African Journal of Biotechnology Vol. 7 (22), pp. 3995-4003, 19 November, 2008
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2008 Academic Journals

Full Length Research Paper

Adsorption separation of 3β-D-monoglucuronyl-18β-glycyrrhetinic acid from directional biotransformation products of glycyrrhizin

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Accepted 22 September, 2008

3β-D-monoglucuronyl-18β-glycyrrhetinic acid (GAMG) can be obtained on a large scale by directional hydrolysis of glycyrrhizin (GL) with β-glucuronidases, which has more physiologically potential application than GL in a wide range of biological activities. In this study, the preparative separation of GAMG in hydrolysates of GL by macroporous resins (NKA-9, D4020, HZ-803, 1300, HP-20 and X-5) were studied systematically, and the column packed with selected resin was used to perform dynamic adsorption and desorption tests to optimize the separation process. The results shown that NKA-9 resins was the appropriate resin for the separation of GAMG and GL under pH 5, which adsorption data fitted to the Freundlich isotherm equation and pseudo-second-order kinetic model at 25ºC. While with desorption of 70-90% ethanol-water solution, GAMG with the purity of 85.02% was obtained on a NKA-9 resins packed column. Comparing with conventional methods, the developed method can successfully improve product yield and reduce the consumption of raw materials in the biotransformation process of GL. The structure identification of each pure fraction was carried out by ^{1}H and ^{13}C NMR.

Key words: 3β-D-monoglucuronyl-18β-glycyrrhetinic acid (GAMG), glycyrrhizin (GL), adsorption separation, macroporous resin, directional bio-transformation.

INTRODUCTION

Glycyrrhizin (GL) as a triterpenoid saponin derived from the root of licorice (*Glycyrrhiza glabra*) which is one of the traditional medical herbs most popular around China, is composed of one molecule of glycyrrhetic acid (GA) as aglucon and two molecules of glucuronic acid (Shiraki et al., 2004; Hennell et al., 2008). With cleavage of one terminal glucuronic acid by hydrolysis, GL can be transformed into glycyrrhetic acid monoglucuronide (GAMG) which has several advantages over GL (Mizutani et al., 1998; Lu et al., 2006). For example, GAMG has high sweetness and low calorie; sweetness is 941 times of the sucrose, and 5 times of the GL (Kuramoto and Yamamoto, 1990; Akao et al., 1991; Kuramoto et al., 1992; Feng et al., 2006). In addition, pharmacological researches have shown that LD_{50} of GAMG is 5000 mg/kg while that of GL was 805 mg/kg (Feng et al., 2006), which means that GAMG has negligible toxicity and higher safety than GL. On the other hand, GAMG possesses a wide range of biological activities better than GL, such as anti-cancer (Kozuka et al., 1993; Mizutani and Tamura, 1995; Park et al., 2004), anti-anaphylaxis (Park et al., 2004), anti-virus (Ito et al., 1988), and anti-inflammatory (Akao et al., 1992). Therefore, GAMG is expected to be a potential substitute of GL in wider applications.

In our previous work (Lu et al., 2006), we had produced GAMG from GL by hydrolysis of β-glucuronidase, which was prepared from liver of domestic duck. The biocatalytic reaction is illustrated in Figure 1. The enzymatic transformation system includes GAMG and non-transformed GL. In order to obtain highly purified GAMG, a suitable method to separate GAMG from hydrolysates of GL is required for the further pharmacological investi-

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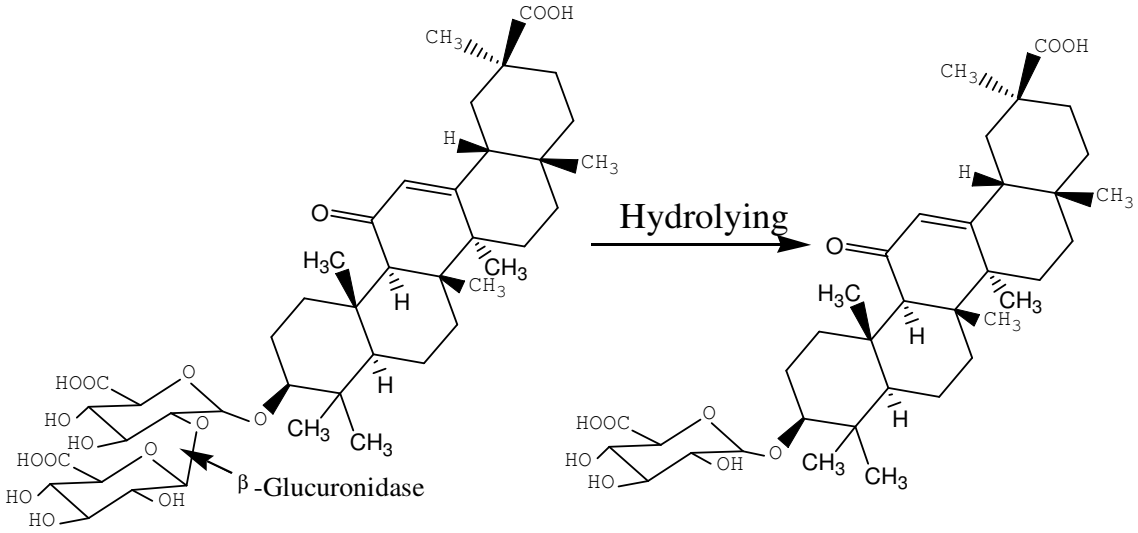

