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Multifactorial design principles applied for the simultaneous separation of local anesthetics using chromatography modeling software

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This study describes the development of liquid chromatographic methods for the simultaneous separation of some of the most popular local anesthetics in pharmaceutical preparations and medical praxis (benzocaine, bupivacaine, chloroprocaine lidocaine, oxybuprocaine, prilocaine, procaine, propipocaine and tetracaine) based on a systematic approach using experimental design methodology in which one or more factors are changed at the same time. The strategy employs a chromatography modeling software for the simultaneous optimization of critical chromatographic parameters, which are gradient time t_G, temperature T and the ternary composition of the organic eluent B. Three different stationary phases were investigated, which are: Kromasil C_{18}, Prontosil C_{18}-AQ and Luna Phenyl-Hexyl. To build the design space for each column, 12 initial experiments were carried out by systematical variation of the selected critical parameters simultaneously. The chromatographic conditions of these initial runs are based on two different gradient times (t_G = 20 and 60 min, linear gradient system from 10–90% organic modifier *B*) each at two different temperatures (T = 25 and 40 °C) repeated at three different ternary composition of the eluent B (a) 100% acetonitrile, (b) acetonitrile–methanol (50 : 50) (v/v), and (c) 100% methanol. In all experiments the pH value of the eluent A (20 mM phosphate buffer) was kept at 3.0. The mixture of local anesthetics could be well separated on all three stationary phases. Although not demonstrated in this paper, this method should be suitable for the analysis of LAs in pharmaceutical preparations or to detect them in some illegal cosmetics. The results showed that the selectivity and the elution order were similar on Kromasil C_{18} and Prontosil C_{18}-AQ. On the other hand, a unique selectivity is resulted on Luna Phenyl-Hexyl, which shows, depending on the analytes, some additional interactions, since the separation mechanism on this column is influenced by its different steric and polar properties compared to the separation mechanism of alkyl-bonded phases. The predictions and real experiments were strongly correlated with an average absolute error (Δt_R) of 0.13 min (<8 s).

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1. Introduction

Local anesthetics (LAs) are drugs that have the ability to induce reversible local anesthesia. Most LAs are weak bases, with a pK_a between 7.0 and 9.0. These compounds are structurally related molecules and are made up of 3 structural motifs, a lipophilic group "the aromatic moiety", a hydrophilic group "usually a tertiary, in rare cases, a secondary amine" and the so-called link chain. The type of linkage as well as structural differences in LAs molecule affects potency, duration of action, rate of metabolism and toxicity.[1] Ester LAs are rapidly hydrolyzed in plasma by plasma cholinesterase, which results in a shorter half-life compared to amides. Amide LAs are not broken down by plasma cholinesterase, but are subjected to biotransformation in the liver, and that leads to a longer half-life. In the case of propipocaine, the link chain between the aromatic ring and the terminal amino group contains a ketone group. The chemical structures of the LAs described in this study are presented in Table 1. Combinations of LAs could be used in pharmaceutical preparations to achieve the effect of local anesthesia with an appropriate onset time and duration of action.[2,3] These mixtures contain usually a combination of an ester and amide, which is very useful because these two types have generally different times of peak levels. Moreover, this combination gives the advantage of reducing toxicity as it is not additive when two or more LAs are in one mixture.[4]

Several analytical methods have been published over the last years for the determination of single LA or for the simultaneous determination of different LAs in different matrices. The technique of choice, which is most often recommended in the literature for the determination of LAs is high-performance liquid chromatography (HPLC) coupled with various detection methods including UV,[5–11] mass spectrometry,[12–14] and or the application of an amperometric detection method.[15]

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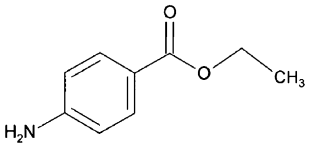
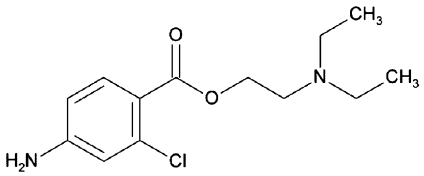
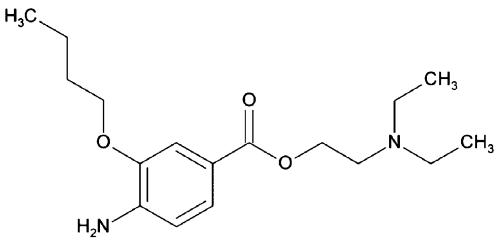
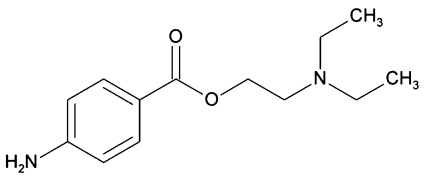
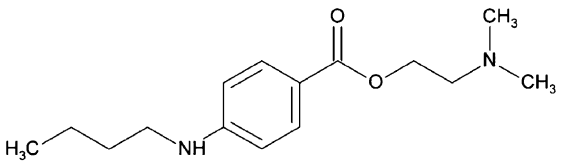
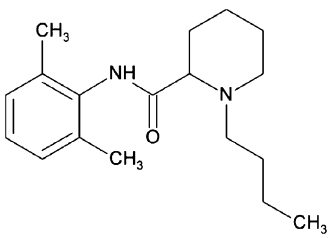
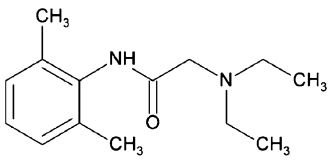
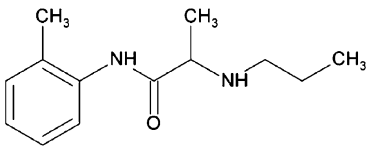
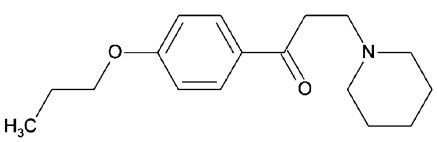
Table 1 Chemical structures of the investigated local anesthetics

