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Liquid chromatographic separation of phenothiazines and structurally-related drugs using a β-cyclodextrin bonded phase column

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Abstract: The retention behaviour of 16 phenothiazines and structurally-related drugs on a β-cyclodextrin bonded phase column is examined with respect to pH, methanol and TEAA concentrations in the mobile phase, and column temperature. The results are utilized to optimize the separation of these compounds. Both isocratic and gradient-elution separation of the 16 phenothiazine derivatives on the β-cyclodextrin bonded phase column are investigated. The successful separation of all of the compounds is achieved in the gradient-elution mode.

Keywords: *Liquid chromatography; retention behaviour; separations; β-cyclodextrin; phenothiazine drugs.*

Introduction

Phenothiazine and its derivatives are an important group of drugs possessing antiemetic, antipsychotic, sedative, antipruritic, antidyskinetic, analgesic and antihistaminic properties [1–3]. More than 100 phenothiazine derivative drugs have been synthesized and pharmacologically tested during the past few decades [1, 2]. Separation and quantification of these compounds and their metabolites is necessary in clinical studies and in analytical toxicology to diagnose possible intoxication.

Although these compounds are chemically closely related, there is no uniformity in their separation and quantification. A number of investigators [4–7] have described TLC systems that are useful in the separation and identification of phenothiazines and their structurally-related compounds. A TLC system applicable to the separation of 40 phenothiazines and their sulphoxides was developed by Kofoed *et al.* [4]. Separation and detection of some of these drugs using gas chromatography has also been described [8, 9]. A gas chromatography–mass spectrometric (GC–MS) technique for the identification of phenothiazines and analogous neuroleptics was reported by Maurer and Pfleger [10]. Jelinek and Dohnal [11] employed an isotachophoresic system to separate 11 phenothiazine and structurally-related compounds. Liquid chromatography (LC) has also been applied to the separation and detection of some phenothiazine derivatives, such as promethiazine [12], thioridazine [13], and fluphenazine and its esters [14]. However, some of the phenothiazine derivatives are very difficult to separate by LC methods based on adsorption, ion-pair partition, ion-exchange or reversed-phase partition processes.

A new type of bonded stationary phase based on cyclodextrins for LC has been demonstrated to be particularly adept in many difficult separations [15], such as the separation of enantiomers, diastereomers, and positional, geometric and structural isomers. The basic property of cyclodextrin bonded stationary phases that allows them to accomplish many difficult separations is their ability to form selective inclusion complexes with a wide variety of guest molecules. The inclusion complexation depends on the structure of solutes considered as a whole rather than as a function of specific functional groups. Therefore, cyclodextrin bonded stationary phases may substantially improve the separation of phenothiazine and its structurally-related drugs.

In this study, the liquid chromatographic retention behaviour of 16 phenothiazines and structurally-related drugs (Table 1) on a β-

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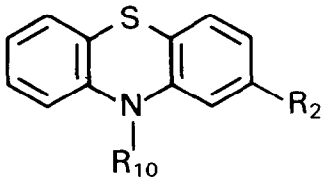
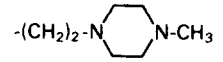
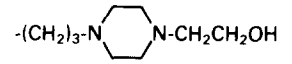
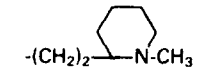
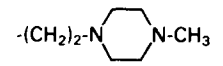
Table 1
Structures of the compounds investigated



No.	Name	R_{10}	R_2
1	Propiomazine	$-CH(CH_3)CH_2N(CH_3)_2$	$-COC_2H_5$
2	Ethopropazine	$-CH_2CH(CH_3)N(C_2H_5)_2$	$-H$
3	Promethazine	$-CH_2CH(CH_3)N(CH_3)_2$	$-H$
4	2-Acetylphenothiazine	$-H$	$-COCH_3$
5	Acetopromazine	$-(CH_2)_3N(CH_3)_2$	$-COCH_3$
6	Triflupromazine	$-(CH_2)_3N(CH_3)_2$	$-CF_3$
7	2-(Trifluoromethyl)phenothiazine	$-H$	$-CF_3$
8	Promazine	$-(CH_2)_3N(CH_3)_2$	$-H$
9	Phenothiazine	$-H$	$-H$
10	Trimeprazine	$-CH_2CH(CH_3)CH_2N(CH_3)_2$	$-H$
11	2-Methoxylphenothiazine	$-H$	$-OCH_3$
12	Chlorpromazine	$-(CH_2)_3N(CH_3)_2$	$-Cl$
13	Trifluoperazine		$-CF_3$
14	Perphenazine		$-Cl$
15	Thioridazine		$-SCH_3$
16	Prochlorperazine		$-Cl$

