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Monolithic columns—a new concept of separation in the sequential injection technique

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Abstract

In this contribution, the coupling of monolithic column with sequential injection technique (SIA), as a new possibility of implementation of a separation step in SIA is described.

Monolithic columns were developed based on a new sol–gel technology. Due to the presence of macropores, the monolithic columns possess a much higher porosity than conventional particulate high pressure liquid chromatography (HPLC) columns. Consequently, monoliths can be coupled to the SIA system without loss of performance or limitations due to the very low column back-pressure.

The aim of our study was to develop and test the new SI separation manifold and to demonstrate its functionality in the simultaneous determination of four different compounds (methylparaben, propylparaben, sodium diclofenac and internal standard butylparaben) in standard solutions and in a topical emulgel.

A Chromolith® Flash RP-18e, 25 mm × 4.6 mm (Merck, Germany) column and a FIAlab® 3000 system (USA) with an eight-port selection valve and 10 ml syringe were used for sequential injection chromatographic separations in our study. The mobile phase used was acetonitrile:water (40:70 (v/v)) + 0.05% triethylamine, pH 2.5, gradient flow rate 8–20 $\mu l\,s^{-1}$.

A novel reversed-phase sequential injection chromatography (SIC) technique with UV spectrophotometric detection was optimised and validated. The validation parameters showed very good results. The analysis time was less than 8 min. The method was found to be applicable for the routine analysis of the active compound sodium diclofenac and preservative propylparaben in a pharmaceutical product, topical diclofenac emulgel 1%.

The coupling of the monolithic columns with sequential injection manifold provides an excellent tool to solve the separation problems without using HPLC instrumentation and with low cost per analysis and low consumption of organic solvents (non-continuous flow).

Keywords: Sequential injection analysis; Separation; Sequential injection chromatography; Monolithic columns; Methylparaben; Propylparaben; Sodium diclofenac

1. Introduction

In recent years, the sequential injection analysis (SIA) has become an important analytical technique, mainly for the determination of drugs in

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pharmaceuticals and for the determination of the environmental contaminants.

SIA, developed by Ruzicka and Marshall in 1990 [1], is based on forward, reversed, and stopped flow of the carrier stream and it has been the subject of several studies aimed to establish its theory and particularities [2–6]. It offers several advantages: the instrumental set-up is very flexible, the components undergo little wear and the hydrodynamic variables can be controlled with high efficiency. Mostly configured as a "single line system", the SIA device consists of a single low-pressure bi-directional syringe pump as flow drive that generates main pulsation-less flow with variable direction and speed, and a multi-position selection valve both as injector of samples and reagents and as a connector with reactors and detector. During measurement, selected volumes of the sample and reagent solutions are drawn into the holding coil by flow reversals and then moved forward through the selection valve into the detector. All the operations connected with liquid handling can be automated using automated syringe pump as a liquid driver and selection valve.

On the other hand, the SIA technique has an important drawback—lack of a possibility to carry out the separation analysis and analysis of the multi-component samples without previous sample pre-treatment and removing the interfering matrices.

The separation process can be achieved by the usual separation methods, such as liquid chromatography (LC), high pressure liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE) or isotachophoresis (ITP). These methods are sensitive and can give the most reliable results, but it requires highly sophisticated apparatus, higher operating cost and expensive instrumentation. The main aim of our study was to develop a new, simple and fully automated separation set-up consisting of the SIA analyser and a monolithic column, and to present its functionality on the simultaneous determination of four different compounds (methylparaben (MP), propylparaben (PP), sodium diclofenac (SD) and internal standard butylparaben (BP). The second aim of this study was validation of this sequential injection chromatography (SIC) method, its comparison with HPLC and application for the determination of the above-mentioned compounds in commercially available pharmaceutical sample—in a topical emulgel.

This system should be able to provide the low organic solvent consumption, low operating costs, low generation of the waste and high sample throughput. It offers several advantages: the eight-port selection valve enables to combine higher number of mobile phases (eluting reagents) or can be used as an autosampler. Due to the simple control of the syringe pump and structure of the monolith, it is possible to use the simple flow gradient for compounds with different retention characteristics.

