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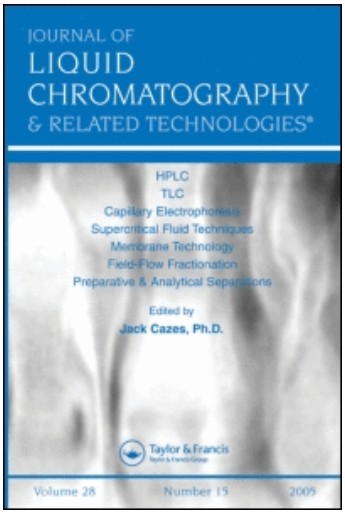

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Evaluation of the Chromatographic Behaviour of Fluoxetine and Norfluoxetine Using Different Cyclodextrins as Mobile Phase Additives and Fluorimetric Detection

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EVALUATION OF THE CHROMATOGRAPHIC BEHAVIOUR OF FLUOXETINE AND NORFLUOXETINE USING DIFFERENT CYCLODEXTRINS AS MOBILE PHASE ADDITIVES AND FLUORIMETRIC DETECTION

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ABSTRACT

The principal goal of this work was to investigate the liquid chromatographic retention behaviour of fluoxetine and norfluoxetine using HPLC with respect to mobile phase composition, pH, flow rate and the amount of the native β-cyclodextrin (β-CD) or the β-hydroxypropyl-cyclodextrin (HP-β-CD), added to the mobile phase. Further, it was of interest to evaluate the effectiveness of β-CD and HP-β-CD to enhance fluorescence detection of these compounds.

Another purpose of this study, was to calculate the formation constants of the inclusion complexes (K_f) of fluoxetine and norfluoxetine within the HP-β-CD, in different mobile

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phase compositions. Based on these findings, the log K_f values of these two compounds referred to pure aqueous mobile phase (log K_{fw}), can be determined by extrapolation.

INTRODUCTION

Fluoxetine is an important new antidepressant drug for the treatment of unipolar mental depression. Both fluoxetine (FL) and its N-desmethylated metabolite norfluoxetine (NR) (fig. 1), enhance serotoninergic neurotransmission through potent and selective inhibition of presynaptic serotonin reuptake.[1-3]

FL, as well as NR, are two compounds of great pharmacological and analytical importance. Several HPLC methods have been reported for the determination of FL and NR in serum or plasma.[4-9] Since FL is marketed as a racemic mixture, a number of methods have been reported for the indirect[10-12] and direct[13] chromatographic separation of the enantiomers of FL and NR.

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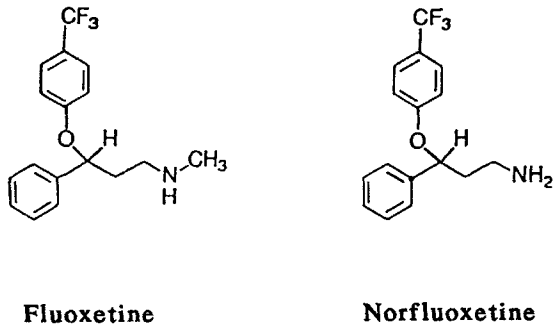
Cyclodextrins (CDs) are cyclic oligosaccharides composed of six or more glycopyranose moieties bonded together via α-(1,4)-linkages. Generally, the external part of the CD molecule is hydrophilic compared to its cavity. The hydrophilic nature of the molecule's external portion is due to the primary and secondary hydroxyl groups, being located on the smaller and larger sides of the CD molecule, respectively. CDs have the ability to form inclusion complexes with a variety of molecules. The formation of an inclusion complex depends on the shape, size and spatial geometry of the solute, the diameter of the CD cavity and other factors.[14]

In this paper, the liquid chromatographic retention behaviour of FL and NR was investigated by using a reversed phase column (RP-Spherisorb-phenyl) with respect to mobile phase composition, pH, flow rate and the amount of the β-cyclodextrin (β-CD) or the 2-hydroxypropyl-β-CD (HP-β-CD) added to the mobile phase. Moreover, the effectiveness of β-CD and HP-β-CD, to serve as fluorescence enhancement agents on the chromatographic detection of these compounds was studied.

