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Implementation of gradients of organic solvent in micellar liquid chromatography using DryLab®: Separation of basic compounds in urine samples

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ABSTRACT

In micellar liquid chromatography (MLC), chromatographic peaks are more evenly distributed compared to conventional reversed-phase liquid chromatography (RPLC). This is the reason that most procedures are implemented using isocratic elution. However, gradient elution may be still useful in MLC to analyse mixtures of compounds within a wide range of polarities, decreasing the analysis time. Also, it benefits the determination of moderately to low polar compounds in physiological fluids performing direct injection: an initial micellar eluent with a low organic solvent content, or a pure micellar (without surfactant) solution, will provide better protection of the column against the proteins in the physiological fluid, and once the proteins are swept away, the elution strength can be increased using a positive linear gradient of organic solvent to reduce the analysis time. This work aims to encourage analysts to implement gradients of organic solvent in MLC, which is rather simple and allows rapid analytical procedures without pretreatment or the need of re-equilibration. The implementation of gradient elution is illustrated through the separation of eight basic compounds (β-blockers) in urine samples directly injected into the chromatograph, the most hydrophobic showing large retention in both conventional RPLC and MLC. The use of the DryLab® software to optimise gradients of organic solvent with eluents containing a fixed amount of surfactant above the critical micellar concentration is shown to provide satisfactory predictions, and can facilitate greatly the implementation of gradient protocols.

1. Introduction

Micellar liquid chromatography (MLC) is a reversed-phase liquid chromatographic (RPLC) mode, where the mobile phase is basically composed of an aqueous solution of an ionic or neutral surfactant above the critical micellar concentration (CMC). This RPLC mode has evolved from laboratory curiosity in Ref. [1], to its current practical utility. There are more than three decades of MLC experience with several hundreds of reports [2,3]. One of the most outstanding features of MLC is its unique selectivity, often attributed to the ability of micelles to organise solutes at the molecular level. However, the association of surfactant monomers to the bonded phase, forming a structure similar to the exterior of open micelles [4], has deep implications in the chromatographic behaviour (analysis time, selectivity and efficiency). The adsorption of an approximately fixed amount of surfactant monomers on the stationary phase [5] gives rise to a stable modified packing, with properties remarkably different from those of the underlying bonded phase [6,7].

MLC was first proposed as a chromatographic mode with a mobile phase containing only water, buffer, micelles and a small amount of surfactant monomers [1]. Therefore, micelles played the role of the organic modifier. However, solutions containing only surfactant are too weak and yield poor peak shape. To remediate these problems, Dorsey et al. suggested the addition of a small amount of organic solvent to the mobile phase [8]. Since then, most reported analytical procedures in MLC make use of aqueous solutions of surfactant, mixed with organic solvent (mainly 1-propanol, 1-butanol, 1-pentanol and acetonitrile) [2,3], where the concentration of organic solvent is maintained low enough to allow the formation of micelles. The advantage of increasing the concentration of organic solvent to values producing micelle breakdown has been also addressed in terms of reduced analysis time, larger selectivity and improved peak shape, with respect to MLC

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and conventional RPLC [9–11]. The technique has been called high submicellar liquid chromatography (HSLC). In HSLC, hydrophobic interaction may be dominant due to the reduction of the ionic surfactant coating on the stationary phase, the disaggregation of micelles and the decrease in mobile phase polarity.

The unique selectivity of MLC, together with the smaller consumption of organic solvent and lower toxicity, with respect to conventional hydro-organic RPLC, may not be compelling reasons for a shift to the use of this chromatographic mode. Perhaps, the major reason of the increasing interest in MLC is the possibility of performing the direct injection of physiological samples through the solubilisation of the protein components by interaction with the micelles in the mobile phase, and the protection of the stationary phase by coating with surfactant monomers to avoid clogging [12–14]. The proteins are thus swept away, eluting with or shortly after the solvent front. Micelles also release protein-bound drugs, which results in higher concentrations in the mobile phase for partitioning to the stationary phase and detection. These features simplify the procedures and increase the sample throughput.

In conventional RPLC without additive, there is a linear dependence between the logarithm of the retention factor ($\log k$) and the solute polarity, usually measured as the logarithm of the octanol–water partition coefficient ($P_{o/w}$) [15]. This relationship gives rise to one of the main problems of the technique, called the "general elution problem of chromatography": if the polarity range is too wide, it will be difficult to find a set of chromatographic conditions able to balance satisfactory resolution for the least retained solutes and reasonable retention time for the most retained ones [16]. A logical solution to overcome such a situation is gradient elution, where the elution strength is increased gradually as the analysis progresses, by altering at least one experimental factor (usually the organic solvent content).

In MLC, the situation is different due to the more effective removal of highly hydrophobic compounds from the stationary phase transported by the micelles. This gives rise to linear k versus $\log P_{o/w}$ relationships [17], which is translated in a larger number of compounds being eluted per time unit in the isocratic mode with regard to conventional RPLC. Chromatographic peaks are thus more evenly distributed, with longer retention times for the least retained compounds [18]. This behaviour has been called "gradient effect" and is the main reason that almost all reported MLC procedures use isocratic conditions. In spite of this, gradient elution can be useful in MLC to expedite some analyses, or even its application may enhance the separation capability of the technique.

However, after the seminal reports by Dorsey, Khaledi and coworkers [19–22], describing the capability and usefulness of gradient MLC to speed up the elution of strongly retained compounds, only very few authors have been concerned with the development of gradient elution procedures in MLC. Paleologos et al. determined biogenic amines in fish, chicken and wine samples using an eluent with fixed 0.40 M sodium dodecyl sulphate (SDS) and increasing acetonitrile (from 30 to 50% v/v) [23,24]. Ghorbani et al. determined water-soluble vitamins in multi-vitamin tablets with fixed 0.016 M SDS and increasing 1-butanol (from 3.5 to 10%) [25]. Bryant and Altria determined basic drugs and neutral compounds using gradients with increasing SDS and 1-pentanol (a simultaneous increase in both surfactant and organic solvent from 0.006 M SDS/0.7% 1-pentanol to 0.08 M/9.8%, and from 0.011 M/1.4% to 0.11 M/14%, respectively) [26]. Cao et al. separated phenolic compounds with fixed 0.05 M SDS and increasing acetonitrile (from 0 to 30%) [27].

Particularly interesting is the work of Nakao et al., who determined several radio-metabolites in plasma samples during positron emission tomography (PET) to evaluate the pharmacokinetics of the PET ligands using gradients with fixed SDS and increased 1-butanol or acetonitrile [11,28,29]. The high-speed analysis of short-lived radioligands is essential, together with the possibility of processing a large amount of samples to derive correct metabolite pharmacokinetic functions. The authors demonstrated that MLC overcomes the limitation of conventional RPLC, which only allows metabolite analysis for a limited number of plasma samples. That work was developed guided by the principle of direct injection of the physiological sample in the chromatographic column, in conditions where it is protected (MLC conditions), and the rapid elution of highly retained analytes (after the protein separation, a rapid increase of organic solvent reaching HSLC conditions).

