Reversed phase liquid chromatography without organic solvent for determination of tricyclic antidepressants

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Abstract

The chromatographic behavior of seven tricyclic antidepressants (amitryptiline, clomipramine, doxepin, imipramine, maprotiline, nortryptiline and trimipramine) was examined with micellar mobile phases containing the non-ionic surfactant Brij-35. Acetonitrile-water mixtures were also used for comparison purposes. Tricyclic antidepressants are moderately polar basic drugs, which are positively charged in the usual working pH. This gives rise to a strong association with the alkyl chains and residual ionized silanols in silica-based stationary phases, which is translated in a high consumption of organic solvent to get appropriate retention times. Brij-35 modifies the surface of the stationary phases creating a neutral bilayer that masks silanols and reduces the polarity. Consequently, the retention times are decreased. A simple chromatographic procedure for the control of tricyclic antidepressants in pharmaceutical formulations was developed, using 0.02 M Brij-35 at pH 3 and UV detection. Satisfactory recoveries were achieved, with intra- and inter-day relative standard deviations usually below 1% and 2%, respectively. The preparation of the samples was simple and only required solubilization and filtration steps previous to injection. The proposed procedure has the advantage of not using an organic solvent in the mobile phase, and the biodegradable character of Brij-35. This makes an example of "green" liquid chromatographic analysis.

Keywords: Brij-35; Micellar liquid chromatography; Green HPLC; Pharmaceuticals; Tricyclic antidepressants

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1. Introduction

Tricyclic antidepressants (TCAs) are a group of drugs traditionally used in the treatment of psychiatric disorders. Although a second generation of antidepressant drugs has recently been commercialized, TCAs are still widely prescribed due to their high efficiency against depression [1], especially for children and adolescents [2]. Owing to their wide use, the pharmacological and toxicological profiles, and the side effects of TCAs are well documented.

Several reports on the analysis of TCAs in both physiological and pharmaceutical samples have been published, being reversed-phase liquid chromatography (RPLC) [3–6], or capillary electrophoresis [7–9], the preferred techniques. The versatility of RPLC and its simplicity in use facilitates the development of analytical procedures. TCAs possess a chemical structure with three rings and a side chain derived from *N*-alkylmethylamine or *N*-alkyldimethylamine. The ring structure determines a relatively low polarity with octanol-water partition coefficients (log $P_{o/w}$) in the range 3.9–5.3 [10]. Therefore, TCAs are strongly associated with the alkyl chains of conventional C18 stationary phases in RPLC. On the other hand, the amine groups confer them a basic character with pK_a values in the range 9.0–9.7 [10]. This favors the interaction with the residual ionized silanols present on the silica-based stationary phases, which is a slow process [11]. All these features are translated in a strong retention and a high consumption of organic solvent (usually methanol or acetonitrile) in RPLC analysis, which generates toxic wastes that require later disposal. Also, peak shapes are poor.

The use of different types of additives in the aqueous-organic mobile phases is a common practice to solve the problem of the chromatographic performance of basic drugs [12–14]. The additives minimize the additional ion-exchange interaction of the positively charged

basic drugs with residual silanols. Consequently, the retention times are shortened and the amount of organic solvent in the mobile phase decreased.

The anionic surfactant sodium dodecyl sulfate (SDS) has been shown to enhance the peak shape of basic drugs [15–17]. The existence of surfactant monomers or micelles in the bulk solution has important implications in the elution strength and selectivity. However, the main responsible for all changes observed in the chromatographic performance (i.e. retention, selectivity and peak shape) is the hydrophobic interaction of the SDS monomers with the stationary phase, which creates a negatively charged bilayer. In previous work, we observed a strong retention for a group of TCAs in an SDS micellar system, which forced the use of a C8 column and the addition of a strong organic solvent (pentanol) to the mobile phase, to obtain practical retention times [18]. A single procedure (using the same column and mobile phase composition) was developed for the control of several TCAs in pharmaceutical formulations. The cationic surfactant cetyltrimethylammonium bromide (CTAB) has been also considered for TCAs separation, in the presence of 5–10% propanol [19].