Generally, considerable attention has been focused on the use of CDs in luminescence applications.[15-17] The presence of CDs can dramatically enhance the fluorescence signal of complexed solutes. The factors thought of being responsible for such intensified luminescence, include shielding of the CD-



Fluoxetine **Norfluoxetine**

Figure 1. Molecular structure of Fluoxetine and Norfluoxetine.

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complexed analyte molecule from quenching by water molecules or solvent-borne quenchers. In addition, the included solute molecule experiences a less polar and more rigid local microenvironment.[15,18]

Chromatographic separation, using CDs, is mainly the result of variation in the stability of inclusion complexes of the analytes with the CD. The elution time of a solute is a function of the stability of these complexes. Several intermolecular interactions are responsible for the formation of these complexes. These driving forces act synergistically and are related to the physicochemical properties of the guest molecule. Since the formation constant (K_f) of the complex is dependent on many factors the use of CDs as mobile phase additives provides a high degree of selectivity.

From this point of view, in this work special attention was devoted to determine the apparent formation constants of FL and NR complexes (K_f) within the HP-β-CD. The effect of the organic modifier, e.g., acetonitrile, on the inclusion process has been investigated by using mobile phases with different amounts of the organic solvent. Finally, the K_f values of these two compounds referred to pure aqueous mobile phase (K_{fw}) have been found experimentally by extrapolation based on the above mentioned data.

MATERIALS

All solvents were of HPLC-grade and were purchased from Tech-line (Athens, Greece). Triethylamine and glacial acetic acid were of analytical grade and were purchased from Aldrich. Fluoxetine and Norfluoxetine in the form of their hydrochloride salts were kindly provided by Eli Lilly and used as received.

METHODS

Apparatus

The liquid chromatographic system consisted of a Waters model 501 pump, a Rheodyne model 7125 injector with 5μL, 20μL and 100μL loops, as well as a Perkin Elmer LS30 fluorimetric detector with an 8μL flow cell. The chromatograms were obtained by using a Hewlett Packard integrator model HP3394A.

A Spherisorb-phenyl S5 column (150 X 4.6-mm I.D.) was obtained from Hellamco (Athens, Greece). When not in use, the column was stored in 100% methanol. pH readings were obtained by using a Metrohm Herisau pH-meter (model 654). All experiments were performed at room temperature (about 25°C). The compounds were detected at an excitation wavelength of 235nm and an emission wavelength of 315nm.

Chromatographic Conditions

The mobile phases, consisting of triethylammonium acetate buffer and the appropriate amount of the organic modifier, were freshly prepared, filtered and degassed under vacuum by using a Millipore system.

Buffers were prepared using triethylamine solutions of different concentrations (i.e., 0.1%-2.5%) which were adjusted by addition of glacial acetic acid to the desired pH (i.e., 3.5-7.0).

The effect of the organic modifier in this study was examined by preparing organic/aqueous mobile phase systems. The organic modifiers used were: methanol, acetonitrile, tert-butanol, cyclopentanol.

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Solutions

The stock standard solutions of the compounds (1.00mg/mL) were accurately prepared by dissolving an appropriate amount of the compound in HPLC-water and kept in amber-coloured bottle in a refrigerator and renewed every week. Working standard solutions of each compound (1.00μg/mL) were prepared every day in mobile phase. Typically a volume of 5μL of each solution was injected. The void volume of the column was determined by injecting 5μL of pure methanol. To evaluate the reproducibility of the retention times each run was performed three times.

A standard curve and five validation samples at five concentrations of each compound were assayed, i.e., 50-, 100-, 200- 350- and 500 ng/mL. The standards were prepared by diluting the appropriate aliquots of the stock solution with mobile phase. Typically, a volume of 20 μL or 100 μL of each standard was injected. The standard curves were generated by a linear least-squares regression analysis of the peak height of each compound versus its concentration.

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RESULTS AND DISCUSSION

Preliminary studies

The effect of mobile phase composition on the retention time and the resolution of FL and NR, using CDs as mobile phase additives, was investigated by changing the organic modifier/buffer ratio in the mobile phase from 10:90 to 50:50. Acetonitrile, MeOH, t-BuOH and cyclopentanol have been used as organic modifiers in binary or ternary mobile phase mixtures. Further, the presence of the organic modifier in the mobile phase, leads to an enhancement or a reduction of the fluorescence signal, with respect to its ability to compete with solutes for the β-CD cavity. The best results were obtained by using ACN as organic modifier.