In our opinion, in spite of the advantage of using isocratic elution in MLC, more analysts should consider the improvement of some procedures through the application of gradient elution. To encourage the implementation of gradient MLC, in this work, we show in detail the development of a gradient procedure for the separation of a set of basic compounds (β-blockers) in urine samples with direct injection, using a fixed amount of SDS and a linear increase of organic solvent. The use of the DryLab® software to predict the optimal separation conditions is described.

2. Experimental

2.1. Reagents and columns

The following basic compounds (β-blockers) were analysed (see Table 1): acebutolol, atenolol, carteolol, labetolol, metoprolol and propranolol from Sigma (St. Louis, MO), celiprolol from Rhône-Poulenc Rorer (Alcorcón, Spain), and oxprenolol from Ciba-Geigy (Barcelona, Spain). The drugs were dissolved in a small amount of the organic solvent added to the eluent, and diluted with water. The concentration of the injected solutions was 20 μg/ml for the aqueous mixtures, and 5 μg/ml for the fortified urine samples, which were obtained from human volunteers.

The mobile phases contained sodium dodecyl sulphate from Merck (99% purity, Darmstadt, Germany), and acetonitrile or 1-propanol from Scharlau (Sentmenat, Barcelona). The pH was buffered at 3 with 0.01 M anhydrous sodium dihydrogen phosphate (Fluka, Steinheim, Germany). All experiences were performed with nanopure water, obtained with a Barnstead ultrapure water purification system from Thermo Scientific (Dubuque, IA). The drug solutions and mobile phases were filtered through 0.45 μm Nylon membranes from Micron Separations (Westboro, MA).

Two chromatographic columns were used: Zorbax Eclipse XDB-C8 and Zorbax Eclipse XDB-C18 (both 150 mm × 4.6 mm i.d., 5 μm particle size) from Agilent (Waldbronn, Germany).

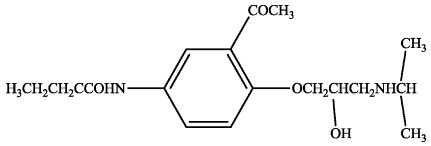
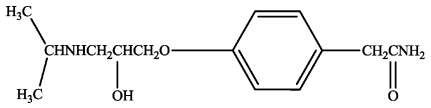
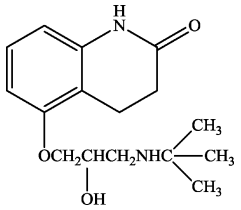
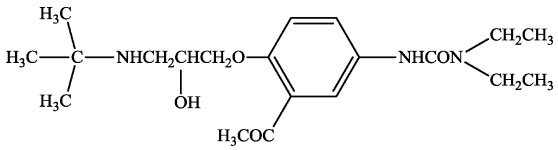
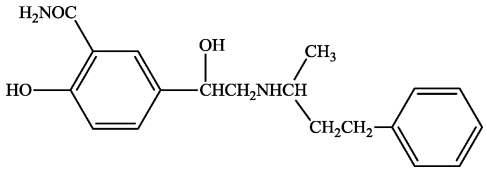
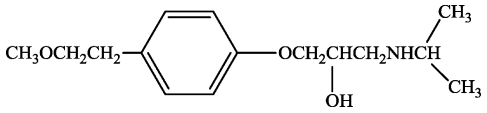
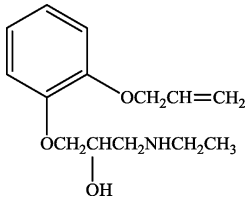
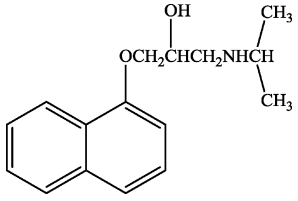
2.2. Apparatus and software

An Agilent liquid chromatographic system was used, which was equipped with the following modules: a quaternary pump (HP 1200) run at 1 ml/min, an autosampler (HP 1100) with 2 ml vials, and a UV–visible detector (HP 1100) set at 225 nm. The temperature was controlled at 25 °C with a thermostated column compartment (HP 1100). The maximal operating pump pressure was 400 bar.

The system was controlled by an OpenLAB CDS LC ChemStation (Agilent B.04.03). The separation conditions were optimised with the assistance of the DryLab® software (Molnár Institute, Berlin, Germany). The chromatograms were exported into DryLab® with the PeakMatch® software from Agilent in AIA format (*.cdf) for "peak tracking".

The dwell volume (total volume of plumbing in a gradient system between the point where the gradient is formed and the column inlet) was determined by removing the column from the system and connecting the injector directly to the detector. The midpoint of the signal obtained with a 0–100% linear gradient, formed by mixing water and 0.1% acetone, indicated the dwell

Table 1
Structures, dissociation constants (p*K*a) and octanol–water partition coefficients for the β-blockers.

Compound	Structure	p*K*a[a]	log $P_{o/w}$[b]
Acebutolol		9.2	1.19
Atenolol		9.6	−0.026
Carteolol		NA[a]	1.42
Celiprolol		NA[a]	1.93
Labetolol		8.7; 7.4	2.41
Metoprolol		9.7	1.69
Oxprenolol		9.5	1.83
Propranolol		9.5	2.60

[a] Ref. [30]; NA = Not available.
[b] Ref. [31]: Octanol–water partition coefficients calculated from the compound structures using the on-line interactive LOGKOW program of the Environmental Science Centre of Syracuse Research Corporation.

time (t_D). The mean value from several runs was t_D = 0.89 min. The dead time (t_0) in conventional RPLC was obtained from the peak of uracil (t_0 = 1.42 min). The first perturbation in the chromatograms allowed the determination of the dead time in MLC (t_0 = 1.30 min).

3. Results and discussion

Based on our previous experience with the MLC and HSLC isocratic separation of β-blockers, we have developed this work, which examines and optimise the gradient elution capability of

