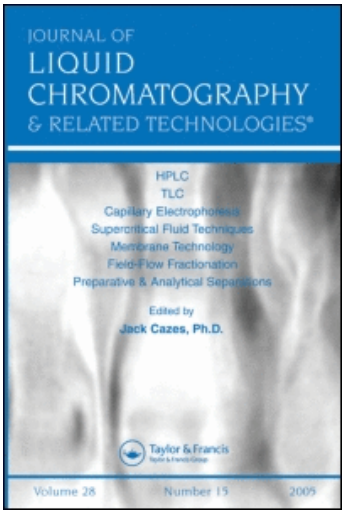

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HPLC Separation and Determination of Phenolphthalein and its Glucuronid as Markers of Enterohepatic Circulation Using Acetonitrile-Methanol as Organic Modifier and Application to Rat Plasma

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HPLC SEPARATION AND DETERMINATION OF PHENOLPHTHALEIN AND ITS GLUCURONID AS MARKERS OF ENTEROHEPATIC CIRCULATION USING ACETONITRILE-METHANOL AS ORGANIC MODIFIER AND APPLICATION TO RAT PLASMA

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ABSTRACT

An isocratic-HPLC method for the separation and determination of phenolphthalein and its metabolite phenolphthalein-glucuronide, using bromocresol purple as an internal standard, a mobile phase methanol-cetonitrile-50 mM phosphate buffer (pH 7.6) (35:10:55, v/v/v) and a 3 μm reversed-phase C_{18} column (50 x 4.6 mm i.d.), is described. The flow-rate was 1 ml/min and the UV detector wavelength 230 nm. This method extends a prior study of the most important variables that can affect the retention of these compounds (i.e., concentration of acetonitrile as organic modifier, buffer concentration and pH). The proposed method has been applied to the determination of phenolphthalein and phenolphthalein-glucuronide in rat plasma using solid phase extraction.

INTRODUCTION

Phenolphthalein (P), its metabolite phenolphthalein-glucuronide (PG), and bromocresol purple (BCP) are compounds derived from triphenylmethane skeleton. The presence of polar subtituent groups enhance their water solubility and their existence as neutral, anionic or cationic species depending on the pH of the medium[1].

In a previous paper[2], a HPLC separation of P and PG as markers of enterohepatic circulation, using BCP as an internal standard (IS), was reported. This method uses a mobile phase MeOH-50 mM phosphate buffer (pH 7.7) (47.5:52.5, v/v) and a reversed-phase C_{18} column, with UV detection. Moreover, an inversion in the elution order of BCP and P was observed depending on the pH buffer and phosphate buffer concentration used or when other additives such as triethylamine or acetic acid were added to the mobile phase. In this paper a HPLC method with isocratic elution for the separation and determination of P and PG using a C_{18} column, is described. This method involves a previous study of the most important variables that can affect separation of this compounds, using acetonitrile (AcCN) as organic modifier. After optimizing these variables and taking into account the behavior of these compounds in MeOH[2], a mobile phase MeOH-AcCN-phosphate buffer (pH 7.6) was finally used. This method

has some advantage with regard to the method developed for methanol[2] as organic modifier (the elution order of these compounds is independent of pH buffer and buffer concentration, the peak resolution is better, the number of possibilities to be applied to biological samples increases with regard to using MeOH or AcCN alone, the expent of solvent are lower and the analysis times are very similar). The proposed method has been applied to the determination of P and PG in rat plasma using solid phase extraction (SFE).

MATERIALS AND METHODS

Apparatus

The chromatographic system consisted of the following components: a Rabbit-HP solvent delivery system equipped with two pumps and a pressure module (Rainin Instrument Co Inc., Woburn, MA, USA); a Rheodyne 20-μl loop injector (Rheodyne, Berkeley, CA, USA); a reversed-phase guard column (Rainin Microsorb C_{18}; 15 x 4.6 mm i.d.; 3μm) and a reversed-phase Rainin Microsorb C_{18} column (50 x 4.6 mm i.d., 3μm); a Knauer UV-VIS variable-wavelength monitor operating between 190 and 400 nm (Knauer, Hambourg, Germany).

An Apple Macintosh SE 30 Computer interfaced to the HPLC equipment using the Dynamax HPLC Method manager, Version 2.1 (Rainin Instrument Co. Inc. Woburn, MA, USA), was also used.

A Jouan centrifugal concentrator vacuum system (Jouan, Inc. Winchester, VA, U.S.A.) was used to evaporate samples under reduced pressure in centrifuge tubes.

