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Two-column sequential injection chromatography for fast isocratic separation of two analytes of greatly differing chemical properties

Dalibor Šatínský*, Petr Chocholouš, Olga Válová, Lucia Hanusová, Petr Solich

Department of Analytical Chemistry, Faculty of Pharmacy, Charles University, Heyrovského 1203, Hradec Králové 500 05, Czech Republic

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ABSTRACT

This paper deals with a novel approach to separate two analytes with different chemical properties and different lipophilicity. The newly described methodology is based on the two column system that was used for isocratic separation of two analytes with very different lipophilicity—dexamethasone and cinchocaine. Simultaneous separation of model compounds cinchocaine and dexamethasone was carried under the following conditions in two-column sequential injection chromatography system (2-C SIC). A 25 × 4.6 mm C-18 monolithic column was used in the first dimension for retention and separation of dexamethasone with mobile phase acetonitrile:water 30:70 (v/v), flow rate 0.9 mL min^{-1} and consumption of 1.7 mL. A 10 × 4.6 mm C-18 monolithic column with 5 × 4.6 mm C-18 precolumn was used in the second dimension for retention and separation of cinchocaine using mobile phase acetonitrile:water 60:40 (v/v), flow rate 0.9 mL min^{-1} and consumption 1.5 mL. Whole analysis time including both mobile phase's aspirations and both column separations was performed in less than 4 min. The method was fully validated and used for determination of cinchocaine and dexamethasone in pharmaceutical otic drops. The developed 2-C SIC method was compared with HPLC method under the isocratic conditions of separation on monolithic column (25 × 4.6 mm C-18).

Spectrophotometric detection of both compounds was performed at wavelength 240 nm. System repeatability and method precision were found in the range (0.39–3.12%) for both compounds. Linearity of determination was evaluated in the range 50–500 μg mL^{-1} and coefficients of determination were found to be r^2=0.99912 for dexamethasone and r^2=0.99969 for cinchocaine.

1. Introduction

Sequential injection chromatography (SIC) was introduced in 2003 by Šatínský et al. [1] as a following generation in the development of sequential injection analysis technique (SIA). The principles upon which SIC is based are the same to those of SIA, namely controlled sample and reagent injection, and reproducible sample handling. The SIC technique was developed as an original research based on integration low-resistance monolithic separation columns to SIA manifold. SIC has facilitated low-pressure chromatographic separation of multi-component mixtures while offering the advantage of flexible flow programming and possibility of on-line sample manipulation [2]. Characteristic advantages of SIC remains its versatility, full computer compatibility, high sample throughput, and low sample, reagent and mobile phase consumption. In recent years, it became apparent that the scope of SIC was extended to encompass a variety of practical applications for separation of more complex samples in pharmaceutical and environmental matrices [3–7]. Next possibility how to spread out the analytical potential of SIC technique was the similar modification of multi-syringe flow injection analysis (MSFIA) to multi-syringe chromatography (MSC) introduced by Cerda-Martin et al. [8]. The contribution of multi-commuted flow analysis combined with monolithic column to the family of low-pressure chromatographic techniques was mentioned in a comprehensive review where advantages and drawbacks of HPLC, SIC and MSC were discussed in detail [9].

Complex samples often contain components widely differing in retention times on one column, hence programmed gradient elution of mobile phase significantly improves the peak resolution and peak capacity over the isocratic elution mode [10,11]. Gradient elution is widely used in liquid chromatography for reasonable time-consuming analysis. Its main purpose is to keep enough resolution of analytes weaker retained on column and to accelerate elution of analytes stronger retained on column [12]. Chromatographic procedure similar to gradient elution or stepwise gradient elution in a low-pressure manifold was proposed firstly by Cerda [13], and Masini and co-workers [14]. In this article [13] a dual isocratic elution protocol was described using a MSC system for separation of a model mixture of vitamin B. Advantages

* Corresponding author. Tel.: +420 495067228; fax: +420 495067164.
E-mail address: satinsky@faf.cuni.cz (D. Šatínský).

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of easy manipulation of mobile phases and generation of reproducible concentration gradients that were obtained through multiple isocratic elution steps were well documented [13,14]. The procedure of gradient elution with respect to efficient separation easily attained by programming changes of the ratio of the individual mobile phases and flow rate of their aspiration into the mixing coil was devised by Sklenářová et al. for separation of indomethacin and its two degradation products [15]. One of weak sides of gradient elution methods is necessity of column equilibration after analysis back to the initial conditions before next analysis. This step prolongs the time, decreases sample throughput and increases solvent consumption of whole chromatographic run.