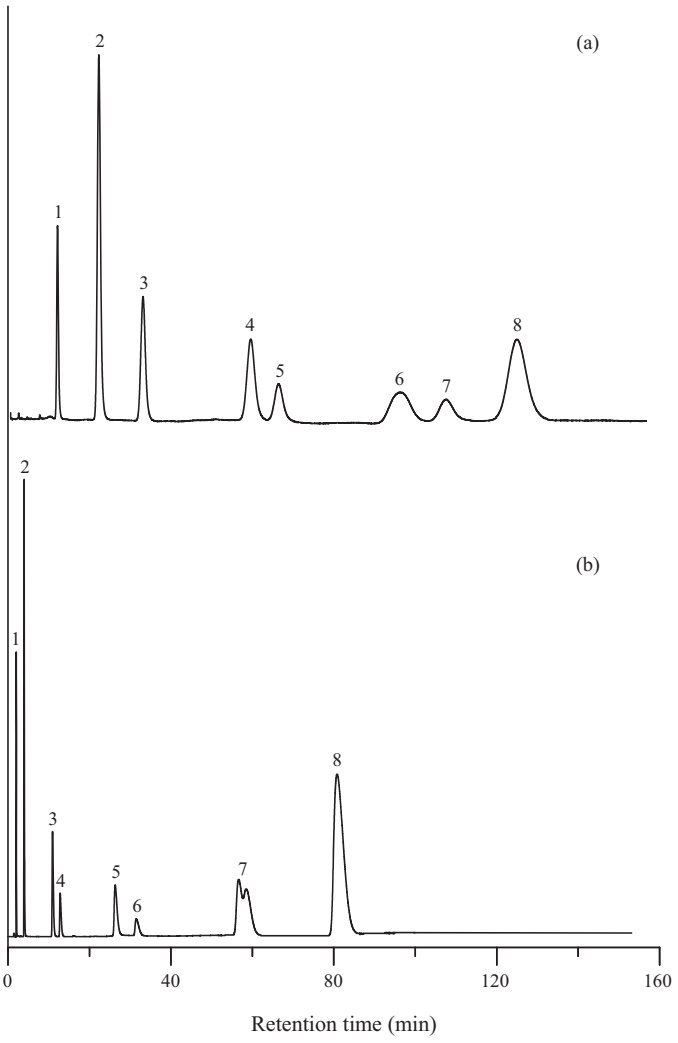


Fig. 1. Chromatograms for an aqueous mixture of the set of β-blockers eluted isocratically using 15% v/v acetonitrile in the presence of 0.10 M SDS (a), and without SDS (b). Column: Zorbax Eclipse XDB-C8. Peak identity: (1) atenolol, (2) carteolol, (3) acebutolol, (4) metoprolol, (5) celiprolol, (6) oxprenolol, (7) labetolol, and (8) propranolol.

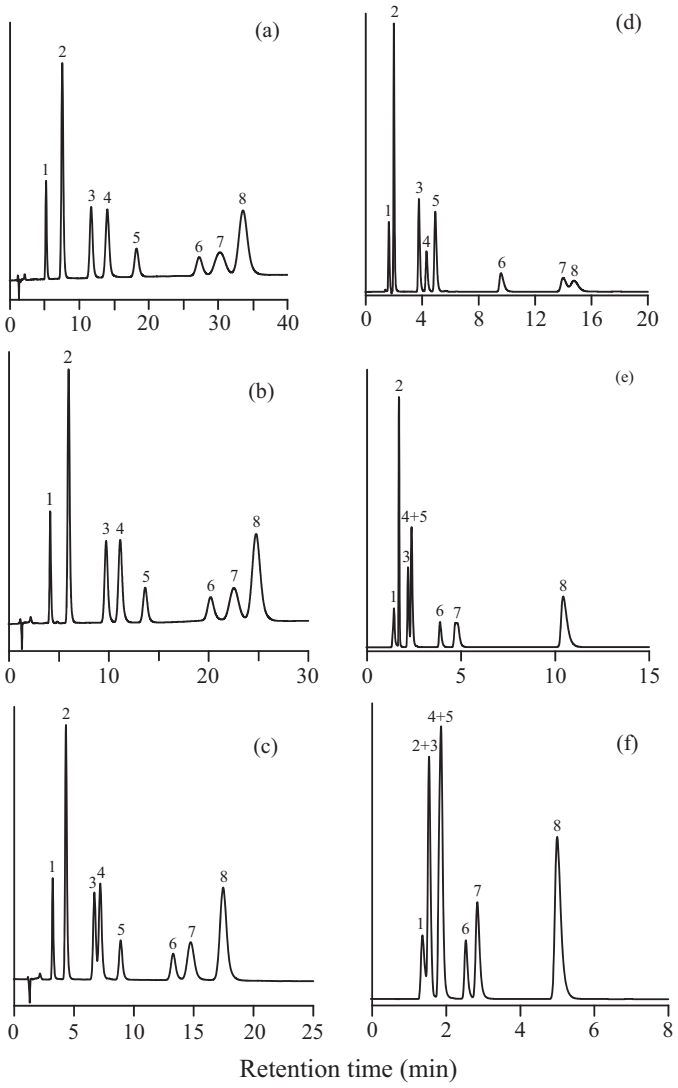


Fig. 2. Chromatograms for an aqueous mixture of the set of β-blockers eluted isocratically with micellar-organic (a–c), and conventional hydro-organic (d–f) mobile phases, containing 10% (a and d), 15% (b and e), and 20% (c and f) 1-propanol. The SDS concentration for the micellar-organic mobile phases was 0.10 M. See Fig. 1 for column and peak identity.

solutions containing SDS and organic solvent. For this purpose, we selected a group of eight β-blockers with different polarities as probe compounds: acebutolol, atenolol, carteolol, celiprolol, labetolol, metoprolol, oxprenolol, and propranolol. Along this study, the behaviour in MLC was compared with hydro-organic RPLC using the same organic solvent. The aim of this work is not the implementation of a specific procedure to analyse β-blockers, but to show a useful methodology for analysts to implement gradients of organic solvent in MLC for β-blockers (parent drugs and/or metabolites), or other compounds (basic and other types of compounds).

3.1. Isocratic separation of the β-blockers

3.1.1. Preliminary assays

Before implementing gradient elution, it is often convenient to assess the chromatographic behaviour using isocratic conditions. This is especially true for MLC. Thus, we carried out preliminary experiences to separate the eight β-blockers, using a Zorbax Eclipse XDB-C18 column and mobile phases containing 15% acetonitrile, in the presence of 0.10 M SDS. The retention was too long, especially for the most hydrophobic compounds (oxprenolol, labetalol and propranolol), with retention times reaching 300 min or above. We decided to change the column by a similar less hydrophobic (Zorbax Eclipse XDB-C8), for which the retention times decreased significantly, but keeping still too large values, as depicted in Fig. 1a.

For comparison, in Fig. 1b, the chromatogram obtained with 15% acetonitrile (without SDS) is shown. Although the elution order is the same for both chromatographic modes, the particular behaviours for MLC (Fig. 1a) and conventional RPLC (Fig. 1b) are observed: peak distribution is more homogeneous along the chromatogram for the MLC run. Also, the tailing behaviour observed with the acetonitrile-water mixture is less evident with the micellar-organic mobile phase, but in this case the peaks are wider and the retention for all peaks shifted to longer times. The double peak for labetalol in the conventional RPLC chromatogram is noteworthy. This double peak was also observed in other chromatograms obtained with hydro-organic mixtures in this work.

3.1.2. Use of hybrid mobile phases of SDS and 1-propanol

Although 1-propanol is not a usual solvent in conventional RPLC, it has become the solvent of choice in MLC since the early

years of the technique. This solvent gives a solution to the weak elution strength of methanol and acetonitrile in MLC, being particularly useful for moderately polar compounds. Fig. 2a–c depict the chromatograms obtained for the β-blockers with 10, 15 and 20% 1-propanol, respectively, in the presence of 0.10 M SDS. For comparison, in Fig. 2d–f, the chromatograms obtained with 1-propanol at the same concentrations in the absence of surfactant are shown. The elution patterns are different in the two chromatographic modes.

As observed, in the conventional RPLC mode, 1-propanol is too strong: the separation is adequate for 10% 1-propanol, but 15% 1-propanol resulted in coelution of metoprolol and celiprolol. For 20% 1-propanol, carteolol and acebutolol also coeluted. In contrast, the peaks in the chromatograms obtained with the combination of SDS and 1-propanol (Fig. 2a–c) were well or sufficiently well resolved for the three assayed concentrations of organic solvent, although the retention times were still long. Note that the selectivity for 10% and 15% 1-propanol in the MLC mode was similar.