Type	Name	Chemical structure
Esters	Benzocaine	
	Chloroprocaine	
	Oxybuprocaine	
	Procaine	
	Tetracaine	
Amides	Bupivacaine	
	Lidocaine	
	Prilocaine	
Ketone	Propipocaine	

Since the late 1970s, a considerable progress in the development of chromatography modeling software has been achieved. These software reduce the time spent in data processing, developing, and optimizing separations methods in chromatography, pivotal issues in any chromatographic method development.[16]

Currently, a number of chromatography modeling software is made commercially available. The prediction accuracy of these programs is established in many studies and application notes. Two groups of chromatography modeling software are available, based on different operational principle. Software of the first group depends on changing of one or more chromatographic variables and predicts the optimal separation based on these variables. To this group of software belongs: DryLab® (Molnár Institute, Berlin, Germany), which was introduced in the late 1985. Another principle based on molecular structure allows predicting the best initial separation conditions. The second group includes software like: ChromSword® (http://www.chromsword.com)[17] and ACD/LC&GC Simulator® (Advanced Chemistry Development ACD/Labs, Toronto, Canada).[18]

DryLab is one of the most established software for chromatography modeling, which allows for modeling of chromatographic separations based on input data from two or more experimental runs.[19] The use of DryLab for HPLC modeling to facilitate methods development was well documented in the last 27 years. In this time a continuous development occurred to the software which enabled it to cope more with the ongoing technological progress. On the other hand, a number of published studies exist that deal with the use of DryLab in different chromatographic modes and wide application ranges. DryLab is applied to solve different analytical problems in pharmaceutical analysis, which deal mostly with the separation of active pharmaceutical ingredients (APIs) in the presence of their impurities and/or their degradation products.[20–28] In the field of phytochemical analysis many applications on complex plant extracts are also available.[29–34] Moreover, DryLab has been successfully applied to optimize the separation of different groups of environmental pollutants,[35–37] peptides and proteins,[38] and metabolites.[39]

The common practice in the development of analytical method in high performance liquid chromatography is the so-called step-by-step optimization, which is based on a trial-and-error approach by varying one-factor-at-a-time (OFAT) and investigating the resolution of one or more critical pair of peaks till best results are achieved. This traditional approach has many disadvantages; for most it is time-consuming and involves a large number of experiments and manual data interpretation. Moreover, this approach leads in many cases to non-robust analytical methods, especially when transferred into another lab, as it don't consider the possible interactions between the critical factors in the separation.[40]

In this study, a more systematic approach using experimental design methodology was carried out in which one or more factors were changed at the same time. This approach was applied for the identification of optimum parameters to develop suitable liquid chromatographic methods for the simultaneous separation of some of the most popular local anesthetics in pharmaceutical preparations and medical praxis (benzocaine, bupivacaine, chloroprocaine lidocaine, oxybuprocaine, prilocaine, procaine, propipocaine and tetracaine).

2. Materials and methods

2.1. Reagents and chemicals

Benzocaine, bupivacaine hydrochloride, lidocaine hydrochloride, prilocaine hydrochloride, procaine hydrochloride and tetracaine hydrochloride were purchased from Sigma-Aldrich (Steinheim, Germany). Chloroprocaine hydrochloride and oxybuprocaine hydrochloride were obtained from VWR International (Leuven, Belgium). Propipocaine hydrochloride was kindly provided by Jenapharm GmbH & Co. KG (Jena, Germany).

Phosphoric acid (85%), potassium hydroxide (85%) and potassium dihydrogen phosphate (99.5%) were obtained from Merck (Darmstadt, Germany).

Acetonitrile and methanol HiPerSolv CHROMANORM® for HPLC gradient grade were purchased from VWR International (Leuven, Belgium). Water was obtained by bi-distillation.

2.2. Columns

Three different columns were used in this study: Kromasil C_{18} (125 × 4.6 mm, 5 μm) pore size 100 Å from VDS optilab (Berlin, Germany); Prontosil C_{18}-AQ (125 × 4.6 mm, 5 μm) pore size 120 Å from Bischoff (Leonberg, Germany) and Luna Phenyl-Hexyl (150 × 4.6 mm, 5 μm) pore size 100 Å from Phenomenex (Aschaffenburg, Germany).