cyclodextrin bonded phase column was examined with respect to methanol and triethylammonium acetate (TEAA) buffer concentration in the mobile phase, pH, and column temperature. The liquid chromatographic separations of these 16 compounds were investigated in both isocratic elution and gradient elution modes.

Experimental

Apparatus

Chromatography was performed using a liquid chromatographic system that consisted of two Model 590 pumps (Waters Associates, Milford, MA, USA), a Model 660 solvent programmer (Waters Associates), a Model 7125 injector equipped with a 10-μl loop (Rheodyne, Cotati, CA, USA), and a Model 440 UV detector (Waters Associates). The chromatograms were recorded on a Model SE120 strip chart recorder (Goerz Electro, Austria). The column temperature was controlled by a HETO 623 water bath (Bach-Simpson Limited, London, Ontario, Canada).

A Cyclobond I, 250 × 4.6 mm column was purchased from Advanced Separation Technologies (Whippany, NJ, USA). The Cyclobond I column contains β-cyclodextrin molecules chemically bonded to spherical silica gel through a five-atom, non-nitrogen containing spacer. When not in use, the column was stored in 100% methanol.

Chemicals

2-Acetylphenothiazine, 2-(trifluoromethyl)-phenothiazine and 2-methoxyphenothiazine were obtained from Chemical Dynamics Co. (South Plainfield, NJ, USA). All other phenothiazine derivatives were obtained from Sigma (St. Louis, MO, USA). HPLC grade methanol and triethylamine were purchased from Fisher (Fair Lawn, NJ, USA). Glacial acetic acid was obtained from Allied Chemical (Pointe Claire, Quebec, Canada). Water was deionized by passing distilled water through a Barnstead water purification system.

Procedures

Mobile phase was prepared by mixing meth-

anol with TEAA buffer. The mobile phase was degassed by bubbling helium through it for about 10 min before use. Sample solutions were prepared by dissolving each compound in methanol to give a concentration of about 1 mg ml^{-1}. Typically, 2 μl of sample solution was injected. The chromatography was performed at a flow rate of 1.0 ml min^{-1} and the column pressure at this flow rate ranged from 1500 to 2500 psi. Absorbance of the column effluent was monitored at a wavelength of 254 nm.

All data points on the graphs were obtained by averaging at least three separate determinations. A careful reproducibility study involving five injections determined the relative standard deviation of the capacity factor to be <2%.

Results and Discussion

Effect of mobile phase composition

As stated previously, cyclodextrins form inclusion complexes with various compounds. However, inclusion selectivity is usually found only in the presence of water, although inclusion complex formation also takes place in certain organic solvents, such as acetonitrile, methanol, ethanol, dimethylformamide, dimethylsulphoxide and other dipolar solvents [16]. Therefore, an aqueous solution is usually selected as the mobile phase in liquid chromatographic separations on cyclodextrin bonded phase columns.

Preliminary studies of separation conditions showed that most of the compounds of interest could not be eluted within a reasonable time from the β-cyclodextrin bonded phase column by water alone. This indicated that an aqueous–organic eluent was essential for the present separation. However, mobile phases that consisted of acetonitrile–water or tetrahydrofuran–water exhibited poor selectivity, as the capacity factors of these compounds on the β-cyclodextrin column were nearly the same. Methanol–water was found to provide much better selectivity than acetonitrile–water and tetrahydrofuran–water systems and was therefore chosen as the mobile phase in all subsequent experiments.

