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Short communication

Phloroglucinol: novel synthesis and role of the magnesium cation on its binding with human serum albumin (HSA) using a biochromatographic approach based on Langmuir isotherms

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Abstract

In this paper, a new and efficient method for synthesis of phloroglucinol with an overall yield of 60% was described. As well, the phloroglucinol association on an immobilized human serum albumin (HSA) column was analyzed in biochromatography by the determination of its Langmuir distribution isotherms. The role of the magnesium cation Mg^{2+} on the phloroglucinol–HSA binding process was as well analyzed. The results showed that in the Mg^{2+} concentration range (0.7–2 mM) (including its biological concentration range, i.e. 0.75–0.90 mM), increasing the Mg^{2+} concentration increased the fraction of free phloroglucinol (not linked with HSA) and thus its biological effect.

Keywords: Phloroglucinol; Magnesium cation; Langmuir isotherms

1. Introduction

Phloroglucinol (P) and its trimethoxy derivative have been known for several years. Many processes have been described for the synthesis of this compound [1–3]. Among these processes, only two ways can be used for an industrial application. The first one consisted in an amination of 3,5-diaminochlorobenzene [3] followed by an acid hydrolysis and the second one consisted in both a reduction and a decarboxylation of trinitrotoluene (TNT). As for magnesium, phloroglucinol had antispasmodic properties [4–6]. Human serum albumin (HSA) is the most abundant protein in blood and can reversibly bind a large number of pharmacological substances such as phloroglucinol. HSA was the model ligand used in a great number of studies. The main advantage of using HSA is that data are available for its interaction with a wide range of organic and inorganic compounds [7]. Biochromatography with HSA immobilized on the

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support is specially suited to the study of drug–protein interactions. The association constants of many ligands have been determined by zonal elution [8] or frontal analysis [9]. The thermodynamic process involved in the binding have already been studied [10–15]. In this paper, a novel method to obtain phloroglucinol in two steps from halogenobenzene was presented. As well, the association constant between phloroglucinol and HSA and the role of the magnesium cation on its association using a biochromatographic approach based on Langmuir adsorption isotherms were analyzed.

2. Materials and method

2.1. Chemistry

Infrared spectra were taken in potassium bromide pellets on a Shimadzu FTIR-8201PC spectrometer.

^{1}H NMR spectra were recorded on a Bruker AC 300 spectrometer at 300 MHz using tetramethylsilane as the internal standard. Chemical shifts are reported in parts per million and signals are quoted as *s* (singlet), *d* (doublet), *t* (triplet), *q* (quadruplet) and *m* (multiplet).

Elemental analyses were carried out at the Central Service of Analysis, Centre National de la Recherche Scientifique (CNRS), 69390 Vernaison, France.

The new synthetic procedure to obtain phloroglucinol in two steps is shown in Fig. 1. Compound (1) was obtained by nitration [18] of halogenobenzene with sulfuric acid and fuming nitric acid. The reduction in presence of tin [1,19] and hydrochloric acid gives a non-isolated compound (2), which leads to phloroglucinol (3) after hydrolysis.

2.1.1. 1,3,5-Trinitrobromobenzene (1a)

A solution of (5 ml, 4.75 mmol) of bromobenzene in 60 ml of concentrated sulfuric acid and 20 ml of fuming nitric acid was heated to reflux during 17 h. After cooling, the precipitate product is collected by filtration and then heated at 140 °C during 12 h in a mixture of 30 ml of sulfuric acid

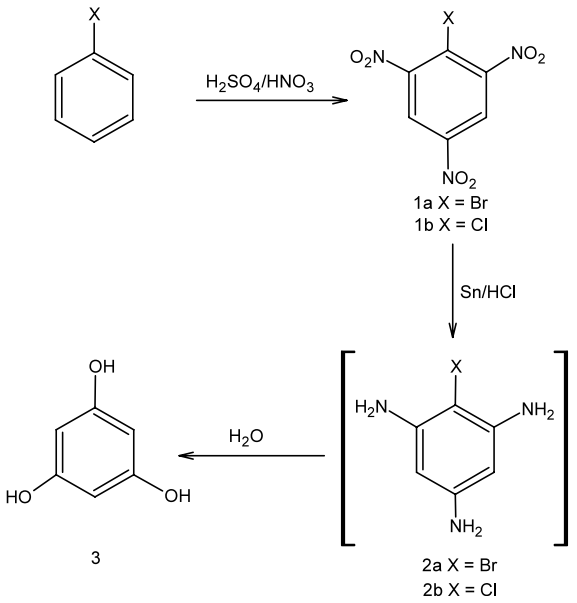

Fig. 1. Synthetic route to compounds 1–3.

and 10 ml of fuming nitric acid. The resulting mixture was poured in ice. The precipitate was filtrated and the product was re-crystallized from 50 ml of ethanol to give yellow crystal (9 g; 65%); m.p. 122 °C, IR (KBr): ν 1360 cm^{-1} (NO_2); 1550 cm^{-1} (NO_2), ^{1}H NMR ($CDCl_3$): δ 8.2 (*s*, 2H).