Consequently, the separation of the β-blockers in both chromatographic modes should be conveniently performed in the gradient mode to allow the resolution of the early eluting compounds and/or reduce the analysis time. In the conventional RPLC mode, acetonitrile seems to be more adequate than 1-propanol. Also, the gradients should reach concentrations well above 15% acetonitrile, and considering the distribution of peaks in the isocratic chromatograms, rather complex gradients are needed.

3.2. Preliminary assays in MLC gradient elution

Since the aim of this work was to implement a direct injection procedure, we concentrated our effort to optimise a gradient in MLC. The chromatogram in Fig. 2c indicated the need of 1-propanol contents of at least 20% to elute the most hydrophobic β-blockers in sufficiently short times, but acebutolol and metoprolol showed a small overlapping at this mobile phase composition. On the other hand, the column is better protected from clogging when the physiological sample is injected in an eluent with low organic solvent contents (or without organic solvent). We decided, therefore, to run gradients starting with 0.10 M SDS and increasing 1-propanol linearly from 0 to 30% over different time periods (t_G = 15 to 45 min). The gradients were built by mixing gradually a 0.10 M SDS aqueous solution (reservoir A) with 1-propanol (reservoir B). In further runs, we added a 2 min isocratic step with 0.10 M SDS before starting the gradient, in order to increase the protection of the column against the proteins in the physiological fluid.

The use of a pure micellar solution, or a hybrid solution of SDS containing a rather small amount of 1-propanol, at the beginning of the gradient, forced to increase the 1-propanol content in the eluent above 20% at the end of the gradient. The final 1-propanol content was limited to 30% to maintain the system pressure sufficiently below the maximal operating pump pressure. Along the gradients, the pressure ranged between 100 and 250 bar, without apparent differences between the hydro-organic and micellar modes.

It should be noted that above 22% 1-propanol, SDS micelles are disrupted [32]. Therefore, the behaviour is changed to the HSLC mode. However, in previous work we checked that within certain organic solvent ranges, a unique retention model can be used to describe the retention in both MLC and HSLC modes [7]. With the selected gradients (0–30% 1-propanol with or without a previous isocratic step of 2 min with 0.10 M SDS), t_G > 20 min was needed to elute all β-blockers. As observed in Figs. 3a–c and 4a and b, in these conditions the chromatograms contain a

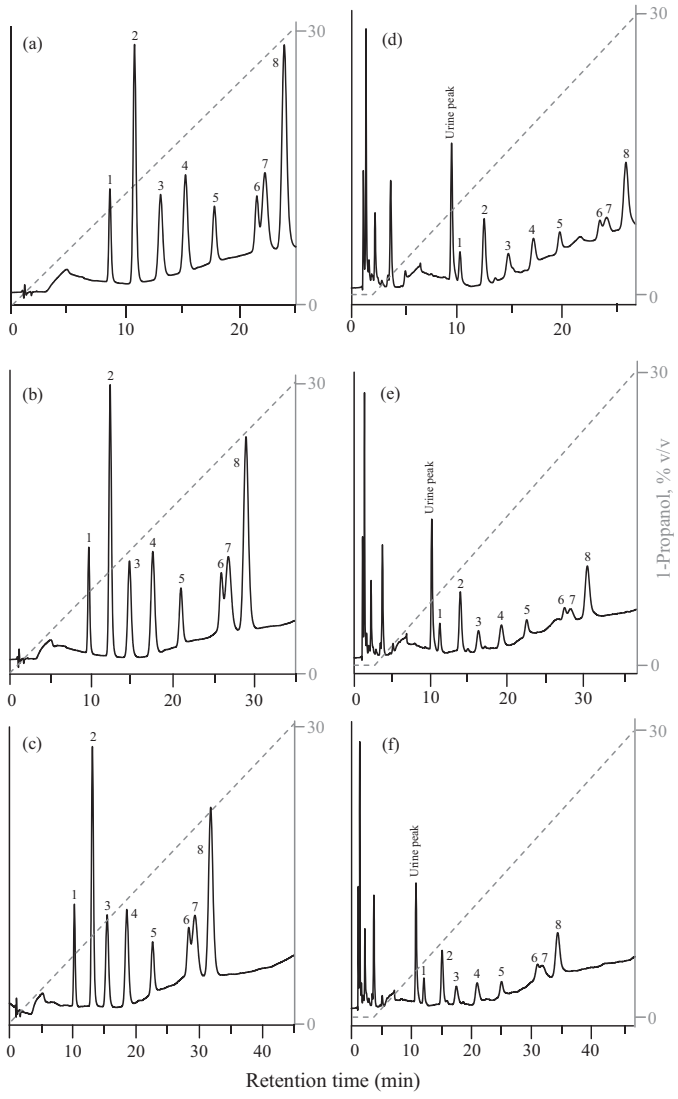


Fig. 3. Chromatograms obtained using gradient elution for an aqueous mixture of the β-blockers (a–c), and a urine sample fortified with the β-blockers (d–f). The concentration of SDS in reservoir A was fixed at 0.10 M, and the 1-propanol content was linearly increased from 0 to 30% over 25 min (a and d), 35 min (b and e), and 45 min (c and f). For the fortified sample, an isocratic step of 2 min with 0.10 M SDS preceded the gradient. See Fig. 1 for column and peak identity. The peaks at retention times shorter than that marked as "Urine peak" (an endogeneous compound) correspond to the elution of matrix proteins and other endogeneous compounds.

critical pair (oxprenolol and labetalol), which in the isocratic mode eluted at close times, but was base-line resolved. It should be noted that at increasing t_G (from 25 to 45 min, Fig. 3a–c), the analysis time increased, but the resolution of the critical pair decreased.

We examined also the effect of the concentration of SDS on the resolution. As observed in Fig. 4a and b, an increase from 0.10 to 0.20 M SDS in reservoir A decreased the analysis time, but the resolution of the critical pair was deteriorated. A smaller SDS concentration would allow better resolution of the critical pair, but we decided to use 0.10 M SDS to guarantee column protection with surfactant monomers. Fig. 4c shows a chromatogram obtained with a similar gradient in the absence of SDS (i.e. conventional RPLC mode). The elution profile is significantly different to the MLC mode. As observed, acebutolol and metoprolol coeluted, and labetolol produced a double peak.

