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Simple automated generation of gradient elution conditions in sequential injection chromatography using monolithic column

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ABSTRACT

The paper deals with the concept of simple automated creation of gradient profile of the mobile phase for gradient-elution sequential injection chromatography (GE-SIC). The feasibility and merits of this concept are demonstrated on the separation and simultaneous assay of indomethacin as active principle and of its two degradation products (5-methoxy-2-methylindoleacetic acid and 4-chloro-benzoic acid) in a topical pharmaceutical formulation.

The GE-SIC separation was performed with a FIAlab® 3000 SIC set-up (USA) equipped with an Onyx™ Monolithic C18 (25 mm × 4.6 mm, Phenomenex®) column, a six-port selection valve, a 5-mL syringe pump and a fiber-optics UV CCD detector. Ketoprofen was used as an internal standard (IS). The gradient elution was achieved by automated reproducible mixing of acetonitrile and aqueous 0.2% phosphoric acid in the holding coil of the SIC system. Different profiles of the gradient elution were tested. The optimal gradient using two mobile phases 30:70 and 50:50 of acetonitrile/0.2% phosphoric acid (v/v) was achieved under the optimum flow rate 1.2 mL min^{-1}. The chromatographic resolution *R* between the peaks of all solutes (including the IS) was >2.00. The repeatability of retention times was characterized by the RSD values 0.18–0.30% ($n = 6$). Net separation time was 3.5 min and the mobile phase consumption was 4.5 mL for a single GE-SIC assay. The figures of merit of the novel GE-SIC method compared well with those of conventional HPLC.

1. Introduction

Sequential injection chromatography (SIC) is an original technique [1] established by integrating a short monolithic separation column into the sequential injection analysis (SIA) flow system. The 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. SIC has already proved to be an acceptable alternative to high performance liquid chromatography (HPLC) as for fast analysis of relatively simple mixtures. Benefits of SIC consist in automation including possible on-line sample pretreatment, miniaturization and low consumption of samples and mobile phases.

In the recent seven years a number of papers dealing with practical applications of SIC and multi-syringe chromatography (MSC) systems involving integration of monolithic columns and isocratic elution in the analysis of pharmaceuticals have been published [2–10].

The contribution of multi-commuted flow analysis combined with monolithic columns 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 [11].

Chromatographic procedure similar to gradient elution in a low-pressure environment was proposed by Cerda and coworkers [12]; here a dual isocratic elution protocol was used in a multi-syringe flow system for the separation of a model mixture of vitamins B. In this article common features of gradient techniques in HPLC separations were discussed including resolution enhancement, shortening the time of analysis and accelerated stabilization of monolithic columns. Advantages of easy manipulation of mobile phases in flow systems were pointed out, such as generation of reproducible concentration gradients that could be obtained through multiple isocratic elution steps.

The same procedure based on five successive steps of isocratic elution was used for the separation of free intracellular amino acids with fluorescence detection [13]. HPLC gradient elution was compared with two-column approach in the SIC system for fast and effective analysis. Two columns of different retention activity were proved to be efficient tool for the separation of paracetamol, caf-

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feine, salicylic acid and propyphenazone; the results compared well with those of gradient analysis [14]. A method for the generation of high-precision linear gradients by pulse modulation (gradients were created by computer-controlled mixing of two solutions with a total volume as low as 75 μL under incremental or continuous flow conditions) in flow systems was devised by Herbelin and Růžička [15].

Indomethacin [1-(4-chlorobenzoyl)-5-methoxy-2-methylindoleacetic acid, IND], is a non-steroidal antiinflammatory (NSAID), analgetic and antipyretic drug. Recently a number of reports dealing with various analytical methods for the determination of IND, such as capillary electrophoresis [16,17], capillary zone electrophoresis [18,19], nonaqueous capillary electrophoresis [20], micellar electrokinetic chromatography [21], liquid chromatography–mass spectrometry [22], high performance liquid chromatography [23,24], ultra performance liquid chromatography [25], and sequential injection analysis with chemiluminescence detection [26] have been published.