2.3. Equipment

Chromatography was performed on a Shimadzu Prominence HPLC equipped with on-line degassing unit (DGU-20A), two solvent delivery units (LC-20AD) with low pressure gradient unit, autosampler with cooling function (SIL-20AC), column oven (CTO-20A), photo-diode Array detector (SPD-M20A) and system controller (CBM-20A) (Shimadzu Europe, Duisburg, Germany).

The dwell volume of the chromatographic system was determined using an established method[41] and was 360 μl.

2.4. Chromatographic conditions

For all chromatographic runs in this study, an injection volume of 5 μl was used. The detection was performed at 254 nm. Flow rate of 1 ml min^{-1} was applied for all experiments.

Initial runs, which were used to build the 3-D resolution models "cubes", were carried out under the following chromatographic conditions: gradient times (t_G) of 20 min and 60 min (linear gradient system from 10–90% organic modifier B), temperatures (T) of 25 °C and 40 °C and pH value of eluent A (20 mM phosphate buffer) of 3.0.

Ternary eluent compositions B (organic modifier) were changed between (a) 100% ACN, (b) ACN–MeOH (50 : 50) (v/v), and (c) 100% MeOH. For each organic modifier four initial runs were necessary to build the 2-D resolution model.

As a result, the experimental design for the simultaneous optimization of three chromatographic parameters required twelve experimental runs, which were performed to obtain the 3-D resolution models "cube" for each column in our study, as illustrated in Fig. 1.

The chromatographic data of the initial runs were exported then automatically to PeakMatch® to carry out the peak tracking process of the different local anesthetics in the mixture. The last step was to transfer the processed data from PeakMatch® to DryLab®.

2.5. Calculations

2.5.1. The Snyder–Dolan characterization approach. The Snyder–Dolan characterization approachis a model-based evaluation method, which uses the hydrophobic-subtraction model and acetonitrile–water (50 : 50) (v/v) as mobile phase. Solute retention factors k are given by the following model:[42]

$$\log k = \log k_{EB} + \eta' H - \sigma' S^* + \beta' A + \alpha' B + \kappa' C$$

2.5.2. Column comparison function. The F_s value of a column relative to the reference column (Kromasil C_{18} in this study; see Table 2). F_s is the distance separating two columns in the plot (of values of H, S^* and so on) in five-dimensional space. The F_s value is the basis for ranking the similarity or difference between two columns in the Snyder–Dolan approach:[42]

$$F_s = \{[12.5(H_2 - H_1)]^2 + [100(S_2^* - S_1^*)]^2 + [30(A_2 - A_1)]^2 + [143(B_2 - B_1)]^2 + [83(C_2 - C_1)]^2\}^{1/2}$$

2.6. Software

LabSolutions® chromatography software (Shimadzu Europe, Duisburg, Germany) was used to control the chromatographic separation, acquire data and to convert them to Analytical Instrument Association (AIA) format, which could be used in the following steps. The simulated chromatograms were then generated based on the results of the experimental runs utilizing DryLab®2010, which includes PeakMatch® v. 3.60 and DryLab® v. 3.95 (Molnár-Institute, Berlin, Germany).

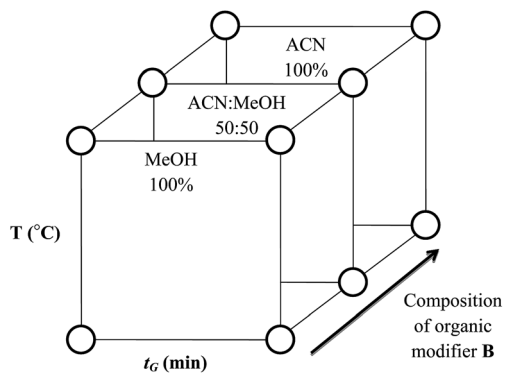


Fig. 1 Design of experiments (DoE) for the simultaneous multifactorial optimization of three critical chromatographic parameters: gradient time (t_G), temperature (T) and composition of organic modifier (B). Each 3-D resolution model, *i.e.* the cube, is based on three different 2-D resolution models measured for gradient time (t_G), temperature (T), and requires twelve initial chromatographic runs. Each circle in this experimental design represents, therefore, one oft he twelve input experiments for the 3-D model.

3. Results and discussion

The chromatographic columns used in this study were formerly characterized in accordance with two of the most widely used and acceptable approaches for the characterization of reversed-phase columns, which are Tanaka and Snyder–Dolan.[43] Based on the results this study only three columns were chosen to the separation of LAs. The first one was the "reference" column Kromasil C_{18}. The second column is Prontosil C_{18}-AQ, which should give comparable chromatographic separation, and the last column is Luna Phenyl-Hexyl, which should have a different selectivity based on the radar plots resulted from Tanaka approach and the F_s values relative to Kromasil C_{18}.

We aimed with the aid of the resulting parameters from these tests for each column (see Table 2) to check the applicability of the radar plots and the F_s value when choosing either an "equivalent" column in our study Prontosil C_{18}-AQ, or a column with "very" different selectivity, *i.e.* different radar plot and a large value of F_s, (in our study Luna Phenyl-Hexyl).

