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Complexation in the retention of benzoic acids on a bonded xanthine HPLC stationary phase

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Summary

The retention of a number of benzoic acids was determined using a bonded xanthine stationary phase (XSP). Retention of benzoate on XSP was studied as a function of pH and organic modifier composition of the mobile phase. The measured retention for 10 substituted benzoic acids was correlated with the complexation constants previously reported for these compounds with theophylline.

Introduction

Previously, the complexing tendencies of substituted xanthines such as caffeine and theophylline have been studied and characterized using a number of hydroxybenzoic and hydroxynaphthoic acid derivatives and numerous other compounds in aqueous solutions (Higuchi and Connors, 1965 and refs. therein). Although no single physicochemical parameter can be used to describe the complexation of benzoic acids and naphthoic acids with xanthines, it has been concluded (Higuchi and Kristiansen, 1970) that hydrogen bonding, Van der Waals interactions, London forces, hydrophobic interactions and π–π interactions are all implicated. Additionally, Connors et al. (1969) have found that a correlation exists between the planar area of cinnamic acid esters and their complexation constants with theophylline, indicating planar stacking of the substrate and ligand. The extent of such interactions are maximized in water and have been shown to be altered (decreased) by the

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presence of organic cosolvents (Kristiansen et al., 1970), increased temperature (Higuchi and Zuck, 1953) and the degree of ionization of the interacting species (Higuchi and Zuck, 1953; Kreilgård et al., 1975; Higuchi and Drubulis, 1961).

It was postulated that if a xanthine derivative was employed as a stationary phase in HPLC, retention of appropriate analytes would be related to their association constants with the xanthine and could be controlled by judicious choice of temperature, solvent composition and pH of the mobile phase to effect resolution of complex mixtures.

In addition, the capacity factors of these analytes determined by HPLC on such specialized phases might be relatable to published values of association constants determined in non-dynamic systems. If such were the case, one might be able to use such an HPLC technique for rapid estimation of complexation of various compounds with the appropriate xanthine derivative. Alternatively, it would also be possible to predict HPLC retention characteristics from such published data, thereby expediting the development of chromatographic systems for the resolution of mixtures of suitable analytes.

The present study represents initial attempts to evaluate the above considerations through preparation of a stationary phase (XSP) in which theophylline-7-acetic acid is covalently attached to a commercially available amine-bonded phase (i.e. aminopropylsilicate) through an amide linkage. Specifically, the work is concerned with the application of liquid chromatography to the investigation of the interaction of aromatic acids with theophylline covalently bonded to silica gel through an amide linkage.

Materials and Methods

Apparatus

Chromatography was performed using a Waters Associates (Milford, MA) model 6000A pump, model U6K injector and a model 440 ultraviolet detector. The wavelength for the UV detector was set at 254 nm. Separations were carried out on 100×4.5 mm (i.d.) columns slurry packed with either: (a) Chromosorb LC-9 (Johns-Manville, Denver, CO), an irregular 10 μm particle diameter, amine-bonded stationary phase (ASP); or (b) Chromosorb LC-9 which had been derivatized by covalently bonding theophylline-7-acetic acid through an amide linkage. For analytical evaluation and comparison, columns were operated at ambient temperature ($\sim 20°C$). The flow rate was maintained at 1.2 ml/min.

Materials

Theophylline-7-acetic acid was purchased from Pfaultz and Bauer (Stanford, CT). All chemicals were of reagent grade and available from commercial sources. HPLC solvents were purchased from Fisher (Fair Lawn, NJ) and mono- and dibasic sodium phosphate from Mallinckrodt Chemicals (St. Louis, MO). Water was deionized and distilled prior to use.

Procedure

(a) Synthesis of the xanthine-modified stationary phase (XSP). A suspension of

theophylline-7-acetic acid (3.0 g) in redistilled thionyl chloride (18 ml) and dried benzene (40 ml) was refluxed until (~ 6 h) a solution was obtained which was orange-red in color. The excess benzene and thionyl chloride were removed in vacuo to obtain an orange-brown solid identified by elemental analysis and mp (155–157°) as theophylline-7-acetyl chloride (Maslankiewicz et al., 1975).