For pH measurements, an Orion Digital pH-meter equipped with a Fisher 13/620/91 combined glass calomel electrode was used.

Reagents

P, PG and BCP were provided by Sigma Chemical Co (St. Louis, MO, U.S.A.). β-Glucuronidase was provided from Boehringer Mannheim Biochemicals (IN, U.S.A.). HPLC-grade Potassium Phosphate Monobasic, Sodium Acetate, AcCN and MeOH were purchased from Fisher Scientific (Fair Lawn, NJ, USA). The water used to prepare the mobile phase and all aqueous solutions was purified using a Milli-Q water purification system from Millipore (Bedford, MA, USA).

Mobile Phase

The mobile phase was prepared daily by mixing the acetate or phosphate buffer solution with AcCN (or the

mixture of MeOH and AcCN) at the required volume ratio. The pH of buffer solutions was adjusted by the addition of 1 M sodium hydroxide or 1 M hydrochloric acid.

After mixing of buffer solution and AcCN (or the mixture of AcCN and MeOH), the mobile phase was filtered and degassed using vacuum and ultrasonication, and the corresponding pH was also monitored.

In all experiments % AcCN (or % AcCN and % MeOH) and % Buffer equal 100 (v/v) (or v/v/v).

Chromatographic Analysis

After conditioning the column with mobile phase, HPLC chromatograms were obtained at room temperature with a injection of a solution of 20 μl containing 10 μg/ml of an aqueous solution of PG, and P and 20 μg/ml BCP(IS). The flow-rate was 1.0 ml/min; UV detection: 230 nm.

The composition of mobile phase, control of pumps and data acquisition for each experiment was coordinated using a computer program (see apparatus). [Two different reservoirs containing AcCN - phosphate buffer (pH fixed) (10:90, v/v), and AcCN - phosphate buffer (pH fixed) (60:40, v/v), were used. In the case of employing mixtures of AcCN and MeOH as organic modifier the reservoirs contain 10% AcCN and 40% AcCN respectively and a fixed concentration of MeOH in each one, were used].

All chromatographic measurements were performed in triplicate.

RESULTS AND DISCUSSION

Effect of AcCN Concentration

The effect of organic modifier, AcCN on the retention of the three compounds under study PG, P and BCP has been carried out using phosphate buffer (pH 6.7). Phosphate buffer concentration was varied from 10 to 70 mM. Fig. 1 shows a plot of capacity factors [$k' = (t_R - t_o)/t_o$, where t_R is the retention time of the compound and t_o the retention time of an unretained compound] for the compounds versus AcCN concentration at 50 mM phosphate buffer concentration. Other plots k' versus AcCN concentration at different phosphate buffer concentration were very similar behavior and depends slightly on the AcCN concentration.

In Fig.1 can be observed that the elution order was P/BCP/PG, with PG and BCP overlapping at higher concentrations of AcCN. A gentle increase of k' values for P and BCP, and a exponential increase for P when the AcCN concentration is decreased, were also observed.

Different separations can be carried out specially at lower AcCN concentrations, though the k' values for P are very large.

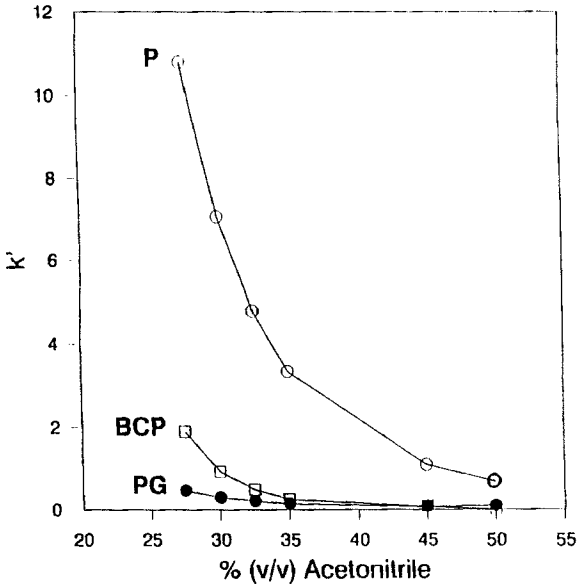

FIGURE 1. Effect of AcCN concentration on the retention of PG, P and BCP at 10 mM phosphate buffer (pH 6.7). P and PG 10 μg/ml, and BCP 20 μg/ml. Conditions: Column (see Experimental); Flow-rate, 1.0 mil/min; UV detection at 230 nm; Sample size 20 μl.