The aim of this study was to develop and validate a new low-pressure concept of the gradient-elution SIC method (GE-SIC) and to demonstrate its efficiency on the GE-SIC determination of indomethacin (IND) and its two degradation products, namely 5-methoxy-2-methylindoleacetic acid (Impurity A) and 4-chlorobenzoic acid (Impurity B) in a topical pharmaceutical formulation. Ketoprofen (KTP) was used as an internal standard. The generation of the mobile phase gradient was studied with respect to the volume ratio of the two individual mobile phases employed as well as to the flow rate of their mixing due to dispersion in the holding coil of the SIA manifold. Repeatability of retention times of individual solutes and the effect of the parameters mentioned above on the separation quality were examined in detail. Novel GE-SIC and conventional HPLC techniques with UV spectrophotometric detection for the assay of IND and its main degradation products were optimized and validated.

2. Experimental

2.1. Chemicals and reagents

Working standards of indomethacin, 5-methoxy-2-methylindoleacetic acid, 4-chlorobenzoic acid and ketoprofen (internal standard) were provided by Sigma–Aldrich (Prague, Czech Republic). Analytical grade 85% phosphoric acid was obtained from Merck (Darmstadt, Germany). HPLC grade acetonitrile was purchased from Sigma–Aldrich (Prague, Czech Republic). The deionised water purified by a Milli-Q system (Millipore, MA, USA) was used throughout.

2.2. The SIC system

A FIAlab® 3000 device (FIAlab® Instruments, USA), was used in this study. It consisted of a syringe pump (syringe volume 5 mL), a Cheminert (Valco Instruments Co., USA) six-port selection valve and fiber optics UV detector with a 10 mm flow-through Z-cell. A D-2000 CE UV light source and USB 2000 UV–vis fiber optic CCD detector (OceanOptics, USA) were connected to the flow system via 600 μm I.D. optical fibers (Avantes, USA).

The detection wavelengths were set to 224 nm (for Impurity A), 237 nm (for Impurity B and KTP) and 305 nm (for IND). The flow connections were made of 0.50 mm I.D. PTFE tubing. The SIA set-up was controlled by a FIAlab® for Windows 5.0 software indicating only the retention time and peak height data. The peak areas were computed separately as the integral of the absorbance vs. time curve by using Cauchy–Riemann equations [27]. This integral was approximated by the sum according to equation:

$$\int f(x)dx \approx \sum (f(x) \cdot \Delta x)$$

where x is the value of retention time and $f(x)$ is functional value at point x.

A mixing coil (length 460 cm, volume 3.6 mL) allowing simple generation of the mobile phase gradient profile was placed between the selection valve and the syringe pump.

The GE-SIC separation was carried out by using Onyx™ Monolithic C18 (25 mm × 4.6 mm, Phenomenex®) column linked to a Chromolith® RP-18 pre-column (10 mm × 4.6 mm) at ambient temperature. The separation column was placed between the 6-port selection valve and the 10 mm detection Z-cell. The mobile phase was degassed by helium. The sample injection volume was 20 μL. The scheme of the GE-SIC set-up is shown in Fig. 1.

2.3. HPLC apparatus

Comparative conventional HPLC analyses were performed on the Breeze HPLC System (Waters Corp., USA), consisting of a binary pump (Waters 1525 Binary HPLC Pump), an autosampler (Waters 717 plus), a UV detector (Waters 2487 Dual λ Absorbance Detector) and a PC for data processing. The data were collected and analyzed by using the Breeze software.