Monolithic supports have become the subject of extensive study in the past years. In principle, monoliths could be made of synthetic organic materials (e.g. acrylate resins), natural polymers (cellulose), or inorganic materials, such as silica. The monoliths were developed based on a new sol–gel process, which includes the hydrolysis and polycondensation of alkoxysilanes (e.g. tetramethoxysilane or tetraethoxysilane) in the presence of water-soluble polymers (e.g. poly(ethyleneoxide) or polyethylene glycol). The different procedures for the preparation of monolithic porous silica or other polymer rods are described in the literature [7–30]. The part of these studies has been interested in the development of the monolithic supports for capillary electrochromatography (CEC) applications [9,15,18–28] and second direction was the area of a new monolithic HPLC columns [7–14,16,17,19,26,28–30]. On the other hand, the only commercial available monoliths are from Merck (Darmstadt, Germany), and BIA Separations (Ljubljana, Slovenia), at present.

Despite their advantageous features and many successful chromatographic applications in the analytical scale, only a few examples of using monoliths were described. On the other hand, the number of these works shows increasing trend [31–47]. Fast separations of biological macromolecules, such as proteins and oligonucleotides, peptides or amino acids [31,37,41], the fast analysis of organic acids [34], the rapid enantiomeric separation of dansyl amino acids and hydroxy acids [26], the fast separation of drug fenfluramine, temazepam and tamoxifen in plasma [36], or determination of ochratoxin A and phenolic compounds in different wines [35,44] have been successfully carried out.

In contrast to conventional HPLC columns, the Chromolith® column is formed from a single piece of porous silica gel (monolith). Due to the presence

of macropores, the monolithic columns possess a much higher porosity, about 15% higher than of conventional particulate HPLC columns. The resulting column back-pressure is therefore much lower, allowing operation at higher flow rates (HPLC) or low flow rates (SIA) including flow gradients. Thus, they can be incorporated into the SIA system regardless of the limits of syringe pump.

The performance of the reversed-phase monoliths is equivalent to a typical C18 5 μm particulate HPLC column (monoliths from Merck, Darmstadt, Germany). The comparative studies on the column performance of microparticulate C18-bonded and monolithic C18-bonded reversed-phase columns in high-performance liquid chromatography were described [48–52]. The repeatability and reproducibility of retention data and band profiles on a series of six Chromolith® Performance RP-18e columns from Merck (Darmstadt, Germany) were carried out by Kele and Guiochon [53].

Diclofenac, as the sodium salt, is a benzene acetic acid derivative, designated chemically as 2-[2,6-dichlorophenyl)amino]benzene acetic acid, monosodium salt. Diclofenac is a non-steroidal anti-inflammatory drug (NSAID). In pharmacological studies, SD has shown anti-inflammatory, analgesic and antipyretic activity. Methylparaben and propylparaben are effective antibacterial and anti-fungal agents, which are commonly used as the preservatives in foods, beverages, cosmetics and pharmaceuticals. Methylparaben and propylparaben are used together since they have a synergistic effect [54].

Recently, there have been a number of reports dealing with various analytical techniques for the determination of SD, such as capillary electrophoresis [55–58], thin-layer chromatography [59,60], flow injection analysis [61–63], gas chromatography–mass spectrometry (GC–MS) [64,65], supercritical fluid chromatography [66,67] and liquid chromatography [68–72], but were not applied in the presence of preservatives methylparaben and propylparaben.

LC analysis of MP and PP is frequently described [54,73,74], but there is only one LC method [75] for determination of SD in the presence of preservatives MP and PP.

The purpose of this study was to develop a new low-pressure concept of the chromatography—sequential injection chromatography—for the determination of three compounds of a topical emulgel, active component sodium diclofenac and two preservatives methylparaben and propylparaben, and butylparaben as an internal standard. Thereafter, this method was applied for the separation and quantification of the compounds in the pharmaceutical formulation diclofenac emulgel 1%.