Usually, the theory of multidimensional chromatography is based on combinations of two or more different separations systems, which can significantly increase the number of separated compounds in complex samples. In comprehensive two-dimensional liquid chromatography (2D-HPLC), all sample compounds are subject to separation in both dimensions (columns) [11,16]. The fractions from the first dimension are transferred on-line in small aliquots to the second dimension, using either two 6-port or a single 8-port switching valve equipped with two sample loops or small enrichment columns working in alternating cycles [11,17]. Dissimilarity of the separation mechanism, different stationary and mobile phases, in the first and in the second dimension is the critical parameter for the orthogonality of the two-dimensional comprehensive separation systems in 2D-HPLC technique [11,18].

Our study was based on different compositions of two mobile phases (weak and strong (low percentage and high percentage of acetonitrile in water)) and on two columns with different lengths used in parallel in one SIC system. Both columns (the same chemistry of stationary phase) were used separately for parallel separation and determination of two analytes. The first analyte (dexamethasone) was separated on the first column and the second analyte (cinchocaine) on the second column. No sample fraction transfer from the first column in the mobile phase with lower elution strength onto the second column was carried out. For these reasons, the term orthogonality of the 2-C SIC system was not fulfilled and no comparing with comprehensive two-dimensional LC theory in 2D-HPLC systems is possible. However, the aim of this study was not to develop comprehensive two dimensional SIC system, but to show further direction how to increase the number of resolved compounds in complex samples using simple SIC manifold with two columns.

Our new approach was using 2-C SIC for fast isocratic separation as an alternative to gradient elution chromatography. In our earlier work, we described and compared SIC setup for gradient elution chromatography with two-column SIC setup for isocratic elution of paracetamol, caffeine and propyphenazone. Pros and cons of the 2-C SIC approach were experimentally verified and widely discussed [12].

The aim of the presented work was to examine further possibilities of the SIC system for fast and isocratic separation of two analytes with different lipophilicity. Dexamethasone and cinchocaine, structures as shown in Fig. 1A and B, were chosen as model analytes showing different chromatographic properties. Dexamethasone as a synthetic corticosteroid with glucocorticoid activity represents relatively polar substance with coefficient of lipophilicity (log $P=1.93$), while cinchocaine from the group of long-term acting topical anesthetics belongs to non-polar compounds with high lipophilicity showing log P value 4.75. Combination of these two substances is used in clinical practice in the treatment of otitis, different kinds of local pain, local skin inflammation and local skin allergy.

No analytical procedure has been reported for the simultaneous determination and separation of dexamethasone and cinchocaine yet. The first method for separation of both compounds based on use of two mobile phases and two monolithic columns in one SIC system was proposed and advantages vs. disadvantages of such technique are discussed in the presented paper.

2. Experimental

2.1. Reagents

All chemicals used were of analytical grade quality. Dexamethasone, cinchocaine and organic solvents (methanol, acetonitrile) were obtained from Sigma-Aldrich, Germany. Chemicals for sample preparation (triethylamine, 1,3-butanediol and glycerol) were obtained from Sigma-Aldrich, Germany. Millipore Milli-Q RG (Millipore s.r.o., Prague, Czech Republic) ultra-pure water was used for preparing the solutions and mobile phases. Mobile phases and sample solutions were degassed by sonication before use.

2.2. Apparatus

A FIAlab® 3500 system (FIAlab® Instruments, USA), commercially produced instrument consisting of a glass syringe pump (Cavro-Tecan, syringe reservoir 5 mL) and 8-port selection Cheminert valve (Valco Instrument Co., USA) was used in our study. FIAlab® 3500 was equipped with fiber-optic UV–vis CCD array detector USB2000 (Ocean Optics Inc., USA) with deuterium UV light source DH-2000 (Ocean Optics Inc., USA). The optic fibers and 10 mm Z-flow cell were from Avantes Inc. (Colorado, USA). The whole SIA system was controlled by the FIALab® software. Flow connecting lines were made of 0.50 mm i.d. PTFE tubing. Holding coil was reduced to volume 50 μL. Mobile phases and samples were aspirated through the selection valve and then delivered to the monolithic columns and to the detector. Compounds separations were performed on Chromolith® Flash RP-18e, 25 × 4.6 mm column and on 10 × 4.6 mm C-18 monolithic column with 5 × 4.6 mm C-18 monolithic precolumn (Merck, Germany). Both monolithic columns were placed between the 8-port selection valve and flow cell of the detector. Both lines were put together with T-piece before the flow cell inlet. Mobile phases were aspirated through the filter ending (10 μm). A schematic of the 2-C SIC system is depicted in Fig. 2.