More recently, the use of micellar mobile phases containing the non-ionic surfactant polyoxyethylene(23) lauryl ether ($(C_2H_4O)_{23}C_{12}H_{25}OH$, known as Brij-35) has been explored as an alternative to separate basic compounds by RPLC [20]. Due to the neutral bilayer created on the stationary phase, and the smaller polarity of the polyoxyethylene chain, the retention times for basic drugs are shorter with regard to those obtained with SDS. This allows the elution of moderately polar compounds (such as many TCAs) in appropriate retention times from C18 columns, without the need of adding an organic solvent. Brij-35 is a biodegradable surfactant, which gives rise to a promising example of “green” liquid chromatography to separate compounds that usually require aqueous-organic mobile phases with a high amount of organic solvent. In the literature, however, only few reports have been published on the analytical use of this or other non-ionic surfactants (Brij-35 [20–24],

Tween 80 [25], and Triton X-100 [26]). Recently, extensive use of Brij-35 has been reported in the field of quantitative structure-activity relationships (QSAR) [27–30], due to its capability to mimic biopartitioning processes.

In this work, a detailed study of the chromatographic behavior of seven TCAs is presented, using a C18 column and micellar mobile phases that only contain water, Brij-35 and citric buffer at pH 3 (micellar RPLC). For comparison purposes, acetonitrile-water mobile phases (classical RPLC) have been considered. Special attention has been paid to the peak shape behavior, which has been evaluated through the measurement of the peak half-widths [17]. With all this information, a simple environmentally friendly RPLC procedure has been developed for the determination of commonly prescribed TCAs in pharmaceutical formulations.

2. Experimental

2.1. Reagents

The following TCAs were examined: amitriptyline, clomipramine, doxepin, imipramine, maprotiline, nortriptyline and trimipramine (all from Sigma, St. Louis, MO, USA). The drugs were dissolved in a small amount of ethanol with the aid of an ultrasonic bath (Elma 15h Elmasonic, Singen, Germany), and diluted with water. For the micellar mode, dilution of the samples before injection was made with 0.02 M Brij-35 (Fluka, Buchs, Switzerland). The concentration of the stock solutions was 500 μg/mL. These remained stable during at least two months at 4^{o}C. Uracil (Acros Organics, Geel, Belgium) was used as dead time marker.

The concentration ranges for Brij-35 and acetonitrile (HPLC grade, Scharlab, Barcelona, Spain) in the mobile phases were 0.01–0.04 M and 25–50% (v/v), respectively. The pH of the mobile phases was buffered at 3.0 with 0.01 M citric acid monohydrate and sodium hydroxide (Panreac, Barcelona). The drug solutions and mobile phases were filtered through 0.45 μm

Nylon membranes with a diameter of 47 mm (Osmonics Magna, Herental, Belgium) and 17 mm (Osmonics Cameo, Herental, Belgium), respectively. Nanopure water (Barnstead, Sybron, Boston, MA, USA) was used throughout.

2.2. Apparatus

The HPLC system was equipped with an isocratic pump (Agilent Series 1200, Waldbronn, Germany), an autosampler (Series 1200), a temperature controller module set at 25ºC (Series 1200), and a UV detector (Series 1100), set at 254 nm. Duplicate injections were made, with an injection volume of 20 μL and a flow-rate of 1 mL/min. Data acquisition was carried out with an HPChemStation (Agilent, B.03.02).

Two different Zorbax columns with the same characteristics (150 × 4.6 mm i.d., Agilent) were used for both micellar and aqueous-organic modes. The columns were preceded by C18 guard columns (30×4.6 mm i.d.) from Scharlab. The chromatographic systems were periodically rinsed, first with water and then with methanol.

2.3. Procedure

The pharmaceuticals were commercialized in tablets and capsules. The average weight per tablet or capsule was calculated from 10 units. The contents were ground and reduced to a homogeneous fine powder in a mortar. Several portions of powder were taken and sonicated in the presence of a 1:1 mixture of ethanol and 0.02 M Brij-35 for the micellar mode, and with a small amount of ethanol for the aqueous-organic mode. Dilution was made with 0.02 M Brij-35 and water, respectively. The excipients were not soluble in the assayed media, hence the sample solutions should be filtered through 0.45 μm Nylon membranes before injection into the chromatograph.

3. Results and discussion

A comparative study of the chromatographic behavior and analysis of the TCAs was carried out, using micellar and classical RPLC with two different Zorbax columns (which are type B columns, made of highly purified silica). The mobile phases were buffered at pH 3 to enhance the peak shape through the protonation of the residual free silanol groups on the stationary phase. In order to follow in detail the change in behavior of the drugs with mobile phase composition, chromatographic data (retention factors and peak half-widths) were modelled from experimental designs that involved four mobile phases.