This solid was then suspended in dried benzene (150 ml) and added to Chromosorb LC-9 (3.0 g) in a brown reagent bottle. The suspension was shaken gently at ambient temperature for 24 h. The stationary phase was recovered by filtration through a sintered-glass Buchner funnel. The solid was then washed sequentially with chloroform (200 ml), methanol (300 ml) and 50% acetone–water (500 ml). The solid was suspended in 0.02 M Na_2CO_3, separated by filtration, and washed sequentially with water (400 ml), 50% acetone–water (500 ml), methanol (200 ml), chloroform (100 ml) and methanol (100 ml). The solid was then dried in vacuo and submitted for elemental analysis.

(b) The retention of the model compound, benzoic acid (BA), was studied as a function of pH and organic modifier composition on both XSP and Chromosorb LC-9 stationary phases. Mobile phases were prepared using either methanol, acetonitrile or tetrahydrofuran and phosphate buffers (5×10^{-3} M, pH 7.01 and pH 3.00). The study was extended to pH 7.00, 6.00, 4.50 and 3.00 phosphate (5.0 mM) buffers containing varying volume fractions of methanol. The retention time (t_0) of an unretained component was determined using D_2O; and capacity factors (k′) were obtained from retention times of solute (t_R) measured in triplicate (Eqn. 1):

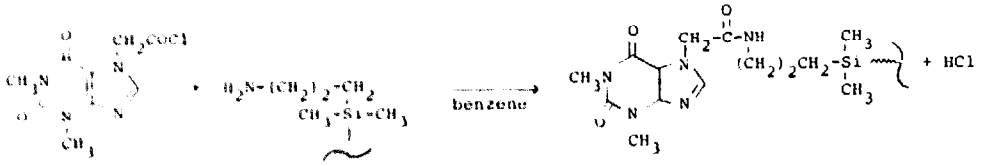
$$k' = \frac{t_R - t_0}{t_0} \tag{1}$$

Retention values for 9 substituted benzoic acids (see Fig. 6) on XSP were also determined using pH 7.00 phosphate (5 mM) buffer as the mobile phase.

Results and Discussion

Stationary phase selection and preparation

The stationary phase developed for this study was prepared by bonding theophylline-7-acetic acid through an amide linkage to a commercially available 10 μm (irregular particle) amine-bonded stationary phase (ASP). The reaction may be described as shown in Scheme I:



The extent of coverage of theophylline-7-acetic acid bonded to the stationary phase was determined by elemental analysis of the stationary phase prior and

subsequent to reaction with the theophylline-7-acetyl chloride. Elemental analysis for ASP was N, 1.35; C, 3.93; and H, 1.29. A typical analysis of a batch of the synthesized XSP gave the following results: N, 3.20%; C, 7.39%, and H, 1.33%, indicating that ~ 36% of the amine groups had reacted with theophylline-7-chloride. Extensive washing of the support without loss of xanthine content verified the covalent nature of attachment.

Preparation of several small batches of XSP yielded coverage in the range of 30–36%. Efforts to achieve greater coverage were not pursued. While the coverage obtained was incomplete, it was felt that such a stationary phase would still allow for evaluation of the viability of the hypothesis that the bound xanthine moiety would alter the retention of various suitable analytes through complexation. The relative contributions of the xanthine–analyte interactions were ascertained by comparison of chromatographic behavior of test analytes on the XSP support with that on the unmodified amine stationary phase (ASP).