Figure 1. Schematic illustration of hydrolysis process of GL.

gation and application research as natural sweetening agent.

Although many methods such as organic solvent extraction (Ong and Len, 2003; Eng et al., 2007) or adsorption separation by macroporous resins (Fu et al., 2005) had resulted in purification of GL from liquorice extract, the preparative separation of GAMG had not been carried out. In recent years, macroporous resins have been extensively used for the separation and purification of bioactive components from many crude extracts of herbal raw materials (Fu et al., 2005; Wang et al., 2008a,b). It is an ideal method with high adsorption capacity, less solvent consumption, relatively low cost and easy resin regeneration. Purification through adsorption is based on differences in molecular weight, polarity, or shape of molecules in the solution, which leads to differences in affinity to the adsorbent (Wan et al., 2008).

GAMG and GL have difference in molecular polarity because GAMG is produced by selective removal of one molecule of glucuronic acid from GL, so the adsorption separation of GAMG from hydrolysates of GL can be realized by macroporous resins. Therefore, the objectives of the present study were to investigate the adsorption and desorption properties of GAMG from directional biotransformation products of GL on different macroporous resins, and develop an efficient method for the preparative separation of GAMG with selected resin. The results of this study are significant for the preparative separation of important products with high additional value from directional hydrolysis system from other biotransformation system in general.

MATERIALS AND METHODS

Reagents

Standard glycyrrhizinic acid was purchased from Sigma (USA). Authentic GAMG sample was a kind gift from Prof. Dong-hyun Kim (College of Pharmacy, KyungHee University, Korea). Glycyrrhizinic acid (monoammonium salt) was purchased from Gansu Lante Phytochemical Co. Ltd. Methanol was of chromatography grade. Freshly deionized water (18.2MΩ) purified by a Millipore Milli-Q® was used in HPLC. All other chemicals were obtained commercially.

Adsorbents

Macroporous resins including NKA-9, D4020 and X-5 were purchased from Chemical Plant of Nankai University (Tianjin, China). HZ-803 resin was obtained form Zhenghua Technology and Trade Co. Ltd. (Shanghai, China). Resin 1300 was provided by pharmaceutical factory of Yangzhou (Jiangsu, China). HP-20 resin was bought from Mitsubishi Co. Ltd. (Japan). The physical properties measured to correlate with the adsorption capacity of the adsorbents were summarized in Table 1.

These macroporous resins were pre-treated with 95% ethanol, shaken for 24 h until they swelled and then washed with ethanol until there was no white cloudiness, even when some water was added to the elute. Subsequently, the resins were washed with distilled water to remove ethanol, then by 5% (v/v) hydrochloric acid, followed by 2% (mass fraction) sodium hydroxide to eliminate other impurities. The washed resins were immersed in ethanol for reserve.

Analytical methods

HPLC was performed using LB-5 pump with a UV detector and N2000 workstation (Lu et al., 2006). Sample analyses were performed on a Apollo C_{18} column (250 × 4.6 mm i.d., 5 μm) with a liner elution using methanol/water/acetic acid (75:25:5, v/v/v) solution at a flow rate of 1.0 mL/min, and at a column temperature of 25ºC. The detection wavelength was set at 254 nm and the sample injection volume was 20 μL. In addition, all of samples were filtered through a 0.45 μm one-off filtration membrane prior to injection into the HPLC system. Concentrations of GAMG were calculated from peak areas using a calibration curve. ^{1}H NMR and ^{13}C NMR spectra were recorded in CD_3OD–D_2O (1:1, v/v) using a Bruker 400 Hz spectrometer.