Data of the Tanaka test are usually normalized and presented through radar plots, allowing a simple visualization of the properties and represent multidimensional data simply in two dimensional diagrams. However, that can be used only to compare a limited number of columns on the same diagram. Radar plots allow therefore a rapid and simple assessing of columns to find similarities with or differences from other columns. However, to make it easier for visual display, it is better to use different scales as seen in Fig. 2.

On the other hand, the so called column comparison function (F_s) as a single parameter allows defining the difference between a reference column and other columns. Values of F_s using Kromasil C_{18} as a reference are summarized in Table 2.

It is clear from the radar plot for Tanaka's parameter in Fig. 2 and the F_s values from Snyder–Dolan test, that both Kromasil C_{18} and Prontosil C_{18}-AQ columns are fairly similar and should give therefore comparable chromatographic separation based on their comparable selectivity. On the other hand, the last column (Luna Phenyl-Hexyl) seems to have a different selectivity and should have as a result a different chromatographic behavior based on its F_s values (23.41) relative to Kromasil C_{18} (compared to $F_s = 9.68$ of Prontosil C_{18}-AQ). These predictions are in good agreement with the observed chromatographic behavior of these columns with the mixture of local anesthetics.

Molnár *et al.*[44] presented the first 3-D resolution model, *i.e.* the so-called "cube", using DryLab® chromatography modeling software. The work flow to build a 3-D resolution model for the simultaneous optimization of three chromatographic parameters was fully discussed and the advantages of this 3-D model were introduced. Many scientific papers utilized this method in the meantime to solve different analytical problems especially in the field of pharmaceutical analysis.[40,45,46]

Table 2 List of the investigated stationary phases and their properties including the characterization parameters from Tanaka and Snyder–Dolan chromatographic test approaches

		Tanaka						Snyder–Dolan						
Abbr.	Stationary phase	k_{PB}	$\alpha_{PB/BB}$	$\alpha_{T/O}$	$\alpha_{C/P}$	$\alpha_{B/P}$ (pH 2.7)	$\alpha_{B/P}$ (pH 7.6)	H	S^*	A	B	C (pH 2.8)	C (pH 7.0)	F_s-Krm
Krm	Kromasil C_{18}	7.95	1.49	1.51	0.37	0.07	0.31	1.04	−0.04	0.06	−0.03	0.19	0.37	
AQ	Prontosil C_{18}-AQ	5.63	1.47	1.25	0.55	0.07	0.33	0.98	0.04	0.00	0.01	0.18	0.35	9.68
PhH	Luna Phenyl-Hexyl	2.80	1.34	1.10	1.21	0.08	0.54	0.76	0.07	−0.61	−0.04	0.14	0.57	23.41

3.1. Investigations about the retention behavior of local anesthetics

For a good design of experiments (DoE), it is critical to investigate the chromatographic retention behavior of the analytes in the mixture. As most of local anesthetics have pK_a between 7.0 and 9.0, it is preferable to work at lower pH values of the mobile phase. In our study a pH value of 3.0 was chosen for the simultaneous chromatographic separation of LAs.

Since the retention of LAs depends strongly on their chemical structures, it is expected that the more hydrophobic LAs with long alkyl chains attached to the aromatic ring (oxybuprocaine, propipocaine and tetracaine) will elute at the end of the chromatogram. In contrast, the more polar LAs, *i.e.* procaine, chloroprocaine and lidocaine, tend to have shorter retention times under the applied chromatographic conditions of the mobile phase. Consequently, the simultaneous separation of all these LAs in a reasonable analysis time requires the use of gradient system due to the broad range in the retention behavior of this group of compounds.

3.2. Design of initial experiments

The main goal of the multifactorial optimization was to develop a robust method, with which the best separation of all compounds in the mixture within a reasonable analysis time could be achieved.

Previous to designing the initial experiments, it was of importance to choose the parameters, which will be then optimized.

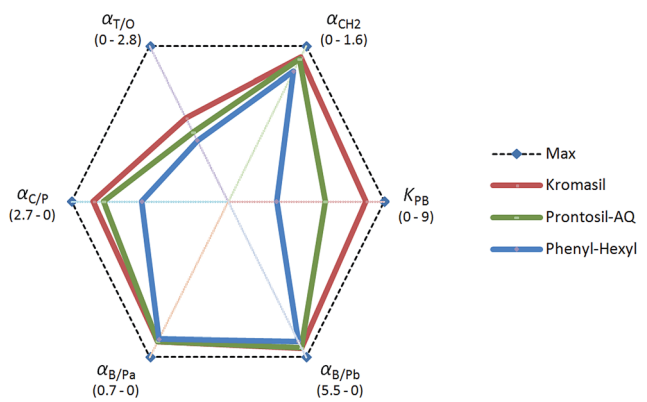


Fig. 2 Radar plots for Tanaka's parameter illustrating the similarities and differences for Prontosil C_{18}-AQ and Luna Phenyl-Hexyl; using Kromasil C_{18} as a reference.

These parameters should play an important role in the selectivity. In order to keep the number of the initial experiments acceptable, three parameters were varied, whereas other parameters such as pH value of the mobile phase, buffer type and concentration *etc.* were kept constant during the initial experiments.