2. Methods and materials

2.1. Reagents

The standard of sodium diclofenac was obtained from Amoli Organics Ltd. (Mumbai, India); methylparaben and propylparaben were from Galena a.s. (Opava, Czech Republic), internal standard butylparaben was from Fluka (Prague). Standard solutions were prepared in methanol. The final concentrations of the sample or reference standards were 250 μg ml^{-1} sodium diclofenac, 25 μg ml^{-1} methylparaben, 12.5 μg ml^{-1} propylparaben and 50 μg ml^{-1} internal standard butylparaben.

Methanol and acetonitrile (Chromasolv, for LC) were obtained from Sigma–Aldrich; orthophosphoric acid, 85% p.a. was from Merck (Darmstadt, Germany). Triethylamine was obtained from Fluka. All other chemicals used were of analytical grade quality. Diclofenac emulgel 1% was supplied from Herbacos-Bofarma Ltd. (Bochemie Group, Pardubice, Czech Republic). The deionised water was purified by a Milli-Q system (Millipore Corp., Bedford, MA) and meets the European Pharmacopoeia specifications.

2.2. Sequential injection system

An overall schematic view of the sequential injection system with the monolithic column is shown in Fig. 1. A FIAlab® 3000 system (FIAlab® Instruments, USA) is a commercially produced instrument and consists of a syringe pump (syringe reservoir 10 ml) and eight-port selection Cheminert valve (Valco Instrument Co., USA). FIAlab® 3000 was equipped with fibre-optic UV-Vis diode array detector S2000 (Ocean Optics Inc., USA) with UV-Vis tungsten lamp LS-1 (Ocean Optics Inc., USA). The solarisation optic fibres and 10 mm Z-flow cell were from Avantes

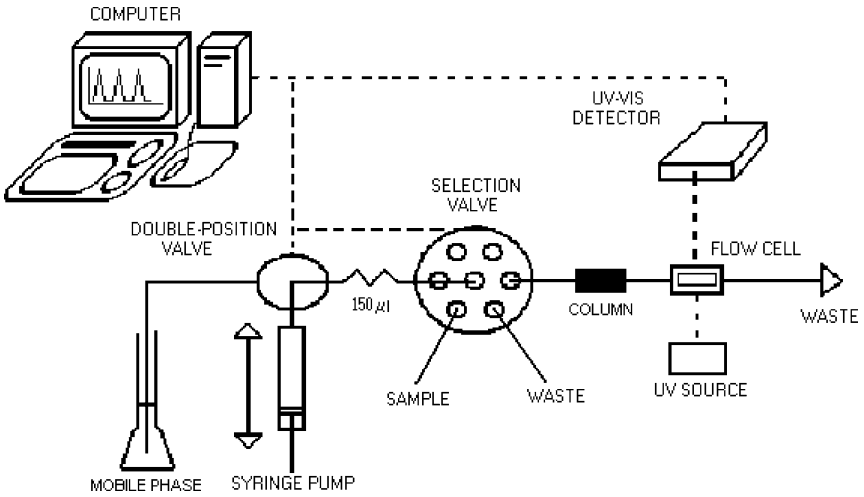

Fig. 1. Scheme of SIC set-up for the chromatographic separation and determination of sodium diclofenac, methylparaben and propylparaben.

Inc. (Colorado, USA). The whole SIA system was controlled by the latest version of program FIAlab® for Windows 5.0. Flow lines were made of 0.75 mm i.d. PTFE tubing. Mobile phases and samples were aspirated through the selection valve and then delivered to the monolithic column and to the detector. Sample compounds separation was performed on Chromolith® Flash RP-18e, 25 mm × 4.6 mm column (Merck, Germany). The monolithic column was placed between the selection valve and flow cell of the detector. For safety reasons, a special replaceable in-line filter (2–5 μm sieve, Merck) was installed prior to the column and mobile phase was aspirated through the filter end (10 μm).

The comparative HPLC system, consisting of a binary pump LCP 4100 (Ecom, Prague), Waters autosampler 717 plus, variable wavelength UV detector Waters 486 (Waters, Milford, MA) and a PC for data processing, was controlled by chromatographic software CSW v.1.7 for Windows (Data Apex s.r.o., Prague). Analyses were performed on the same above-mentioned column.