The methanol concentration in the mobile phase also affected the values of the capacity factors on the β-cyclodextrin bonded stationary phase. The effect of the methanol concentration on the capacity factor was investigated by changing the methanol–water ratio in the mobile phase stepwise from 30:70 to 80:20 (v/v). In this set of experiments, the column temperature was controlled at 20°C, and no TEAA buffer was present in the mobile phase. Figure 1 shows the plots of capacity factors of some studied compounds versus methanol content in the mobile phase. As can be seen from Fig. 1, both retention time and selectivity decreased with an increase in the methanol concentration. The effect of changing the methanol concentration on the retention time was not linear. When the methanol content reached 80%, there was almost no retention of most of these compounds. If the logarithm of capacity factors is plotted against the methanol concentration, linear plots (see Fig. 2) can be obtained for most of these compounds, as is the case with most reversed-phase columns [17]. This fact suggests that a reversed-phase mechanism dictates the interaction between the solutes and β-cyclodextrin stationary phase.

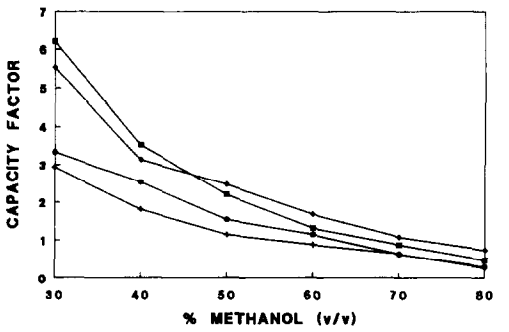


Figure 1
Effect of the methanol content in the mobile phase on retention. ◆, Chlorpromazine; ■, acetopromazine; ●, promethazine; +, propiomazine; column, Cyclobond I; mobile phase, methanol–water (v/v); T = 20°C; flow rate, 1 ml min^{-1}.

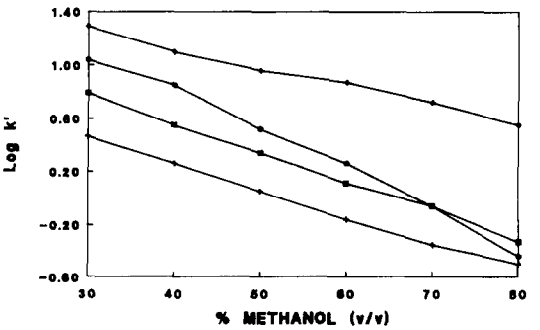


Figure 2
Plots of the logarithm of capacity factors versus methanol content. ■, Acetopromazine; ◆, prochlorperazine; ●, trimeprazine; +, 2-(trifluoromethyl)-phenothiazine.

Effect of pH

The β-cyclodextrin bonded phase column is stable over the pH range 3.5–7.5. Therefore, the effect of pH on the retention time of these compounds was investigated by changing the pH of mobile phase from 3.5 to 7.2. The pH values were obtained by using TEAA buffer (0.02 M) and the column temperature was held at 22°C. In this set of experiments, the mobile phase contained 40% methanol.

Table 2 lists the capacity factors of these compounds at different pH values as well as those pK_a values that can be found in the literature [2]. The capacity factors for almost all of these compounds are nearly constant within the range of pH investigated. This means that there is no pH effect on retention time over this range. This fact may be rationalized in terms of the pK_a values of these solutes and of β-cyclodextrin. Although phenothiazine itself has a pK_a value of 2.52, most of phenothiazine derivatives substituted in the 10- and/or 2-position have pK_a values of 8.10–9.58, and β-cyclodextrin has a pK_a value of about 12.0 [18]. In the pH range examined, the chemical form of neither phenothiazine and its derivatives nor of the β-cyclodextrin stationary phase can be changed. Therefore, the retention time of all of these compounds was unaffected by pH changes.

Effect of TEAA buffer concentration

The capacity factors of these compounds at different TEAA concentrations in the mobile phase are listed in Table 3. The retention of all of these compounds decreased with the addition of TEAA buffer and more or less stabilized at TEAA >0.01 M. This type of retention behaviour was previously encountered with the retention behaviour of chlorophenols and chlorobiphenols as well [19, 20].