To build the 3-D resolution model for each column three sets of chromatographic runs were necessary, as illustrated in Fig. 1.

Each of these sets contains four experiments (two gradient times and two temperatures), which were done for three ternary eluent compositions. In this way, three different parameters (t_G, T, B) could be optimized at the same time using the so-called "DryLab-cube".

3.3. Building the 3-D resolution models

Once the initial chromatographic runs were designed and the chromatograms were acquired, the raw data were converted to Analytical Instrument Association format (AIA).

The next step then was to carry out a peak tracking to match the peaks in each of the chromatograms by using the PeakMatch®, which is a part of the DryLab® software.

The peaks are identified and matched based on peak areas, which are quite constant when injecting the same mixture and the same injection volume in all runs. Moreover, the results of peak matching were also confirmed based also on the UV-spectra of the LAs acquired using PDA.

Data were then automatically transferred to DryLab® to create first the 2-D resolution maps, which were used to build the 3-D resolution model, *i.e.* the cube. In this way one can simultaneously evaluate the effect of three critical parameters on the chromatographic separation, and optimize them to choose the best working point in the design space, which gives the optimal separation.

DryLab-cubes for Kromasil C_{18}, Prontosil C_{18}-AQ and Luna Phenyl-Hexyl are shown in Fig. 3.

The robust regions ($R_{s,crit} > 1.7$) could be identified by editing the scales of the resolution values, which are shown in the resolution maps. This scale is based on different colors, in which "red" areas present high resolution values and "blue" ones present low resolution values (overlaps) for the critical pair of peaks in the chromatogram. Using these benefits of DryLab® modeling software, optimal and robust working points within a created design space including their chromatographic conditions and predicted retention times and resolution values of the critical pair of peaks ($R_{s,crit}$) could be readily identified.

3.4. Selection of the optimal working points from the 3-D resolution model and verification of their robustness

The selection of the working point from the 3-D resolution model for each stationary phase in this study was based generally on three criteria: (I) the point with the highest value of $R_{s,crit}$ from the most robust region in the cube, *i.e.* the center of an enough large "red body" in the 3-D model; (II) the retention time of the last eluted peak in the chromatogram resulted from the selected working point should be as short as possible to produce an analytical method with reasonable analysis time and (III) the working point with less acetonitrile and more methanol as an organic modifier in the mobile phase will be employed. If possible, the replacement of acetonitrile by methanol in the mobile phase was one of our goals.

The 3-D resolution models resulted on Kromasil C_{18} and Prontosil C_{18}-AQ columns displayed in Fig. 3 show that, a good separation of all peaks in the mixture could be achieved using only methanol as an organic modifier in the mobile phase within an acceptable analysis time. The working points are: $t_G = 63$ min and $T = 25$ °C for Kromasil C_{18} (the predicted $R_{s,crit} = 2.08$), and $t_G = 65$ min and $T = 25$ °C for Prontosil C_{18}-AQ ($R_{s,crit} = 1.85$).

At the same time, the 3-D resolution model for Luna Phenyl-Hexyl shows that a potential separation of all LAs in the mixture is only possible when using a mixture of methanol and acetonitrile in the mobile phase. However, this separation would not be very robust as shown that the area in red in the cube is rather small (Fig. 4). Moreover, according to the criteria for the selection of the working points, it was intended, to use eluent compositions, which reduce the amount of acetonitrile in the mobile phase. To assess the availability of a working point in the design space, which leads to a base-line separation of all peaks using 100% methanol as an eluent B in the mobile phase,

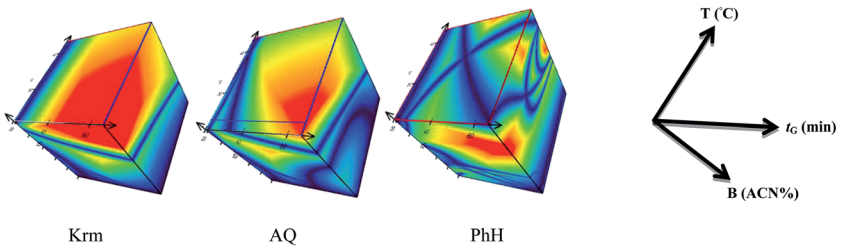


Fig. 3 3-D resolution spaces of gradient time (t_G min), temperature (T °C) and composition of organic modifier (B ACN%) for Kromasil C_{18} (125 × 4.6 mm, 5 μm); Prontosil C_{18}-AQ (125 × 4.6 mm, 5 μm) and Luna Phenyl-Hexyl (150 × 4.6 mm, 5 μm), from left to right. Red areas within the resolution spaces indicate high resolution values for the critical pair of peaks in the chromatogram ($R_{s,crit} \geq 1.7$) and "blue" ones present low resolution values (overlaps) ($R_{s,crit} = 0$).

