The Behavior of Some Phenothiazines and Their Demethylated Derivatives in Reversed-Phase Liquid Chromatography

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Abstract

Three selected phenothiazines and their demethylated derivatives are chromatographed on different C_{18} bonded reversed-phase liquid chromatography columns. A quadratic equation fits the relationship log *k* versus the organic modifier percentage. When acetonitrile is the organic modifier, the demethylated derivative is eluted before the parent compound, whereas it is eluted after when methanol is the organic modifier. The log k_w values are therefore different. Selectivity between the metabolite and the parent compound is higher with methanol.

Introduction

Phenothiazines and their derivatives are an important group of pharmaceuticals that are used for the treatment of psychic diseases. Determination of phenothiazines in pharmaceutical formulations or in body fluids is important. Phenothiazines are a group of basic drugs with different substituents attached at the 2-position (R_2) and 10-position (R_{10}) of the phenothiazine ring. The R_{10} substituent is either an alkyl piperazine group, piperidine moiety, or aliphatic chain containing an amino group.

As far as this last group is concerned, when two methyl groups are attached at the nitrogen atom, phenothiazines can undergo a loss of one methyl group. When this happens, a mono demethylated derivative or nor-derivative is produced, which is the main circulating metabolite and exhibits psychotic properties. Further demethylation gives rise to a primary amine at the end of the alkyl chain

Some phenothiazines have been separated by thin-layer chromatography (TLC) (1,4), gas chromatography (GC) (5–7), or capillary electrophoresis (8,9). GC methods suffer from thermal instability and the low volatility of such solutes, and TLC does not generate the necessary selectivity for separation. First attempts in normal-phase liquid chromatography (NPLC) have been carried out on high surface area silica (10) or cyanopropyl-bonded phase (11). Reversed-phase liquid chromatography (RPLC) is well suited for phenothiazine analysis because direct injection of the aqueous solution can be carried out. Karst et al. (12,13) used a C_{18} column with a gradient consisting of acetonitrile (ACN)– ammonium formate buffer with post column oxidative derivation electrochemistry–mass spectrometry (MS) or electrochemistry–fluorescence. Phenothiazines are sensitive to oxidation or desulfurization, which allows enhanced detection (14,15).

Other authors utilized LC–MS, which provides low limits of detection (16–21), and a screening method based on electrospray ionization–MS was devised (22) in which the sole levomepromazine was detected among 70 psychoactive drugs. Nevertheless, in most cases, UV detection (23) with photodiode array detection is utilized because it is sensitive (24).

From the literature one can find lists of phenothiazine retention factors (*k*) on different stationary phases (25,26), but infor-

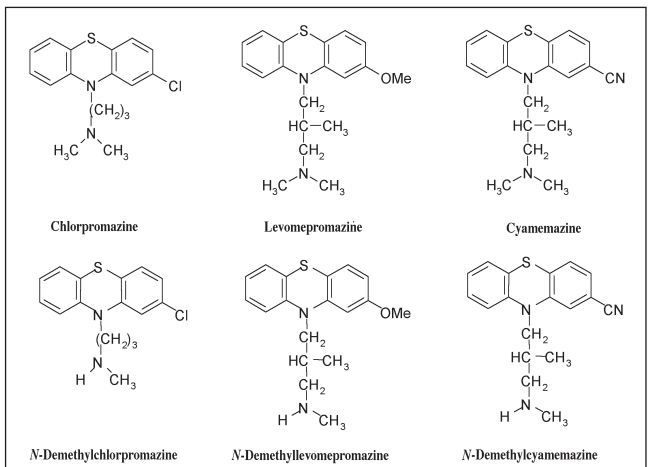

Figure 1. Structural formulas of the three phenothiazines and their *N*-demethyl derivatives.

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mation on the chromatographic behavior of metabolites is scarce. Boehme and Strobel (27) separated chlorpromazine metabolites on an Ultrasphere cyano column with a very complex mobile phase (ACN–methanol–sodium acetate–ammonium acetate–diethyl amine–triethyl amine at pH 9.5), which can be detrimental to the stationary phase. Chetty et al. (28) used NPLC to separate the chlorpromazine metabolites. Loennechen and Dahl (29) utilized ion pair formation to separate the metabolites of levomepromazine. In a previous paper (30), the chromatographic behavior of two phenothiazines and their demethylated derivative on different alkyl-bonded phases with a binary eluent (ACN–phosphate buffer) was studied. In an attempt to optimize the separation of levomepromazine from its demethylated derivative, it was noticed that a ternary eluent (ACN–methanol–phosphate buffer) may be either detrimental to the separation or conversely enhance the selectivity.