3.1. Retention behavior

The only factor that differentiated both chromatographic modes was the reagent added to the mobile phase: Brij-35 (0.01–0.04 M) in micellar RPLC, and acetonitrile (20−50%) in classical RPLC. The studied concentration ranges gave rise to enough retention for the TCAs in both chromatographic modes, and guaranteed micelle formation in the micellar mode. The retention behavior in the micellar and aqueous-organic systems is shown in Fig. 1, where the retention factor (*k*) is plotted against the molar concentration of Brij-35, and the acetonitrile content in percentage. As observed, the changes in retention for the TCAs were smaller in the micellar mode. Also, the aqueous-organic mode required high amounts of acetonitrile (> 30%, v/v) to obtain practical retention times, whereas a small concentration of Brij-35 (< 0.03 M) was enough to achieve similar retention times.

The linear plot obtained from the correlation between 1/*k* and the surfactant concentration suggested the saturation of the Zorbax stationary phase by the Brij-35 monomers, according to the following equation [31]:

$$\frac{1}{k} = \frac{1}{K_{AS}} + \frac{K_{AM}}{K_{AS}}[M] \qquad (1)$$

where K_{AS} and K_{AM} are the solute-stationary phase and solute-micelle association constants, respectively, and [*M*] the molar concentration of surfactant forming micelles. The extrapolation of the linear segment (1/*k* vs. [*M*]) gives a measurement of the strength of the interaction between solute and stationary phase, expressed as $1/K_{AS}$. The slope of the fitted equation allows the estimation of K_{AM}.

The determination coefficient r^2 for the fitted straight-lines 1/*k* versus [*M*], obtained from the experimental data for the set of TCAs was above 0.975. The K_{AS} and K_{AM} values were: amitryptiline (35.7, 148.9), clomipramine (98.0, 306.5), doxepin (14.7, 74.8), imipramine (21.9, 91.8), maprotiline (62.9, 204.6), nortriptyline (57.5, 188.9), and trimipramine (36.4, 151.4). These constants correlated satisfactorily with the log $P_{o/w}$ values for the tertiary amines: doxepin (3.9), imipramine (4.4), amitryptiline (4.6), trimipramine (4.7) and clomipramine (5.3). The secondary amines did not follow the same trend: nortriptyline (4.3) and maprotiline (4.2). In previous work with the set of TCAs eluted with SDS [18], similar trends were observed between the retention factor and log $P_{o/w}$. The association constants (K_{AS} and K_{AM}) could not be however determined, due to the strong association of TCAs with the stationary phase modified with SDS.

The selectivity for the two chromatographic modes was different. The elution order of TCAs in the Zorbax column with acetonitrile-water correlated well with the log $P_{o/w}$ values of the drugs. In contrast, as commented, for the micellar mode using Brij-35, considering both secondary and tertiary amines, a poor correlation between retention and polarity was found. The tertiary TCAs imipramine, amitryptiline and trimipramine presented a relative reduction of their retention times in the micellar mode with regard to the aqueous-organic mode.

3.2. Peak shape behavior

The examination of the changes of efficiency and asymmetry with mobile phase composition can be carried out through the construction of plots that represent the left (*A*) and right (*B*) half-widths of the chromatographic peaks (measured at 10% peak height) versus the retention time [17].

$$A = m_A\, t_R + A_0 \quad (2)$$

$$B = m_B\, t_R + B_0 \quad (3)$$

where m_A and m_B are the slopes of the linear correlations, and A_0 and B_0 the intercepts.

The peak half-width plots isolate the intra-column contribution to the peak broadening. The combination of the parameters obtained from Equations (2) and (3) characterize the chromatographic system: the sum of slopes ($m_A + m_B$) represents the peak broadening rate inside the column and their ratio (m_B/m_A) the asymmetry of a peak eluting at a time where the extra-column contribution is non-significant. The extra-column contributions are associated to the intercepts ($A_0 + B_0$ and B_0/A_0). These equations also allow the prediction of the half-widths for peaks eluting at different retention times, and from these the corresponding efficiencies. The approach is advantageous against the common practice of using mean values of efficiencies and asymmetries for one or more compounds eluted at different mobile phase compositions.

Peak half-width plots were built considering the data obtained with all mobile phases in the experimental design, in order to compare the peak performance. In all cases, the correlations were satisfactory. Figs. 2a and b depict the plots for the TCAs eluted with 0.02 M Brij-35 and 32% acetonitrile-water, respectively, which were the mobile phases used in the analysis of the pharmaceuticals (see Section 3.4). The slope of the straight line for the right half-width (*B*) was always significantly larger than for the left half-width (*A*), indicating tailing peaks. On the other hand, the slope for the right half-width changed with the

concentration of Brij-35, whereas for the left half-width remained unchanged. Meanwhile, for the aqueous-organic mobile phases, the slope for both half-widths did not change with mobile phase composition. The change in slope for the right half-width with Brij-35 was previously explained by the continuous stationary phase modification at increasing surfactant concentration [17]. The different interaction of secondary and tertiary amines with the C18 stationary phase modified with Brij-35 was also revealed in the plots (Fig. 2a).