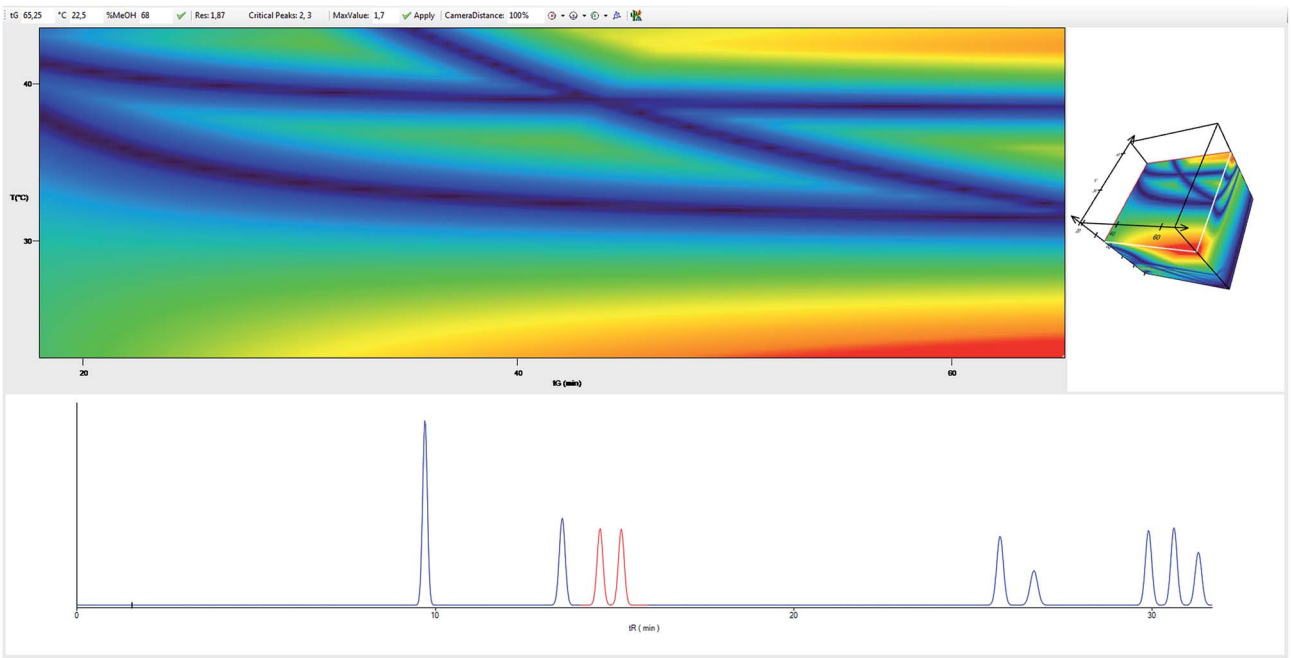


Fig. 4 2-D and 3-D resolution model for Luna Phenyl-Hexyl, which shows that a potential separation of all LAs in the mixture is only possible when using a mixture of methanol and acetonitrile in the mobile phase ACN–MeOH (32 : 68) (v/v). The area in red in the cube is rather small and this separation would not be very robust.

the 2-D resolution model of LAs on Luna Phenyl-Hexyl was created, which gives a more comprehensive overview. Fig. 5 shows the 2-D resolution map for this column and it is clear that a robust working space is only available using a gradient time more than 60 min. A good working point could be at $t_G = 90$ min and $T = 35$ °C ($R_{s,crit} = 2.24$).

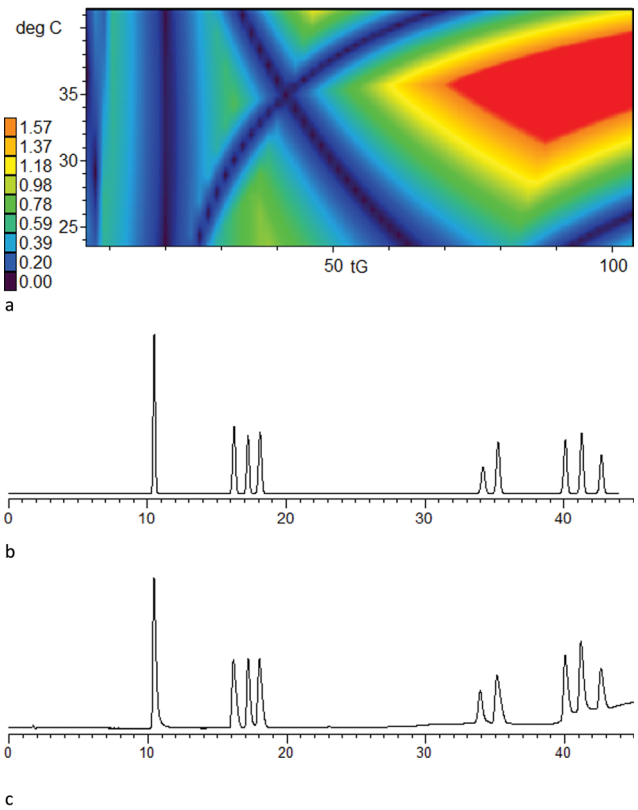


Fig. 5 (a) 2-D resolution model of LAs on Luna Phenyl-Hexyl, with gradient time (t_G) from 0 min to 100 min. This 2-D resolution map contains a very robust working space using a gradient time more than 60 min, with broad warm "red" region. The predicted (b) and experimental (c) chromatograms for the working point (10–90% MeOH, $t_G = 90$ min and $T = 35$ °C) with $R_{s,crit} = 2.24$ are shown.