The efficiency of the separations was also substantially increased with the addition of TEAA buffer in the mobile phase. For these compounds, a TEAA solution (0.02 M, pH 5.0) substituted for water in a methanol–water (40:60) system produced a three- to four-fold increase in efficiency. The selectivity of the separation was rarely affected by the TEAA concentration as evidenced by the absence of any changes in the elution order of these studied compounds with increasing TEAA concentrations. Since the presence of TEAA in the mobile phase did not decrease the selectivity, the β-cyclodextrin bonded phase column could be coupled with electrochemical detectors. As an electrolyte, the presence of TEAA in the mobile phase would increase the conductivity and minimize the *iR* drop between the reference and working electrodes, thus facilitating the efficient operation of an electrochemical detector.

Effect of temperature

The effect of column temperature on the retention was examined by changing the tem-

Table 2
Effect of pH on the retention of phenothiazine and structurally-related compounds

Compounds	Capacity factors at different pH values*					pK_a†
	3.50	4.50	5.50	6.50	7.20	
Acetopromazine	2.20	2.22	2.23	2.27	2.28	—
Chlorpromazine	3.83	3.83	3.86	3.86	3.90	9.30
Promazine	2.83	2.86	2.86	2.86	2.89	9.40
Ethopropazine	1.67	1.70	1.70	1.73	1.73	9.58
Thioridazine	7.43	7.43	7.46	7.46	7.47	9.50
Phenothiazine	2.57	2.57	2.60	2.63	2.63	2.52
Prochlorperazine	10.54	10.57	10.60	10.63	10.69	8.10
Promethazine	1.87	1.89	1.93	1.96	1.96	9.10
Propiomazine	1.67	1.71	1.74	1.74	1.77	—
Perphenazine	6.60	6.63	6.66	6.70	6.73	—
Trifluoperazine	6.80	6.83	6.83	6.86	6.89	8.10
Trimeprazine	2.70	2.70	2.73	2.76	2.76	9.00
2-Methoxyphenothiazine	2.93	2.96	2.99	3.02	3.02	—
2-Acetylphenothiazine	2.17	2.20	2.20	2.23	2.23	—
Triflupromazine	2.37	2.40	2.40	2.42	2.43	9.41
2-(Trifluoromethyl)-phenothiazine	2.70	2.73	2.76	2.76	2.81	—

*Conditions: column, Cyclobond I; mobile phase, methanol–0.02 M TEAA buffer (40:60), T = 22°C; flow rate, 1 ml min^{-1}.

†Data from ref. 2.

Table 3
Effect of TEAA buffer concentration on the retention of phenothiazine and structurally-related compounds

Compounds	Capacity factors at different TEAA concentrations*				
	0.00	0.01	0.02	0.05	0.10
Acetopromazine	3.53	2.26	2.23	2.22	2.18
Chlorpromazine	4.60	3.93	3.86	3.86	3.83
Promazine	3.13	2.86	2.86	2.86	2.83
Ethopropazine	1.93	1.75	1.74	1.72	1.72
Thioridazine	8.33	7.49	7.46	7.46	7.45
Phenothiazine	3.00	2.63	2.60	2.57	2.54
Prochlorperazine	12.67	10.72	10.60	9.90	9.55
Promethazine	2.53	2.00	1.73	1.67	1.58
Propiomazine	1.80	1.77	1.74	1.68	1.59
Perphenazine	6.80	6.75	6.66	6.33	6.03
Trifluoperazine	7.06	5.56	5.33	5.27	5.17
Trimeprazine	2.79	2.76	2.73	2.63	2.51
2-Methoxyphenothiazine	3.27	3.05	2.99	2.80	2.56
2-Acetylphenothiazine	2.27	2.26	2.20	1.93	1.73
Triflupromazine	2.43	2.33	2.33	2.26	2.22
2-(Trifluoromethyl)-phenothiazine	2.80	2.76	2.47	2.35	2.28

*Conditions: column, Cyclobond I; mobile phase, 40% methanol; pH 5.0; T = 22°C.