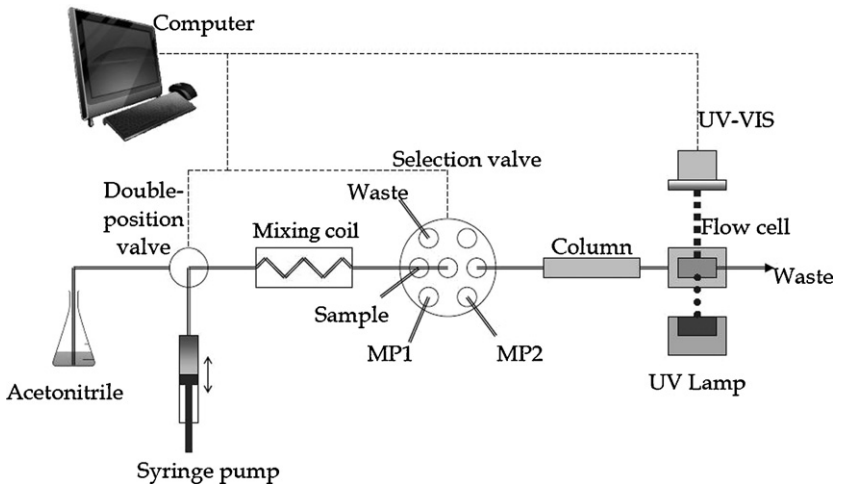

Fig. 1. SIA system for simultaneous determination of indomethacin and its impurities. MP1, mobile phase 1; MP2, mobile phase 2.

Separation testing was carried out with use of Onyx™ Monolithic C18 column (25 mm × 4.6 mm, Phenomenex®) with pre-column Chromolith® RP-18e (10 mm × 4.6 mm) at ambient temperature. The sample injection volume was 10 μL.

2.4. Preparation of standards

The stock solution of internal standard was prepared by dissolving 50 mg of KTP in 100 mL of methanol. Working solutions of KTP were prepared by a 50-fold dilution of the stock solution with methanol to get the final concentration 10 mg L^{-1}. The stock solutions of the impurities were prepared by dissolving 10.0 mg of Impurity A or Impurity B, in 20 mL of methanol. The stock solution of IND was prepared by dissolving 50 mg of the substance in 20 mL of methanol.

Reference standard solution used for the optimization and validation procedures was prepared by mixing 1.0 mL of IND stock solution, 400.0 μL of KTP stock solution and 100.0 μL of each impurity stock solution in 10 mL volumetric flask and adjusting the volume to 10.0 mL with methanol.

Stock and standard solutions were stored at 4 °C to avoid decomposition.

2.5. Mobile phase preparation

Mobile phases were prepared by mixing individual components (acetonitrile and aqueous 0.2% phosphoric acid) and filtering the mixture with a Millipore filtration device.

2.6. Sample preparation

A 0.5 g amount of topical gel (corresponding to 5.0 mg of IND as an active substance) was weighed and transferred to a 50 mL centrifuge tube. A 20 mL volume of internal standard working solution (10 mg L^{-1} of KTP in methanol) was added. This mixture was sonicated for 10 min and then centrifuged at 1300 × *g* (3000 rpm) for 15 min by using an EBA 21 laboratory centrifuge (Hettich, Germany). The supernatant was filtered through ValuPrep® 25 mm syringe filters (pore size 0.45 μm, Biotech, Czech Republic) and injected directly into the GE-SIC or HPLC system.

2.7. Optimization of chromatographic parameters

The aim of this study was to find optimal SIC separation conditions for quick determination of IND, Impurity A, Impurity B and KTP. KTP was used as internal standard. Original separation conditions were adopted from the validated method [23] considering also data obtained in a previous study [28]. To compare quality of separation conventional HPLC method was chosen.

Comparative HPLC separation with gradient elution was carried out under optimized conditions: acetonitrile/aqueous 0.2% phosphoric acid (30:70, v/v) from 0 min to 3 min, thereafter acetonitrile/0.2% phosphoric acid (50:50, v/v) from 3 min to 5 min and then acetonitrile/0.2% phosphoric acid (30:70, v/v); the flow rate was 1.2 mL min^{-1}.

Optimal conditions found in the HPLC system were useful to be adapted for the GE-SIC system. Aspirated volumes of each mobile phase, total volume of both mobile phases and flow rate for mixing (gradient generation) were studied. In the next step the effect of different flow rates used for aspiration of the mobile phases into the mixing coil was tested with respect to repeatability of the retention times of analytes and peak resolution.

Mobile phases consisted of acetonitrile and 0.2% phosphoric acid in different ratios (MP1: 30/70, v/v; MP2: 50/50, v/v). Total volume of MP1 and MP2 was sufficient for achieving complete separation of all analytes under study. Optimization of the ratio of MP1 and MP2 was carried out at the flow rate of 20 μL s^{-1}.

