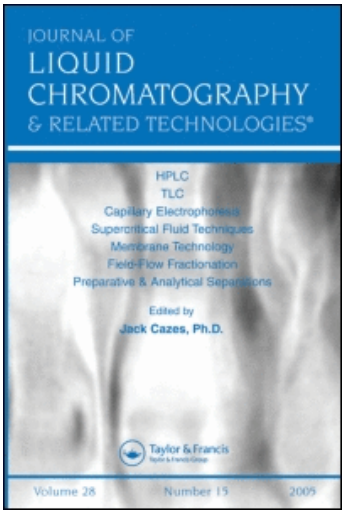

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A REVERSED-PHASE HPLC METHOD DEVELOPMENT FOR THE SEPARATION OF NEW ANTIDEPRESSANTS

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ABSTRACT

A RPLC method with UV detection (225 nm) is developed for the separation of five SSRIs (fluvoxamine, fluoxetine, sertraline, paroxetine, and citalopram), two SNaRIS (venlafaxine and milnacipran), one NaSSA (mirtazapine), and four active metabolites (norfluoxetine, desmethylcitalopram, desmethylvenlafaxine, and desmethylmirtazapine). A standard solution (20 μg/mL) of the twelve compounds is analysed under isocratic conditions on two new-generation RP columns (Satisfaction® RP 18 AB and Satisfaction® C8+, 250 mm × 4.6 mm, 5 μm). Mobile phase composition (acetonitrile content, pH of the aqueous buffer) and temperature are varied and the effect of these parameters on the retention factors of the antidepressants is examined. Similar

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elution profiles are observed with the two stationary phases, but the separation of all the solutes is only possible on the RP 18 AB column. It can be achieved at 45°C (or 50°C) with a mobile phase consisting of a mixture of potassium dihydrogen phosphate (pH 4.8, 25 mM)-acetonitrile (65:35, v/v) (flow rate: 1 mL/min). The run time is 20 min and a baseline resolution is obtained for all the analytes allowing this procedure to be well suited for a rapid toxicological screening.

INTRODUCTION

Antidepressants are widely used in the treatment of depressive disorders. Before 1980, antidepressant treatment principally consisted of the tricyclics, monoamine oxydase inhibitors and lithium. Since the early nineties, a new generation of compounds is available having a different pharmacological profile and generally better tolerated adverse effects. (1,2) The first class introduced was the selective serotonin reuptake inhibitors (SSRIs), which includes fluvoxamine (FLUV), fluoxetine (FLUO), sertraline (SER), paroxetine (PAR), and citalopram (CIT).

A second class consists of venlafaxine (VEN) and milnacipran (MIL). They have a very similar activity to the SSRIs at low doses where serotonin reuptake inhibition predominates but, at higher doses, noradrenaline reuptake inhibition is prominent and they were called, for this reason, serotonin noradrenergic reuptake inhibitors (SNaRIs). Mirtazapine (MIR) belongs to the chemical group of compounds known as piperazinoazepines. It is a noradrenergic and specific serotoninergic antidepressant (NaSSA). Even if many undesirable side-effects of the earlier classes have disappeared while offering equivalent or better efficacy, new antidepressants can lead to intoxications.

The development of rapid and specific screening methods in biological fluids allowing the simultaneous determination of several antidepressants and associated metabolites could be of great interest for the toxicologist. (3,4) Most of the existing methods involve gas (GC) or high performance liquid chromatography (HPLC), but few allow the simultaneous determination of many molecules for drug monitoring or toxicological purposes. (5–9) LC methods mainly involve phenyl, (10) C18 or C8 columns with acetonitrile (ACN)/acidic potassium phosphate buffered mobile phases. (11–14) Sometimes, amine modifiers like triethylamine (TEA) (15,16) or octylamine (17) are used to improve peak symmetry. These conditions are related to the basic properties of these compounds leading to ion-exchange interactions with acidic silanols and poor peak shapes. To avoid these problems, several solutions are offered to analysts, and much progress has been made in manufacturing suitable columns. (18)

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New-generation reversed-phase columns based on high-purity B silica are particularly designed for the analysis of such solutes.