The extra- and intra-column contributions to the peak broadening rate and peak asymmetry for the micellar and aqueous-organic RPLC modes with the Zorbax column were: $A_0 + B_0 = 0.14$ and 0.09, $B_0/A_0 = 2.24$ and 0.43, $m_A + m_B = 0.155$ and 0.107, and $m_B/m_A = 4.98$ and 8.09, respectively (see Equations (2) and (3)). It is observed that the peak shape at the column entrance was poorer for Brij-35 (the peaks were broader), but the intra-column contributions to the peak broadening rate were smaller. Consequently, at short retention times, the peaks were wider with Brij-35 (Fig. 3), but at increasing retention times the widths tended to become similar to those obtained with the acetonitrile-water mixtures. The asymmetry factors were also enhanced for Brij-35. As an example, for a compound eluting at 20 min, $A + B = 3.05$ and $B/A = 4.45$ for 0.02 M Brij-35, and $A + B = 2.31$ and $B/A = 7.24$ for 32% acetonitrile. The corresponding efficiencies (measured with the Foley and Dorsey equation [32]) are $N = 314$ and 368.

In the aqueous-organic mode, the reason of the low efficiencies and tailing peaks for basic drugs (as the TCAs in this work) is the interaction of the positively charged solutes with the anionic silanols on the stationary phase. In the micellar mode, the silanols on the stationary phase are protected by the surfactant adsorbed on the alkyl bonded phase. Therefore, the poor mass transfer within the thick layer of Brij-35, which results in poor stationary-phase diffusion, is likely responsible for the low efficiencies [21]. In hybrid micellar mobile phases containing the surfactant SDS and organic solvent, the efficiencies

have been observed to be significantly enhanced, being the peaks almost symmetrical, since the organic solvent reduces the thickness of the surfactant layer. Therefore, solute diffusion is no more a limiting factor in mass transfer, while the surfactant layer efficiently masks the free residual silanols on the column packing. In this work, however, we did not add an organic solvent to improve the peak shape in the micellar mode, since the objective was to develop a green analytical method (without organic solvent). However, the characteristics of the chromatographic peaks with aqueous Brij-35 mobile phases were adequate for the analysis of the TCAs in the pharmaceutical formulations (Fig. 3).

3.3. Figures of merit

The assayed mobile phases in both chromatographic modes allowed appropriate analysis times for the commercial pharmaceutical formulations containing the TCAs. The selected mobile phase compositions were: 0.02 M Brij-35 and 32% acetonitrile in the micellar and classical RPLC modes, respectively. Calibration curves for each TCA were built using the areas of the chromatographic peaks from triplicate injections of standard solutions, at five concentrations in the range 20-60 μg/mL. The slopes did not considerably differ significantly between the micellar and the aqueous-organic systems. In both cases, the determination coefficients were $r^2 > 0.98$.

The repeatability was evaluated by measuring the signals from replicate solutions, at four different concentrations: 20, 30, 40 and 50 μg/mL. Intra-day variation was calculated from six-fold assays, and inter-day variation from triplicate assays performed during five consecutive days. The values are given in Table 1. Intra- and inter-day relative standard deviations were usually below 1% and 2%, respectively.

The limits of detection (LODs) were calculated according to the 3s criterion by injecting five solutions containing 6 μg/mL of each TCA, and were usually below 2 μg/mL. For the

RPLC modes using Brij-35 and acetonitrile-water, LODs (µg/mL) were: amitriptyline (0.16, 0.05), clomipramine (0.38, 0.31), doxepin (0.10, 0.02), imipramine (0.25, 0.09), maprotiline (1.53, 0.21), nortriptyline (0.29, 0.62) and trimipramine (0.08, 1.36).

3.4. Analysis of pharmaceutical formulations

Several formulations prescribed in Europe containing one of the seven TCAs studied in this work were analyzed (Table 2). Parallel analyses of all formulations were carried out using 0.02 M Brij-35 and 32% acetonitrile. Five samples of each formulation were analyzed making duplicate injections to obtain average values of the drug concentrations. For this purpose, an appropriate amount of each sample was weighed to prepare solutions containing approximately 40 µg/mL of the drugs.