The optimal mobile phase for separation of SD, MP, PP and internal standard BP was a mixture

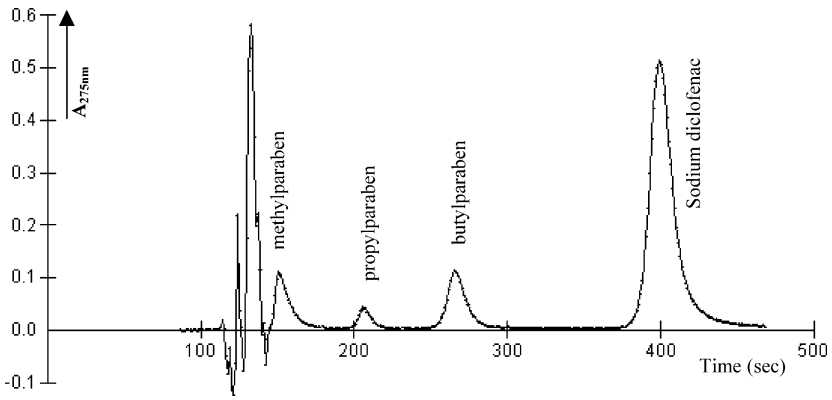

Fig. 2. SI chromatogram of the separation of the standard solutions of methylparaben (25 μg ml^{-1}), propylparaben (12.5 μg ml^{-1}), internal standard butylparaben (50 μg ml^{-1}) and sodium diclofenac (250 μg ml^{-1}).

of acetonitrile:water (40:70 (v/v)) + 0.05% of triethylamine (TEA), pH adjusted to 2.5 by means of orthophosphoric acid (8.5%). Mobile phase was degassed before application by means of helium.

The finally selected optimised conditions were as follows: injection volume 10 μl for sample of emulgel, the isocratic mobile phase was pumped at the flow gradient 8–20 $\mu l\,s^{-1}$ at ambient temperature, and the detection wavelength was 275 nm. The flow gradient was performed sequentially in steps by increasing flow rate after the analyte elution. Fig. 2 shows a sequential injection chromatogram of standard solutions of sodium diclofenac, preservatives and butylparaben as internal standard.

2.3. Sample preparation

An accurately weighed portion (ca. 0.5 g) of the pharmaceutical emulgel was transferred into a 50 ml centrifuge tube and supplemented with 20.00 ml of internal standard (50 $\mu g\,ml^{-1}$ of butylparaben in methanol). The mixture was placed into the ultrasonic bath for 10 min and then centrifuged at 4000 × *g* for 10 min. A volume of 10 μl of supernatant was analysed by SIA chromatography system. Identification of peaks in the emulgel samples was based on comparison of retention times of compounds in standard solutions. Peak identity was confirmed by UV-Vis spectra.

3. Results and discussion

3.1. Method development and optimisation

The main aim of our study was to find an optimal separation condition of the sequential injection cycle, to introduce the simple method of the determination of SD, MP and PP, and to show the possibility of this method application for real pharmaceutical sample. An on-line coupling of the SIA-monolith technique has never been shown in the field of analytical chemistry and we have observed some troubles during the development of this method. The main criteria for developing a successful SIA determination of sodium diclofenac and preservatives were as follows: the method should be able to separate all compounds of the interest and the second aim was to be stability indicating, free of the interference from the excipients, robust and straightforward enough for routine use in the determination of compounds in topical emulgel.

From the UV spectra of all the analysed compounds, the optimal detection wavelength was chosen to be 275 nm—the absorption maximum of SD. On the other hand, this commercial SIA system makes possible measurement of four different wavelengths simultaneously and we used both wavelengths of 256 nm (absorption maximum of parabens) and 275 nm for the determination in emulgel. We used the wavelength of 275 nm during the optimisation of method due to the simple evaluation of the peaks.