Effect of Buffer Concentration

The effect of phosphate buffer concentration on the retention of PG, P (pK_a 9.3)[3] and BCP (pK_a 6.1)[3]has been studied at pH 6.7. The phosphate concentration was varied in the range 10-70 mM at pH. 6.7. At a given buffer concentration in the range studied the elution order was always PG/BCP/P, and the retention can be explained by the rule of hydrophobic interactions when the concentration of AcCN is varied. With these experimental

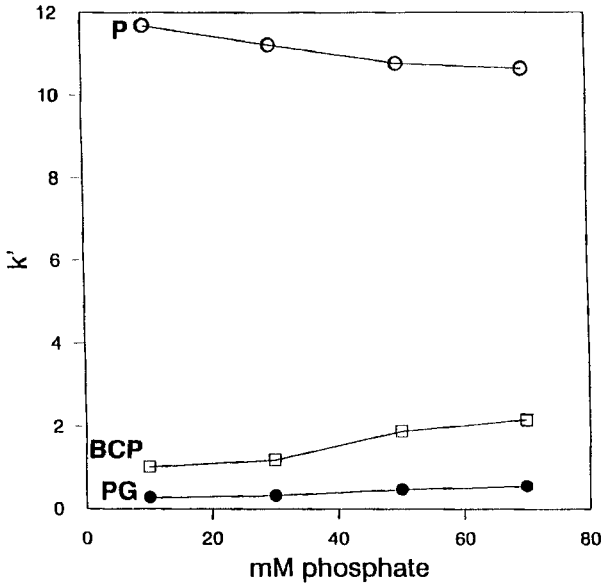

FIGURE 2. Effect of phosphate buffer concentration on the retention of PG, P and BCP. Mobile phase: AcCN-phosphate buffer (pH 6.7) (27.5:72.5, v/v). The remainder conditions as in Figure 1.

conditions different separations can be carried out. As an example of this effect, in Fig. 2 it can be observed that for 27.5% AcCN the k' values of PG and BCP increased slightly when the phosphate concentration is increased (probably due to the low buffering capacity of buffer used). However, the k' values to P decreased (i.e., k' values in the range 10-70 mM phosphate varied between 7.45 and 6.33 for 30% AcCN, and between 11.70 and 10.66 for 27.5% AcCN respectively). This indicates that the retention of P is due to a slight increase in the

dissociation process of P (salt effect) which is more important than the buffering capacity effect.

For the remainder of experiments, a 50 mM phosphate buffer concentration was selected due to the acceptable buffering capacity.

Effect of pH

The pH effect on the retention of P, PG, and BCP was studied at different pH values: pH 4.5 (50 mM acetate buffer), and pH 6.7 and 7.6 (50 mM phosphate buffer). The AcCN concentration was varied for each pH. At any constant pH, the retention decreased when AcCN concentration increased according to hydrophobic interactions.

In Fig. 3 is shown the influence of pH on the retention and separation of the compounds using 30% AcCN as a representative example. It can be observed that the elution order is PG/BCP/P. When the pH value increased a slight decrease of the k' values for PG is observed, however this decrease is more noticeable for P and specially for BCP which is practically linear, as a consequence of its dissociation process. In the case of P, this decrease can be explained by taking into account two simultaneous effects: the proximity of the pK_a value of P and the salt effect mentioned above (i.e., at pH 7.6

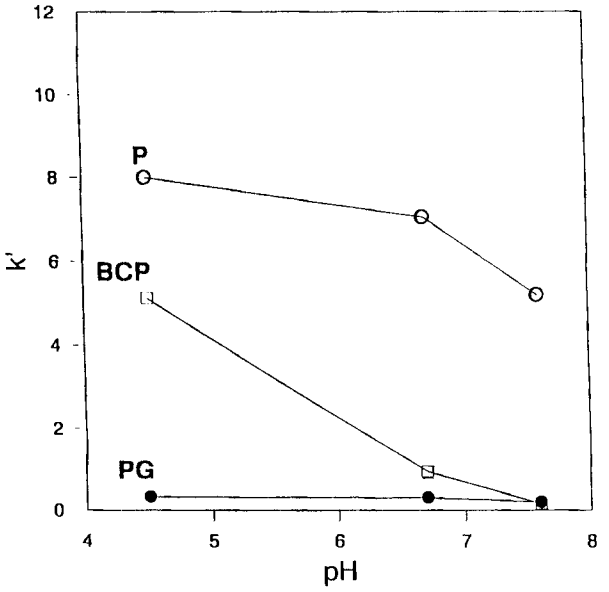

FIGURE 3. Effect of pH on the retention of PG, P and BCP. Mobile phase: AcCN-50 mM phosphate buffer (30:70, v/v). The remainder conditions as in Figure 1.

in comparison to pH 6.7 the concentration of the basic species of phosphate increases and consequently the concentration of its counter-ion).