The effect of the ionic strength on the retention behaviour of FL and NR was studied by varing the concentration of triethylammonium acetate (TEAA) from 0.1 - 2.5% (w/v) at different pH values. The decrease of salt concentration results in an increase of retention and resolution.

On the other hand, the effect of pH in this study was investigated by changing the pH of the aqueous content of the mobile phase from 3.5 to 7.0 (respecting column limitations). As pH increases, the solutes become more retained and hence, the column's inclusion selectivity and solutes' resolution also increase.

Furthermore, the effect of the flow rate on the retention of FL and NR was investigated by decreasing the flow rate (v) from 2.0 to 0.2 mL/min.

The presence of CDs in the mobile phase enhances the fluorescence signal which depends on the CD concentration. This dependence is no doubt due to the increased proportion of the solute molecule which is included in the CD cavity. Excited states of analyte molecules possessing a -NH moiety, as FL and NR, are efficiently quenched by water molecules.[15] Thus, fluorescence enhancement, observed for the molecules of FL and NR can be due to the elimination, or the reduction, of their exposure to water molecules with the added CDs media.

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Typically, addition of β-CD and HP-β-CD in the mobile phase always results in an enhanced fluorescence compared to that observed in bulk water; the relative magnitude of this enhancement was found to be dependent upon the specific β-CD system used. This is presumably due to their different complexing tendencies.

It has been reported that HP-β-CD exhibits an equal or worse complexing ability than native β-CD does; nevertheless, the use of HP-β-CD has two main advantages compared to the β-CD: it presents lower solution viscosity and greater water solubility at each concentration level .

Moreover, it appears that for the molecules of FL and NR, HP-β-CD exhibits greater fluorescence enhancement ability than native β-CD does. Figure 2 presents the typical chromatograms by injecting equal amounts of FL and NR, under the same chromatographic conditions, without β-CD (fig. 2a) and in the presence of 10mM β-CD (fig. 2b) and 10mM HP-β-CD (fig. 2c), respectively. With the addition of native β-CD, as it is presented in Table I, the fluorescence signal has been increased by 22% and 51% for the molecule of NR and FL, respectively. The addition of the same amount of HP-β-CD results in greater fluorescence output and the signal has been increased by 40% and 110% for the molecule of NR and FL, respectively.

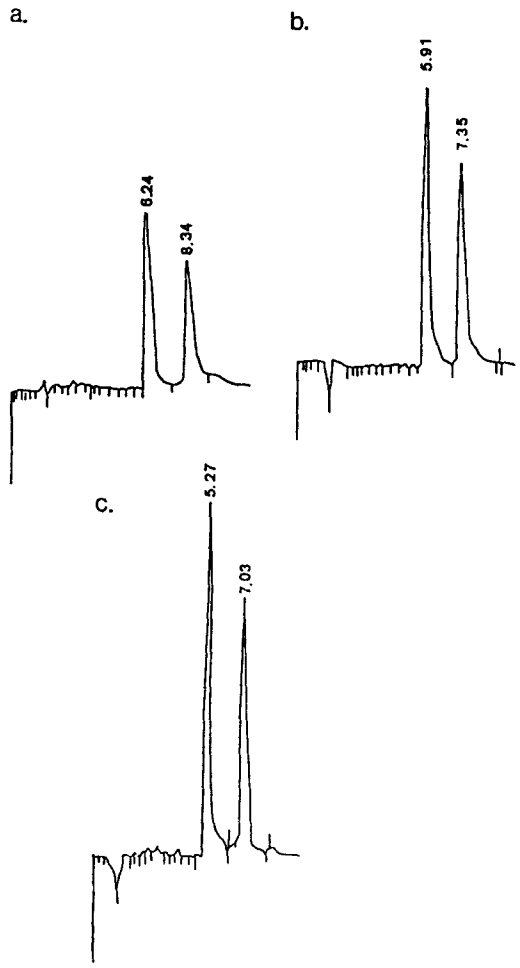

Figure 2. Chromatographic resolution of NR and FL: a. without β-CD b. in the presence of 10mM β-CD, c. in the presence of 10mM HP-β-CD. Chromatographic conditions: 0.5 %TEAA (pH:6.5) /ACN, 60/40, flow rate : 1.5ml/min.