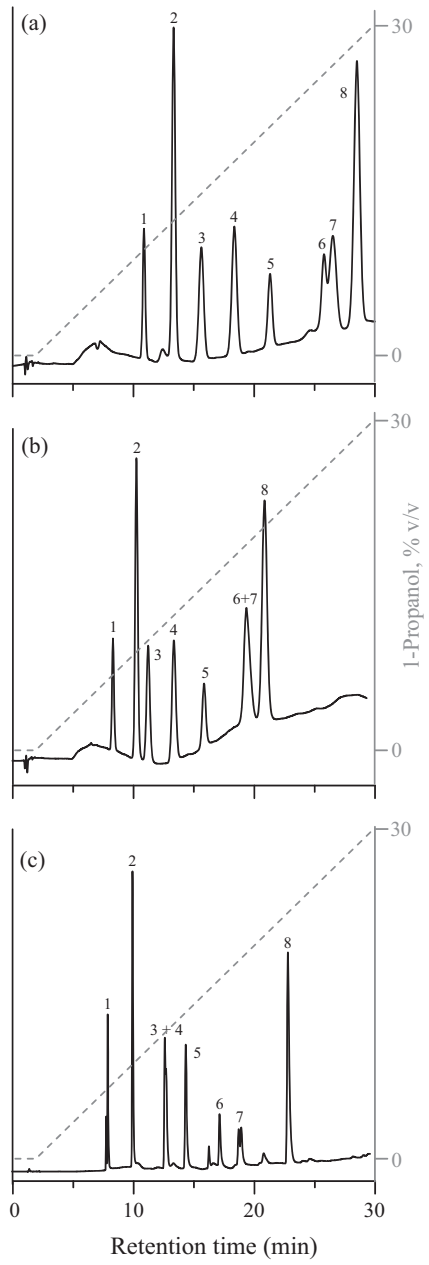


Fig. 4. Chromatograms obtained using a linear increase of 1-propanol from 0 to 30% over 30 min after an isocratic step of 2 min. The gradient was started with: (a) 0.10 M SDS, (b) 0.20 M SDS, and (c) 0 M SDS. See Fig. 1 for column and peak identity.

3.3. Re-equilibration studies

In conventional RPLC, a significant amount of the organic solvent is extracted by the alkyl bonded stationary phase. As the concentration of organic solvent is changed during a gradient run, the composition of the bonded phase may change, being solvated to a varying extent [33,34]. Other possible non-ideal processes are solvent de-mixing due to the preferential uptake of one mobile phase component by the stationary phase, and changes in the column dead time. Failure to regenerate a column completely after a gradient run will produce wide variability in the retention of early eluting peaks, from one injection to the next one. The column will need re-equilibration by flushing through the column some volumes of the initial mobile phase composition, before the next gradient run. Despite the faster gradient separation, the re-equilibration time (which may be significant) will increase the analysis time.

Because of their extensive use as ion-pairing reagents, the adsorption of surfactant monomers on RPLC stationary phases at concentrations below the CMC has received much attention [35]. In these conditions, equilibration times are quite long. However, above the CMC (micellar conditions), the amount of free surfactant in the mobile phase is approximately constant such that any change in the total surfactant concentration will result only in a change in the micelle concentration. Furthermore, adsorption isotherms have shown that, in these conditions, no or little change occurs in the stationary phase, even in the presence of organic solvent [5]. Therefore, it is possible to speed up the elution of strongly retained compounds with surfactant gradients. Starting the gradient above the CMC, no additional surfactant will adsorb onto the stationary phase as the total surfactant concentration is increased and the surfactant will not be either desorbed when returning to the initial conditions. The only re-equilibration necessary before analysing the next sample is that amount of mobile phase needed to flush the mixer and the injector, together with other pre-column volumes. This gradient capability was first reported by Landy and Dorsey in 1984 [19], and further described by Madamba-Tan et al. in 1994 [21]. It was presented as a much faster approach than conventional RPLC, because of the significant reduction in column re-equilibration after a gradient. In 2003, McCormick et al. demonstrated that the column re-equilibration necessary between injections after a surfactant gradient is as rapid on large-pore columns as on small-pore columns [36,37].

When a gradient of organic solvent is used in MLC, however, the composition of the stationary phase might change since the addition of an organic solvent to a micellar mobile phase may result in significant desorption of the surfactant monomers from the stationary phase [38]. In addition, the surfactant CMC in the mobile phase depends on the organic solvent content [39], and a change in the CMC may lead to a change in the concentration of adsorbed surfactant monomers. This would disturb the equilibration of the column. However, several authors have observed that re-equilibration times for gradients of organic solvent are also short [11,22,23]. According to Madamba-Tan et al., the main reason behind this behaviour is the range of organic solvent content along the gradient, which is too limited to produce any significant effect on the composition of the stationary phase [22]. The re-equilibration time in conventional RPLC after gradient elution with propanol is also short, as first described was Cole and Dorsey, and explained by the consistent solvation of the stationary phase by this solvent [40].

It is worth to show here a representation of some data obtained by Madamba-Tan et al. [22] to illustrate the short re-equilibration time in an MLC procedure with a gradient of organic solvent, in comparison to conventional RPLC (Fig. 5a and b, respectively). The authors injected dansylated phenylalanine on a Nucleosil C18 column (150 mm × 4.6 mm i.d., 5 μm particle size) from Phenomenex (Torrance, CA), using a 3–15% 2-propanol gradient, in the presence and absence of 0.15 M SDS. In that study, the system was flushed with different volumes of the initial concentration of organic solvent in the gradient up to 20 ml, at a flow-rate of 1 ml/min. Note that the column can be considered completely equilibrated when the retention time of the probe compound reaches a constant value. As observed, in the work of Madamba-Tan et al., the signal was stabilised at around 11 min for both conventional RPLC and MLC. The first point in each plot was obtained immediately after the gradient reached its top value (a manual injector was used). Since the dwell time (i.e. the time taken to sense the initial conditions) was 3.5 min, the column re-equilibration time was actually 7.5 min.

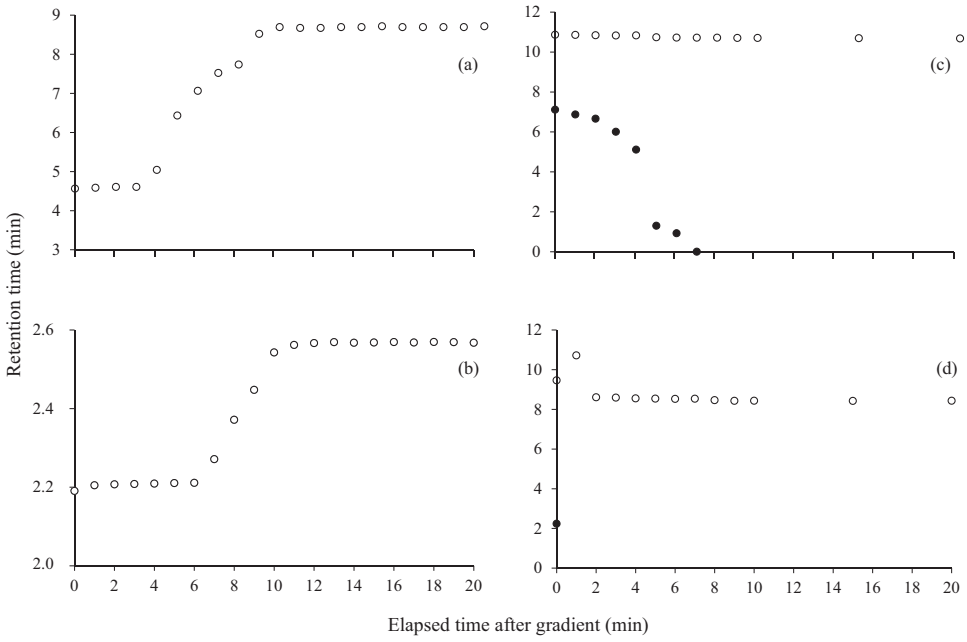


Fig. 5. Experiences carried out to obtain the column re-equilibration time after gradient elution, using a micellar-organic eluent with SDS (a and c), and a hydro-organic eluent (b and d); (a and b) depict data reported by Madamba-Tan et al. [22], and (c and d) correspond to this work. The gradient for (a and b) consisted of a linear increase of 2-propanol from 3 to 15% over 15 min, and for (c and d), a linear increase of 1-propanol from 0 to 30% over 30 min. The plots correspond to: (a and b) dansylated phenylalanine (○), and (c and d) atenolol (○) and the front peak in the chromatogram (●). See text for other details.