Comparative retention behavior on XSP and ASP as a function of mobile phase composition

The retention of benzoic acid in a purely aqueous mobile phase was evaluated over the range of pH 3–7 for both stationary phases. A sample chromatogram is shown in Fig. 1. From the plot of k′ vs pH (shown in Fig. 2), it is clear that the benzoic acid species are retained to a much greater extent on the XSP stationary phase than on ASP. This increased retention was attributed to complexation between the bonded xanthine moieties and the benzoate and benzoic acid species. It should be noted that maximum retention of benzoate on both stationary phases occurred at pH 4.5. Due to the fact that the XSP contained about 65% unmodified amine sites, retention on XSP most probably represents the sum of contributions due to ASP itself as well as the effect produced by the bonded xanthine functionalities. Simple subtraction of the retention values obtained on ASP from those obtained using XSP should reasonably represent the retention due to the xanthine–benzoate interaction. Thus, the intermediate (dashed line) curve C in Fig. 2 presumably reflects the net retention due to complexation.

The retention behavior of benzoic acid species on ASP may be rationalized by considering the ionization of both the stationary phase amino groups ($pK_a \sim 4$ (LC Column Report)) and benzoic acid ($pK_a \sim 4.2$ (Handbook of Biochemistry, 1970)). At pH values of 6 and 7, virtually all the amine groups are unprotonated, while benzoic acid is essentially present totally in the ionized form. Under these conditions, the slight retention observed is probably due to hydrophobic interaction of the analyte with the stationary phase. At pH values of 4.5, most amine groups are protonated and ~ 65% of the benzoic acid is present as the benzoate ion. In this situation, an ion exchange process is operative, resulting in substantially increased retention of the benzoate species. In addition, unionized benzoic acid may also be retained by either hydrogen bonding with the silica support and/or hydrophobic interactions. At pH 3, the ion exchange contribution would expectedly decrease as the fraction of benzoate species decreased, but may be compensated for, in part, by the hydrogen-bonding or hydrophobic interaction at other sites on the stationary

phase. As mentioned previously, the retention of benzoate species on XSP may be attributed to a combination of the retention characteristics on the ASP plus the retention due to complexation with the bonded xanthine. The net retention ascribed to complexation above (as reflected by curve C, Fig. 2) may also be qualitatively explained. At pH 7, the benzoate–xanthine complexation interaction would be expected to be largely responsible for retention under these conditions. The increasing retention exhibited as pH decreased may be attributed to both the greater association constant between benzoic acid and the xanthine (as has been found in homogenous solutions for caffeine–benzoic acid interactions (Higuchi and Zuck, 1953)) as well as enhancement of the xanthine–benzoate interaction through coulombic interaction of the complexed benzoate ion with neighboring protonated amine groups. The slight decrease in retention at pH 3 relative to pH 4 in the XSP system may be accounted for qualitatively by the fact that the fraction of benzoate species existing at low pH is minimal and thus may involve only benzoic

Fig. 1.

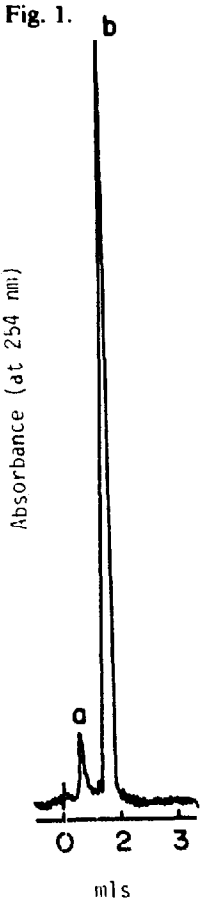


Fig. 1. A sample chromatogram of benzoic acid on XSP using an aqueous phosphate (5×10^{-3} M, pH 7.0) mobile phase. 'a' is the solvent front; 'b' is benzoic acid.

Fig. 2.