In this paper, we propose to check the ability of two new-generation RP columns (Satisfaction® RP 18 AB and Satisfaction® C8+) to separate, under isocratic conditions, the twelve molecules mentioned above. The effect of different mobile phase parameters (% organic modifier, pH) and temperature is investigated with regard to retention time, alteration in retention order and resolution.

EXPERIMENTAL

Chemicals

All chemicals and solvents were of analytical or HPLC grade. ACN was purchased from Baker (Deventer, The Netherlands). Potassium dihydrogen phosphate (KH_2PO_4) and phosphoric acid (H_3PO_4) were obtained from Merck (Darmstadt, Germany) and TEA from Lancaster (Morecambe, UK). Water was deionised and glass-distilled prior to use.

CIT (HBr) and desmethylcitalopram (DMCIT) (HBr) were kindly donated by Lundbeck A/S (Copenhague, Denmark), FLUV (maleate) by Solvay Pharma (Suresnes, France), MIL (HCl) by P. Fabre (Castres, France), MIR and desmethylmirtazapine (DMMIR) by Organon (Oss, The Netherlands), PAR (HCl) by Smith Kline Beecham (Nanterre, France), SER (HCl) by Pfizer (Amboise, France), VEN (HCl) and desmethylvenlafaxine (DMVEN) (HCl) by Lederle (Pearl River, N.Y., USA). FLUO (HCl) and norfluoxetine (NFLUO) (HCl) were purchased from Sigma (Saint Quentin Fallavier, France). The internal standard (IS) F2570 was kindly supplied by P. Fabre (Castres, France). The structures of the twelve molecules are shown in Figure 1.

Individual stock solutions of each antidepressant and the IS were prepared at a concentration of 1 mg/mL in methanol and stored at −20°C. Working solutions were prepared by dilution of the stock solutions with mobile phase to a final concentration of 20 µg/mL and filtered through a 0.45 µm nylon filter before injecting, in triplicate, onto the column. A stock solution and a working solution of the mixture of all the compounds were prepared in the same way.

Apparatus and Chromatographic Conditions

The HPLC system consisted of a SpectraSystem P1000 pump (San Jose, CA, USA), a 20 µL Rheodyne 7125 model injector, and a Waters 990 photodiode array detector operating at 225 nm. The separation was performed on

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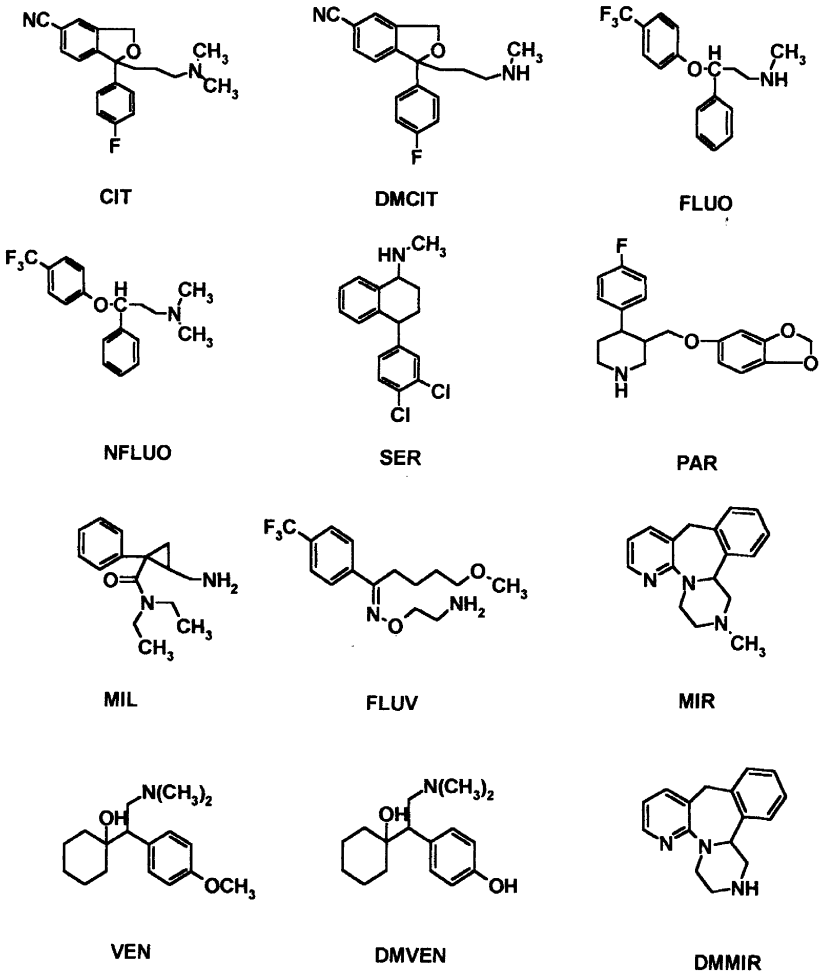