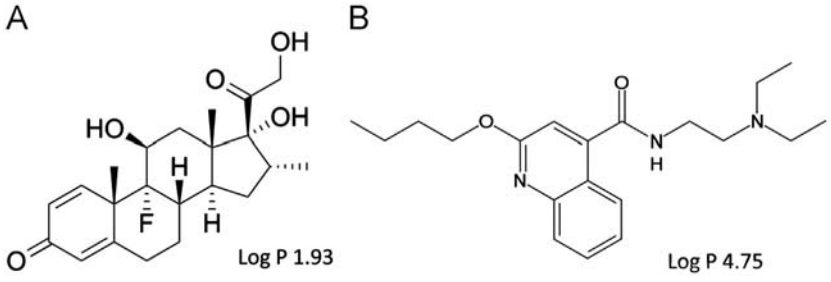

Fig. 1. Chemical structures of dexamethasone (A) and cinchocaine (B) with coefficients of lipophilicity.

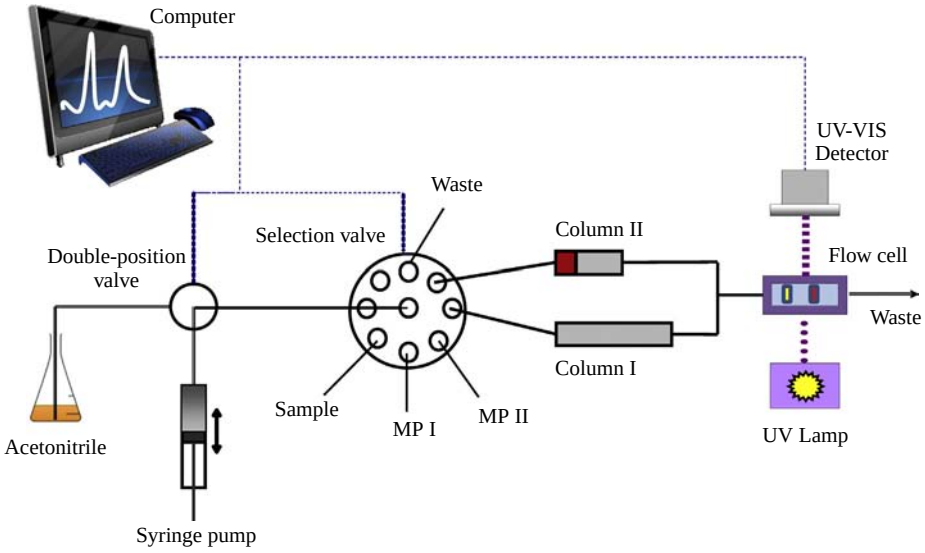

Fig. 2. Schematic view of the two-column sequential injection chromatography system for separation of dexamethasone and cinchocaine.

2.3. Mobile phase preparation

Mobile phases were prepared by mixing individual components (acetonitrile and ultra-pure water) and filtering the mixtures with a Millipore filtration device. Mobile phase I used on the first column for dexamethasone elution consisted of acetonitrile/water 30:70 (v/v) mixture. Mobile phase II used on the second column for cinchocaine elution consisted of acetonitrile/water 60:40 (v/v) mixture.

2.4. Standard solutions and sample preparation

Dexamethasone and cinchocaine hydrochloride standard stock solutions were prepared by dissolving 50.0 mg of dexamethasone and cinchocaine into 50 mL of acetonitrile/water solution 35:65 (v/v) with addition of 0.1% triethylamine separately. Standard stock solutions of both substances were stored at 4 °C in dark. Working solution (mixture of both compounds) was prepared by dilution 2 mL of each stock solution to 10 mL volumetric flask with mobile phase I to obtain working mixed standard solution in concentration 200 $\mu g\ mL^{-1}$ of each substance for further method development and validation. The calibration standard solutions were prepared over the concentration range of 50–500 $\mu g\ mL^{-1}$, using seven calibration points.