With the availability of monolithic silica-based columns and the commercially available Chomolith, it is easier to perform separations at a higher speed than on columns packed with conventional particles. Monolithic high-performance liquid chromatography (HPLC) columns exhibit both a high permeability and high separation efficiency at high flow velocities. The behavior of three selected phenothiazines (e.g., cyamemazine, levomepromazine, and chlorpromazine) together with their demethylated derivatives (Figure 1) on C_{18} bonded silica from Chromolith together with a conventional column packed with 5-µm particles (Purospher) and other RP columns from different manufacturers has been investigated. Two types of binary mixtures (ACN–buffer and methanol–buffer) were used. The differences of the behavior of the demethylated metabolites are reported, according to the type of organic modifier.

Experimental

Chromatography was carried out with a Lachrom (Merck, Darmstadt, Germany) instrument equipped with a Hitachi L 6200A pump (VMR, Fontenay Sous Bois, France) and a Rheodyne injection port (Rheodyne, Cotati, CA) with a 20-µL sample loop. A Hitachi L-4200 UV detector was used at the selected wavelength of 254 nm (0.005 AUFS). Data acquisition was carried out with a D2500 (Merck) data system.

Columns were Chromolith Performance RP18e (100- × 4.6-mm) (UMC 126/048) (Merck) or Purospher C18 (100- × 4.6-mm, 5-µm particle size) from Merck. The Symmetry column (100- × 4.6-mm, 5-µm particle size) was from Waters (St. Quentin, Yvelines, France). Columns were thermostated at 30°C in an oven (Cluzeau, Ste Foy la Grande, France). The retention time of the unretained solute was measured by the injection of either uracil solution or sodium nitrate solution.

Samples of cyamemazine, chlorpromazine, levomepromazine, and their demethylated derivatives were provided by Specia Rhône Poulenc Rorer (Aventis, Paris, France). Stock solutions were prepared at 20-mg/L concentrations.

ACN and methanol were HPLC grade from Merck. HPLC-grade water was from SDS (Peypin, France). Phosphate buffer (pH 3.2) was prepared according to the procedure of the French Pharmacopea.

Results and Discussion

Chromatography was carried out with two types of binary mixtures as the eluent: ACN–phosphate buffer and methanol–phosphate buffer.

Table I lists the retention factors (as log *k*) of the selected phenothiazines and their demethylated derivatives on both the Chromolith column and the Purosher column with different percentages of ACN as organic modifier. As expected, the retention times were very long when the percentage of the organic modifier is low. The retention factor of the demethylated cyamemazine, which was the first eluted solute, was 492 when the ACN percentage was 10%. However, the main advantage of the Chromolith column is the possibility to explore a wide range of mobile phase compositions because the monolith column allows the use of very high flow rates (5 mL/min or even more). The external porosity of Chromolith is nearly twice that found in conventional particle-packed columns. Phenothiazines are basic solutes (pK ~ 9), and the chromatographic peaks were asymmet-

Table I. log *k* Values of the Selected Solutes at Different Percentages of Acetonitrile in the Mobile Phase*

Percentage of ACN	25		30		40		50		60	
	log *k*									
Solute	Chromolith column	Purospher column	Chromolith column	Purospher column	Chromolith column	Purospher column	Chromolith column	Purospher column	Chromolith column	Purospher column
Demethyl cyamemazine	2.211	3.015	1.112	2.147	–0.241	0.560	–1.253	–0.321	–1.931	–1.041
Cyamemazine	2.294	3.078	1.344	2.234	–0.154	0.664	–1.113	–0.218	–1.836	–0.936
Demethyl levomepromazine	2.308	2.969	1.329	2.084	–0.241	0.537	–1.253	–0.376	–1.931	–1.099
Levomepromazine	2.572	3.211	1.593	2.350	0.069	0.731	–0.953	–0.125	–1.669	–0.887
Demethyl chlorpromazine	2.868	3.542	1.812	2.604	0.182	0.939	–0.881	0.000	–1.595	–0.796
Chlorpromazine	2.994	3.713	1.968	2.820	0.347	1.136	–0.814	0.211	–1.401	–0.565

* Mobile phase consisted of ACN–phosphate buffer (pH 3.2).

rical. The higher the percentage of buffer in the eluent, the more asymmetrical the peaks.

As far as the parent compounds are concerned, the elution order was cyamemazine < levomepromazine < chlorpromazine, according to the hydrophobicity of the substituent CN < OMe < Cl.