Table 2 gives the found contents, together with the label claim percentages, which were usually in the 90-100% range. The label claim was below 80% for Norfenazin (nortryptiline) using both micellar and aqueous-organic systems. This result was checked by repeating the analyses in different days.

Fig. 3 shows the chromatograms for some formulations. For the other formulations, the chromatograms were similar. The excipients were eluted at the dead time or did not absorb at the measuring wavelength.

4. Conclusions

Brij-35 covers the stationary phase with surfactant monomers, creating a neutral bilayer that modifies the polarity of the stationary phase. This allows the elution of basic drugs of intermediate polarity at appropriate retention times, using mobile phases without organic solvent. Brij-35 is an interesting alternative to the use of SDS in micellar RPLC for the

determination of these compounds in pharmaceuticals, since the anionic character of SDS yields excessive retention times and forces the addition of a strong organic solvent.

The chromatographic procedure with Brij-35, applied to the control of commercialized formulations, yielded satisfactory results, comparable to those obtained with the aqueous-organic procedure. Since there was no interference from common additives, excipients or other drugs, previous extraction of the TCAs was not needed. The preparation of the samples (solubilization and filtration) was simple, requiring similar effort for both modes (micellar and classical RPLC). The procedure with Brij-35 has, however, the advantage of not requiring the addition of an organic solvent to the mobile phase to increase the elution strength (against the use of 32% acetonitrile for the aqueous-organic mode). Brij-35 is a biodegradable surfactant, making the described procedure an example of the interest of micellar RPLC in “green” chromatographic analysis.

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The authors have declared no conflict of interest

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346 **FIGURE CAPTIONS**

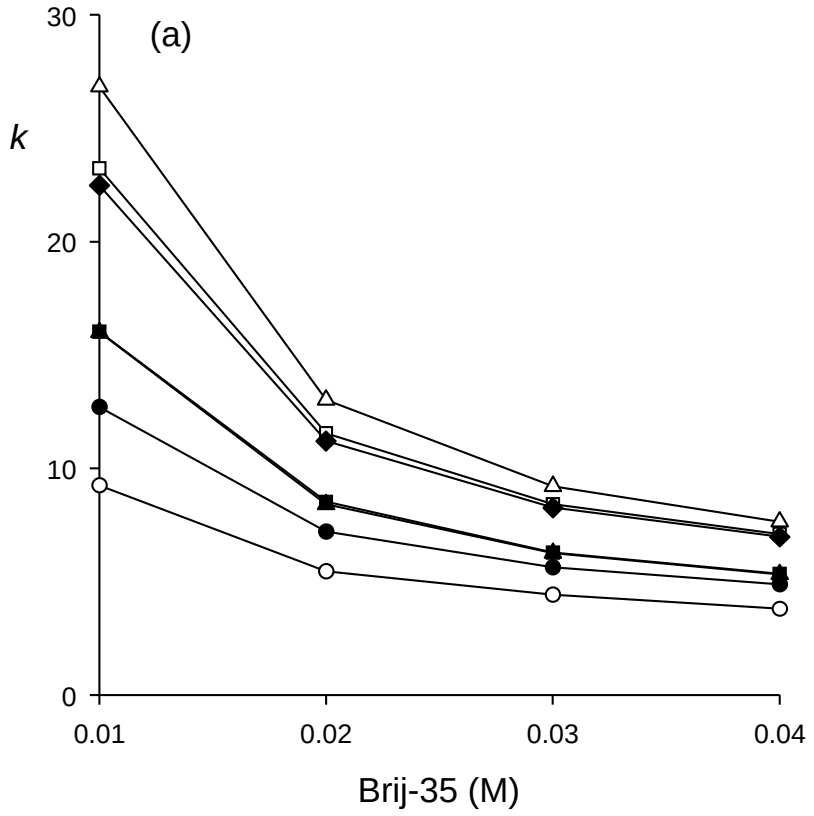
347 **Figure 1.** Retention behavior with mobile phases containing Brij-35 and acetonitrile.
348 Solute identity: (○) doxepin, (●) imipramine, (▲) amitryptiline, (■) trimipramine,
349 (♦) nortryptiline, (□) maprotiline, and (Δ) clomipramine.

350

351 **Figure 2.** Half-width plots for the TCAs eluted with: (a) 0.02 M Brij-35, and (b) 32%
352 (v/v) acetonitrile-water. Half-widths: (○) right, *B*, and (●) left, *A*. The arrows in (a)
353 point the half-widths for the secondary TCAs.