3. Results and discussion

3.1. Optimization of SIC chromatographic parameters

The commercial SIA system employed allows measurement at four different wavelengths simultaneously. To avoid any problems that might arise from lower sensitivity of the UV detector the detection wavelengths were selected by preliminary determination of the absorption maxima in the UV spectra of the analytes: 224 nm for Impurity A, 237 nm for Impurity B and KTP, and 305 nm for IND. The sample injection volume was set to 20 μL for the sake of sufficient sensitivity.

Generation of gradient profile in the GE-SIC system was based on program-controlled mixing of mobile phases MP1 and MP2 during their aspiration into the mixing coil as well as during the transport of these partially mixed phases from the mixing coil to the separation column via the selection valve (initially the reservoir of the syringe pump was filled with acetonitrile serving as the propelling liquid). Owing to precisely controlled mixing of the aspirated zones of MP1 and MP2 on their way into the mixing coil and back to the separation column repeatable gradient was created (see Fig. 2). Flow rate of the mobile phase passing through the separation column was 1.2 mL min^{-1} (the same value as that used in comparative HPLC experiment). Typical sequence of particular steps of the GE-SIC program is indicated in Table 1.

Three different gradient profiles were studied. The optimization was performed by changing the ratios of the aspirated volumes of MP1 and MP2 through the control program of the SIC system. The results are shown in Fig. 3 where the effect of gradients generated on the resolution parameter is documented. Since all generated profiles of gradient provided similar results with respect to quality of resolution and repeatability of retention times of the analytes, the medium profile was chosen for achieving higher level of robustness.

Subsequently the effect of different flow rates (20–100 μL s^{-1}) of aspiration of the two mobile phases was tested with respect to repeatability of the retention times in the case of quick or slow gradient generation (and thus mobile phases mixing). The lowest value of peak resolution $R_{KTP/IND}$ was observed for the lowest aspiration flow rate (20 μL s^{-1}) of the mobile phases. On the other hand higher flow rate of aspiration resulted in increased peak resolution. The resolution of other peaks ($R_{A/B}$, $R_{B/KTP}$) was not significantly influenced by the aspiration flow rate. Optimum aspiration flow rate of

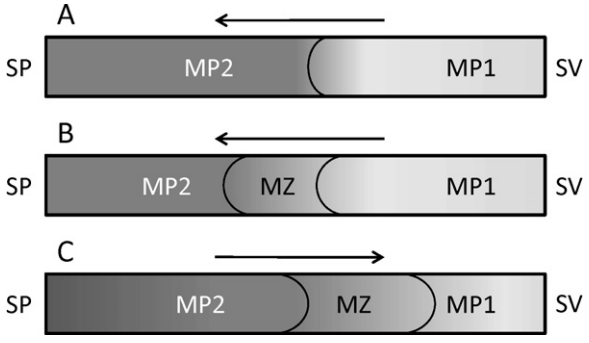

Fig. 2. Gradient generation in the GE-SIC system. MP1, mobile phase 1; MP2, mobile phase 2; MZ, mixed zone with gradient character created by mixing MP1 and MP2; SP, syringe pump; SV, selection valve. A: aspiration of mobile phases through the selection valve. B: formation of the mixed zone of mobile phases by dispersion during the movement in tubes of the flow system. C: increased volume of mixed zone after changing flow direction from mixing coil to the selection valve and monolithic column.

Table 1
The sequence of particular steps of the GE-SIC control program.

Unit	Command	Parameter	Action	Fig. 2
Syringe pump	Valve in			
	Flow rate (μL s^{-1})	70		
	Aspirate (μL)	1000	Acetonitrile aspirated	
Syringe pump	Valve out			
Valve	Port 3			
Syringe pump	Aspirate (μL)	2500	Mobile phase aspirated	
Valve	Port 5			
Syringe pump	Flow rate (μL s^{-1})	20		
	Dispense (μL)	2000	Washing the column	
Syringe pump	Valve in			
	Flow rate (μL s^{-1})	70		
	Aspirate (μL)	1000	Acetonitrile aspirated	
Syringe pump	Valve out			
Valve	Port 4			
Syringe pump	Flow rate (μL s^{-1})	70		
	Aspirate (μL)	1900	Mobile phase (2) aspirated	
Valve	Port 3			
Syringe pump	Aspirate (μL)	1600	Mobile phase (1) aspirated	A and B
Valve	Port 2			
Syringe pump	Flow rate (μL s^{-1})	10		
	Aspirate (μL)	20	Sample aspirated	
Valve	Port 5			
Syringe pump	Flow rate (μL s^{-1})	20		
	Empty		Analytes eluted	C
Spectrometer	Reference scan			
	Absorbance scanning		Peaks recorded	