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Table 1

Enhancement of Fluorescence* of Norfluoxetine and Fluoxetine in the Presence of Different Cyclodextrins Media.

Compound	Without CD	With 10mM β-CD	With 10mM HP-β-CD
NR	26257 (±78)	32244 (±66)	45108 (±67)
FL	18086 (±56)	27367 (±45)	39708 (±85)

*Representative peak height corresponding to 5μL injections of 1μg/ml of each compounds; integrator attenuation 4; n=5 (runs performed).

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Table 2

Equations Relating Peak Height (H) with β-CD Concentration
$H = a + b \cdot [\beta\text{-CD}]$

Compound	a	b	r	n
NR	9094 (±2377)	2547040 (±228170)	0.990	5
FL	9547 (±1099)	1863680 (±105522)	0.995	5

a : intercept, b : slope, r : correlation coefficient, n : number of points

Fluorescence Chromatographic Detection of FL and NR by Using β-CD as Mobile Phase Additive

It has already been mentioned that the enhancement of the fluorescence signal with the addition of CDs media depends on the CD concentration. As it is shown in Table 2, the peak height of FL and NR increases linearly by increasing the β-CD concentration from 5mM to 17mM, under the same chromatographic conditions (i.e.,mobile phase: 0.5% TEAA (pH:6.5)/ACN: 60/40, flow rate : 1.7mL/min).

Table 3

Equations Relating Peak Height (H) with Flow Rate (v) :
$1/H = a + b \cdot v$

Compound	a	b	r	n
NR	$5.84 \cdot 10^{-6}$ ($\pm 1.26 \cdot 10^{-6}$)	$3.78 \cdot 10^{-5}$ ($\pm 1.62 \cdot 10^{-6}$)	0.997	5
FL	$5.29 \cdot 10^{-6}$ ($\pm 1.74 \cdot 10^{-6}$)	$4.49 \cdot 10^{-5}$ ($\pm 2.23 \cdot 10^{-6}$)	0.996	5

a : intercept, b : slope, r : correlation coefficient, n : number of points.

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Moreover, an increase of the fluorescence signal (H) has been observed when the flow rate (v) of the mobile phase decreases from 1.3 to 0.3mL/min, keeping the other chromatographic conditions constant, i.e., mobile phase:0.5% TEAA (pH:6.5)/ACN, 60/40 and β-CD 10mM. Table 3 shows the dependence of peak height on flow rate.

On the other hand, a considerable decrease of flow rate (v) results in an approximately 3.5-fold enhancement of fluorescence which leads to a better sensitivity. The difference in slope values presented in Table 3 indicates that the influence of flow rate is greater for the molecule of FL.

It seems that by decreasing the flow rate the molecules have greater opportunity to interact with the CD cavity and thus fluorescence signal is further intensified, possibly due to a greater tendency of solutes to be encapsulated into the protective microenvironment of CD cavity.

Fluorescence Chromatographic Detection of FL and NR by Using HP-β-CD as Mobile Phase Additive

HP-β-CD presents greater water solubility than β-CD and the use of more concentrated HP-β-CD medium in lieu of native β-CD, leads to a better sensitivity. As shown in tables 4 and 5, the peak height of FL and NR increases as the concentration of HP-β-CD increases from 5mM to 50mM under the same

Table 4

Equations Relating Peak Height (H) of NR with [HP-β-CD]
$H = a + b \cdot [HP\text{-}\beta\text{-}CD]$

Buffer/ ACN	a	b	r	n
90/10	24759 (±2401)	1243870 (±87680)	0.992	5
80/20	39235 (±1431)	1521310 (±52289)	0.998	5
70/30	51595 (±939)	2226310 (±34301)	0.999	5
60/40	101219 (±3872)	2188430 (±141413)	0.994	5
50/50	159686 (±1885)	1838690 (±68866)	0.998	5

a : intercept, b = slope, r = correlation coefficient, n = number of points

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Table 5

Equations Relating Peak Height (H) of FL with [HP-β-CD]
$H = a + b \cdot [HP\text{-}\beta\text{-}CD]$