The analyzed pharmaceutical preparation was Otobacid® otic-drops (containing 0.2 mg of dexamethasone and 5 mg of cinchocaine hydrochloride in 1 g of 1,3-butanediol/water/glycerol 48:2:50 (w/w/w) solution). Sample preparation for determination of the active substances in otic-drops was done by the following procedure. Amount of 200 mg of otic-drops for cinchocaine analysis was transferred to the 5 mL calibrated flask; sample was subsequently filled up to the mark with acetonitrile/water solution 35:65 (v/v) with addition of 0.1% triethylamine. Amount of 2500 mg of otic-drops for dexamethasone analysis was transferred to the 5 mL calibrated flask; sample was subsequently filled up to the mark with acetonitrile/water solution 35:65 (v/v) with addition of 0.1% triethylamine. Both sample solutions were homogenized and dissolved by 5 min. sonication. A volume 20 μL of prepared sample for dexamethasone analysis was analyzed by 2-C SIC system with mobile phase I on the longer column (the first dimension), while a volume 10 μL of prepared sample for cinchocaine analysis was analyzed by 2-C SIC system with mobile phase II on the shorter column (the second dimension). Standards and samples were measured in triplicate and the mean peak height values were used for data evaluation.

2.5. Assay protocol

The following procedure was carried out in the proposed 2-C SIC system. Samples were aspirated through the 8-port selection valve and then delivered to the monolithic columns and to the flow cell of detector. Retention and elution of dexamethasone was performed on the first column (first dimension) with mobile phase acetonitrile:water 30:70 (v/v), with flow rate 0.9 mL min^{-1}. Total volume of mobile phase 1.7 mL was used for elution of dexamethasone. Retention and separation of cinchocaine from the peak of dexamethasone was achieved on the second column (the second dimension) with mobile phase acetonitrile:water 60:40 (v/v), with flow rate 0.9 mL min^{-1}. Total volume of mobile phase 1.5 mL was used for retention and separation of cinchocaine. Whole analysis time including both mobile phases aspiration and both column separations was less than 4 min. The resulting signals were recorded in the form of peaks; the peak heights were calculated automatically by FIAlab® software and the data were stored by the PC for subsequent processing. Each measuring cycle was carried out in triplicate and the mean peak height values were used for data evaluation. All measurements were performed at ambient temperature.

3. Results and discussion

3.1. Optimization of the 2-C SIC—investigation of mobile phases

A procedure based on sequential aspiration of two different mobile phases and their propelling through the different columns was proposed. This approach in SIC was called two-column sequential injection chromatography. The priority of the presented study was to optimize the composition of each mobile phase for each column and analyte separately to obtain the peak of the analyte that is differentiated from peak of system void volume and from the second analyzed compound. The weak and strong mobile phase used in the particular steps of separation influenced retention of the both analytes on both columns during the whole procedure and the optimization of their composition was a complex process. Optimization of the single steps discussed in the following text was based on finding the optimum composition of the mobile phases for different column lengths. The analytical cycle involved two main steps: (1) loading the sample onto the first column and following

separation, elution, and detection of the less lipophilic analyte; and (2) loading the same sample onto the second column and following separation, elution, and detection of the more lipophilic analyte. No column equilibration was required before next analysis because still the same mobile phase was used for each column. The mobile phase carry-over effect was eliminated by using small-volume holding coil (50 μL). The composition of mobile phases was optimized for each column separately.

The experiments concerning the mobile phases composition were started by searching the optimal mobile phase I for sufficient retention of dexamethasone (log P=1.93) on the first column. The next important requirement for the mobile phase I used in the first dimension was very strong retention of cinchocaine (log P=4.75). This parameter was crucial, because very weak elution and strong diffusion of cinchocaine zone was required to avoid the interferences from this retained analyte in next steps analysis of dexamethasone. This parameter was fulfilled with using mobile phase I acetonitrile: water 30:70 (v/v) and flow rate 0.9 mL min^{-1} in the first dimension. Peak of dexamethasone was sufficiently retained and separated from the peak of the void volume of system, while peak of cinchocaine was strongly retained and eluted in extremely long time (50 min) as a strong diffused zone on the chromatogram. Detailed description of cinchocaine zone diffusion and sufficient retention of dexamethasone in the first dimension is depicted in Fig. 3. The tested mobile phase I composition was in the range from 30% to 45% of acetonitrile in water. Concentration of acetonitrile higher than 35% showed insufficient retention of dexamethasone (peak was eluted with void volume peak of the system) and early elution of cinchocaine (retained peaks showed potential interferences to next analysis). Therefore, optimal mobile phase I was found to be acetonitrile:water 30:70 (v/v) and flow rate 0.9 mL min^{-1}.