Table 1. Physical property of the test macroporous resins.

Trade name	Polarity	Particle diameter (mm)	Specific area (m^2/ g)	Mean pore size
D4020	Non-polar	0.3-1.25	540-580	100-105 nm
HP-20	Middle-polar	0.3-1.25	600	>200Å
HZ-803	Non-polar	0.3-1.25	550-600	60Å
NKA-9	Non-polar	0.3-1.0	570-590	——
X-5	Non-polar	0.3-1.25	500-600	290-300 nm
Resin 1300	Non-polar	0.2-1.25	600	——

The working calibration curve based on GL and GAMG standard solutions showed good linearity within the range of 0.2-20 μg. The regression line for GL and GAMG were $\rho=3.31\times10^{-7}A-4.40\times10^{-3}$, $R=0.9996$, $\rho=4.41\times10^{-7}A-8.50\times10^{-3}$ and $R=0.9984$, respectively, where A is the peak area of GL and GAMG, and ρ is the concentration of GL and GAMG (mg/mL).

Preparation of GAMG from directional biotransformation products of GL

Crude GL hydrolasate was prepared with the method in our early experiment (Lu et al., 2006). GL was weighed exactly (~30 g) and dissolved into 3000 mL pH 5.8 phosphate buffer solution, supplied with 20 g crude GL hydrolasate, and then put in thermostatic water bath at 55ºC for 72 h with the electromagnetic stirring. When the reaction was finished, the crude GL hydrolasate was further purified by extraction method. In the condition of pH 4.0~5.0, 25ºC, phase ratio=1.5/1, extraction phase was combined and dried in a vacuum evaporating equipment after the solution of enzymatic transformation was extracted three times with ethyl acetate. The obtained dry powder was ground and stored in refrigerator at 0-4ºC.

Static adsorption and desorption tests

The static adsorption tests of GL and GAMG were carried out with following procedure. A changeless amount of the test resins, equaling to 1.0 g wet resins, were placed into 50 mL air-tight Erlenmeyer flask. And 30 mL of aqueous solution of crude GAMG was added into each flask. The flasks were then shaken (130 rpm) in a water bath shaker at 25ºC for 12 h. The solution after adsorption was analyzed by HPLC. After the adsorption equilibrium was reached, the resins were desorbed with 10 mL of 90% ethanol solution. The flasks were shaken for 12 h at 25ºC, and desorption solutions were also analyzed by HPLC. The preliminary choice of these resins was evaluated by adsorption capacity and the ratio of adsorption and desorption.

The following equation was used to quantify the capacities of adsorption and desorption. Adsorption evaluation is:

$$E = \frac{(C_0 - C_e)}{C_0} \times 100\% \quad (1)$$

Where E is the adsorption ratio (%), which is the percent of the mass of total adsorbate being adsorbed after reaching equilibrium; C_0 and C_e are the initial and equilibrium concentrations of solutes in the solutions, respectively (mg/mL) (Wang et al., 2008a). Desorption evaluation is:

$$D = \frac{C_d V_d}{(C_0 - C_e)V_i} \times 100\% \quad (2)$$

Where C_d is the concentration of the solute in the desorption solution (mg/mL); V_d the volume of the desorption solution (mL); V_i the volume of the initial sample solution (mL); D the desorption ratio (%); C_0 and C_e are the same as described above (Wang et al., 2008b).

The adsorption capacity of different resins towards GL and GAMG were evaluated under different solution pH. The adsorption isotherm of GL and GAMG on the selected resins were investigated by contacting 30 mL of aqueous solution of crude GAMG at different concentrations with pre-weighed amount of hydrated resins on a water bath shaker at 25ºC for 12 h, and their degrees of fitness to Henry, Langmuir and Freundlich isotherms were evaluated (Wang et al., 2008a). And then, for pseudo-second-order kinetic model study, the selected resins were investigated (Noroozi et al., 2007). 1.0 g (wet weight) resins were kept in contact with 30 mL of aqueous solution of crude GAMG. The initial concentration of GL and GAMG were 1.3 and 2.4 mg/mL, respectively, and with pH 5. The respective concentration of GAMG and GL were monitored at the certain intervals till adsorption equilibrium.