354

355 **Figure 3.** Chromatograms for some formulations containing TCAs, eluted with 0.02
356 M Brij-35 (left) and 32% (v/v) acetonitrile-water (right): (a,d) Anafranil, (b,e) Sinequan
357 and (c,f) Tonofranil. Compounds: CLOMI = clomipramine, DOXE = doxepin, and
358 IMI = imipramine.



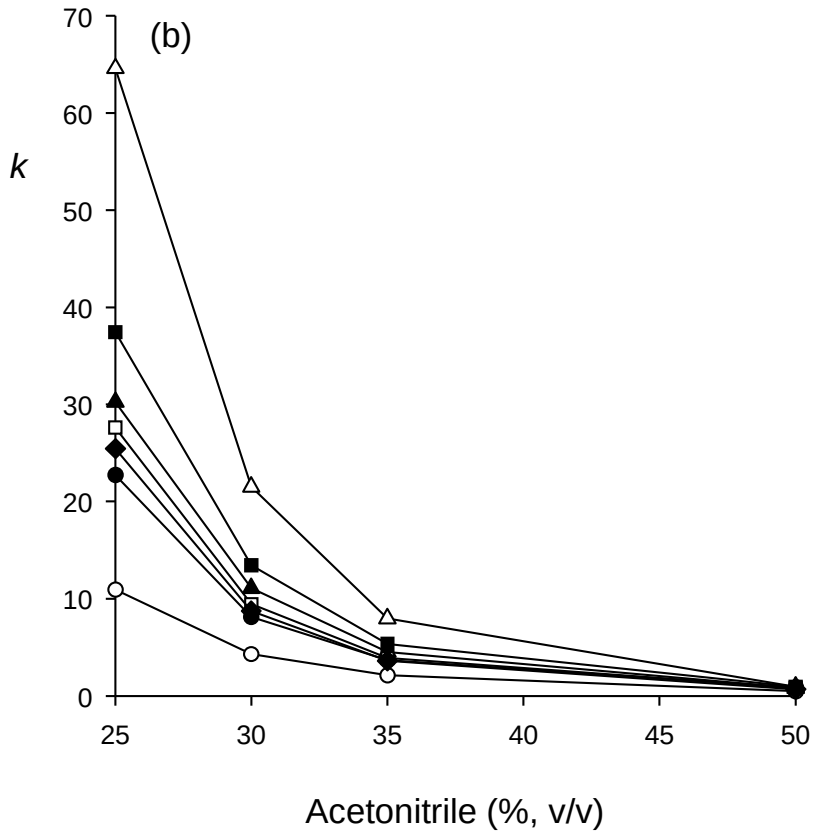


Figure 1

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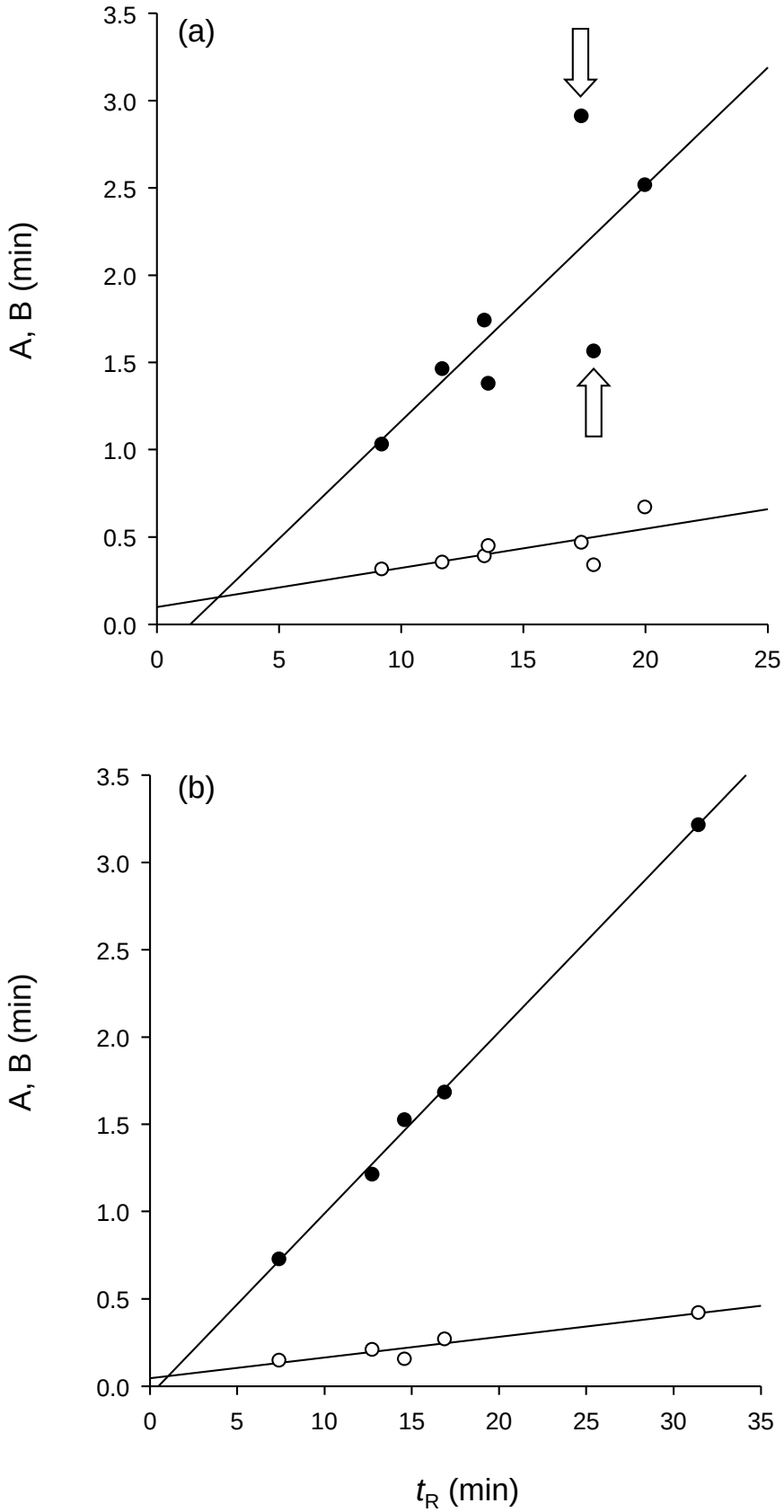