Effect of Mixtures of AcCN/MeOH and Other Modifiers

From the results obtained above using AcCN as organic modifier it is possible to establish different analytical procedures for the separation and determination of these type of compounds. As it can be observed throughout this study, the k' values of PG are very short from the point

of view of analyzing this compound in biological samples. For increasing k' values of PG it was necessary to decrease pH or % AcCN (v/v). However, the retention of P increased greatly. The use of AcCN and MeOH mixtures as organic modifier was studied because AcCN as mobile phase elutes PG and BCP at pH 7.6 (see pH effect) at the same k' values while MeOH does not[2].

This study was performed using 50 mM phosphate buffer (pH 7.6), by fixing a given % MeOH in the range 20-35%, and varying the %AcCN in the range 10-22%. By combining simultaneously MeOH and AcCN effects on the separation of these compounds at pH 7.6, the elution order did not change in any case.

In this way, a large number of complete separations were obtained, which were always better than using MeOH or AcCN alone. In Table I is shown a summary of the k' values for PG, BCP and P at pH 7.6, at several concentrations of mixtures MeOH and AcCN. With the purpose of analyzing biological samples a mobile phase consisting of MeOH-AcCN-50 mM phosphate buffer (pH 7.6) (35:10:55, v/v/v) was finally chosen. In Fig.4, a typical chromatogram for the separation of PG, BCP and P is shown.

Appropriate mobile-phase modifiers, such as triethylamine (TEA) or acetic acid (AcOH) were added to the buffer solution above chosen to eliminate problems

TABLE 1

Capacity Factors, k', of PG, BCP and P in MeOH:AcCN: [50 mM Phosphate buffer(pH 7.6) and 50 mM Phosphate Buffer Containing AcOH* or TEA(pH 7.6)**] Mobile Phase.

Compounds	%(v/v)MeOH	%(v/v)AcCN							
		10	11.5	13	14.5	16	17.5	19	20.5
PG BCP P	20						0.90 2.31 10.0	0.70 1.41 7.21	0.41 0.92 5.61
PG BCP P	25				0.77 2.22 7.41	0.63 1.51 5.80	0.36 0.82 3.21		
PG BCP P	30			0.68 2.20 6.06	0.55 1.74 5.06	0.35 1.04 3.80			
PG BCP P	35	0.81 3.93 7.10	0.72 3.11 6.10	0.50 2.16 4.62	0.43 1.41 3.62				
PG BCP P	35*	0.82 4.12 6.52	0.65 2.92 5.52	0.53 2.21 3.61	0.41 1.60 3.01				
PG BCP P	35**	0.90 5.30 6.93	0.83 4.32 5.94	0.63 3.40 4.80	0.52 2.43 4.01				

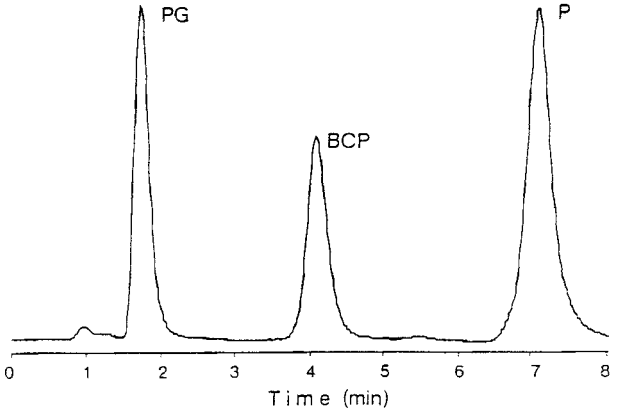

FIGURE 4. HPLC-UV chromatogram of PG, P and BCP standards. P and PG 10μg/ml, and BCP 20 μg/ml. Mobile phase MeOH-AcCN-50 mM phosphate buffer (pH 7.6) (35:10:55, v/v/v). The remainder conditions as in Figure.1.

due to secondary interactions that cause broad and tailing peaks[4]. After adding TEA or AcOH to buffer, the pH was adjusted again to pH 7.6. In this situation the effects about broad and tailing peaks are similar to those observed when only MeOH was used as organic modifier[2]. However using mixtures of AcCN and MeOH, there was no inversion in the retention order of P and BCP (see also data of k' in Table 1).