The next step was optimization of mobile phase II in the second dimension. Fast elution of dexamethasone (with void volume of the system) was required while cinchocaine should be sufficiently retained on the shorter column. This part was focused on finding an appropriate concentration of acetonitrile for fast elution of strongly retained cinchocaine from second column to achieve reasonable short time of separation in the second dimension. The tested ranges of acetonitrile or methanol concentrations were from 55% to 70% (v/v) in water. Methanol based mobile phases were tested with less satisfactory results than acetonitrile based ones that showed faster elution of cinchocaine and better peak symmetry. The optimal mobile phase II for separation of cinchocaine from low retained matrix (1,3-butanediol with glycerol) and dexamethasone together in one peak on the second short column was found acetonitrile:water 60:40 (v/v) and flow rate 0.9 mL min^{-1}. From the UV spectra of both analyzed compounds, the optimal detection wavelength was 240 nm that provides sufficient sensitivity.

3.2. Optimization of the 2-C SIC—choice of columns

The experiments concerning the design of 2-C SIC system included the elution of two compounds from two columns. Searching the optimal stationary phase—different lengths of monolithic columns had to be combined directly with mobile phases optimization. A brief study of different column lengths was carried out. A 5 × 4.6 mm C-18 monolithic precolumn; 10 × 4.6 mm C-18 monolithic column without precolumn; 10 × 4.6 mm C-18 monolithic column coupled with 5 × 4.6 mm C-18 precolumn were tested separately in both dimensions, and Chromolith® Flash RP-18e, 25 × 4.6 mm C-18 column was tested for separation in the first dimension only. Both columns of total lengths 5 mm and 10 mm and its combination showed insufficient retention, and partial co-elution of dexamethasone with peak of the void volume was observed during the mobile phase optimization. Therefore longer Chromolith® Flash RP-18e, 25 × 4.6 mm C-18 column was found optimal for sufficient retention of dexamethasone in the first dimension. Moreover, cinchocaine was strongly retained under these conditions and thus the interference from cinchocaine accumulation was not observed due to its very slow elution (well documented in Fig. 3.). It was eluted as late eluting broadened peak observed as very small drift of the baseline. Experimentally it was verified by multiple sample injection that the retention of

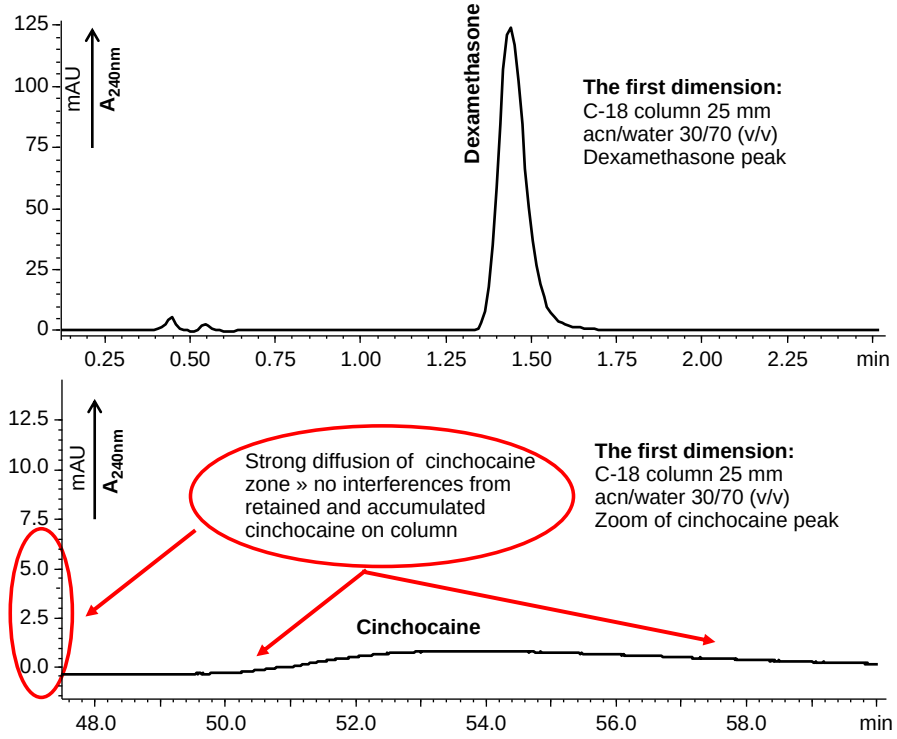

Fig. 3. Detailed view of sufficient dexamethasone retention and cinchocaine zone diffusion in the first dimension.

cinchocaine does not decrease the efficiency of column by occupation of active places of stationary phase.