A plot of log $k = f(\phi)$ with F = percentage of ACN is displayed in Figure 2. For the sake of simplicity, the plot of levomepromazine and its demethylated derivative is only displayed. The log $k = f(\phi)$ plot is adequately described by a three parameter quadratic equation.

$$\log k = a\phi^2 + b\phi + c \qquad \text{Eq. 1}$$

If only a limited range of mobile phase compositions is considered one can write (31)

$$\log k = \log k_w - S\phi + a\phi^2 \qquad \text{Eq. 2}$$

where ϕ is the volume fraction of the organic modifier, k_w is the extrapolation of k for water as mobile phase ($\phi = 0$), and S is a constant for the solute.

Table II presents the regression equations for the six solutes on the Chromolith column. It is obvious that the quadratic term (a) was very small. A simple linear regression ($\log k = \log k_w - S\phi$) will suffice when considering low percentages of ACN. The main feature was that, in any case, the demethylated derivative always elutes before the parent compound (Figure 3).

The a and S coefficients were very similar, and the selectivity between the phenothiazine and its derivative remains constant over the whole range of ϕ. The curves were all parallel. The selectivity between levomepromazine and its demethylated derivative is a little bit higher than the one observed with the pair of other solutes.

The log k_w values were in accordance with the elution order and the hydrophobicity of the substituents in the parent compounds. It would be possible to use a very low percentage of organic modifier but a "phase collapse" would occur. The observed log k_w values looked high and did not compare with the reported values from Detroyer et al. (26), who measured the retention factors of 22 phenothiazines on different columns at different pHs and determined the log k_w at pH 7.3 and 11.7.

In order to compare the Chromolith column with the conventional ones, the same experiments were carried out on two "classical" commercial columns. Data are compiled in Table III. The same trend was observed with similar coefficients. The log k_w values were high and differ according to the type of column. Nevertheless, it has been pointed out by Poole (32) that different log k_w values cannot be used for column characterization, as was evidenced in data compiled by Detroyer (26). Nevertheless, the log k_w values were much higher than those previously published.

The situation changed completely when methanol was used as the organic modifier. Because of the possible precipitation of the phosphate buffer in methanol, a limited range of percentages was considered. In any case, the demethylated derivative was eluted after the parent compound (Figure 4).

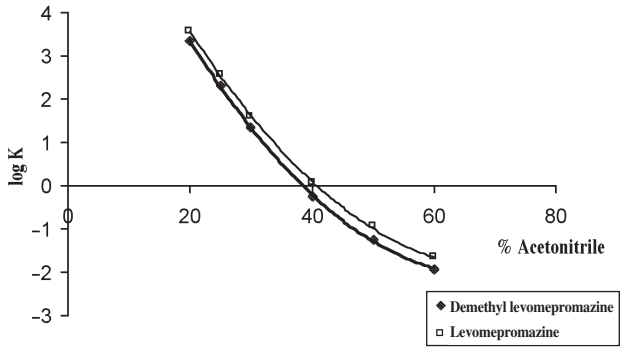

Figure 2. Plot of log k versus percentage of acetonitrile (ϕ) for levomepromazine and *N*-demethyl levomepromazine (plot was generated from experimental data obtained with a Chromolith column and binary mixtures of acetonitrile–phosphate buffer as mobile phase).

Table II. Coefficients of the Quadratic Regression Equation Showing the Relationship Between log k and ϕ* Calculated for Three Phenothiazines and Their *N*-Demethyl Derivatives*

Solutes	Coefficients of the quadratic regression equation $\log k = \log k_w - S\phi + a\phi^2$		
	log k_w	S	a
Cyamemazine	8.3539	–0.2963	0.0021
Demethyl cyamemazine	8.3304	–0.3029	0.0022
Levomepromazine	8.8255	–0.3057	0.0022
Demethyl levomepromazine	8.8008	–0.3167	0.0023
Chlorpromazine	10.024	–0.3461	0.0026
Demethyl chlorpromazine	9.6993	–0.3360	0.0025

* Percentage of acetonitrile in binary mixture acetonitrile–phosphate buffer used as mobile phase.