Dynamic adsorption and desorption tests of NKA-9 resins

Dynamic adsorption experiments were carried out in a glass column (Φ1.0 cm×30 cm) wet-packed with the selected NKA-9 resins (10.0 g wet resin). The bed volume (BV) of the resin was 20 mL. The feed flow rate was 3 BV/h. The GL and GAMG in the eluent were monitored by HPLC analysis of the eluted aliquots collected at 6.4 mL of intervals. Breakthrough point was indicated when GAMG was detected in the eluent. The column was washed first with deionized water, and then desorbed with aqueous-ethanol (30-50%- 70-90%) solution. The eluents were concentrated and dried under vacuum before further analyses.

RESULTS AND DISCUSSION

Adsorbents capability of different resins

Selectivity of resins was the difference of capable of binding to adsorbed substance. The selectivity of resins focuses on exchange equilibrium constant (K), the equation is as follow:

$$K_{GL}^{GAMG} = \frac{[R-GAMG][GL]_S}{[R-GL][GAMG]_S} \quad (3)$$

Table 2. The test result of adsorption rate.

Trade name	Adsorption rate (%)					
	D4020	HP-20	HZ-803	NKA-9	X-5	1300
GL	6.86	31.53	46.86	5.57	18.54	32.85
GAMG	51.56	96.50	55.15	90.56	91.92	88.03
Adsorption exchange coefficient	14.49	59.84	1.39	162.69	50.00	15.02

GL: Glycyrrhizin; GAMG: glycyrrhetic acid monoglucuronide.

Table 3. The effect of the pH value of the solution on the adsorption ratio of three macroporous resins.

pH	Adsorption ratio for GL (%)			Adsorption ratio for GAMG (%)		
	HP-20	NKA-9	X-5	HP-20	NKA-9	X-5
4	45.81	8.65	36.92	90.78	78.97	85.73
5	30.42	4.78	18.08	95.49	89.67	91.32
6	16.52	4.47	10.09	91.02	85.35	84.64
7	11.13	3.20	10.88	67.20	64.18	64.67
8	6.45	1.50	5.31	66.19	58.20	61.05

Where [R-GL], [R-GAMG] are the concentrations of GL and GAMG which combine with resins; $[GL]_S$, $[GAMG]_S$ the equilibrium concentrations of GL and GAMG in solutions, respectively.

The results of adsorption rate of six macroporous resins for GL and GAMG were listed in Table 2. As can be seen from Table 2, the results showed the adsorption capacities of HP-20, NKA-9 and X-5 resins towards GAMG were considerably higher than those of the other resins; moreover the 3 resins, ranked according to their absorption and exchange capacity (exchange equilibrium constant K) toward GAMG, were as follows: NKA-9 > HP-20 > X-5. On the contrary, the non-polar resins NKA-9 also showed rather low adsorption than all other resins, and the X-5 was secondary. The result showed that resins with non-polarity show stronger adsorption ability to weak-polar substance. GAMG is weak-polar, which is composed of non-polar glycyrrhetic acid and polar glycoside. Hence HP-20, NKA-9 and X-5 resins were selected to further study their adsorption behavior towards GAMG and GL.

Influence of initial solution pH value

The pH value is an important factor in the process of adsorption and desorption of resins, because the pH determines the extent of ionization of GAMG and GL molecules, thereby affecting their adsorption affinity.

As shown in Table 3, for the three resins selected, a decrease of pH value results in an increase of adsorption ratio for GAMG, and the highest adsorption ratio appeared at the pH values of 5. Meanwhile, the adsorption ratio for GL was lower nearby the pH 5. At higher pH, the hydrogen bonding interactions are reduced because the phenolic hydroxyl groups in GAMG and the carboxyl groups in GL both dissociate to form H^+ and their corresponding anions. Such ionization processes also reduced their adsorption on those resins. Thus, the pH of the solution was adjusted to 5 for the following tests.

Adsorption isotherms

Isotherms play a crucial role in model prediction and design of adsorption systems. Considering static equilibrium adsorption, adsorption dynamics and equilibrium rate constant, HP-20, NKA-9 and X-5 resins were adaptable for adsorbents of GAMG. Therefore, adsorption isotherms were obtained by contacting 10 mL of aqueous solution of crude GAMG at different concentrations with 3 types of resins on a water bath shaker at 25ºC. The C_0 of aqueous solution of crude GAMG were 0.6, 1.2, 1.8, 3.6, 4.8 and 6.6 mg/mL, respectively.