As mentioned before, the robust regions, which contain suitable working points, could be visually identified in the 3-D resolution model as irregular geometric bodies. Moreover, the so-called robustness map for the selected working point on each stationary phase is generated using DryLab®. Fig. 6 shows that, at this working point small changes of the critical parameters, *i.e.* gradient time t_G (±2 min), temperature T (±1 °C) and even the ternary composition of the eluent B, should not have any negative impact on the resolution values of the critical pair of peaks and on the total chromatographic separation of all peaks in the mixture. From the robustness maps in Fig. 6, one could see that the robustness of the separation is not the same on all stationary phases, and the descending order would be [Kromasil C_{18} (left) > Prontosil C_{18}-AQ (middle) > Luna Phenyl-Hexyl (right)]. However, the chromatographic separation at the workings points is robust enough on all of columns over a wide range of changes in the critical parameters, where the resolution of the critical pair of peaks in the chromatogram $R_{s,crit} \geq 1.7$.

The accuracy of the resulting experimental chromatograms compared to the predicted ones for the selected working points for each column in this study were also evaluated as seen in Table 3. The absolute error ($\Delta t_R = |t_{pre} - t_{ex}|$) as well as the percent absolute error [$\%\Delta t_R = (|t_{pre} - t_{ex}| \times 100)/t_{pre}$] were calculated for each analyte, where: t_{pre} is the predicted retention time and t_{ex} is the experimental retention time.

The average difference between predicted and experimental retention times is 0.13 min (7.8 s) and the largest difference was 0.29 min (17.4 s) as can be seen from Table 1, confirming that an excellent accuracy in predictions of retention times was obtained for all peaks in the mixture of LAs.

Fig. 7 shows that the selectivity and the elution order were to a large extent similar on Kromasil C_{18} and Prontosil C_{18}-AQ. On the other hand, unique results in term of chromatographic selectivity were obtained on Luna Phenyl-Hexyl column. Two differences in the elution order on this column are due to: (a) higher selectivity between procaine and chloroprocaine (b) longer retention of oxybuprocaine and propipocaine and shorter retention of tetracaine. This behavior of Luna Phenyl-Hexyl results from some additional interactions compared to

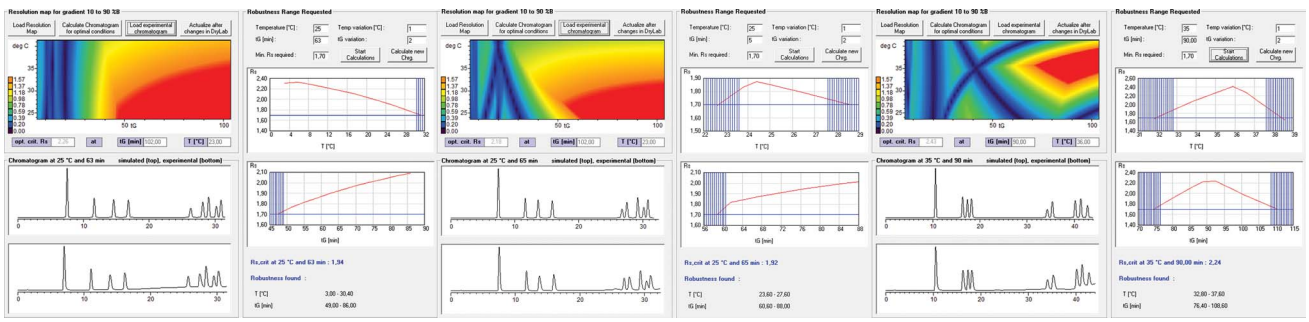


Fig. 6 Robustness maps for the selected working point on Kromasil C_{18} (10–90% MeOH, $t_G = 63$ min and $T = 25$ °C); Prontosil C_{18}-AQ (10–90% MeOH, $t_G = 65$ min and $T = 25$ °C) and Luna Phenyl-Hexyl (10–90% MeOH, $t_G = 90$ min and $T = 35$ °C), from left to right. All robustness maps indicate that small changes of the critical parameters, *i.e.* gradient time t_G (±2 min), temperature T (±1 °C) and even the ternary composition of the eluent B, around the selected working point should not have any negative impact on the resolution values of the critical pair of peaks ($R_{s,crit}$) and on the total chromatographic separation of all peaks in the mixture.