Subsequently, the searching of column length for strongly retained cinchocaine in the second dimension was based on the same chromatographic principles. Short column lengths 5, 10, and 5 +10 mm of total length were tested together with different mobile phases containing higher ratio of acetonitrile. The shortest precolumn 5 mm showed too low number of theoretical places for required chromatography separation. Better results were observed with 10 mm precolumn, however sufficient resolution between cinchocaine peak and void volume peak with dexamethasone was not reached. Compromise between sufficient retention of cinchocaine and fast elution of dexamethasone with void volume interfering peak was achieved with coupled 5 mm and 10 mm precolumns to one column length. Suitable peak symmetry, fast elution of cinchocaine free of interferences from matrix compounds and dexamethasone is depicted in Fig. 4. Typical sequence of particular steps of the proposed 2-C SIC procedure is indicated in Table 1.

3.3. Validation

The method was validated with respect to the linearity, repeatability, precision, accuracy, and selectivity in order to evaluate the reliability of the results provided by the method. Within the method validation, the parameters of the chromatography system suitability test (SST) were measured and evaluated. The samples of standard solution were six times injected into the 2-C SIC system. Mean values and relative standard deviations of retention time, number of theoretical plates, peak asymmetry, resolution, and repeatability of analytical run were calculated according to the European Pharmacopoeia recommendations [19]. Obtained validation results and chromatography system suitability parameters are summarized in Tables 2 and 3. Method validation results showed the good method linearity, repeatability and system precision. Small and deliberated changes in mobile phase compositions ($\pm$ 5% deviation of volume content of acetonitrile in each mobile phase) are possible without loss of the separation efficiency.

3.3.1. Linearity and selectivity

Under the optimal 2-C SIC chromatographic conditions, linear relationships of standard solutions were verified for both compounds. Seven test solutions ranging 50–500 μg mL^{-1} of dexamethasone and seven test solutions ranging 50–400 μg mL^{-1} of cinchocaine were prepared by dilution of stock solutions and analyzed for linearity test. The detector response at 240 nm for both compounds was found linear in whole range of calibration curve. The linear regression curve was obtained by plotting peak height of standards at each level against the concentration of each injection in mentioned range—500, 400, 300, 200, 100, 75, and 50 μg mL^{-1} (for dexamethasone) and 400, 300, 200, 150, 100, 75, and 50 μg mL^{-1} for cinchocaine. Suitable linearity (coefficients of determination (r^2=0.99912 and 0.99969)) were achieved in the investigated range for dexamethasone and cinchocaine, respectively. Linear regression parameters were described by the following equations: $A=(0.00216\pm0.00004)c-(0.03995\pm0.01153)$, where A is the absorbance and c is the analyte concentration for dexamethasone, and $A=(0.00200\pm0.00002)c-(0.02106\pm0.00489)$ for cinchocaine. All samples were measured in triplicates.

The selectivity of the validated method was evaluated for placebo of pharmaceutical preparation—otic-drops by checking the chromatogram. No interferences were observed in retention

Table 1
The sequence of particular steps of the 2-C SIC control program for dexamethasone and cinchocaine separation on both monolithic columns (15 and 25 mm)—a single cycle.

Action	Unit	Parameter
Dexamethasone analysis		
Mobile phase aspiration	Selection valve	Valve port 2
	Pump	Flow rate 6.0 mL min^{-1}
	Pump	Volume 1.70 mL
Sample aspiration	Selection valve	Valve port 4
	Pump	Flow rate 1.8 mL min^{-1}
	Pump	Volume 0.02 mL
Mobile phase elution	Selection valve	Valve port 7
	Pump	Flow rate 0.9 mL min^{-1}
	Pump	Volume 1.72 mL
Dexamethasone detection	Detector	240 nm
Cinchocaine analysis		
Mobile phase aspiration	Selection valve	Valve port 3
	Pump	Flow rate 6.0 mL min^{-1}
	Pump	Volume 1.50 mL
Sample aspiration	Selection valve	Valve port 5
	Pump	Flow rate 1.8 mL min^{-1}
	Pump	Volume 0.01 mL
Mobile phase elution	Selection valve	Valve port 8
	Pump	Flow rate 0.9 mL min^{-1}
	Pump	Volume 1.51 mL
Cinchocaine detection	Detector	240 nm