Anal. Calc. for $C_6H_2BrN_3O_6$: C, 24.68; H, 0.69; Br, 27.36; N, 14.39. Found C, 24.76; H, 0.68; Br, 27.39; N, 14.27.

2.1.2. 1,3,5-Trinitrochlorobenzene (1b)

Prepared from chlorobenzene in a same manner as described for 1a. The resulting mixture was poured in ice. The precipitate was filtrated and the product was re-crystallized from 50 ml of ethanol to give yellow crystal (8.3 g; 70%); m.p. 84 °C, IR (KBr): ν 1350 cm^{-1} (NO_2); 1548 cm^{-1} (NO_2), ^{1}H NMR ($CDCl_3$): δ 8.35 (*s*, 2H).

Anal. Calc. for $C_6H_2ClN_3O_6$: C, 29.11; H, 0.81; Cl, 14.32; N, 16.97. Found C, 29.23; H, 0.82; Cl, 14.26; N, 16.92.

2.1.3. Phloroglucinol (3) = (P)

Compound (3) (24 g, 200 mmol) of tin were added by small portions to (5 g, 20 mmol) of

chlorotrinitrobenzene with 50 ml of concentrated hydrochloric acid at 60 °C. The reaction mixture was heated at 110 °C during 4 h. The resulting mixture was cooling and 10 N aqueous sodium hydroxide was added until persistence of the precipitate. The reaction mixture is heated in 100 °C during 24 h. The mixture was then filtered, and the filtrate is saturated with sodium chloride and extracts three times with 50 ml of ether. The organic layer was dried over sodium sulfate and evaporated. The 2.3 g of product obtained were recrystallized to give (1.5 g, 60%) of phloroglucinol; m.p. 216 °C, IR (KBr): ν 3200 cm^{-1} (OH), ^1H NMR (DMSO d_6): δ 5.70 (*s*, 3H); 9.00 (*s*, 3H).

Anal. Calc. for $C_6H_6O_3$: C, 57.14; H, 4.80. Found C, 57.38; H, 4.72.

2.2. Chromatographic study

2.2.1. Theory

The non-linear chromatography determinates the phloroglucinol adsorption isotherms using the perturbation technique [16,17] which consists in the determination of the retention times of small phloroglucinol amounts injected onto the column equilibrated with phloroglucinol solutions at different concentration levels. The well known Langmuir theoretical approach relates the total concentration of phloroglucinol in the HSA stationary phase (C_s) and that in the mobile phase (C_m) [16,17]:

$$C_s = \frac{\alpha K C_m}{1 + K C_m} \quad (1)$$

where α is the column saturation capacity and K is the association constant between phloroglucinol and the HSA. The phloroglucinol retention factor k was directly proportional to the slope of its adsorption isotherm and can be thus given by the following equation [16,17]:

$$k = \frac{\phi \alpha K}{(1 + K C_m)^2} \quad (2)$$

where ϕ is the column phase ratio (volume of the stationary phase divided by the volume of the mobile phase). By plotting the k value versus the phloroglucinol concentration in the bulk solvent, the constant K can be determined using Eq. (2).

2.2.2. Reagents and apparatus

Sodium chloride, sodium hydrogen phosphate and sodium dihydrogen phosphate were supplied by Prolabo (Paris, France). Water was obtained from an Elgastat option water purification system (Odil, Talant, France) fitted with a reverse osmosis cartridge. $MgCl_2$ was obtained from Sigma Aldrich (Saint-Quentin, France). The chromatographic system consisted of a HPLC Waters pump 501 (Saint-Quentin, Yvelines, France), an Interchim Rheodyne injection valve model 7125 (Montluçon, France) fitted with a 20 μl sample loop and a Merck 2500 diode array detector (Nogent-sur-Marne, France). The column temperature was controlled in an Interchim oven, TM No. 701 (Montluçon). The mobile phase was fixed at 0.80 ml min^{-1}. An HSA protein chiral Shandon column 150 mm × 4.6 mm I.D. (Cergy-Pontoise, France) was used with a controlled temperature in an Interchim oven, TM No. 701 (Montluçon) for high temperature and an Osi Julabo FT200 cryoimmerser (Elancourt, France) for low temperature. Sodium nitrate (Merck, Nogent-sur-Marne, France) was used as a dead time marker. It was recently demonstrated that the electrostatic attractions played a major role on the Mg^{2+} binding mechanism on HSA [20,21]. Then in order to visualize the Mg^{2+} effect on the phloroglucinol retention mechanism, a low sodium phosphate buffer concentration in the mobile phase was used (7×10^{-4} M) at pH 7.3 (pH of the plasma) [20,21]. The $MgCl_2$ concentration in the mobile phase, x, varied from 0.70 to 2 mM (including its biological concentration range (0.70–0.90 mM)). Ten x values were included in this range i.e. 0.7, 0.8, 0.9, 1, 1.1, 1.2, 1.4, 1.6, 1.8 and 2 mM. The equilibration of the column was carried out with 16 different concentrations of synthesized phloroglucinol (0–7.5 mM) in each mobile phase used to obtain a stable detection. 20 μl of the most concentrated phloroglucinol sample was injected three times and the retention times were measured.