Figure 2

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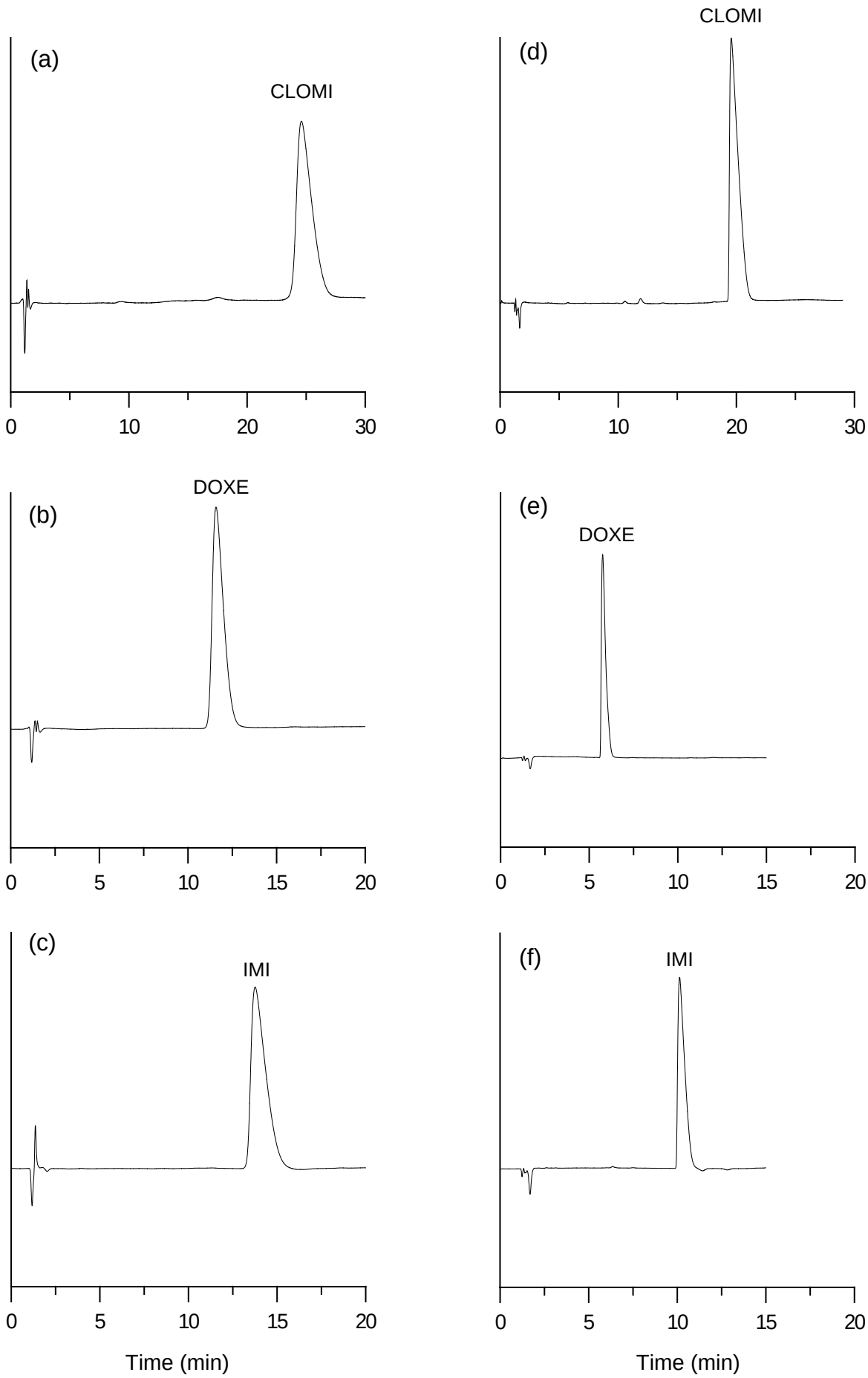