Figure 1. Molecular structures of the antidepressants.

Satisfaction® RP 18 AB and Satisfaction® C8+ columns (250 × 4.6 mm, 5 µm) (CIL, Sainte Foy la Grande, France). The mobile phase was a mixture of ACN-25 mM KH_2PO_4/10 mM TEA. Before mixing to ACN, the aqueous buffer was prepared by first dissolving TEA and KH_2PO_4 in water, then adding H_3PO_4 to the desired pH value. Prior to use, the mobile phase was filtered through a 0.45 µm nylon filter and degassed in an ultrasonic bath. Chromatography was achieved under isocratic conditions at a flow-rate of 1 mL/min, using different percentages

of ACN varying from 25% to 50%. Temperature was maintained at a fixed value in the range 25–50°C with a CROCO-CIL column heater (CIL).

RESULTS AND DISCUSSION

First, the separation of the 12 antidepressants and the IS was performed on the RP 18 AB column and optimised according to mobile phase composition and temperature. At the beginning, the pH of the aqueous buffer was fixed to 3.50. A low pH mobile phase is generally favoured because silanols are mostly uncharged, minimising interactions with the positively charged basic compounds. Of the more generally used buffer cations, K^+ is preferred to Na^+ and at low pHs, ACN is very often the best organic solvent modifier.(18) The temperature was 25°C and the relative amount of ACN was varied from 30% to 50%. The plot of the retention factor values (k) of each antidepressant vs ACN content is shown in Figure 2. For the calculation of k, mean retention time values were used and the dead time was measured from the baseline disturbance caused by the presence of methanol in the sample solution. Its value was found to be 2.40 ± 0.03 min for all experiments. This value is in good agreement with those generally encountered for this type of column. (19)

The goal of solvent strength adjustment is to position all the bands within a k' range of roughly 0.50 (to avoid problems from the initial baseline disturbance overlapping the first band) to 10 (to avoid too long run times and excessive band broadening). (19,20) In this respect, 35% ACN is a correct value, even if k is

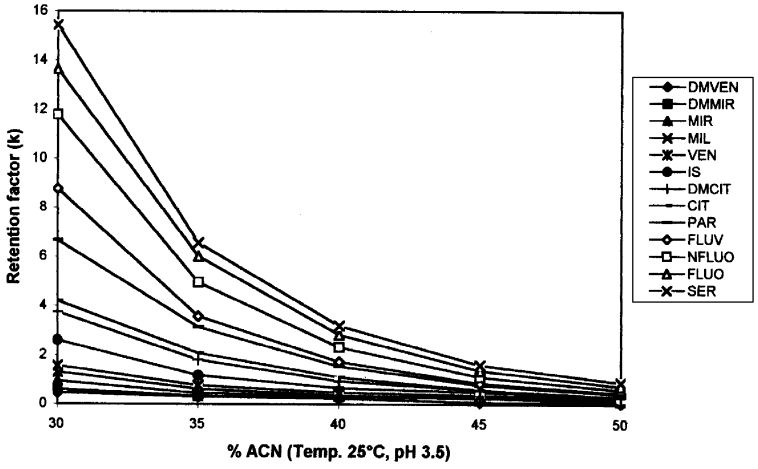

Figure 2. Effect of acetonitrile content on retention factors.

slightly low for DMVEN (0.31), DMMIR (0.34), and MIR (0.46). The last band (SER) eluted at $k = 6.63$ ($t_R = 18.3 \pm 0.06$ min).