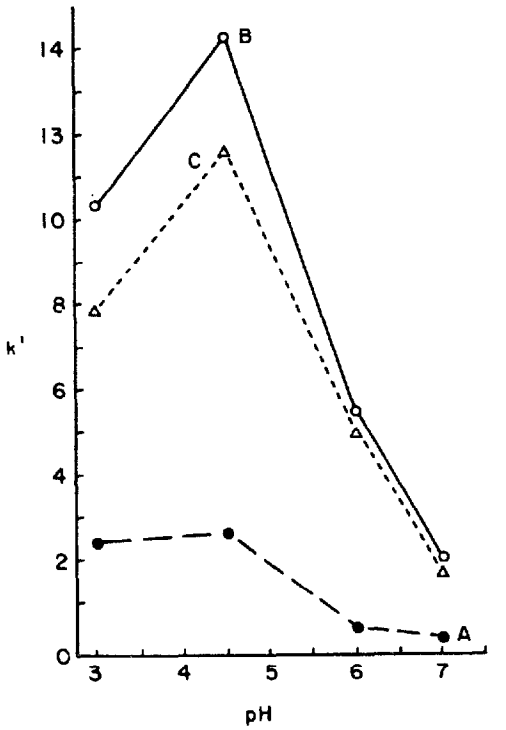


Fig. 2. Plot of k' values of benzoic acid on XSP (○) and ASP (●) as a function of pH of the aqueous mobile phase. The net retention attributed to complexation is shown as △.

acid–xanthine interaction (which should not be influenced substantially by electrostatic forces).

Effect of organic modifiers

The effects of the concentration of organic modifiers (methanol, acetonitrile and tetrahydrofuran) on the retention of benzoic acid were also evaluated at several pHs (Figs. 3–5). The addition of methanol, acetonitrile or tetrahydrofuran to an aqueous mobile phase buffered at pH 3 or 7 dramatically reduced the retention of benzoic acid. Decreasing retention with increasing organic solvent composition is consistent with observations made previously in the determination of association constants between xanthines and small molecules by spectrophotometric and solubility techniques (Higuchi and Kristiansen, 1970). Ion exchange contributions to retention also decrease with increasing organic modifier concentration as observed independently on columns packed with ASP. In pH 3 buffer, a slight increase in retention was