perature from 20 to 60°C with a mobile phase of methanol–water (50:50, v/v). Changes in temperature were seen to have a substantial effect on the retention of the solutes on the β-cyclodextrin column. The retention time of all of these compounds decreased with increasing temperature. When the temperature was increased to 60°C, there was no retention at all for most of these compounds. These results indicate that the binding constant of a solute to β-cyclodextrin decreases with increasing temperature and that inclusion formation is effectively prevented for most solutes at a temperature higher than 60°C.

van't Hoff plots (see Fig. 3), plots of the logarithm of capacity factors versus the reciprocal of the absolute temperature, are non-linear for most of these compounds. According to Horvath [21], non-linear van't Hoff plots can be expected whenever one of the following three conditions holds: (i) the eluate exists in two or more forms having different retention; (ii) there exist two or more retention mechanisms due to the heterogeneity of the stationary phase surface containing more than one type of binding site; and (iii) the eluate exists in more than one form and the surface is heterogeneous. The studies of Otagiri *et al.* [22] suggest that the interaction of β-cyclodextrin with phenothiazine and its derivative takes place at different bonding sites; the aromatic portion of phenothiazine drugs is incorporated into the cavity of β-cyclodextrin through hydrophobic interactions while the N-substituents of the drug interact with the outside groups of the β-cyclodextrin cavity by hydrogen bonding. Thus, the non-linear van't Hoff plots observed in this study are likely to be due to condition (ii) being in effect.

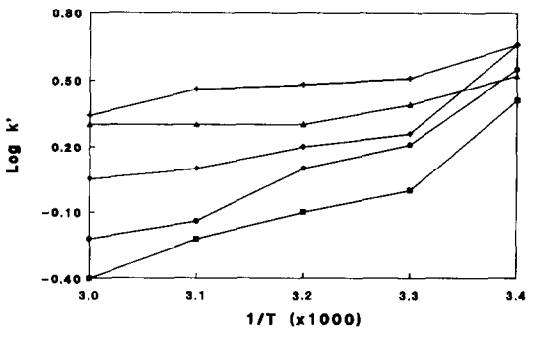


Figure 3
van't Hoff plots of some studied compounds. ■, 2-Acetylphenothiazine; ◆, chlorpromazine; ●, promazine; ▲, trifluoperazine; +, perphenazine; column, Cyclobond I; mobile phase, methanol–water (50:50); T = 22°C; flow rate, 1 ml min^{-1}.

The selectivity was very slightly affected by changing temperature. In this respect, β-cyclodextrin columns appear to behave like other reversed-phase columns [23].

Separations

After studying the liquid chromatographic retention behaviour of these 16-compounds on the β-cyclodextrin bonded phase column with respect to mobile phase composition, pH, TEAA buffer concentration and column temperature, the following isocratic conditions were chosen for the separation of these compounds: methanol–water (35:65, v/v); 0.05 M

TEAA; pH 4.5; and T = 20°C. Under these conditions, all 16 compounds eluted within a reasonable time (43 min). Figure 4 shows the chromatogram obtained under these isocratic conditions. As can be seen from the chromatogram, most of these 16 compounds can be separated with reasonable resolution. However, because a certain degree of overlap existed for these compounds, the complete separation using an isocratic elution technique was impossible.

In order to achieve the complete separation of all of the compounds, a gradient elution technique was used. Figure 5 shows the chromatogram achieved with a linear mobile phase gradient. The separation was carried out at an initial condition of 30:70 of solvent A (methanol) to solvent B (0.05 M TEAA buffer