The choice of the appropriate internal standard was made from the several compounds (flurbiprofen, ketoprofen and butylparaben), all having similar characteristics to the compounds determined. The retention time of ketoprofen was similar to that for PP and retention time of flurbiprofen was similar to that for SD, so these compounds were unsuitable. Butylparaben, as we expected, was better than the others, and it was sufficiently separated from the other compounds of interest. The second criterion of this choice was its good sensitivity.

For good separation of SD, MP and PP, the different mobile phases were tested (methanol, acetonitrile and water). Using different column lengths, Chromolith® Flash RP-18e, 25 mm × 4.6 mm and Chromolith® SpeedROD RP-18e, 50 mm × 4.6 mm were tested. The total separation length of 25, 35, 50 and 60 mm was possible to test due to the possibility of connection with 10 mm pre-column. The simple flow gradient was chosen due to the different lipophility characteristics of the compounds analysed. The high lipophility of SD resulted in the long retention time, mainly for longer column (35 mm and longer). The more polar compound, MP, showed very low retention characteristic, which was not significantly influenced with the increasing length of the column. Therefore, there was necessary to find the compromise between retention of SD and MP, and the optimal condition for the separation was achieved under the flow rate gradient and mobile phase acetonitrile:water (40:70 (v/v)) + 0.05% of TEA, pH 2.5 and total column length 25 mm. The addition of TEA was resulted in decreasing of SD retention time and better peak symmetry. The gradient flow

Table 1
The typical sequence of the particular steps of the SIC programme for the separation analysis

Unit	Command	Parameter	Action
Loop start (#) 1			Start of analysis
Syringe pump	Valve in		Set valve position
Syringe pump	Flow rate ($\mu l\,s^{-1}$)	100	
Syringe pump	Aspirate (μl)	6000	Mobile phase aspirated
Syringe pump	Delay until done		
Syringe pump	Valve out		Set valve position
Valve	Port 2		Set port position
Analyte new sample			
Analyte name: MP			
Syringe pump	Flow rate ($\mu l\,s^{-1}$)	10	
Syringe pump	Aspirate (μl)	10	Sample aspirated
Syringe pump	Delay until done		
Valve	Port 8		Set port position
Syringe pump	Flow rate ($\mu l\,s^{-1}$)	8	
Syringe pump	Dispense (μl)	900	Sample propelled through the column, MP eluted
Spectrometer	Reference scan		
Spectrometer	Absorbance scanning		Peak of MP recorded
Syringe pump	Delay until done		
Spectrometer	Stop scanning		
Analyte name: PP			
Syringe pump	Flow rate ($\mu l\,s^{-1}$)	15	
Syringe pump	Dispense (μl)	750	PP eluted
Spectrometer	Absorbance scanning		Peak of PP recorded
Syringe pump	Delay until done		
Spectrometer	Stop scanning		
Analyte name: BP			
Syringe pump	Flow rate ($\mu l\,s^{-1}$)	15	
Syringe pump	Dispense (μl)	1000	BP eluted
Spectrometer	Absorbance scanning		Peak of BP recorded
Syringe pump	Delay until done		
Spectrometer	Stop scanning		
Analyte name: SD			
Syringe pump	Flow rate ($\mu l\,s^{-1}$)	20	
Syringe pump	Empty		SD eluted
Spectrometer	Absorbance scanning		Peak of SD recorded
Syringe pump	Delay until done		
Spectrometer	Stop scanning		
Loop end			End of analysis

rate used was carried out under the following conditions:

- elution of MP (flow rate 8 $\mu l\,s^{-1}$, volume 900 μl);
- elution of PP (flow rate 15 $\mu l\,s^{-1}$, volume 750 μl);
- elution of BP (flow rate 15 $\mu l\,s^{-1}$, volume 1000 μl);
- elution of SD (flow rate 20 $\mu l\,s^{-1}$, volume 3350 μl).

Total volume for one analysis was 6000 μl of mobile phase. Due to the flow gradient used, we also improved the resolution of MP from the noise of the baseline caused by dead volume and finally, the analysis time for standards of all compounds was decreased to 8 min. The typical sequence of particular steps of the programme is mentioned in Table 1.