The Henry, Langmuir and Freundlich isotherms are the best known and the most often used isotherms for the adsorption of solutes from a solution in the bio-separation process (Wang et al., 2008a). The Henry, Langmuir and Freundlich parameters were obtained from the Henry, Langmuir and Freundlich equations, and summarized in Figure 2. The correlation coefficients of the Henry, Langmuir and Freundlich equations on NKA-9 resin were slightly higher than HP-20 and X-5 resins, especially the equilibrium adsorption isotherm of NKA-9 that highly fit to the Langmuir and Freundlich models. The Langmuir model assumes monomolecular layer adsorption with a homogeneous distribution of adsorption energies and without mutual interaction between adsorbed molecules, while the Freundlich model can be used to describe the adsorption behavior of monomolecular layer as well as that of the muli-molecular layer (Wang et al., 2008a). The Freundlich equations are the most popular ones frequently used in description of the experimental data of adsorption isotherms because of their relative simplicity

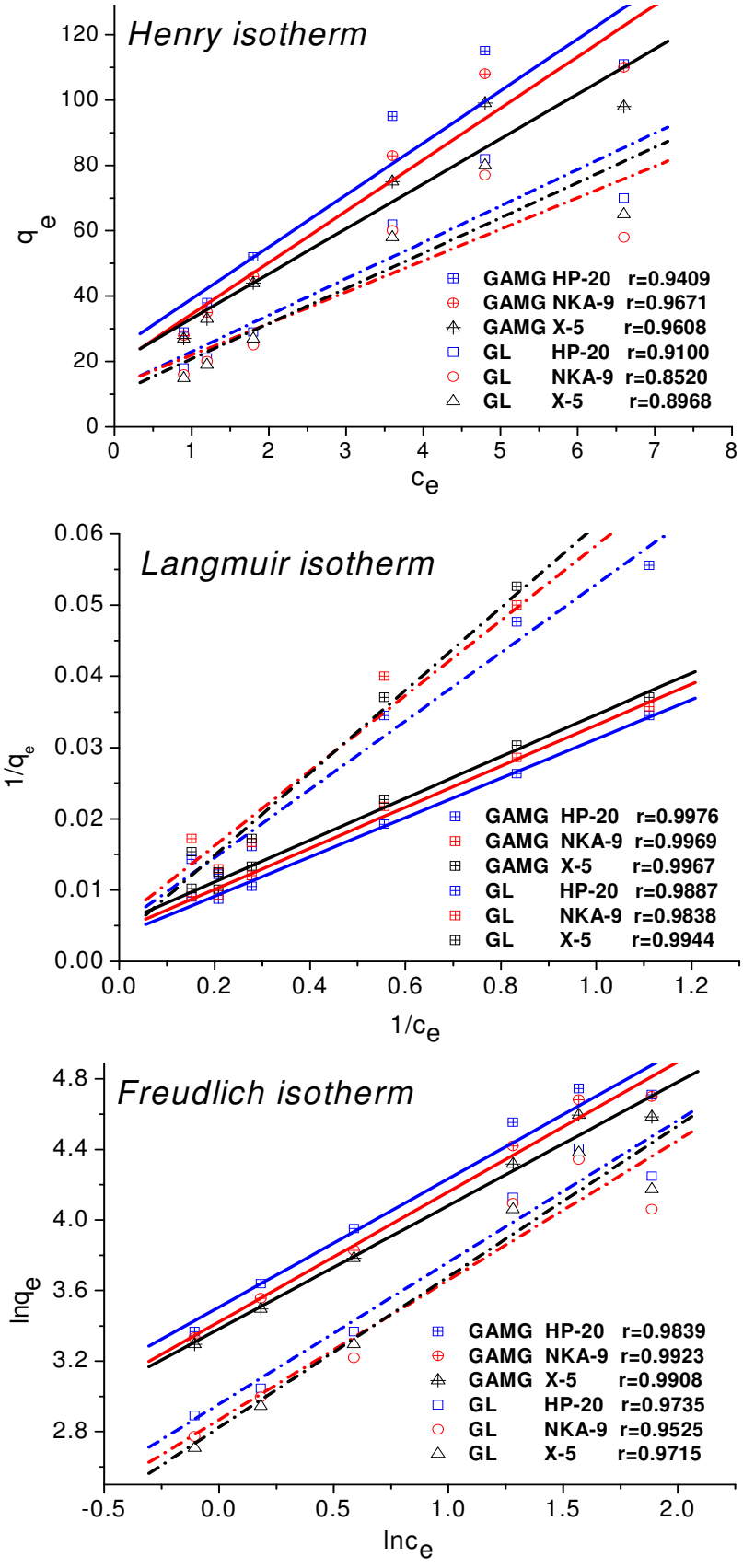

Figure 2. Adsorption isotherms for GL and GAMG on HP-20, NKA-9 and X-5 resins at 25ºC.