Calibration Graphs

Calibration samples were prepared in the mobile phase. Standards containing mixtures of P, PG and BCP

(IS) were made in triplicate at four different concentrations in the ranges P (0.2-20 μg/ml) using 20 μg/ml BCP. These solutions were analyzed using a mobile phase composed of MeOH - AcCN - 50 mM phosphate buffer (pH 7.6)(35:10: 55, v/v/v), a flow-rate of 1 ml/min, and UV detection at 230 nm.

The results were analyzed by linear regression. Plotting the peak area ratio (PAR) of PG or P to BCP (IS) versus the concentration (c) of PG or P, the calibration equations can be expressed in the following way: PG, PAR = 0.0142 + 0.129 c [regression coefficient (r^2 = 1.000)] and P, PAR = 0.0899 + 0.221 c (r^2 = 0.996). In both cases intercepts were not significantly different from zero.

Precision and Accuracy

The precision was examined by analysis of ten samples (n=10) of four concentrations of P and PG in the range (2-20 μg/ml), which were calculated by means of the calibration graphs. The standard deviation (SD) were in the range 0.05-0.17 μg/ml (mean 0.10 μg/ml) for PG and 0.08-0.15 μg/ml (mean 0.13 μg/ml) for P. The relative standard deviation (RSD) were in the range 0.9-2.8% (mean 1.8%) for PG and 1.1-4% (mean 2.2%) for P.

The accuracy[5-6] was assessed in the range 2-20 μg/ml for PG and P. The Barlett and Harley test[7] was applied to

the results to corroborate the randomness of the variances. A linear regression analysis was carried out on the values obtained on known concentrations and the corresponding calculated values obtained. A t-test was applied to the results and the value of the intercept was obtained. This study confirmed that the present method does not present a systematic error (i.e. it has slope value equal to unity) and does not require a blank correction (i.e. it has an intercept equal to zero).

Plasma Analysis

Samples containing P, PG and BCP (IS) were evaporated to dryness. 1 ml blank rat plama was added to the resulting residue. After vortexing for 1 min, to the sample containing plasma and compounds, 3 ml of acetone acidified with acetic acid solution was added to precipitate proteins. After vortexing for 1 min, the plasma proteins were pelleted by centrifugation for 15 minutes. An aliquot of supernatant (3 ml) was transferred to a clean tube and evaporated to dryness. The residue was resuspended in 1 ml of 0.1 M phosphate buffer (pH 7.4) and sonicated for 10 min. The resulting solution was further processed by SFE using a C_{18} (1 ml) column that was previously conditioned with two aliquots of MeOH (1 ml) followed by two aliquots of water (1 ml). The buffer

solution containing the analytes was aspirated through the conditioned column and washed with two aliquots of water (1 ml). Elution of the analytes into a clean test tube was accomplished using 1 ml of MeOH. After evaporating the methanolic eluate to dryness, the final residue was resuspended in 1 ml of HPLC mobile phase, centrifuged to remove particulates, and 20 μl injected onto HPLC.

Using this procedure, four rat plasma samples were analyzed and recoveries were in the range (81.6-88.6%) [mean 83.6% ± 2.4% (RSD)] for BCP, and 91.4-101.2% [mean 93.6% ± 5.4 (RSD) for P (based on 10 μg/ml for P and 20 μg/ml for BCP mobile phase standards and corrected for volume changes). PG was not evaluated by this extraction procedure because the blank of plasma contains a serious interference (there is a endogenous compound in plasma blank chromatograms which coelutes with the peak of PG). In order to obtain separate P and PG levels, the analysis of rat plasma samples was performed for P before and after treatment with β-glucuronidase[8]. The PG levels can can be obtained by sustraction. The recovery for PG was 87% ± 3.2 (RSD).

The method previously developed[2] and the proposed method may be applied to the study of enterohepatic recirculation in other biological fluids such as rat urine or bile, and for developing other chromatographic methods especially for determining PG an P in rat plasma

within a single run (i.e. gradient elution of PG, and isocratic one for P and BCP).

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