skeleton. The increase of K_{av} values turned out to be slow at high buffer concentration on the three gels. This is easily interpreted to mean that the electrostatic repulsive interactions cannot be decreased endlessly, so that the increase of hydrophobic interactions becomes to be slow in high buffer concentration.

Effect of Temperature

Figure 6 shows the chromatograms of PGS on gels of G-25 and HW-40F at temperature range of 5 to 35°C. The retention of macromolecular impurities was hardly influenced by temperature, and the elution volume of PGS on gels decreased slightly with increasing temperature. Temperature could greatly affect the behavior of small molecular weight

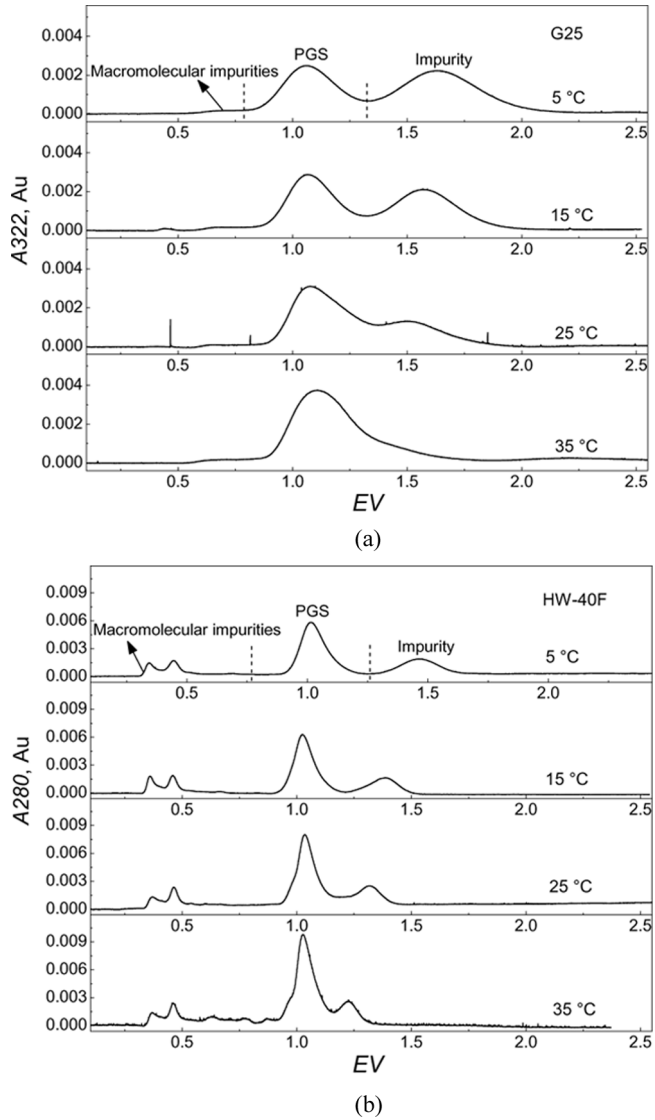

Figure 6. Chromatograms of PGS on the gels of G-25 and HW-40F in the temperature range of 5–35°C. PGS sample concentration: 20 mg·mL^{-1}; injection volume: 0.5 mL.

impurities which were eluted after PGS. The retention of these degraded products decreased as the temperature increased on both columns. At high temperature, these impurities incorporated into the peak of PGS

as shown in Figure 6. High temperature hardly affected the separation of macromolecular impurities from PGS, but resulted in a poor separation between PGS and degraded products. Considering the stability and purification of PGS, temperature below 15°C was preferred.

Detection of Penicilloylated Impurities by Penamaldate Method

Penicilloylated impurities collected from the column of G-10 were detected by the penamaldate method (Figure 7). High *P* values in the fractions corresponding to the elution volumes of macromolecular impurities indicated the existence of penicilloylated impurities, like penicilloylated proteins, penicilloylated peptides, polymers, or penicilloic acids. The penamaldate stability was smallest (low PS_{10} value) in the fraction with the elution volume of 0.5. This should be caused by penicilloic acids as they were eluted at the same position as shown in Figure 6. Penicilloic acids, which were degraded products of PGS and isomeric with PGS, were eluted earlier than PGS. This might be caused by the increasing steric hindrance of side chain adsorption resulting from opening of the β-lactam ring, or the increasing electrostatic repulsive interaction resulting from the additional carboxylic group formed by hydrolysis of PGS.[23] The *P* value in fractions at the elution volume of PGS might

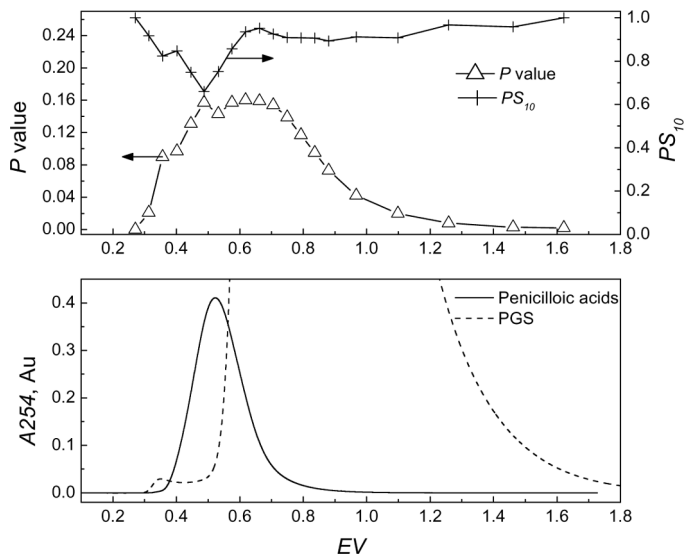

Figure 7. Detection of Penicilloylated impurities separated on the column of G-10 using 5 mM citrate buffer as mobile phase.

be caused by PGS degradation, since it took hours from collection to detection. PGS was liable to degrade, thus would interfere with detection.