Table 3 Absolute error (Δt_R) and percent absolute error ($\%\Delta t_R$) in the predicted retention times for local anesthetics (LAs) on the tested chromatographic columns

	Krm (t_G 63 min, T 25 °C)				AQ (t_G 65min, T 25 °C)				PhH (t_G 90 min, T 35 °C)			
	t_{pre}	t_{ex}	Δt_R	$\%\Delta t_R$	t_{pre}	t_{ex}	Δt_R	$\%\Delta t_R$	t_{pre}	t_{ex}	Δt_R	$\%\Delta t_R$
Procaine	7.09	7.03	0.06	0.92	7.38	7.43	0.05	0.70	10.49	10.43	0.05	0.70
Chloroprocaine	11.17	11.07	0.10	0.89	11.59	11.67	0.07	0.65	16.24	16.15	0.07	0.65
Lidocaine	14.06	13.94	0.12	0.86	13.59	13.67	0.07	0.55	17.24	17.21	0.07	0.55
Prilocaine	16.32	16.17	0.16	0.95	15.80	15.89	0.09	0.59	18.12	18.02	0.09	0.59
Benzocaine	26.03	25.81	0.22	0.83	26.71	26.79	0.08	0.30	34.18	33.91	0.08	0.30
Bupivacaine	27.72	27.46	0.26	0.93	27.47	27.59	0.12	0.44	35.26	35.12	0.12	0.44
Oxybuprocaine	28.66	28.40	0.26	0.90	29.17	29.30	0.13	0.44	40.10	40.03	0.13	0.44
Propipocaine	29.84	29.56	0.29	0.96	30.11	30.25	0.14	0.48	41.29	41.16	0.14	0.48
Tetracaine	30.53	30.27	0.26	0.84	30.76	30.88	0.12	0.39	42.71	42.60	0.12	0.39
$\bar{X}$			0.19	0.90			0.10	0.50			0.11	0.42

alkyl-bonded phases. Moreover, as methanol was the organic modifier in the mobile phase, it is predicted that π–π interactions between the solute and the aromatic π–π active moiety of the stationary phase play an important role in the separation mechanism of Luna Phenyl-Hexyl.[47]

Kromasil C_{18} was used in the study as a standard C_{18} column for the separation of LAs-mixture, and at the same time as a reference column in the radar plots and for the calculating of F_s values for the other columns.

Our results in term of chromatographic selectivity of the stationary phases are in good agreement with predicted behavior based on the resulted parameters from Tanaka and Snyder–Dolan tests.

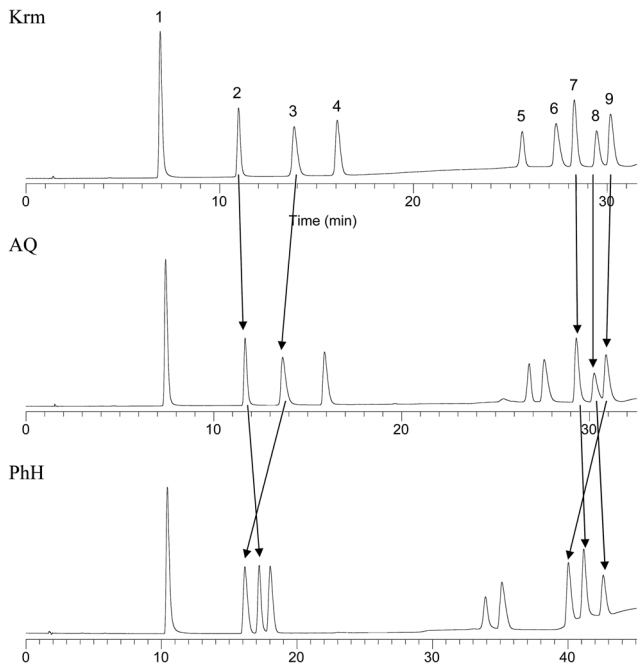


Fig. 7 Experimental chromatograms from the selected working point for each column (experimental conditions see Fig. 6). The selectivities are comparable between Kromasil C_{18} (Krm) and Prontosil C_{18}-AQ (AQ) and all peaks are baseline separated. Different elution order of (procaine and chloroprocaine) and (oxybuprocaine; propipocaine and tetracaine) are shown on Luna Phenyl-Hexyl (PhH). (1) Procaine; (2) chloroprocaine; (3) lidocaine; (4) prilocaine; (5) benzocaine; (6) bupivacaine; (7) oxybuprocaine; (8) propipocaine and (9) tetracaine.

4. Conclusions

A systematic approach for multifactorial HPLC method optimization for the simultaneous separation of local anesthetics is presented. Although not demonstrated in this paper, this method should be suitable for the analysis of LAs in pharmaceutical preparations or to detect them in some illegal cosmetics.

For each column three sets of chromatographic runs containing four experiments each (two gradient times and two temperatures, which were done for three ternary eluent compositions) were carried out. After a stepwise strategy for creating the 3-D resolution models, one working points for each stationary phase, which give a base-line separation of all analytes, was selected with respect to defined criteria. These three working points serve as a practical example for the significant advantages of optimization software in chromatography to solve challenging analytical problems, in this case, the simultaneous separation of a group of structurally related substances, which is commonly used as a mixture in different pharmaceutical preparations.

The advantages of using Drylab® for rapid method development utilizing multidimensional screening allowed determining the optimum chromatographic conditions without conducting a large number of trial-and-error laboratory experimentation, which make this strategy ecologically favorable, since it saves the consume of organic solvents, and is time-saving in comparison to the traditional manual approach.

Overall, a design space is defined and visualized for the investigated local anesthetics, which enables a better understanding of the factors influencing chromatographic separation. The excellent robustness of the developed methods facilitates an effective method transfer to other laboratories. The predicted retention times are, as shown, in excellent correlation with experimental values for each column studied.

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