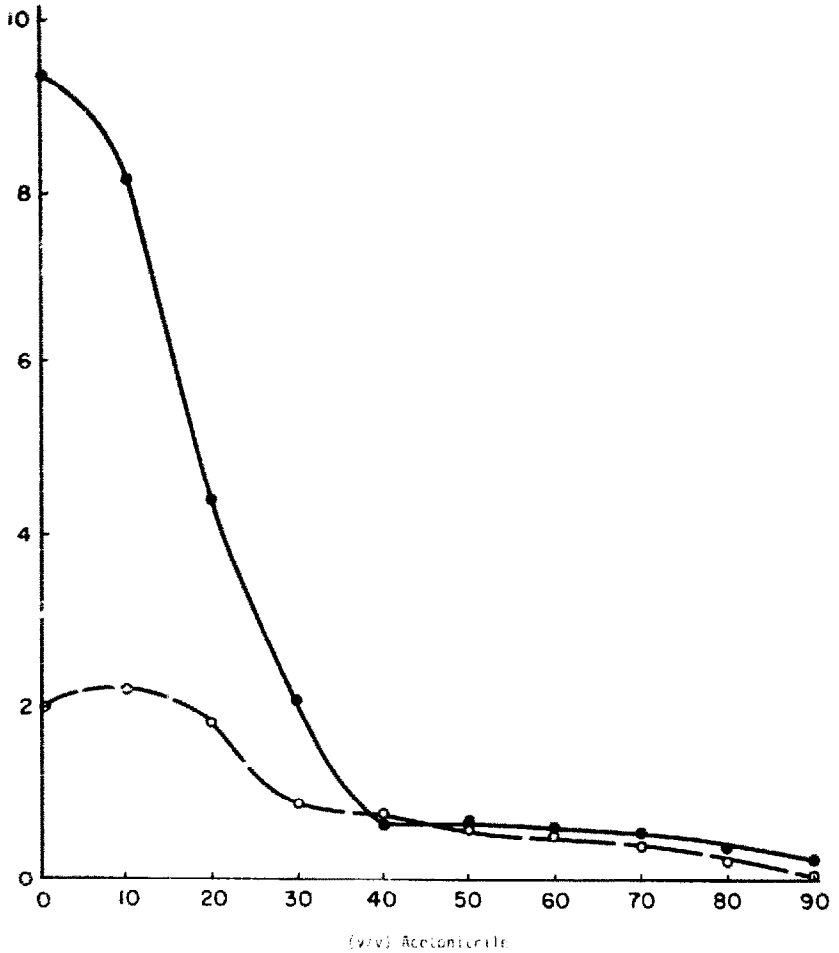


Fig. 3. Plot of k' values for benzoic acid on XSP as a function of % (v/v) acetonitrile in the aqueous mobile phase at pH 7.0 (●) and pH 3.0 (○).

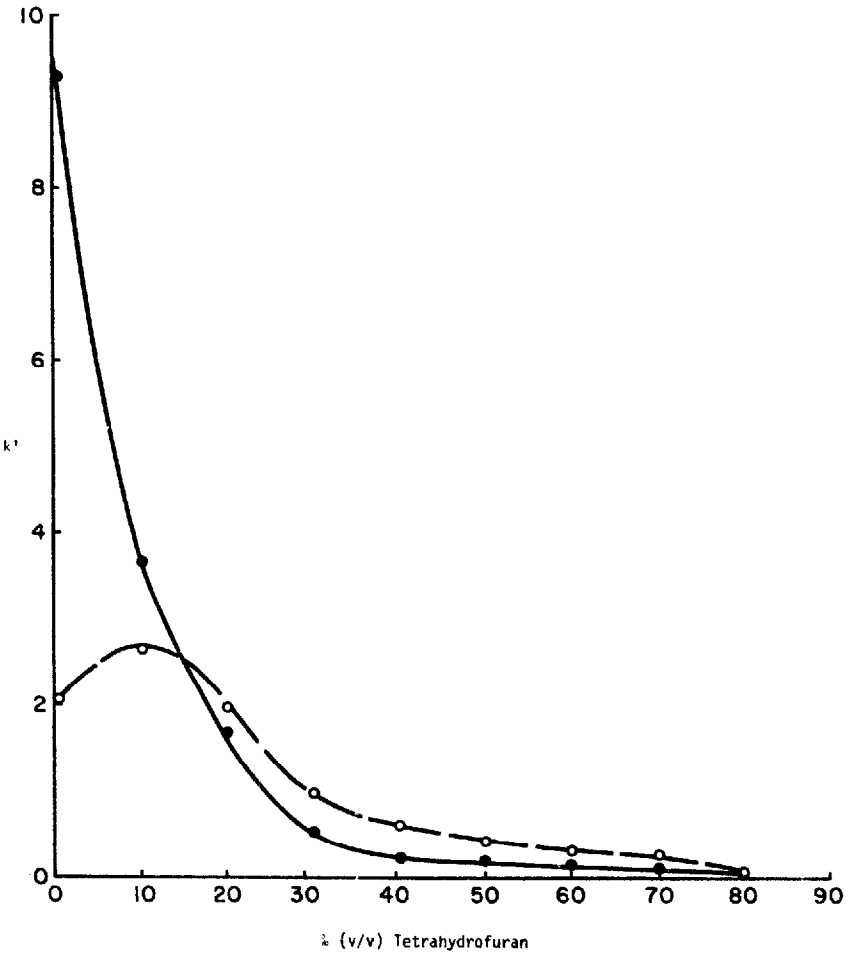


Fig. 4. Plot of k′ values for benzoic acid on XSP as a function of % (v/v) tetrahydrofuran in the aqueous mobile phase at pH 7.0 (●) and pH 3.0 (○).

noted with addition of methanol up to 10% v/v, after which the expected reduction of V_R was noted with further addition of methanol. This anomalous behavior may reflect the increased partitioning of the undissociated acid onto the stationary phase modified by the adsorption of methanol on its surface.