Figures 8 and 9 were the results of the penamaldate method on the column of HW-40F with the mobile phases of 5 and 20 mM citrate buffers, respectively. The separations on HW-40F were better compared with that on G-10. PGS could well be separated from penicilloylated impurities when 5 mM citrate buffer was used as the mobile phase. Penicilloylated impurities and penicilloic acids were both eluted earlier than PGS. The prepared penicilloic acids contained more than one peak in the mobile phase of 5 mM citrate buffer (Figure 8). This might be caused by different steric structures of penicilloic acids. Penicilloic acids prepared from PGS in alkaline media were mixtures of isomers epimerizing at C-5 and C-6. Different steric structures of penicilloic acids might result in unequal electrostatic repulsive interactions with gel at low buffer concentration of 5 mM. The low molecular weight impurity eluted after PGS (Figure 1) was also detected by the penamaldate method. Confirmation of its structure needed a further study.

Increased buffer concentration could not improve the separation of penicillyolated impurities. As shown in Figure 9, the peaks of macromolecular impurities were obviously broadened, and the region corresponding to penicilloylated impurities overlapped slightly with that of PGS.

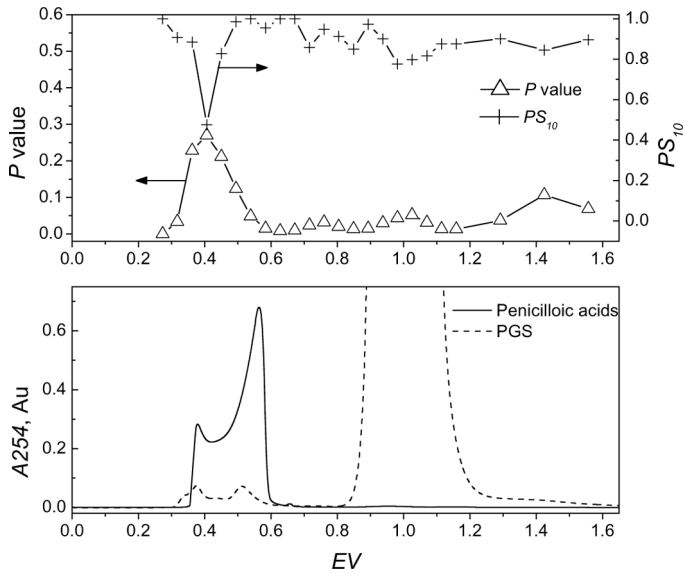

Figure 8. Detection of Penicilloylated impurities separated on the column of HW-40F using 5 mM citrate buffer as mobile phase.

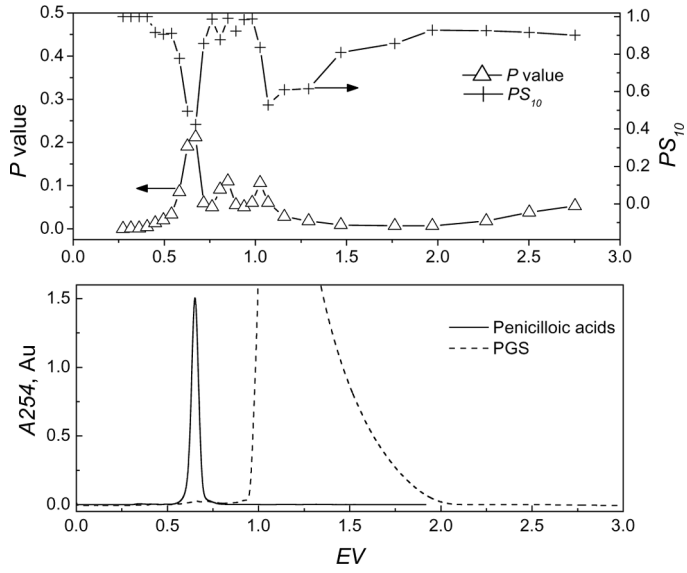

Figure 9. Detection of Penicilloylated impurities separated on the column of HW-40F using 20 mM citrate buffer as mobile phase.

Although PGS had higher adsorption interactions with gels at higher buffer concentrations, its adsorption capacity was low. On one hand, the elution volume of PGS decreased with a larger amount of sample (0.2 g at this experiment); on the other hand, the elution volume of penicilloylated impurities increased with increasing buffer concentration. These led to a poor separation with 20 mM citrate buffer. At high buffer concentration, penicilloic acids were eluted later than in low buffer concentration. Isomers were incorporated into one peak.

CONCLUSIONS

Macromolecular impurities in PGS were well separated by gel filtration chromatography. Factors related to the separation were studied, and mechanism of interactions between PGS and gels was discussed. The results demonstrated that the behavior of PGS on the gels of G-25, G-10, and HW-40F was a combination of the molecular sieving mechanism and gel solute interactions. The chromatographic behavior was greatly affected by the cross linking degree of gel, the ion strength and type of mobile phase, and the structure of solute. Temperature had little effect on the chromatography of macromolecular impurities and PGS, while it obviously affected the retention of small impurities, which were

degraded from PGS. Compared with other two gels, HW-40F was a better choice for the separation of macromolecular impurities from PGS. The suitable condition was using 5 mM citrate buffer as mobile phase at temperature below 15°C.

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