This is necessary to make a critical mention of the FIAlab® software. SIA is the no-separation method and FIAlab® software is inappropriate and insufficient for the separation analysis evaluation, at present. We

Table 2
Analytical parameters and method validation results

	Methylparaben	Propylparaben	Sodium diclofenac
Calibration range ($\mu g\,ml^{-1}$)[a]	1.5–200	1.5–200	0.75–200
Correlation coefficient	0.999941	0.999217	0.999863
Limit of detection ($\mu g\,ml^{-1}$)[b]	0.5	0.5	0.25
Limit of quantification ($\mu g\,ml^{-1}$)	1.5	1.5	0.75
System precision (%)[c]	0.74	1.16	2.64
Method precision (%)[d]	None	1.13	0.83
Accuracy			
Spike recovery (%)[e]	None	103.29	100.68
Recovery R.S.D. (%)	None	2.02	1.12

[a] $n = 9$ at 0.75, 1.5, 3.0, 6.0, 12.0, 25.0, 50.0, 100.0 and 200.0 $\mu g\,ml^{-1}$ levels, each concentration was measured in triplicate.
[b] Volume of sample injection: 50 μl water standard solution.
[c] Relative standard deviation (R.S.D.) values were calculated for repeated standard injections, $c = 15\,\mu g\,ml^{-1}$ ($n = 8$).
[d] R.S.D. for repeated injections of multiple sample preparations ($n = 6$), two preparation each, two injection of each preparation.
[e] Spiked placebo solutions ($n = 6$), two preparations each, two injections of each preparation.

had a big problem to evaluate four peaks inside one analysis cycle. This software evaluated only the highest peak from the analytical data record and the lower peaks were not evaluated. We solved this problem using the special command in the program. Every peak was marked as "analyte new sample" and for every peak was used "spectrometer absorbance scanning" and "syringe pump dispense" sequentially (Table 1).

The possibility of the solvent gradient elution was tested too. SIA system makes possible very high combination efficiency of the number of mobile phases owing to eight-port selection valve and due to the simple aspiration and mixing these solutions in-line the system. On the other hand, our experiments with the mobile phase gradient elution were not successful through the drift and noise of the baseline.

3.2. Analytical parameters and validation

The optimised method was validated by a standard procedure to evaluate if adequate accuracy, precision, selectivity and linearity had been achieved. The accuracy was determined using spiked placebo solutions, two preparations each, two injections of the each preparation. The relative standard deviation (R.S.D.) values were calculated for the repeated standard injections (system precision) as well as repeated injections of the multiple sample preparations (method precision). The stability of the standards was evaluated by comparison of response factors of the fresh and stored standards. Visual inspection of chromatograms of standards and placebo solutions was conducted to ensure selectivity. None interference peak was found in the retention time of all compounds after the placebo sample injection. The method validation results obtained under the final conditions are shown in Table 2. The method meets all common requirements for accuracy, precision and linearity.

3.3. Parameters of sequential injection chromatography process

The optimised method was evaluated from the parameters of the chromatographic process point of view. To demonstrate the efficiency of SIC technique, the relative standard deviation of the retention times for the intra-day repeated standard injections ($n = 10$), the peak resolution and the separation efficiency (number of theoretical plates) for all standards were calculated. The obtained results are shown in Table 3. The chromatogram in Fig. 2 was obtained using the SIC method with sample of the standards. The comparison with HPLC was carried out under the same conditions as well as the SIC system (identical mobile phase and same flow gradient) and HPLC chromatogram is shown in Fig. 3.

3.4. Determination in a pharmaceutical product

All compounds present in the sample of topical cream diclofenac emulgel 1%, sodium diclofenac, both preservatives and internal standard were clearly

Table 3
Parameters of SIC process

	Methylparaben	Propylparaben	Butylparaben	Sodium diclofenac
Retention time (s)	152	205	266	402
Repeatability of T_r (%)[a]	0.15	1.38	2.01	1.76
Peak resolution	$R_{MP,PP} = 5.4$	$R_{PP,BP} = 5.6$	$R_{BP,SD} = 9.0$	
Peak asymmetry	2.55	1.28	1.29	1.47
Number of theoretical plates	1280	2635	2510	3000

[a] Repeatability of T_r—R.S.D. of retention times for intra-day repeated standard injections ($n = 10$).