Table 4. Freundlich adsorption parameters of GL and GAMG on HP-20, NKA-9 and X-5 resins.

Resins		X-5	NKA-9	HP-20
GL	Freundlich equation	$Q_e = 20.17C_e^{0.7211}$	$Q_e = 20.87C_e^{0.6687}$	$Q_e = 22.49C_e^{0.6917}$
	r	0.8353	0.7829	0.8446
	1/n	0.7211	0.6687	0.6917
GAMG	Freundlich equation	$Q_e = 29.95C_e^{0.7125}$	$Q_e = 33.09C_e^{0.7190}$	$Q_e = 37.30C_e^{0.6472}$
	r	0.9872	0.9879	0.9298
	1/n	0.7125	0.7190	0.6472

and reasonable accuracy (Baskaralingam et al., 2006). The experimental data were fitted to the Freundlich equation (4) to describe how solutes interact with the adsorbents:

$$Q_e = K_F C_e^{1/n} \quad (4)$$

Where K_F is the Freundlich constant that indicates the adsorption capacity, 1/*n* value is obtained from the slope in linear regression result, and the empirical constant also related to the magnitude of the adsorption driving force (Fu et al., 2005). The Freundlich parameters are summarized in Table 4.

Commonly, the extent and degree of favourability of adsorption can be described by the Freunlich constant 1/*n*, the adsorption being favourable for 1/*n* comprising between 0.1 and 1.0 (Silva et al., 2007; Wan et al., 2008). On the contrary, the adsorption was very difficult to occur if *1/n* value is above 1. Furthermore, 1/*n* is also reflected to adsorptive energy or intensity. The results showed that the 1/*n* value of GAMG on NKA-9 resins (0.7190) was higher, and *1/n* value for GL (0.6687) was the smallest compared with those of other resins, which indicate that NKA-9 resins was the more appropriate for separation of GAMG from GL. Moreover, the correlation coefficients for the parameter estimations were consistently over 0.98 which was quite good. Therefore, NKA-9 resin was selected for further study.

Adsorption kinetics on NKA-9 resins

Adsorption kinetics curve was obtained for GAMG and GL on NKA-9 resins. As shown in Figure 3, the adsorption capacity of NKA-9 resins increased rapidly in the first 6 h, and then increased slowly and reached equilibrium at around 10 h with the adsorption capacity of 97% to GAMG. The fast adsorption process of NKA-9 resins in 6 h was due to high diffusivity of GAMG into micropore of the resin in the bulk solution, and the slow adsorption process of NKA-9 resins after 10 h was because of the high intraparticle mass transfer resistance within the macroporous resins. On the other hand, the adsorption rate of GL increased with the extension of

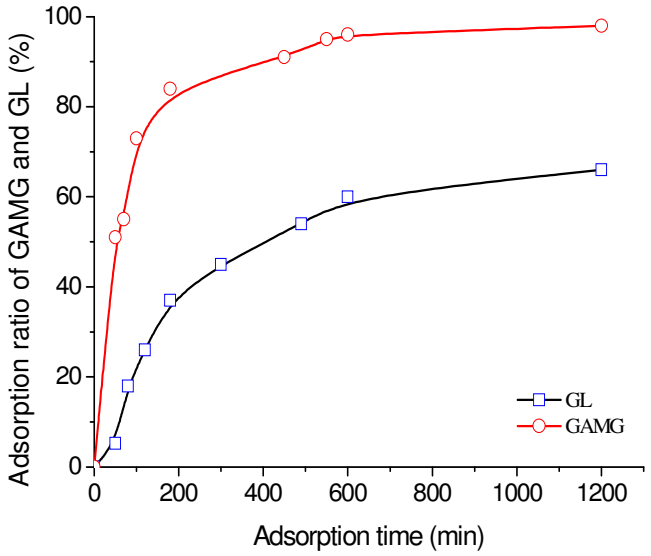

Figure 3. Adsorption kinetic curves on NKA-9 resin of GAMG and GL form glycyrrhizin hydrolysates.

adsorption time, but with a slow speed. After 10 h GL reached equilibrium with adsorption capacity of 75%, and the adsorption rate was slower. Meanwhile, at any time the adsorption capacity of NKA-9 towards GAMG was higher than that of GL.