Fig. 5c and d depict a similar study carried out in our laboratory by injecting atenolol in the Zorbax Eclipse XDB-C8 column, with the same geometrical characteristics as the Nucleosil C18 column used by Madamba-Tan et al. In our experiences, we used an automatic injector with an injection routine that started just at the time the gradient top value was reached and took around 2 min, which is a value above the dwell time (t_D = 0.89 min). It is evident that the re-equilibration time was significantly shorter (less than 2 min) with respect to that reported by Madamba-Tan et al. 20 years ago (around 7.5 min). To appraise this result, it should be noted that the β-blockers are positively charged, and consequently, their retention is very sensitive towards changes in the stationary phase composition, especially the amount of the adsorbed anionic surfactant. Also, note that the gradient starts with pure micellar conditions (a 0.10 M SDS solution) and progressively increases the organic solvent content, which could affect significantly the surfactant thickness on the stationary phase (but this does not seem to happen). The re-equilibration time found in our laboratory with the current technology agrees with the values reported recently by Nakao et al. for MLC, of 1 or 2 min before being ready for the next injection [11,28,29].

Before finishing this section, it is interesting to observe chromatograms taken after several elapsed times once the gradient has finished, between 1 and 10 min (Fig. 6). A sharp signal appears at decreasing times, which corresponds to the front signal. The time at which this signal appears is depicted in Fig. 5c as full circles. A similar signal was observed for the hydro-organic mode, but it appeared always at a time below 1 min (Fig. 5d). Note that the gradient of organic solvent in MLC is built by mixing volumes of a 0.10 M SDS aqueous solution (reservoir A) with volumes of 1-propanol (reservoir B). Specifically, during the gradient, the eluent contained 100–70% of A and 0–30% of B. When the top value was reached, the system was changed immediately to 100% of A (pure SDS), giving rise to the observed front signal.

Therefore, the re-equilibration time in MLC is immediate, but the front signal may interfere in the chromatograms, which would

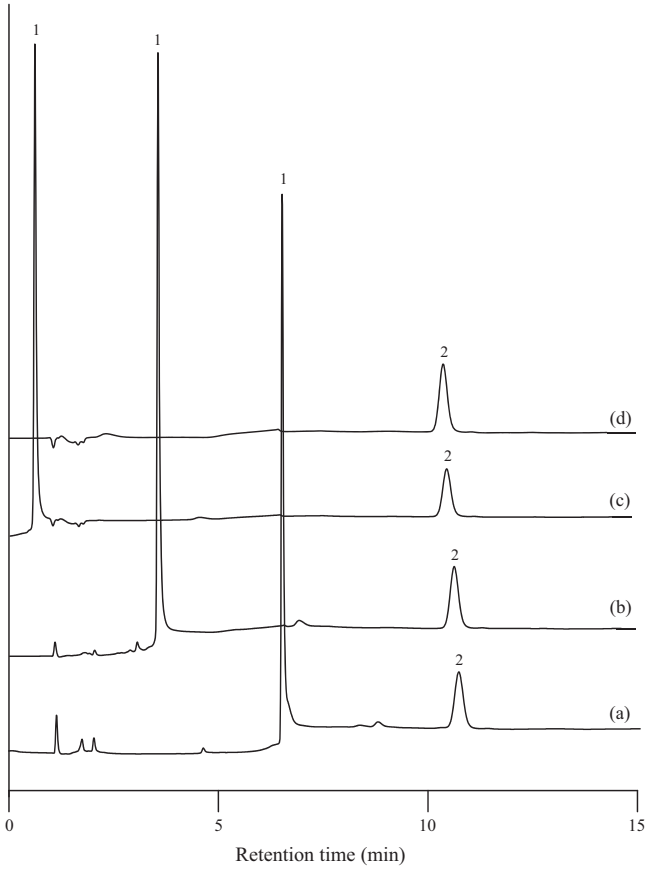


Fig. 6. Chromatograms obtained for the micellar-organic mode with SDS/1-propanol, using different elapsed times after the gradient: (a) 1 min, (b) 5 min, (c) 8 min, and (d) 10 min. Column: Zorbax Eclipse XDB-C8. Gradient: 0.10 M SDS in reservoir A and linear increase of 1-propanol from 0 to 30% over 30 min. Peak identity: (1) front signal, and (2) atenolol.

force to wait ca. 7 min before starting the gradient. However, in the present work, the front signal did not represent any interference, since it appeared in the region before the β-blockers elution (see Section 3.4.2).

3.4. Use of DryLab® to optimise gradients of organic solvent in MLC/HSLC after direct injection

3.4.1. The DryLab® software

DryLab® is a software package extensively used in routine laboratories to optimise protocols in conventional RPLC, using either isocratic or gradient elution [41,42]. In a sufficiently narrow organic solvent concentration range, isocratic elution in RPLC is described by the following linear equation:

$$\ln k = \ln k_w - S\varphi \quad (1)$$

where k_w is the retention factor in water, φ the organic solvent concentration, and S the "solvent strength". This equation has been demonstrated to be also valid in MLC [43,44].

Gradient elution is significantly more complex than isocratic elution. The retention behaviour in gradient elution is described using the so-called "fundamental equation for gradient elution":

$$t_0 = \int_0^{t_g - t_0} \frac{dt}{k(\varphi(t))} \quad (2)$$

where t is a time variable, t_0 the time the mobile phase takes to go through the column (i.e. the dead time), t_g the time a solute requires to reach the column outlet under gradient conditions, and $k(\varphi(t))$ the function that relates the isocratic retention factor with the solvent content, which changes with time according to a gradient program. The simplest approach in gradient elution assumes a linear retention model (Eq. (1)), and a linear gradient program:

$$\varphi = \varphi_0 + mt \quad (3)$$

m being the gradient slope and φ_0 the initial organic solvent content in the gradient. In these conditions, the integration of Eq. (2) is simple, giving rise to the extensively used "linear solvent strength model" [45,46]:

$$t_g = \left[\frac{t_0}{b}\log 2.3 k_0 b(1-f)+1\right] + t_D + t_0 \quad (4)$$

where k_0 is the isocratic retention factor at the initial mobile phase condition, f the column fraction the solute has already travelled before the gradient reaches it, t_D the dwell time, and b the gradient steepness, calculated as:

$$b = \frac{S t_0 \Delta\varphi}{t_G} \quad (5)$$

where $\Delta\varphi$ is the change in the volume fraction of organic modifier during the gradient, and t_G the total gradient time.

Madamba-Tan et al. applied Eqs. (1), (4) and (5) to predict the retention in MLC with satisfactory results [22]. These equations are also used in the DryLab® software for HPLC method development to describe gradient elution in conventional RPLC [45,46]. The DryLab® software implements an interpretive approach, where the retention behaviour is modelled using experimental information from RPLC runs, and the retention times at other conditions are predicted in a selected experimental domain. This allows calculating the resolution for the critical pair, and accordingly, the optimal separation is shown. Also, the software informs about the chromatograms that may be obtained by modifying the recommended gradient for selected linear and multi-linear gradients.