3. Results and discussion

3.1. Phloroglucinol (P)–HSA binding/magnesium effect

For each phloroglucinol and magnesium concentration in the mobile phase, the most concentrated phloroglucinol sample was injected and its retention factor were determined. The variation coefficients of the k values were $<0.6\%$, indicating a high reproducibility and a good stability for the chromatographic system. The variation of the k values versus phloroglucinol concentration in the bulk solvent was similar for all x values and for two studied temperature (5 and 37 °C). An example of plot was given in Fig. 2. Using a non-linear regression and for each x value the non-linear regression coefficients of Eq. (2) were determined. Their values were given in Table 1. From the full regression model a Student's *t*-test was used to provide the basis for the decision as to whether or not the model coefficients were significant. Results of the Student's *t*-test show that no variables can be excluded from the model. These results showed that the Langmuir model describes accurately the association behavior of phloroglucinol with HSA. For each x and temperature values the corresponding K values were calculated. For example, for x = 0.7 mM and at T = 37 °C (respectively, 5 °C) the K value was equaled to 4.6 M^{-1} (respectively, 8.3 M^{-1}). For example, the K values were plotted against the magnesium chloride concentration, x, in the bulk solvent at T = 37 °C (Fig. 3). The K values decreased significantly with salt concentration increasing (Fig. 3). This variation can be analyzed taking into account the ability of HSA to bind divalent inorganic cation such as Mg^{2+} [20–22]. Indeed the Mg^{2+} bound to HSA by electrostatic interactions between its charge and the negatively charged surface of HSA (HSA at pH 7 was negatively charged) [20]. Then, the non-specific binding mode of Mg^{2+} led a competition effect between phloroglucinol and this divalent cation to bind on HSA and consequently a decrease of phloroglucinol–HSA affinity. Then the bioavailable phloroglucinol form increased and thus its pharmacological effect.

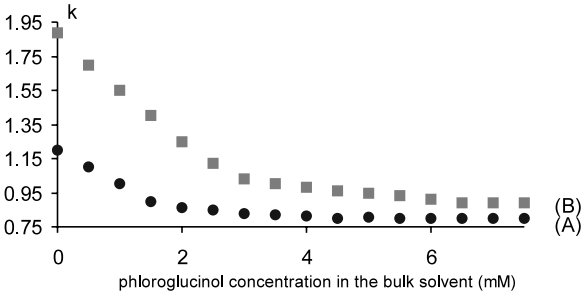

Fig. 2. Plot of k against phloroglucinol concentration in the bulk solvent at a column temperature equal to (A) 37 °C and (B) 5 °C for x = 0.9 mM.

Table 1
Non-linear regression coefficients for Eq. (2)

X (mM)	R^2	F
0.70	0.9970	5
0.80	0.9998	23
0.90	0.9975	12
1.00	0.9996	21
1.10	0.9997	21
1.20	0.9981	18
1.40	0.9984	19
1.60	0.9979	18
1.80	0.9978	14
2.00	0.9967	5

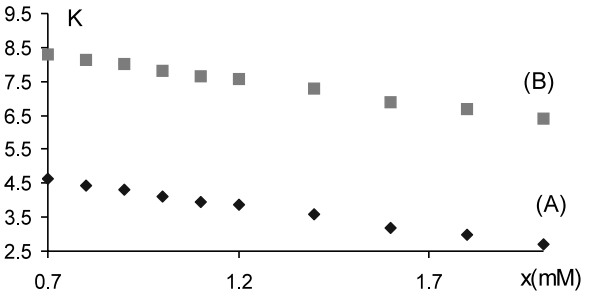

Fig. 3. Plot of K (M^{-1}) against magnesium concentration x (mM) in the bulk solvent at a column temperature equal to (A) 37 °C and (B) 5 °C.

4. Conclusion

In this manuscript, a novel and simple synthesis of phloroglucinol was presented. In addition, the

role of the magnesium cation on the phloroglucinol binding mechanism on HSA was examined. The results showed that in the studied Mg^{2+} concentration range (0.70–2 mM) an increase in the Mg^{2+} concentration produced a decrease in the phloroglucinol–HSA binding affinity. Therefore, a better understanding in the Mg^{2+} effect on the phloroglucinol–HSA association allow to increase the active pharmacological phloroglucinol concentration and thus lead to a reduction in the dose required.

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