The kinetic adsorption data can be processed to understand the dynamics of the adsorption reaction in terms of the order of the rate constant. The process of adsorption separation of GAMG from aqueous phase by a certain adsorbent may be represented by pseudo-first-order kinetics (reversible or irreversible) or pseudo-second-order kinetics (Noroozi et al., 2007). The pseudo-second-order kinetic model is expressed as:

$$\frac{t}{q_t} = \frac{1}{k_{ad2} q_e^2} + \frac{t}{q_e} \quad (5)$$

where k_{ad2} (g/min mg) is the rate constant of pseudo-secondorder model adsorption. $k_{ad2}q^2_e$ considered as the initial adsorption rate (mg/g min).

If pseudo-second-order kinetics is applicable, a plot of

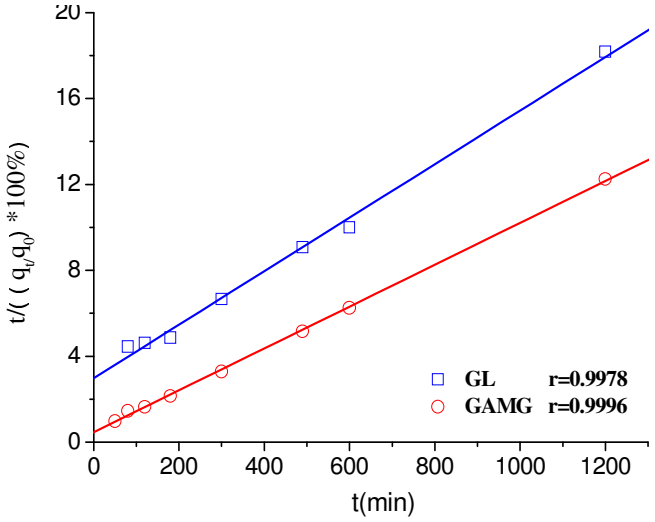

Figure 4. Adsorption rate constants in pseudo-second-order adsorption kinetics model on NKA-9 resin.

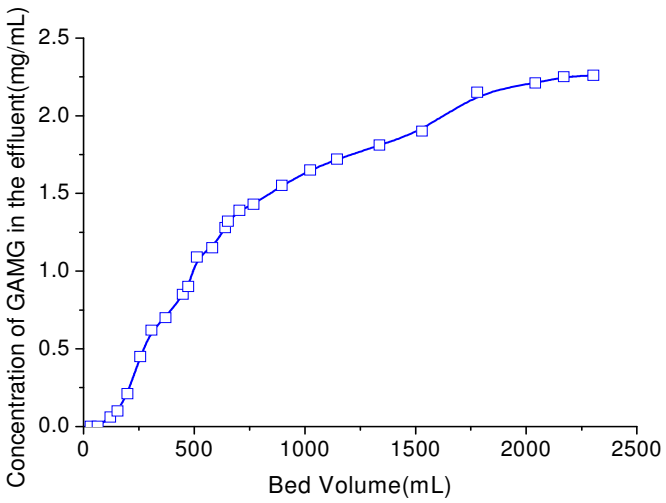

Figure 5. Breakthrough curve for GAMG on NKA-9 resins.

t/qt versus t should provide a linear relationship (Noroozi et al., 2007). Adsorption rate constants in pseudo-second-order adsorption kinetics model on NKA-9 resin (Figure 4). The calculated regression coefficients of GAMG and GL for reversible pseudo-first-order model were 0.9978 and 0.9996, respectively. This could be due to the greater concentration gradient between the solid and liquid phases at the higher GAMG and GL concentration.

Therefore, in the comprehensive consideration of the adsorption capacity, desorption ratio, adsorption isotherms and kinetics, it is proved once again that the selected NKA-9 resins were the most suitable resins for the separation of GAMG and was used in the following test.

Dynamic desorption and gradient elution experiments on NKA-9 resins

Breakthrough volume is important in solid-phase extraction (Jung et al., 2001). Breakthrough volumes were calculated at which exit solute concentration reached to 1% of the inlet concentration. The results are shown in Figure 5. The breakthrough volume of GAMG was 147.2 mL (approximately 7 BV) on NKA-9 resins. In order to optimize the appropriate ethanol concentration to separate GAMG from glycyrrhizin hydrolysates, and make the separation run efficiently and economically, gradient elution tests were carried out under the following conditions: the column was washed successively with 2 BV deionized water, 30, 50, 70 and 90% ethanol. The result was shown in Figure 6. When the ethanol concentration is over 70%, partially purified GAMG was mainly collected, which accounted for 35.36% of the whole eluted glycyrrhizin hydrolysates. Thus, the 70-90%