Buffer/ ACN	a	b	r	n
90/10	19633 (±1242)	934473 (±45384)	0.996	5
80/20	29020 (±1399)	1477040 (±51109)	0.998	5
70/30	46655 (±1660)	2341910 (±60644)	0.999	5
60/40	81486 (±1084)	1822680 (±39583)	0.999	5
50/50	135523 (±2099)	1101420 (±76658)	0.993	5

a : intercept, b = slope, r = correlation coefficient, n = number of points.

chromatographic conditions (e.g., flow rate : 0.8mL/min, 0.5% TEAA (pH : 5.5)) using different mobile phase ratios.

In reversed-phase HPLC it is common to use aqueous/organic mobile phase systems. In addition to the dilution effect, the presence of the organic modifier in the mobile phase leads to another complication with the CDs

Table 6

Equations Relating the Peak Height (H) with flow rate (v) :
$1/H = a + b \cdot v$

Compound	a	b	r	n
NR	$7.22 \cdot 10^{-6}$ ($\pm 7.44 \cdot 10^{-7}$)	$7.70 \cdot 10^{-6}$ ($\pm 8.70 \cdot 10^{-7}$)	0.981	5
FL	$1.41 \cdot 10^{-5}$ ($\pm 7.61 \cdot 10^{-7}$)	$9.18 \cdot 10^{-6}$ ($\pm 8.89 \cdot 10^{-7}$)	0.986	5

a : intercept, b : slope, r : correlation coefficient, n : number of points.

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media, since the organic solvent will compete with the analyte molecules for the CD cavity binding sites, which reduces the percentage of complexed (and protected) fluorophore. In fact, as shown in Tables 4 and 5, as the ACN concentration in the mobile phase increases, the fluorescence intensity observed also increases.

Using HP-β-CD as a fluorescence enhancement agent, the intensity increases tremendously by decreasing flow rate (v) from 1.3 to 0.3mL/min, keeping the other chromatographic conditions constant i.e., mobile phase:0.5% TEAA (pH: 5.5)/ ACN : 90 / 10, HP-β-CD 15mM. Table 6 describes the dependence of peak height (H) of FL and NR on flow rate.

After studying the liquid chromatographic retention behaviour of NR and FL by using CDs as mobile phase additives and with respect to mobile phase composition, pH, ionic strength, a set of isocratic conditions was chosen for the simultaneous separation of these compounds. The conditions are as follows: acetonitrile / buffer, 60/40 (v/v); buffer 0.5% TEAA (w/v); pH 7.0; flow rate : 1.7 mL/min; detection Ex: 235nm, Em: 310nm, attenuation factor : 3 (fluorimetric detector).

The calibration curve for each compound was constructed from a linear-squares regression of peak-height of the standards versus the concentrations. Typical correlation coefficients were r : 0.9998. The linearity of the curve has