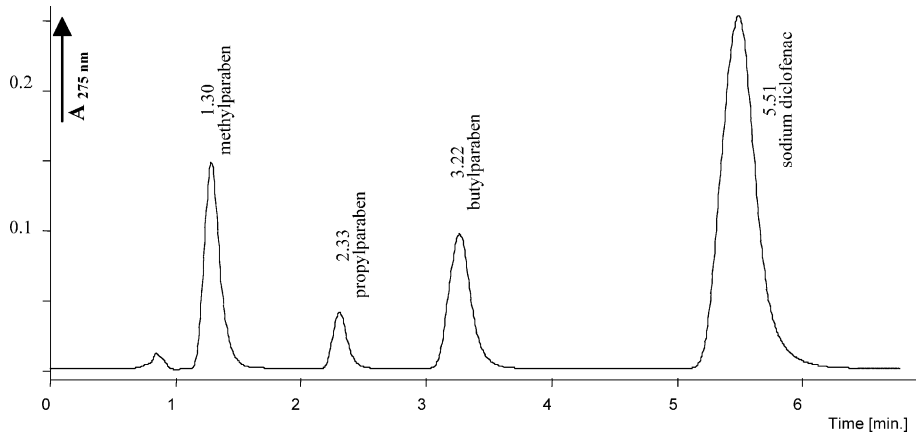

Fig. 3. HPLC chromatogram of the separation of the standard solutions under the same conditions as well as the SIC system.

separated. The average recoveries of SD and PP in the emulgel were 98.12 ± 1.4 and 96.51 ± 0.8% of the labelled amount, respectively. The reliable results of the assay of MP were not achieved due to the short retention time. Eluted matrix components of the emulgel on the start of the SI chromatogram resulted in the partial interference with the peak of MP and the software was not able to evaluate MP quantitatively.

4. Conclusion

In this section, we focus on the critical summary of our work and the advantages and disadvantages of the coupling of these monoliths with SIA, at present. The SIC method with the monolithic column and UV spectrophotometric detection was developed successfully only for the determination of sodium diclofenac and propylparaben in the topical emulgel using butylparaben as the internal standard. The total analysis time was less then 8 min. The method has been validated for PP and SD, and the results obtained were precise and accurate. The method can be used for the routine analysis of the compounds in the pharmaceutical products containing the active compound sodium diclofenac and preservative propylparaben. The proposed method makes possible only the identification of methylparaben without the quantitative evaluation. On the other hand, the main aim of our work was to introduce a new coupling of the SIA with the monoliths to the field of analytical chemistry and to find out the problems, advantages and disadvantages of this new technique.

From the critical point of view, we can say that the simple and automated system that was developed, presents several clear advantages over the usually known methodologies: the possibility of the separation analysis without HPLC instrumentation, the possibility of the simple post- or pre-column derivatisation, the possibility of on-line sample pre-treatment

(solid phase extraction), the simplicity and variability of the separation procedure, low consumption of the organic solvents and waste production due to the non-continuous flow, high variability of optical path length due to the Z-cell and possibility to move the optical fibre inside the flow cell, and low cost per analysis.

The disadvantages of the system, at present, are insufficient software for separation analysis evaluation, limited flow rates of the syringe pump for longer columns (e.g. for 50 mm column, maximum flow rate is $20\,\mu l\,s^{-1}$), limited volume of the syringe pump (commercially available maximum is 10 ml (FIAlab® Instruments, USA) and limited possibility of the separation due to the restriction of the column length and flow rate.

In the comparison between the other analytical separation methods (HPLC, GC, ITP, CE or CEC), SIC showed some restrictions. On the other hand, these conventional separation techniques are reliable and sensitive, but require expensive instrumentation and high cost per analysis.

In summary, due to the number of experiments and testing of the SIC system we get view the power of SIC mainly for the simple separation analysis of low component samples. Moreover, the coupling of the monolithic columns with the sequential injection manifold provides an excellent tool to solve the separation problems without using HPLC instrumentation.

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