Changes in mobile phase composition (involving addition of methanol) similarly affected retention characteristics of salicylic, *m*-hydroxybenzoic, and 2,5- and 2,6-dihydroxybenzoic acids at both pH 4.5 and 7.0 using both XSP and ASP. Again, it was observed that greater retention occurred with XSP than with ASP using similar mobile phases, and in all cases, retention decreased as the organic modifier concentration increased. However, in the case where ASP was used, at pH 4.5, the decrease in retention for all 4 of the substituted benzoic acids was relatively small over the range of methanol concentrations from 0 to 75% (v/v).

When the k′ values obtained for these substituted benzoates on XSP (in aqueous, pH 7 eluant) were compared to the literature values (Higuchi and Connors, 1965) for the $K_{1:1}$ complexation constants for theophylline with the corresponding carboxylate

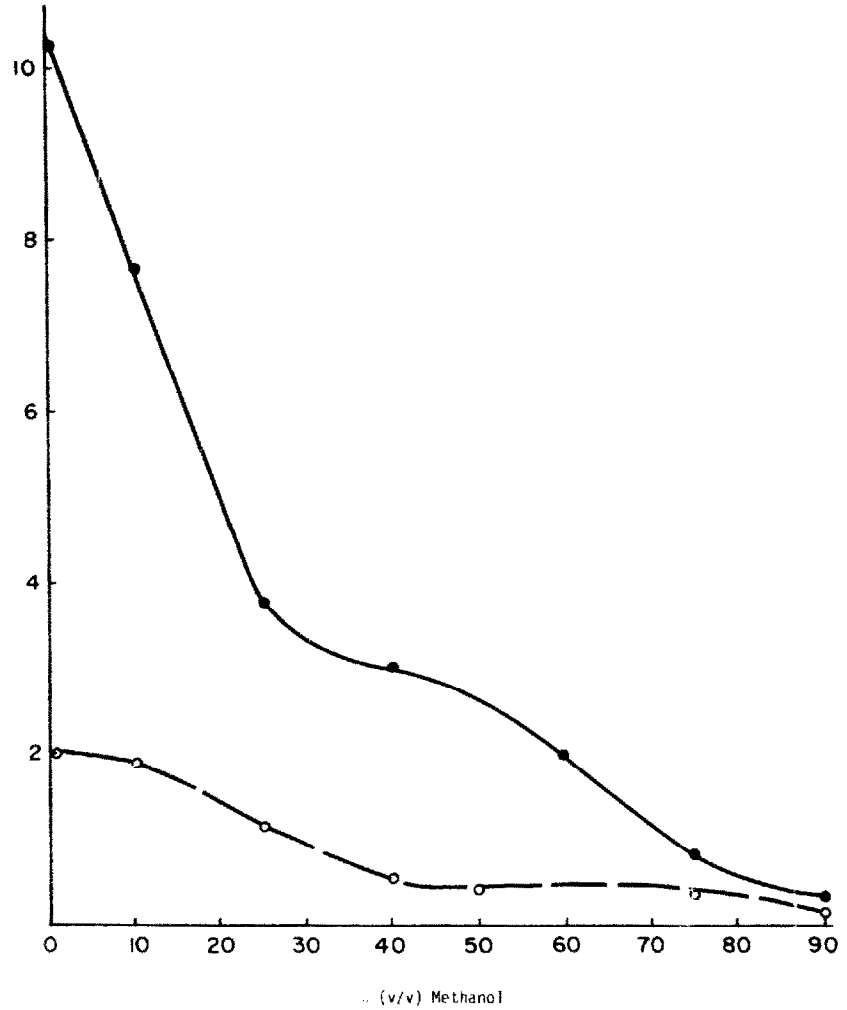


Fig. 5. Plot of k′ values for benzoic acid on XSP as a function of % (v/v) methanol at pH 7.0 (○) and pH 3.0 (●).