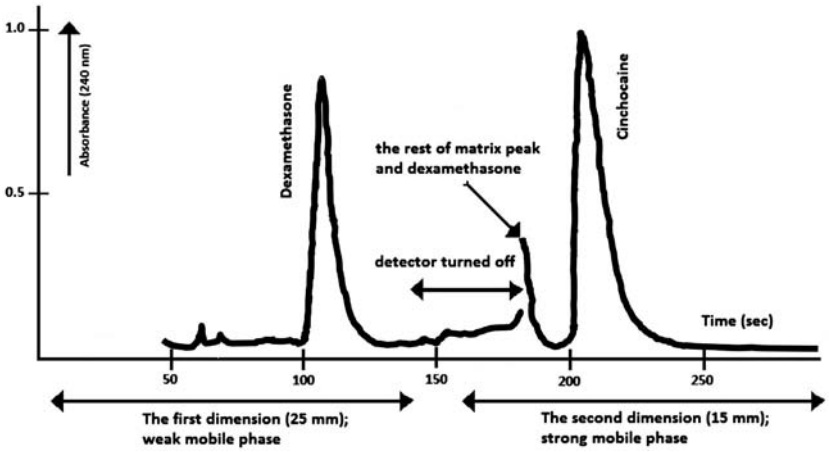

Fig. 4. 2-C SIC chromatogram of dexamethasone and cinchocaine separation on both columns. Conditions—the first column: Chromolith® Flash RP-18e, 25 × 4.6 mm C-18 column, mobile phase I acetonitrile:water 30:70 (v/v), flow rate 0.9 mL min^{-1}; the second column: 10 × 4.6 mm C-18 monolithic column with 5 × 4.6 mm C-18 precolumn, mobile phase II acetonitrile:water 60:40 (v/v), flow rate 0.9 mL min^{-1}.

Table 2
2-C SIC system suitability parameters.

	Retention time (s)	Retention time repeatability RSD (%)[a]	Repeatability of peak heights RSD (%)[a]	Number of theoretical plates	Peak symmetry
Dexamethasone	106.9	0.92	1.40	593	1.75
Cinchocaine	65.9[b]	0.49	0.67	370[b]	2.83

[a] Made in six replicates at one concentration level 200 μg mL^{-1}.
[b] The values for second dimension were calculated separately as a real retention time of cinchocaine on the second column.

Table 3
Analytical characteristics of the validated 2-C SIC method.

Parameters	Dexamethasone	Cinchocaine
Standard calibration—range (μg/mL)[a]	50–500	50–400
Correlation coefficient	0.99912	0.99969
Repeatability RSD (%)[b]	1.40–2.02	0.39–1.72
Method precision—intra-day RSD (%)[c]	3.12	1.72
Accuracy—spike recovery (%) ± SD[d]	99.02 ± 2.63	99.48 ± 1.95

[a] Each concentration of calibration standard was measured in triplicate.
[b] Relative standard deviations (RSD) were calculated from six injections of standard solutions at three concentration levels 50, 200 and 500 μg/mL.
[c] Relative standard deviation (RSD) for repeated injections of multiple preparations of otic drops (n=6), three injections of each sample preparation.
[d] Accuracy was determined as a method recovery using fortified blank pharmaceutical preparation at one concentration level (added amount 100% of active compound content) in six samples from one batch (± minimal and maximal standard deviation of recovery determination).

times of dexamethasone and cinchocaine in the first and in the second chromatography dimension. Interferences of matrix components were observed in the front of the chromatogram together with peak of void volume and did not disturb the quantification of active compounds.

3.3.2. Repeatability, precision and accuracy

Repeatability of the proposed method was characterized by relative standard deviation (RSD, (%)), which was calculated for six consecutive measurements at three concentration levels 50, 200 and 500 μg mL^{-1} of standard solutions during the system suitability test. The results in form of RSD were determined for dexamethasone 2.02%, 1.40% and 1.60%; and for cinchocaine 1.72%, 0.67% and 0.39%. To validate the precision of the method a number of six determinations with the same pharmaceutical preparation—Otobacid® otic-drops were evaluated, which were prepared from the same batches and performed on the same day. This approach provides a means of covering the precision of the entire method, from sample preparation, analyte separation, to data evaluation. The intraday precision values of both compounds were calculated as RSD values which were 3.12% for dexamethasone and 1.72% for cinchocaine.