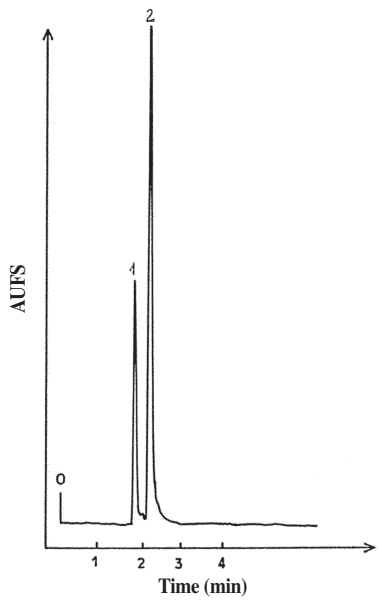

Figure 3. Separation of *N*-demethyl levomepromazine and levomepromazine with acetonitrile as the organic modifier: column, Chromolith C_{18} (100- × 4.6-mm); mobile phase, ACN; phosphate buffer (pH 3.2) (30:70, v/v); flow rate, 2.4 mL/min; detection, UV 254 nm; 0.005 AUFS; peak 1, demethyl levomepromazine; and peak 2, levomepromazine.

Table III. Comparison of the Regression Coefficients with Three Columns (Chromolith, Purospher, and Symmetry) for Three Phenothiazines and Their *N*-Demethyl Derivatives with Mixtures of ACN–Phosphate Buffer as the Mobile Phase

		Coefficients of the quadratic regression equation $\log k = \log k_w - S\phi + a\phi^2$		
Solutes	Columns	$\log k_w$	S	a
Cyamemazine	Chromolith	8.3539	–0.2963	0.0021
	Purospher	8.1411	–0.2775	0.0019
	Symmetry	6.9216	0.2043	0.0009
Demethyl cyamemazine	Chromolith	8.3304	–0.3029	0.0022
	Purospher	8.7469	–0.2775	0.0019
	Symmetry	6.6871	0.2016	0.0009
Levomepromazine	Chromolith	8.8255	–0.3057	0.0022
	Purospher	9.3715	–0.3004	0.0022
	Symmetry	8.1980	0.2664	0.0017
Demethyl levomepromazine	Chromolith	8.8008	–0.3167	0.0023
	Purospher	9.0155	–0.2949	0.0021
	Symmetry	6.9889	0.2133	0.001
Chlorpromazine	Chromolith	10.024	–0.3461	0.0026
	Purospher	9.8213	–0.2956	0.0020
	Symmetry	8.8213	0.2762	0.0018
Demethyl chlorpromazine	Chromolith	9.6993	–0.3360	0.0025
	Purospher	9.6980	–0.2986	0.0021
	Symmetry	8.1302	0.2448	0.0013

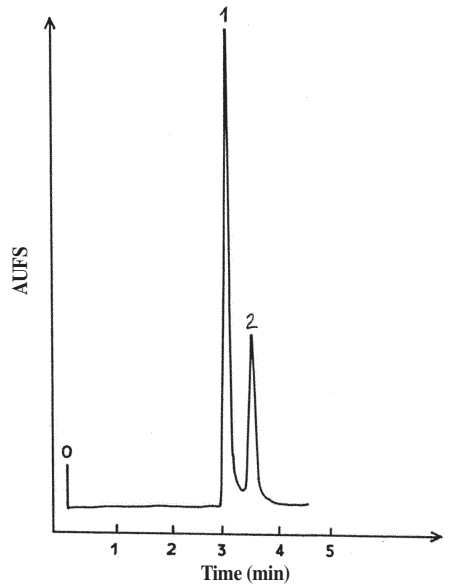

Figure 4. Separation of *N*-demethyl levomepromazine and levomepromazine with methanol as organic modifier: column, Chromolith C_{18} (100- × 4.6-mm); mobile phase, methanol–phosphate buffer (pH 3.2) (50:50, v/v); flow rate, 2.4 mL/min; detection, UV 254 nm, 0.005 AUFS; peak 1, levomepromazine; and peak 2, demethyl levomepromazine.

Table IV displays the retention on both the Chromolith and the Purospher columns with methanol as the organic modifier. It is worth noting that the selectivity between the parent compound and the metabolite was higher, which allowed for a better separation of the six solutes. Figure 5 displays the separation of the six solutes. This result was somewhat disconcerting as the pioneering work of Karger (33) and the Snyder's selectivity triangle (34) show that reversal of the order of retention was observed when different species with different functional groups were chromatographed on an RP column with a different organic modifier in the mobile phase. Different log k_w values can be obtained for the same compound when these log k_w values were derived using a different set of mobile phase compositions, or a different type of organic modifier (methanol or ACN). Guillaume and Guinchard (35) observed that the retention mechanism of 10 benzodiazepines was significantly different in methanol–water and ACN–water mixtures. They argued that the methanol solution is dominated by competitive hydrogen bonding and that the availability of "free" methanol for solute solvation decreases with increasing volumes of water. On the other hand, ACN solution chemistry is governed by clusters of ACM, where the molecules are preferentially solvated. Valko et al. (36) proposed the description of the chromatographic hydrophobicity index (CHI) from a gradient elution. From their data, the CHI indices were higher when ACN was used as the organic modifier than when methanol was the modifier, but the general trend was the same with one single exception. To the best of our knowledge, there are no log k_w data related to metabolites of drugs.