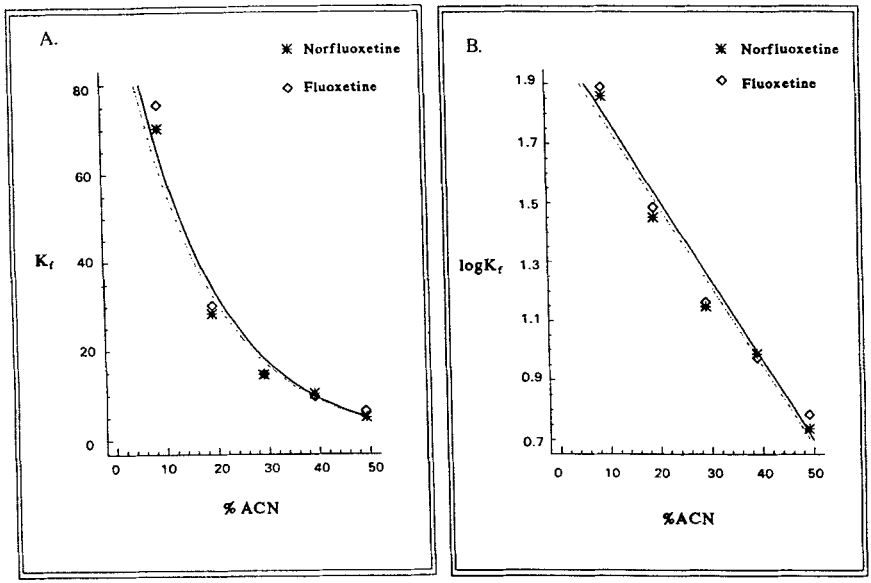

Figure 3. Dependence of the capacity factor (k') of NR (A) and FL (B) on HP-β-CD concentration.

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been demonstrated from 50 ng to 500 ng/mL. These concentrations have been chosen according to the produced plasma concentrations at steady state for both compounds which, after a daily oral dose of 60mg, are between 200 and 500 ng/mL for fluoxetine and 180 and 450 ng/mL for norfluoxetine.[10] The proposed method was tested by analysing five replicates of a series of standard solutions: 50-, 100-, 200-, 350-, 500 ng/mL. The limit of quantitation of the method is 5ng/mL for FL (i.e.,500 pg injected amount) and 4ng/mL for NL (i.e.,400 pg injected amount).

Calculation of the Formation Constants of the Inclusion Complexes (K_f) of Fluoxetine and Norfluoxetine

Another interesting observation is that the capacity factor (k') of FL and NR, in all mobile phase compositions, decreases in the presence of CDs media. In reversed-phase liquid chromatography it is known that the hydrophobic interactions i.e., dispersion forces, between the bonded alkyl moiety of the stationary phase and the non polar part of the molecule plays

Table 7

Equations Relating the Capacity Factor of Norfluoxetine (k') with the Formation Constant (K_f) of the Norfluoxetine-HP-β-CD Inclusion Complex.

buffer/ ACN	$1/k_0'$	K_f/k_0'	$K_f (M^{-1})$	r	n
90/10	0.134 (±0.011)	9.33 (±0.39)	69.63 (±0.014)	0.997	5
80/20	0.2812 (±0.0084)	7.69 (±0.31)	27.63 (±0.011)	0.998	5
70/30	0.4548 (±0.0069)	6.20 (±0.25)	13.630 (±0.0092)	0.998	5
60/40	0.4961 (±0.0062)	4.65 (±0.22)	9.37 (±0.0082)	0.997	5
50/50	0.5428 (±0.0045)	2.86 (±0.16)	5.27 (±0.0060)	0.995	5

r = correlation coefficient, n = number of points.

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Table 8

Equations Relating the Capacity Factor of Fluoxetine (k') with the Formation Constant (K_f) of the Fluoxetine-HPβ-CD Inclusion Complex.

Buffer/ ACN	$1/k_0'$	K_f/k_0'	$K_f (M^{-1})$	r	n
90/10	0.0901 (±0.0075)	6.75 (±0.28)	74.92 (±0.01)	0.997	5
80/20	0.2058 (±0.0050)	6.09 (±0.18)	29.58 (±0.01)	0.998	5
70/30	0.3457 (±0.0064)	4.87 (±0.23)	14.09 (±0.01)	0.996	5
60/40	0.4052 (±0.0064)	3.68 (±0.23)	9.08 (±0.01)	0.994	5
50/50	0.4616 (±0.0034)	2.71 (±0.12)	5.87 (±0.01)	0.997	5

r = correlation coefficient, n = number of points.

an important role in determining the retention of the solutes. Since the hydrophobic interactions are affected by various factors, the addition of CD in the mobile phase is expected to cause a change in the retention value of the solutes owing to the formation of inclusion complex. Thus, the decrease in k' values caused by the addition of CDs in mobile phase is based on the formation of an inclusion complex, resulting in a weakening of the hydrophobic