The influence of pH on the retention of the analytes was examined in the range 3.00–4.95 when the ACN content was fixed to 35% and the temperature to 25°C. A graph of log k vs pH is presented in Figure 3 (the logarithm of k was chosen instead of k, in order to make the diagram more representative). For all the molecules, the k value did not increase very much when the pH was increased (about 20% to 30%), except for MIR, which was much more retained at pH 4.95 than at pH 3.00 (k is multiplied by a factor 2.2). DMMIR had a similar but less significant behaviour due to its poor retention in these conditions (k is only increased by a factor 1.7). Figure 4 shows the evolution of the retention of the five first antidepressants to be eluted (DMVEN, DMMIR, MIL, MIR, and VEN) between pH 3.50 and pH 4.95. At pH 3.50, MIR was eluted near from DMVEN (t_R are 3.52 ± 0.01 min and 3.17 ± 0.02 min, respectively), while it was eluted after VEN at pH 4.95 (t_R are 5.42 ± 0.02 min and 4.69 ± 0.01 min, respectively). When compared to the other antidepressants, MIR and DMMIR exhibit singular acid-base properties.

All the molecules have a primary, secondary, or tertiary aliphatic amine group with a pKa's value typically ranging from 8.50 to 10.50 (e.g., 9.40 and 10.20 were the values obtained from potentiometric data for VEN and SER respectively) (data not shown). For MIR and DMMIR, the presence of a substituted 2-amino pyridine group leads to a second basic centre in the molecule. The related dissociation constant was found to be 3.50 ± 0.10 for MIR from both spectrophotometric and potentiometric measurements (data not shown). More, in

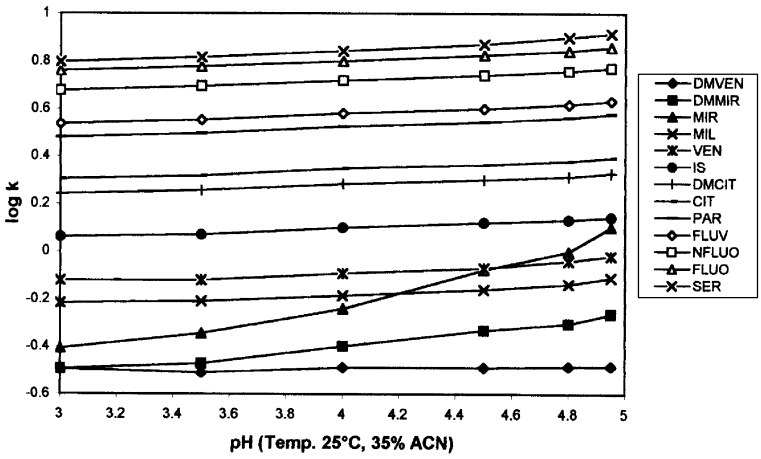

Figure 3. Effect of pH on retention factors.