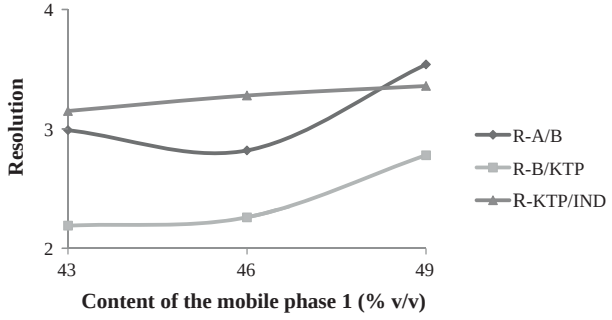

Fig. 3. Effect of different contents of mobile phase 1 in the gradient profile generation on the resolution parameter in GE-SIC. Separation conditions: column Onyx™ Monolithic C18 (25 mm × 4.6 mm, Phenomenex®), mobile phase acetonitrile/0.2% phosphoric acid (gradient described by percentage content of MP1 in the whole volume used for elution), flow rate 1.2 mL min^{-1}.

70 μL s^{-1} was used for all subsequent measurements in order to achieve complete separation and reliability of the syringe pump.

Substantial proof that highly repeatable gradient profiles are generated under the flow rates examined were the RSD values of retention times ranging from 0.28% to 0.88% (n = 6) for all solutes. Thus the gradient profiles are generated with similar repeatability at different flow rates while the gradient created at low flow rate is steeper than that generated at higher flow rate.

3.2. Analytical parameters and validation

The GE-SIC chromatogram demonstrating separation of a standard mixture of the analytes including the internal standard (with 1.6 mL MP1 acetonitrile/0.2% phosphoric acid 30:70 and 1.9 mL MP2 acetonitrile/0.2% phosphoric acid 50:50, flow rate 1.2 mL min^{-1}) is depicted in Fig. 4. Fig. 4 insert shows isocratic SIC

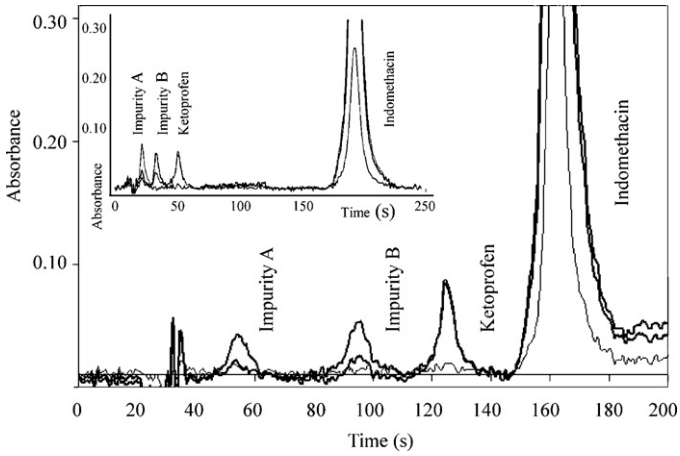

Fig. 4. SIC chromatogram of separation of the standard solutions using column Onyx™ Monolithic C18 (25 mm × 4.6 mm, Phenomenex®), mobile phase acetonitrile/0.2% phosphoric acid (gradient: 1.6 mL of MP1 and 1.9 mL of MP2), flow rate 1.2 mL min^{-1} (each line corresponds to the respective detection wavelength); figure insert: isocratic SIC separation using the same monolithic column.

separation using the same monolithic column and mobile phase consisting of acetonitrile/0.2% phosphoric acid 40:60. It can be clearly seen that the GE-SIC option resulted in shorter time of analysis and more favorite retention time values of all analytes compared to SIC separation with isocratic elution.