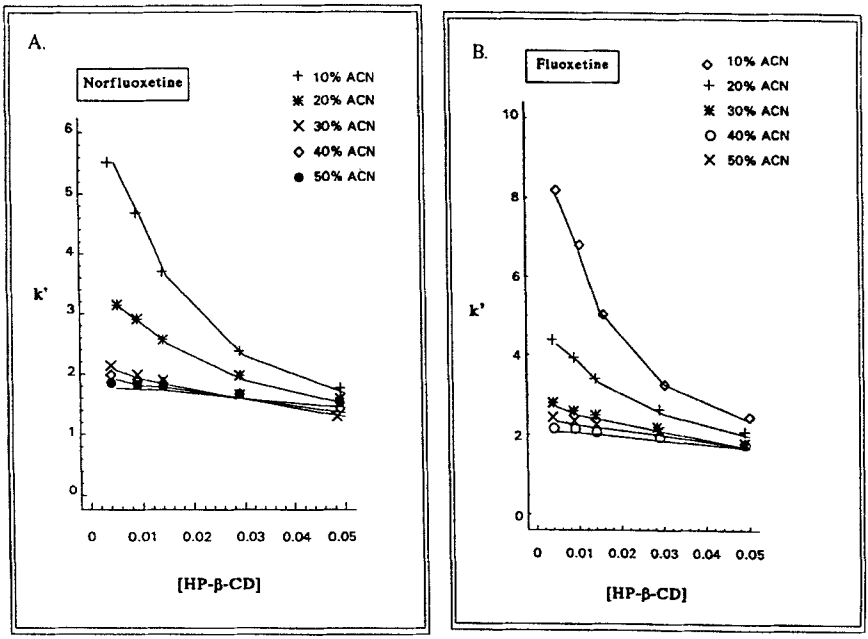

Figure 4. Regression analysis plot of equation 2 (A) and 3 (B) for NR and FL.

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interactions between solutes and the stationary phases. Figure 3 describes the decrease of k' values of FL and NR respectively, by increasing the HP-β-CD concentration and using different mobile phase ratios (TEAA 2% w/v; pH: 5.0; flow rate 0.8mL/min).

In the selection of an organic solvent, e.g. acetonitrile, in a reversed-phase system, retention, resolution and the binding constant of inclusion complexes of the solutes (K_f) are dependent on the type of the organic solvent and its content in the mobile phase. Tables 7 and 8 present the equations which permit the calculation of the K_f values of FL and NR in each mobile phase; it has been shown[13] that the capacity factor k' is related to the equilibrium concentration of CD in the mobile phase [CD]m, as follows :

$$\frac{1}{k'} = \frac{1}{k'_o} + \frac{K_f}{k'_o}[CD]_m \qquad (1)$$

where ko' is the capacity factor of the guest molecule in the absence of CD.

Table 9

Equations Relating the Formation Constants of Norfluoxetine and Fluoxetine with the Organic Modifier Concentration

A. $K_f = e^{(a + b \cdot \%ACN)}$ (2)

Compound	a	b	r	n
NR	4.68 (±0.19)	-0.0623 (±0.0058)	0.987	5
FL	4.75 (±0.21)	-0.0627 (±0.0064)	0.984	5

a : intercept, b = slope, r = correlation coefficient, n = number of points.

B. $\log K_f = a + b \cdot \%ACN$ (3)

Compound	a	b	r	n
NR	2.034 (±0.084)	-0.0271 (±0.0025)	0.987	5
FL	2.061 (±0.092)	-0.0272 (±0.0028)	0.984	5

a : intercept, b = slope, r = correlation coefficient, n = number of points.

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It is useful to investigate a possible relationship between the K_f value of each drug and organic modifier concentration (%ACN). An exponential model (Table 9A) is proposed in order to describe the dependence of K_f on %ACN and figure 4A present regression analysis plots representing the best fitting on the data. As shown in Table 9B, equation 2 can be converted to the corresponding linear equation 3 (fig.4B).

The K_f value of FL and NR in pure aqueous phase (K_{fW}) can easily be determined by extrapolation, as the intercept of equation 3, i.e., norfluoxetine : 108.1 (±1.2) and Fluoxetine: 115.1 (±1.2).

It is known that very often in RPLC the use of pure aqueous mobile phase leads to a decrease of sensitivity and/or resolution due to tailing and peak broadening. Thus, the concept of determining, indirectly, the K_f values in pure

aqueous phase, allows their comparison with the K_f values determined by using other techniques, on which the use of binary aqueous/organic systems is prohibitive.

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