3.4.2. Analysis of urine samples

Because of the facility in obtaining the samples, urine is preferred over other physiological fluids for controlling the intake of drugs. As commented, in MLC, the micelles in the mobile phase solubilise high-molecular-mass proteins in urine samples. Meanwhile, the underlying alkyl-bonded silica phase is protected by a constant layer of surfactant monomers. The proteins are not retained probably due to the formation of protein–surfactant complexes, which are excluded from the pores of the stationary phase packing, appearing as a broad band at the solvent front [13,14]. The

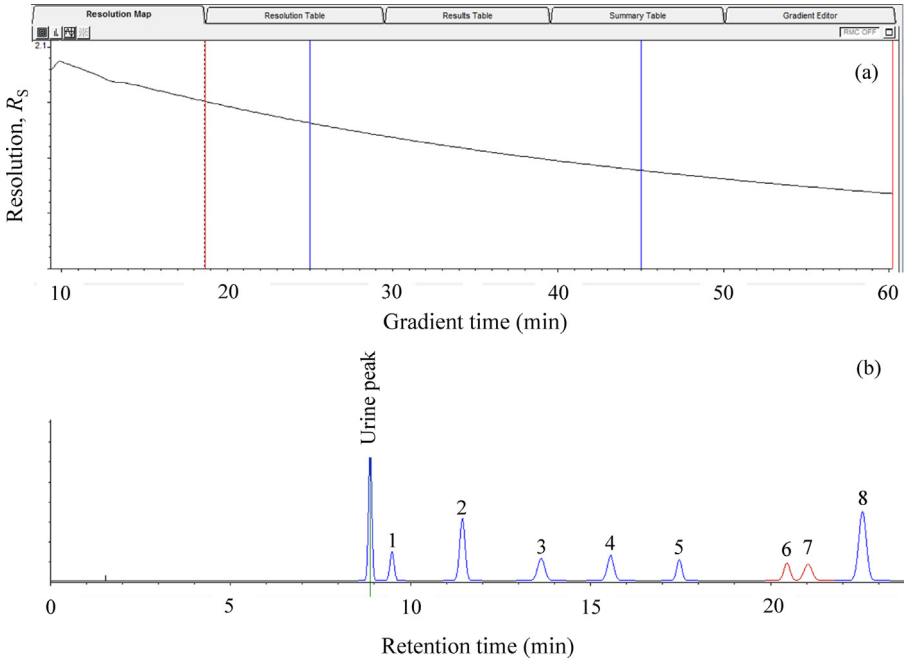


Fig. 7. Optimisation of the gradient separation of the β-blockers in a urine sample assisted by DryLab®: (a) resolution map, and (b) recommended chromatogram. See Fig. 1 for column and peak identity. The vertical lines correspond to the training gradients (25 and 45 min), and the recommended separation (18.6 min).

matrix also contains endogenous compounds with peaks at diverse retention times that may overlap the peaks of analytes, resulting in a useless region in the chromatogram. However, frequently, the analytes elute after this region as shown in the chromatograms in Fig. 3d–f, which were obtained with a 0–30% linear gradient of 1-propanol and different gradient times of t_G = 25, 35 and 45 min, respectively, after an isocratic step of 2 min with 0.10 M SDS. Atenolol, which is the least retained compound in this work, eluted close to a prominent peak corresponding to an endogeneous compound in urine, whose retention time has been checked to vary in less than 4% considering males and females of different ages, diets and weights, although its intensity changes for different individuals [13].

The analyses were performed with 1 ml of urine fortified with the β-blockers, and diluted in a 1:25 factor with water. It should be said here that dilution of the urine sample will keep the column operative for a longer time, but injection of undiluted urine samples to reach smaller limits of detection is in general feasible, provided adequate detection is implemented. The urine solutions were injected into the chromatograph without any other treatment than filtration, made directly in the autosampler vials through a 0.45 μm Nylon membrane. Note that the 1-propanol content at the elution of the urine peak close to atenolol in Fig. 3d–f was 9.0%, 7.0% and 5.8% 1-propanol, respectively.

3.4.3. Optimisation of the gradient protocol

We show in this section the application of the DryLab® software to the optimisation of a gradient of organic solvent in MLC. It should be first indicated that the software needs a training set constituted of two gradients covering the same domain of organic solvent, but different t_G values. These runs allow fitting the retention model (Eq. (4)) for the application.

We should remind that the eluent in MLC contains SDS micelles, which influences the elution strength. Therefore, we checked first the prediction capability of DryLab® in the presence of SDS. The retention times for the chromatograms in Fig. 3a and c, corresponding to a gradient using 0.10 M SDS and a 0–30% linear increase of 1-propanol over 25 and 45 min, respectively, were used to train the software. The input dwell time was t_D = 0.89 min. Next, the retention data for the intermediate t_G = 35 min (Fig. 3b) were predicted. These data are compared with the experimental data for the same conditions in Table 2. As observed, the predicted gradient retention times agreed closely with the experimental values, with small negative errors of up to 0.7 min. We should note that in these experiences, all throughout the gradient, the linear gradient of organic solvent was immediately started. However, the recommended gradient for the analysis of urine samples in this work included a previous isocratic step of 2 min with 0.10 M SDS, which the DryLab® software does not consider. In order to predict this situation, we decided to increase the input dwell time in 2 min (t_D = 2.89 min). The training retention times corresponded to the chromatograms in Fig. 3d and f with t_G = 25 and 45 min, respectively. The data for t_G = 35 min were predicted (Fig. 3e) and compared with the experimental data for the urine sample (Table 2). The predictions were again rather satisfactory, with positive errors of up to 0.8 min.

Table 2
Experimental and predicted retention times (min) obtained with the DryLab® software for a gradient using 0.10 M SDS in reservoir A and a linear increase of 1-propanol from 0 to 30% over 35 min. The results for an aqueous mixture of the standards and a urine sample fortified with the standards are given. For the fortified urine sample, a 2 min isocratic step with 0.10 M SDS preceded the gradient.

Peak identity	Standards[a]		Urine + standards[b]	
	Experimental	Predicted	Experimental	Predicted
Urine peak	–	–	10.16	10.28
Atenolol	9.67	9.52	11.21	11.41
Carteolol	12.29	12.06	13.89	14.18
Acebutolol	14.66	14.42	16.27	16.55
Metoprolol	17.52	17.12	19.27	19.62
Celiprolol	20.97	20.47	22.58	23.09
Oxprenolol	25.92	25.25	27.53	28.27
Labetolol	26.79	26.11	28.32	29.00
Propranolol	28.95	28.23	30.50	31.30

[a] The real dwell time (0.89 min) was input in the DryLab® software.
[b] The input dwell time was the summation of the real dwell time and the isocratic step (in total: 2.89 min).

Fig. 7a shows the resolution of the critical pair as depicted by the DryLab® software. The vertical lines correspond to the two training gradients (t_G = 25 and 45 min), and the recommended gradient, which is the limit of satisfactory extrapolation (t_G = 18.6 min in the case of study). The chromatogram below (Fig. 7b) corresponds to the prediction given by the software for this limit. The input dwell time was again t_D = 2.89 min. Observe that the peaks of oxprenolol and labetolol are predicted to be resolved.