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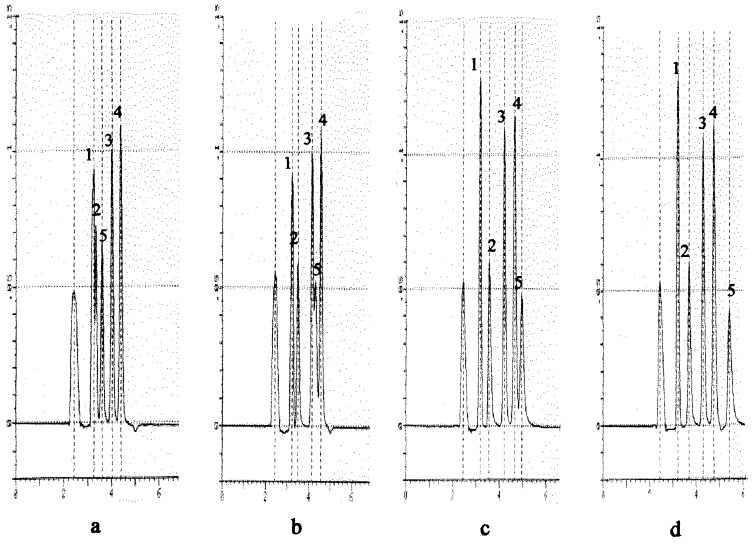


Figure 4. Chromatograms of a mixture of DMVEN (1), DMMIR (2), MIL (3), VEN (4), MIR (5) at different pHs (35% ACN, Temp. 25°C): a) pH 3.5; b) pH 4.5; c) pH 4.8; d) pH 4.95.

comparison with the other antidepressants, the basic character of the intracyclic secondary amine in DMMIR or tertiary amine in MIR is lowered. The corresponding pKa's value deduced from a potentiometric titration in a 0.15 M NaCl solution was 7.60 ± 0.10 for MIR (data not shown). A value of 7.10 was found by KELDER et al. (21) in a mixture of 0.15 M KCl-methanol (53:47, m/m). Consequently, unlike the other compounds, retention and selectivity of MIR (and DMMIR) are very dependent on the pH in the range 3.00–4.95. The best separation for all the analytes was obtained at pH 4.80, but VEN and MIR were not baseline resolved (Figure 4). Above pH 4.85, MIR and the IS began to overlap because of a peak asymmetry factor > 1.7 for MIR.

To solve this problem, the effect of temperature was investigated. Generally, for neutral samples, retention decreases as temperature increases but little changes in selectivity are observed. It may be different for ionic compounds because temperature influence the ionisation characteristics, hydrophobic retention of ionised vs non ionised species, silanol interactions, mobile-phase pH, and the pKas of sample components. (22) In this study, it can be expected that maximum changes in selectivity with temperature will occur for MIR (and DMMIR) for the reason previously reported. The ACN content was fixed to 35%, the pH value to 4.80, and the temperature was varied from 25°C to 50°C. The

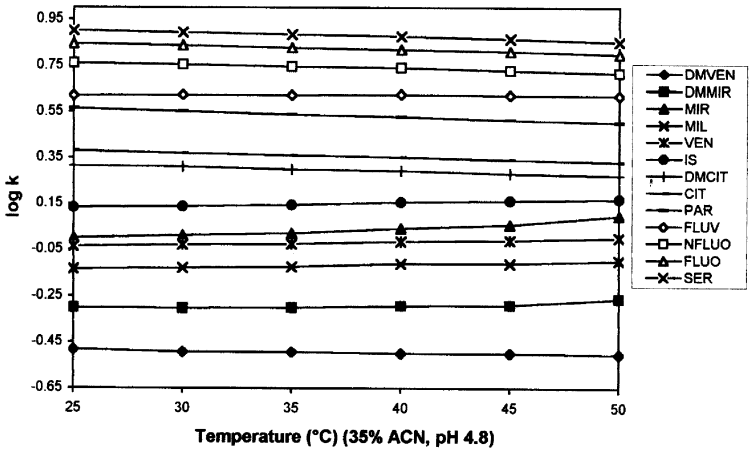
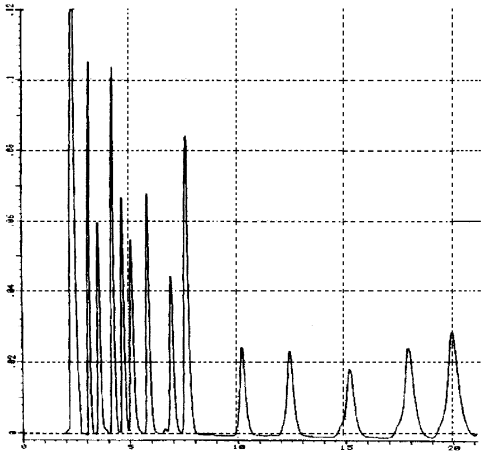