The chromatographic retention of an analyte in RPLC strongly depends on its ionization in addition to its hydrophobicity. From previously described data, the demethylated derivative looked more hydrophobic than the parent compound when methanol was utilized and less hydrophobic when ACN was utilized, which precludes the determination of the hydrophobicity of the demethylated derivatives. Moreover it precluded any attempt of retention optimization with a ternary mixture. It should be pointed out that those solutes were ionizable. At pH 3.2 (the pH of the buffer was measured before addition of the organic modifier) the solutes were protonated; such compounds were prone to secondary interaction mechanisms to the RP. Shifts in $^{s}{}_{w}$pH units caused by the addition of the organic modifier were usually 0.2–0.9 (37). The measured hydrophobicity of the charged

Table IV. Results Obtained with Chromolith and Purospher Columns with Binary Mixture Methanol–Phosphate Buffer as the Mobile Phase

Percentage of MeOH	40	50	60	35	40	50
Solutes	log *k* (Chromolith)			log *k* (Purospher)		
Cyamemazine	2.39	1.00	–0.23	3.26	2.09	1.90
Demethyl cyamemazine	2.62	1.22	–0.08	3.45	2.29	1.86
Levomepromazine	2.87	1.50	0.23		2.61	2.29
Demethyl levomepromazine	3.07	1.67	0.40			
Chlorpromazine	3.46	2.05	0.78			
Demethyl chlorpromazine	3.57	2.17	0.86			

solute was different from the one of the neutral molecule. The hydrophobicity of ionizable molecules depends on the pH (38), but the published data deals with ACN–buffers only. A possible explanation is that alkyl groups in the close vicinity of the basic site may hinder interaction with the column surface or that strong self-association of the solvent may diminish the solvent ability for forming a hydrogen bond with the solute. A possible explanation is the multiplicative interaction. Neue et al. (39) state that "the retention pattern of positively charged analytes is dominated by an interaction mechanism that combines revered-phase interaction of the analyte with the hydrophobic C_{18} layer with ionic bonding to charged silanols in a multiplicative manner". However, this conclusion was drawn from data with ACN and a comparison with methanol would bring further insight.

Conclusion

The demethylated derivatives of phenothiazines, which are the main circulating metabolites, were eluted before the parent compound on an RP column when a binary mixture of ACN and buffer was the mobile phase. On the other hand, they were eluted after ACN was replaced by methanol. The mechanism was not fully elucidated. The selectivity between one single phenothiazine and its demethylated derivative was higher when methanol was the organic modifier in the mobile phase than when ACN was the modifier. Chromolith columns are useful to scan the high percentages of buffer in the eluent because high flow rates can be utilized.

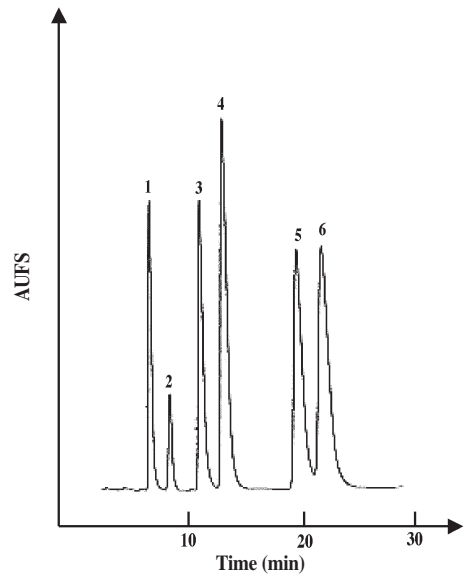

Figure 5. Separation of the six analytes: column, Chromolith C_{18} (100- × 4.6-mm); mobile phase, methanol–phosphate buffer (pH 3.2) (40:60, v/v); flow rate, 2.4 mL/min; and detection, UV 254 nm, 0.005 AUFS. Peak order: 1, cyamemazine; 2, demethyl cyamemazine; 3, levomepromazine; 4, demethyl levomepromazine; 5, chlorpromazine; and 6, demethyl chlorpromazine.

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