We further considered the possibility of modifying the recommended gradient to obtain better resolution and/or smaller analysis time. We changed the gradient time successively to t_G = 15, 13 and 11 min, keeping the dwell time at t_D = 2.89 min, and examined the predicted chromatograms. We also examined a gradient with t_G = 15 min and t_D = 7.89 min, corresponding to an isocratic step over 7 min with 0.10 M SDS before starting the gradient of organic solvent. Table 3 compares the predicted retention times in these conditions with the experimental values. The largest systematic errors were obtained for the gradient including the long isocratic step (i.e. 7 min), with negative errors of up to 2 min for the least retained compounds, and positive errors of up to 0.5 min for the most retained compounds. Therefore, in these conditions, the role of SDS as eluent decreases significantly the accuracy of the predictions.

Table 3
Experimental and predicted retention times (min) obtained with the DryLab® software for a gradient using 0.10 M SDS in reservoir A and a linear increase of 1-propanol from 0 to 30% over 15 min (Experience 1 and 4), 13 min (Experience 2), and 11 min (Experience 3), after an isocratic step of 2 min (Experiences 1 to 3), and 7 min (Experience 4) with 0.10 M SDS. The results correspond to a urine sample fortified with the standards[a].

Peak identity	Experience 1		Experience 2		Experience 3		Experience 4	
	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted
Urine peak	8.36	8.44	8.04	8.16	7.73	7.85	11.53	9.96
Atenolol	8.80	8.92	8.40	8.57	8.00	8.19	12.26	10.78
Carteolol	10.52	10.66	9.97	10.17	9.45	9.63	13.69	11.57
Acebutolol	12.45	12.72	11.78	12.15	11.07	11.50	15.07	13.26
Metoprolol	13.78	14.36	12.88	13.82	11.93	12.78	16.15	14.94
Celiprolol	15.07	15.92	13.99	14.97	12.87	13.93	17.06	16.57
Oxprenolol	17.23	18.41	15.90	17.17	14.60	15.86	18.84	19.11
Labetolol	17.58	18.94	16.25	17.67	14.96	16.37	19.07	19.62
Propranolol	19.16	20.30	17.93	19.06	16.70	17.80	20.90	20.93

[a] The summation of the real dwell time and the isocratic step was input in the DryLab® software: 2.89 min for Experiences 1 to 3, and 7.89 min for Experience 4.

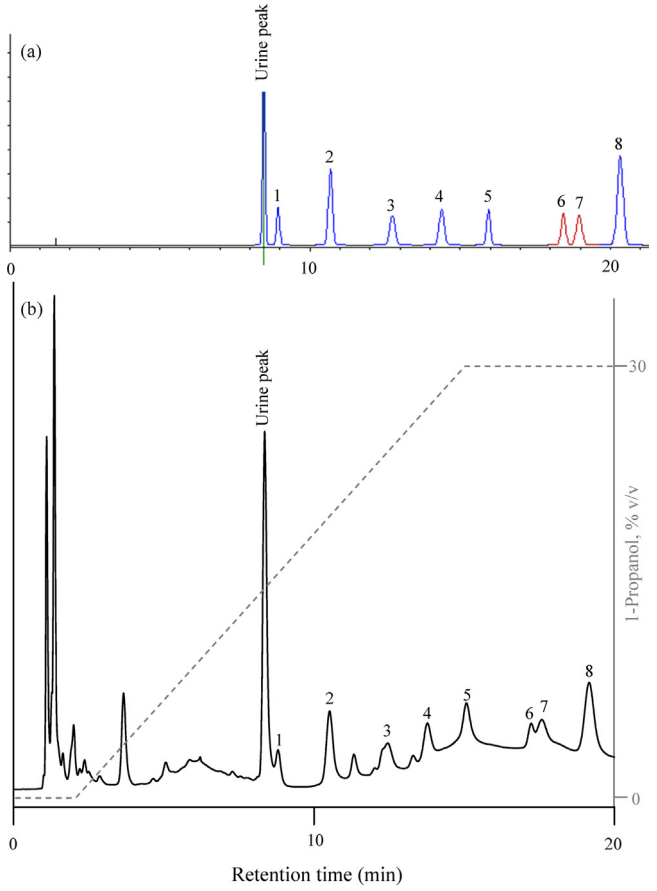


Fig. 8. Predicted chromatogram by DryLab® (a), and experimental chromatogram (b), for a urine sample fortified with the β-blockers at the selected separation conditions. Gradient: 0.10 M SDS in reservoir A and linear increase of 1-propanol from 0 to 30% over 15 min, with a previous isocratic step of 2 min with 0.10 M SDS, followed by an isocratic step of 0.07 M SDS/30% 1-propanol beginning at 15 min. See Figs. 1 and 3 for column and peak identity.

Fig. 8 compares the experimental chromatogram for the 0–30% gradient run over 15 min after a 2 min 0.10 M SDS isocratic step, with the chromatogram predicted by the DryLab® software. Both agree satisfactorily, although the experimental peaks are wider giving rise to larger overlapping of the peaks of oxprenolol and labetolol with respect to the predicted chromatogram.

4. Conclusions

The particular elution behaviour of MLC makes gradient elution often unnecessary. It is, however, surprising that among the hundreds of MLC analytical procedures that have been reported to determine different types of compounds in a variety of samples, only very few make use of gradient elution. This elution mode may be useful to analyse mixtures of compounds within a wide range of polarities, decreasing the analysis time, and as already reported by Nakao et al. [11,28,29], it can benefit the analysis of physiological samples, especially in the case of implementing procedures for moderately to low polar compounds. The use of an initial micellar eluent with a low organic solvent content or just a pure micellar (without surfactant) solution, which would be insufficient to elute moderately to low polar analytes in the isocratic mode, will however provide better protection of the column against the proteins in the physiological fluid. Once the proteins are swept away, the elution strength can be increased using a positive linear gradient of organic solvent.

This work aims to show that the implementation of gradient elution in MLC is rather simple and allows rapid procedures without pre-treatment, or the need of re-equilibration. Also, in the case of performing a gradient of organic solvent, in spite of the presence of surfactant, the optimisation of the separation conditions can be carried out satisfactorily with commercial optimisation software like DryLab®, which has not been reported before in MLC. Therefore, analysts should certainly consider the possibility of using gradient elution in MLC.

The selectivity of the separation in MLC is rather different with respect to conventional RPLC, due to the variety of interactions between solutes, micelles and the adsorbed surfactant monomers on the stationary phase, which give rise to a highly versatile technique, appropriate for a wide range of solutes (from hydrophilic to hydrophobic). Hybrid eluents in MLC have the advantage of requiring significantly smaller amounts of organic solvent with respect to conventional RPLC, especially for apolar compounds, even when using an HSLC procedure where the amount of organic solvent is somewhat larger. This means lower cost, toxicity and environmental impact of hazardous wastes. Also, the stabilisation of organic solvents by micelles in the mobile phase reduces the risk of evaporation. These properties are attractive considering the increasing restriction in the use of organic solvents in the laboratories.

Acknowledgements

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