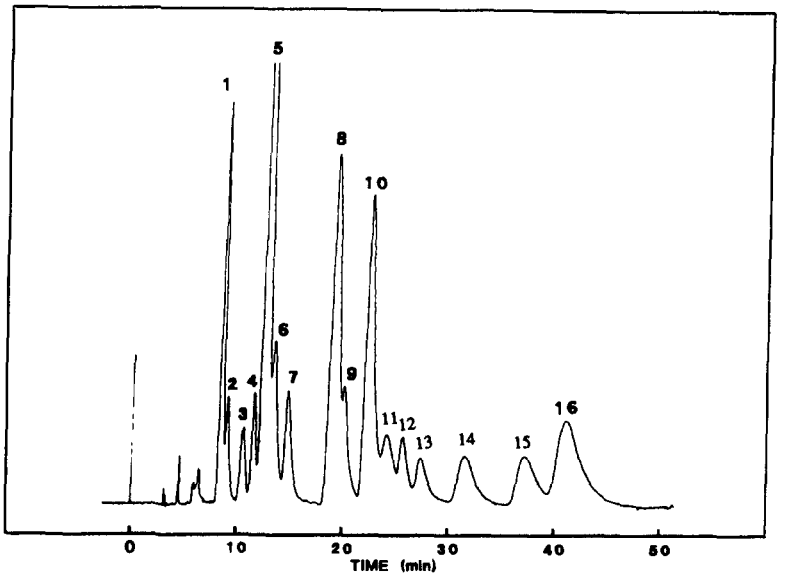


Figure 4
Isocratic separation of phenothiazines and structurally-related drugs. Peaks are identified in Table 1. Conditions: column, Cyclobond I; mobile phase, methanol–0.05 M TEAA, pH 4.5 (35:65); T = 20°C; flow rate, 1 ml min^{-1}; detector, UV at 254 nm.

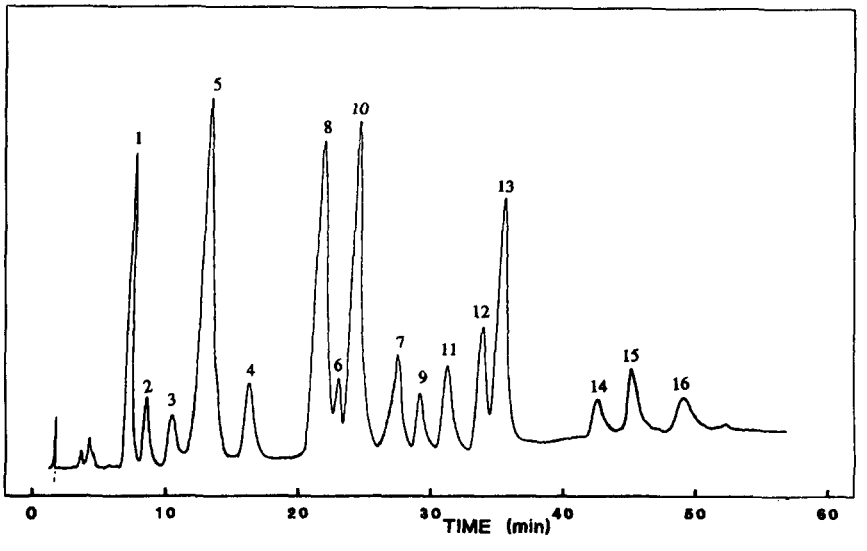


Figure 5
Gradient elution separation of phenothiazines and structurally-related drugs. Peaks are identified in Table 1. Initial mobile phase 30% solvent A (methanol) and 70% solvent B (0.05 TEAA buffer solution, pH 4.1) with linear gradient of 0.5% min^{-1} increase in solvent A; column, Cyclobond I; T = 20°C; detector, UV at 254 nm.

solution, pH 4.1) with a linear increase of 0.5% solvent A min^{-1}. As can be seen from Fig. 5, the resolution was much improved using this gradient elution system.

Previous experience with gradient elution on a β-cyclodextrin bonded phase column has shown that the steepness of the gradient must be less than what is conventionally used in reversed-phase chromatography with C18 columns. In the case of the separation of phenothiazine and its derivatives, if the steepness were greater than a 1% min^{-1} increase in methanol, no separation was observed. This result indicates that the separation is governed by equilibria involving the free and bound forms of the solute as well as solvent molecules from or to the cyclodextrin cavity; the slow kinetics of these equilibria dictates a limit to the steepness of the gradient.

In conclusion, this investigation has demonstrated that the β-cyclodextrin bonded phase column is very selective toward structurally related phenothiazine derivatives. Although the separation of these compounds can be achieved by either an isocratic or a gradient-elution mode, the resolution is much improved with the use of a gradient-elution method.

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