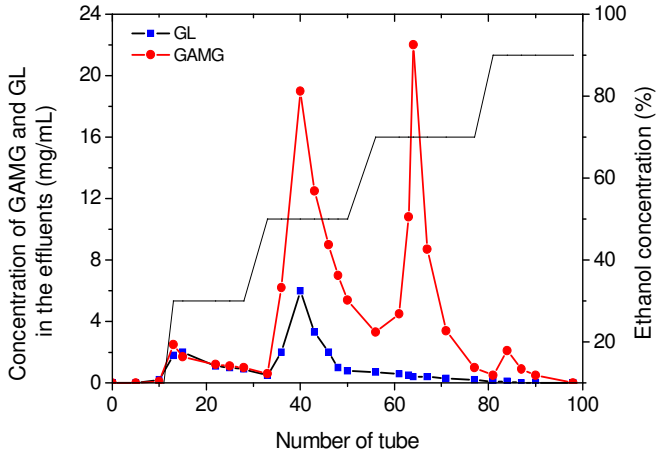

Figure 6. Dynamic desorption curve for GL and GAMG with different concentration effluents.

ethanol eluate was collected as final products, and GAMG with the purity of 85.02% was obtained. The results demonstrated a good selectivity of the adsorption/desorption process for recovering GAMG from the glycyrrhizin hydrolysates.

Finally, the 70-90% ethanol eluate was concentrated to dry by rotary vacuum evaporation. Recrystallization by 85% aqueous methanol, the refinement of GAMG with high purity was obtained.

1H NMR and 13C NMR spectrum of GAMG

^{1}H NMR and ^{13}C NMR spectrum were recorded in $CD_3OD–D_2O$ (1:1, v/v) using a Bruker 400 Hz spectrometer. For GAMG, ^{1}H NMR (400 MHz, CD_3OD-D_2O, 1:1,

v/v) δδ(ppm): 0.66 (s, *CH3*), 0.71 (s, *CH3*), 0.92 (s, *CH3*), 0.98 (s, *CH3*), 1.05 (s, *CH3*), 1.09 (s, *CH3*), 1.26 (s, *CH3*), 4.32 (d, *J* 7.4, anomeric proton), 5.45 (s, *CH*=). ^{13}C NMR (400 MHz, CD_3OD/D_2O, 1:1, v/v) δ(ppm): 39.1 (C-1), 26.5 (C-2), 90.1 (C-3), 39.4 (C-4), 55.1 (C-5), 17.2 (C-6), 32.6 (C-7), 43.6 (C-8), 62.0 (C-9), 36.8 (C-10), 203.1 (C-11), 127.5 (C-12), 175.5 (C-13), 45.8 (C-14), 25.7 (C-15), 26.2 (C-16), 31.9 (C-17), 49.9 (C-18), 41.2 (C-19), 44.1 (C-20), 30.9 (C-21), 37.8 (C-22), 27.4 (C-23), 15.9 (C-24), 16.1 (C-25), 18.3 (C-26), 22.8 (C-27), 27.9 (C-28), 28.2 (C-29), 181.1 (C-30), 105.3 (C-1 '), 82.1 (C-2 '), 76.3 (C-3 '), 74.0 (C-4 '), 72.3 (C-5 '), 173.4 (C-6 ').

From the data of ^{1}H NMR and ^{13}C NMR, it could be concluded that the separation and purification of compounds was 18β-glycyrrhetic acid 3-O-β-D-glucuronide, which was effectively produced by selectively hydrolyzing one glucuronic acid from GL.

Conclusions

The preparative separation process of GAMG with macroporous resin has been successfully developed in this study. Among the six resins investigated, NKA-9 resin offered the best separation efficiency for GAMG from the hydrolysates of GL because of its high surface area, optimum average pore diameter and appropriate surface functional residues, which adsorption data fitted better to the Freundlich isotherm and pseudo-second-order kinetic model at 25ºC. Using the NKA-9 resin at optimal conditions, the purity of 85.02% for partially purified GAMG was higher than the purity of 63.59% for the aqueous solution of crude GAMG, with a recovery yield of 35.36%. Comparing with conventional methods, the developed method can successfully improve product yield and reduce the consumption of raw materials in the biotransformation process of GL. The structure identification of each pure fraction was carried out by 1H and 13C NMR.

ACKNOWLEDGEMENTS

This work was supported by National Basic Research Program (2003CB716000), National Natural Science Foundation of China (Project 2067060) and Specialized Research Fund for the Doctoral Program of Higher Education (20040291004) of China. The authors are grateful to Prof. Dong-hyun Kim (College of Pharmacy, KyungHee University, Korea) for the gift of authentic GAMG.

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