The accuracy of the method was evaluated by analyzing placebo sample solutions fortified with known quantities of active compounds. Spiked placebo solutions (100% addition of active compounds content) and standard solutions were compared for recovery evaluation. Average values of the recoveries and standard deviations (SD) were found as follows: for dexamethasone 99.02% ± 2.63, and for cinchocaine 99.48% ± 1.95. Results of repeatability, recovery, and precision showed satisfactory and reproducible sample preparation as well as chromatography procedure on two columns for determination of both compounds in pharmaceutical preparation.

3.3.3. Determination in pharmaceutical preparation

The newly developed 2-C SIC method has been applied to the determination of dexamethasone and cinchocaine in registered preparation Otobacid® otic-drops. The samples were commercially available on the local market. Otic-drops samples were diluted to mixture acetonitrile–water–triethylamine and dissolved by 5 min. sonication. The problem of very different concentrations of dexamethasone and cinchocaine (concentration level ratio 1:25) in tested pharmaceutical preparation was overcome by using different sample volume injection (20 and 10 μL) and different weight amount of dissolved pharmaceutical preparation for active substance analysis in each chromatography dimension (2500 mg for dexamethasone analysis vs. 200 mg for cinchocaine analysis). This approach was possible to use because of each sample had to be injected to each column separately. The procedure of sample preparation was simple, fast and achieving high precision and low sample and reagent consumption. The average found amounts of dexamethasone and cinchocaine were 98.12% and 101.32% of declared content in Otobacid® otic-drops. The results were in a good agreement with the pharmacopoeia requirements on the active compound content 95–105% [20].

4. Conclusion

A new method using two-column sequential injection chromatography system for fast isocratic separation of two analytes of greatly differing lipophilic properties was designed. The main idea behind this new design was to propose the SIC system by coupling two independent columns, one for retention and separation of less lipophilic analyte and the second for retention and separation of more lipophilic analyte. Aspirating different mobile phases for each column separation and analysis without gradient elution meant avoiding the problems associated with time-consuming stationary phase equilibration back to initial conditions before next sample analysis. Increased consistency in reproducing retention times was achieved, because solvent strength on both columns was constant. The idea of 2-C SIC was based on simple isocratic elution—each of both dimensions was still washed with the same mobile phase and that was the reason of higher stability and robustness of the system. Each mobile phase and also the stationary phase can be tuned or designed separately according to retention characteristic of eluted analyte. High variability to use different column lengths and polarity of stationary phases (in case of complicated mixtures), different pH, buffers and organic-water phase composition for optimal elution, different flow rates of mobile phases and different sample volume injection separately in each dimension are further advantages of the proposed SIC system. Compared to HPLC separation of both compounds under the same conditions as were used in the first dimension of 2-C SIC system, as depicted in Fig. 5, led to huge reduction of analysis time and solvent consumption.

In summary, 2-C SIC with different retention activity can provide a useful alternative tool for the separation and determination of analytes or complex samples with different retention properties. This approach enables easy optimization, fast separation under lower consumption of organic solvents and lower waste production.

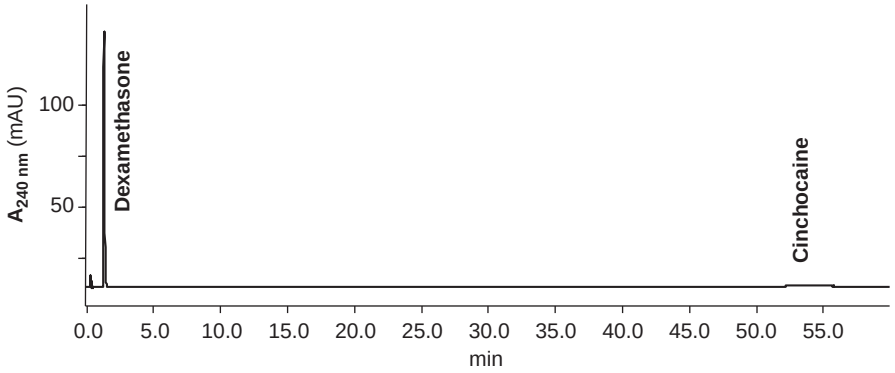

Fig. 5. HPLC separation of dexamethasone and cinchocaine under the same conditions as on the first column of SIC system: Chromolith® Flash RP-18e, 25 × 4.6 mm C-18 column, mobile phase I acetonitrile:water 30:70 (v/v), flow rate 0.9 mL min^{-1}.

Acknowledgments

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