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Table 1. Intra- and inter-day assays (%) for the TCAs eluted with Brij-35 and acetonitrile-water mobile phases.[a]

Compound	Added μg/mL	Brij-35		Acetonitrile-water	
		Intra-day	Inter-day	Intra-day	Inter-day
Amitryptiline	20	0.3	1.9	0.3	1.2
	30	0.4	0.9	0.3	0.2
	40	0.16	0.9	0.16	0.7
	50	0.3	1.6	0.3	1.1
Clomipramine	20	0. 5	1.7	1.5	2.0
	30	2.2	1.7	1.7	0.5
	40	0.4	2.1	0.4	1.1
	50	0.3	1.1	0.3	1.6
Doxepin	20	0.14	1.0	0.6	1.9
	30	0.17	1.0	0.03	0.7
	40	0.2	1.2	0.3	0.6
	50	0.10	1.1	0.09	0.6
Imipramine	20	0.6	2.1	0.14	4.0
	30	0.6	1.9	0.3	3.1
	40	1.1	2.3	0.5	2.9
	50	0.17	2.2	0.4	2.7
Maprotiline	20	1.6	1.8	1.8	1.0
	30	0.8	2.9	1.6	2.6
	40	1.4	1.8	1.7	1.0
	50	0.6	0.9	1.1	1.2
Nortryptiline	20	0.2	1.5	0.9	2.8
	30	0.6	1.2	3.0	1.5
	40	0.8	1.8	2.1	1.4
	50	0.7	1.7	1.8	1.5
Trimipramine	20	1.0	0.9	0.9	0.5
	30	3.4	1.0	0.05	1.2
	40	1.7	0.5	0.3	1.6
	50	0.8	0.7	0.17	1.6

[a] Six-fold replicates.

Table 2. Analysis of several formulations containing TCAs.

Formulation (laboratory)	Composition (mg)	Brij-35		Acetonitrile-water	
		Found (mg)	Label claim (%)	Found (mg)	Label claim (%)
Tryptizol (Merck-Sharp & Dohme, Madrid, Spain)	Per tablet: Amitryptiline chlorhydrate (25), lactose and other excipients	22.5±0.7	90.0	22.3±1.6	89.2
Tonofranil (Amdipharm, Dublin, Irlanda)	Per tablet: Imipramine chlorhydrate(25), glycerol (E422) and other excipients	23.7±0.6	94.8	23.6±0.5	94.4
Anafranil (Defiante farmacéutica, Funchal, Portugal)	Per tablet: Clomipramine chlorhydrate (25), glycerol (E422) and other excipients	23.76±0.16	95.0	23.9±0.3	95.6
Sinequan (Farmasierra, San Sebastián de los Reyes, Madrid, Spain)	Per capsule: Doxepin chlorhydrate (25), excipients	24.0 ±1.0	96.0	24.1±0.9	96.4
Ludiomil (Amdipharm, Dublin, Irlanda)	Per tablet: Maprotiline chlorhydrate (25), wheat starch (27), lactose and other excipients	24.5±0.5	98.0	25.1±0.6	100.0
Norfenazin (Reig Jofré, Barcelona, Spain)	Per tablet: Nortryptiline chlorhydrate (25), perfenazine (2), lactose and other excipients	18.8±0.5	75.2	19.7±0.9	78.8
Surmontil (Sanofi Aventis, Barcelona, Spain)	Per tablet: Trimipramine maleate (100), wheat starch and other excipients	95.5±0.8	95.5	95.2±1.4	95.2