Figure 5. Effect of temperature on retention factors.

effect of temperature on log k is given in Figure 5. Most of the analytes were less retained at 50°C than at 25°C, especially those that exhibited the highest retention times (e.g., 21.50 ± 0.1 min for SER at 25°C and 19.50 ± 0.1 min at 50°C). Conversely, MIR was significantly more retained at 50°C than at 25°C ($t_R = 5.40 \pm 0.03$ min at 50°C and 4.80 ± 0.02 min at 25°C). DMMIR, MIL, VEN, and the IS had the same but less marked behaviour. No influence of temperature on the retention of FLUV was observed. This temperature effect on analyte retention allowed a baseline separation of all the molecules in 20 min at 45°C (or 50°C), the mobile phase consisting of ACN-pH 4.80 aqueous phase (35:65, v/v). A typical chromatogram is presented in Figure 6.

These results are satisfactory, overall, but some little drawbacks can be pointed out. First, DMVEN is poorly retained in these conditions and it is eluted near from the dead time ($k = 0.33$). Consequently, it must be injected in the mobile phase, or a non-absorbing solvent at 225 nm, not to overlap the sample-solvent peak. For this reason, a phosphate buffer was used over the whole range of pH. Even if the mobile phase was not buffered well above pH 3.50, the pH values were stable. A more embarrassing problem arose from MIR. It was said that an important variation of its retention time was noted for a little difference in the pH value. A baseline resolution ($R_S = 1.5$) between VEN, MIR, and the IS can only be obtained at pH 4.80 and 45°C or 50°C. A low difference (0.05) in the pH value of the mobile phase made the baseline resolution of MIR with either VEN or IS impossible. Nevertheless, despite this lack of ruggedness, this method could be useful for a rapid screening in toxicology.



Molecules	t_R (min.)
01 DMVEN	3.16
02 DMMIR	3.63
03 MIL	4.26
04 VEN	4.74
05 MIR	5.14
06 IS	5.89
07 DMCIT	6.98
08 CIT	7.66
09 PAR	10.24
10 FLUV	12.44
11 NFLUO	15.22
12 FLUO	17.94
13 SER	20.00

Figure 6. Chromatogram of an antidepressant mixture solution. Mobile phase: ACN-25 mM aqueous K.

The same method development was carried out on a Satisfaction® C8+ column. A similar elution profile was observed but no complete separation of the 13 solutes could be achieved, whatever the conditions. When the pH was < 5.00, SER and FLUO were coeluted. A baseline resolution of these two analytes was only possible above pH 6.00, but the run time exceeded 26 min. Moreover, a severe band tailing occurred for MIR, which was then coeluted with PAR at 14.3 min. As mentioned above, this delay depends on the major influence of the pH on the retention of MIR ($t_R < 5$ min on the Satisfaction® RP 18 AB column at pH 4.80, 25°C). In the same conditions (pH > 6.00), DMM and MIL were also coeluted ($t_R = 5.04$ min). So, unlike the Satisfaction® RP 18 AB, the Satisfaction® C8+ column is not well suited for the simultaneous determination of new antidepressants in case of intoxications involving these compounds.

CONCLUSION

A simple and rapid RPLC method for the isocratic separation of eight new antidepressants and four active metabolites was developed using two new-generation based on pure silica RP columns, (Satisfaction® RP 18 AB and Satisfaction® C8+). A complete separation of all the analytes could only be obtained on the RP 18 AB column at 45°C (or 50°C) with a mobile phase consisting of a mixture of KH_2PO_4 (pH 4.8, 25 mM)-ACN (65:35, v/v). This

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study emphasized the influence of the acid-base properties of MIR (and DMMIR) on the selectivity and the ruggedness of the separation.

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