Basic chromatographic parameters, such as peak asymmetry, resolution, number of theoretical plates, height equivalent to the theoretical plate and retention factors calculated from experimental data of GE-SIC and HPLC experiments are summarized in Table 2. These results complied with values set by validation authorities [29,30].

The profiles of gradient were different in GE-SIC and HPLC and therefore the results could not be compared directly; similar separation quality could be reached in both systems. In the GE-SIC system equilibration of the column took place before each analysis but in the HPLC system this step occurred at the end of each HPLC run. A single GE-SIC analysis of the mixture took 8.5 min (including all steps of the procedure) while a single HPLC run lasted 8 min (including column equilibration). Net time of separation was 3.5 min for GE-SIC and 5 min for HPLC. The mobile phase consumption was 4.5 mL (GE-SIC) and 6 mL (HPLC) per single analysis.

The repeatability of retention times and peak areas and precision of results of the analyses of real samples (pharmaceutical preparation Indobene gel 1%) for both GE-SIC and HPLC are indicated in Table 3. The RSD values of retention times were considerably lower for GE-SIC while the RSDs of peak areas were worse compared to HPLC. This could be partly explained by the fact that in HPLC more sophisticated commercial software for the peak area integration was employed and also by higher sensitivity of the UV detector included in the HPLC setup and thus better signal-to-noise ratio and lower background. Because of worse sensitivity of the UV detector in the GE-SIC setup and rather low content of Impurity A in the pharmaceutical preparation the Impurity A could not be determined by GE-SIC. To validate the precision of the developed method RSD values of the determined substances compared well with the set limit (<5%).

4. Conclusion

The concept of simple and repeatable generation of mobile phase gradient in SIC through programmable automated mixing of two different mobile phases directly in the sequential injection system was devised. Optimization of the GE-SIC gradient

Table 2
Characteristics of the GE-SIC and HPLC separation processes.

Parameters	Limit	Impurity A		Impurity B		KTP		IND	
		SIC	HPLC	SIC	HPLC	SIC	HPLC	SIC	HPLC
Retention time (s)	–	56	67	97	106	126	164	164	254
Resolution	>1.5			$R_{A/B}$		$R_{B/KTP}$		$R_{KTP/IND}$	
				3.01	4.94	2.54	7.21	3.54	11.13
Asymmetry[a]	0.8–1.5	1.07	1.29	0.97	1.31	0.98	1.19	1.14	1.26
Number of TP	–	242	1329	900	2570	2785	6903	3031	14,798
HETP (μm)	–	110.33	38.12	28.33	19.46	9.22	7.25	8.25	3.38
Retention factor		0.60	0.43	1.77	1.26	2.59	2.49	3.69	4.40

TP, theoretical plate; HETP, height equivalent of the theoretical plate.
[a] Calculated according to the European Pharmacopoeia [31].

Table 3
RSD (%) values of analytical parameters of GE-SIC and HPLC separations of indomethacin and impurities A and B.

	Impurity A		Impurity B		IND	
	SIC	HPLC	SIC	HPLC	SIC	HPLC
Repeatability of t_Ra	0.30	0.92	0.18	0.80	0.24	0.18
Repeatability of peak area[a]	3.64	1.13	3.67	1.15	2.90	1.32
Method precision[b]	–	1.89	3.81	1.72	2.16	1.26

t_R, retention time.
[a] 6 replicate injections of standard solution.
[b] 6 individual samples of the same pharmaceutical preparation; three replicate injections of each individual sample.

profile with respect to separation quality can be relatively easily attained by programming changes of the ratio of the individual mobile phases and flow rate of their aspiration into the mixing coil. The GE-SIC technique was successfully applied to the separation of indomethacin and its main degradation products (5-methoxy-2-methylindoleacetic acid and 4-chloro-benzoic acid) in topical pharmaceutical formulation (Indobene gel 1%).

Both presented methods (GE-SIC and HPLC) could be used for gradient separation of the mentioned compounds with similar results obtained in validation of selected parameters. But SIC system enables easy optimization, quick separation under smaller consumption of organic solvents and generated waste as well.

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