anions, it was noted that there was rank-order correlation between the magnitude of the k′ and $K_{1:1}$ values. Under these conditions, the increased retention on XSP relative to ASP appears to be due to complexation between the xanthine and the various substituted benzoate anions. Consequently, k′ values for 5 additional substituted benzoic acids were determined at pH 7 and these values were compared with the corresponding published (Higuchi and Connors, 1965) $K_{1:1}$ values obtained from theophylline complexation with the carboxylate anion in aqueous solution as determined by partition and solubility measurements. Again these values of $K_{1:1}$ and k′ demonstrated rank-order correlation. In order to determine whether or not there might be some empirical mathematical expressions describing the relationship of $K_{1:1}$ and k′ values, the data were analyzed by regression analysis. The data exhibited excellent linear correlation ($r = 0.997$) using the expression $K_{1:1} = 6.77k' - 5.97$ (see Fig. 6), suggesting the dominance of the complexation component in the retention of aromatic carboxylic acids on XSP at pH 7.0.

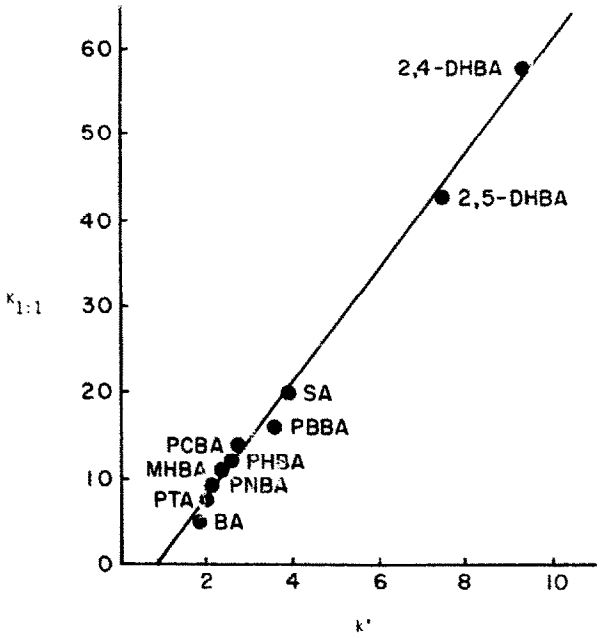


Fig. 6. Plot of $K_{1:1}$ for a series of 10 substituted benzoic acids (chromatographed on XSP at pH = 7). Equation of the solid line is $K_{1:1} = 6.77k' - 5.97$, $r = 0.997$. (Values of $K_{1:1}$ taken from Higuchi and Connors (1965) and references therein.) Acids used: BA = benzoic; PTA = *p*-toluic, PNBA = *p*-nitrobenzoic; MHBA = *m*-hydroxybenzoic; PHBA = *p*-hydroxybenzoic; PCBA = *p*-chlorobenzoic; PBBA = *p*-bromobenzoic; SA = salicylic; 2,5-DHBA = 2,5-dinitrobenzoic; 2,4-DHBA = 2,4-dihydroxybenzoic.

While no physical explanation for the observed good fit of the empirical relationship to the data has yet been developed, the potential usefulness of such a relationship is exciting. While complexation studies using solubility (Higuchi and Connors, 1965) or spectrophotometric (Connors and Mollica, 1966) methods for determination of association constants are laborious, the use of column chromatography using XSP should allow for expediently obtaining k′ values from which $K_{1:1}$ values can be closely estimated. Whether or not the empirical relationship may be extended to analytes other than substituted benzoic acids has not yet been determined.

Conclusions

On the basis of the data obtained, it appears that substituted benzoic acids are retained on XSP through complexation as suggested by the influence of mobile phase pH and composition, and the linear relationship between $K_{1:1}$ and k′ values.

In light of the results obtained, it appears that HPLC stationary phases such as XSP may be useful for both chromatographic purposes, i.e. in resolving certain mixtures, as well as for estimation of complexation constants of suitable interactants of pharmaceutical interest. While no attempts have been made to determine the

chromatographic characteristics or the stability of the XSP used in these studies, efforts are underway to prepare and evaluate a similar stationary phase using 5 μm spherical ASP.

Acknowledgement

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