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Sequential injection chromatographic determination of paracetamol, caffeine, and acetylsalicylic acid in pharmaceutical tablets

In this contribution, a new separation method for simultaneous determination of paracetamol, caffeine, acetylsalicylic acid, and internal standard benzoic acid was developed based on a novel reversed-phase sequential injection chromatography (SIC) technique with UV detection. A Chromolith® Flash RP-18e, 25-4.6 mm column (Merck, Germany) and a FIALab® 3000 system (USA) with an 8-port selection valve and a 5 mL syringe were used for sequential injection chromatographic separations in our study. The mobile phase used was acetonitrile-(0.01 M) phosphate buffer (10:90, v/v) pH 4.05, flow rate 0.6 mL min⁻¹. UV detection was at 210 and 230 nm. The validation parameters showed good results: linearity ($r > 0.999$) for all compounds, detection limits in the range 0.3–0.8 µg mL⁻¹, repeatability (RSD) of peak heights between runs in the range 1.10–4.30% at three concentration levels and intra-day repeatability of the retention times in the range 0.28–0.43%. The analysis time was <6 min. The method was found to be applicable for the routine analysis of the active compounds paracetamol, caffeine, and acetylsalicylic acid in pharmaceutical tablets.

Key Words: Sequential injection analysis (SIA); Sequential injection chromatography (SIC); Monolithic columns; Paracetamol; Caffeine; Acetylsalicylic acid

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1 Introduction

Sequential injection analysis (SIA), developed by Ruzicka and Marshall in 1990 [1], represents advanced form of solution manipulation available to analytical chemists for mixing and transport of samples, reagents, and products of chemical reactions to the measurement point. Fast and intensive development of SIA methodology was due to several factors essential for routine analytical determinations, e.g. simplicity of fundamental principles, inexpensive instrumentation, automated sampling and analytical procedures, limited sample consumption, short analysis time, on-line performance of difficult operations (pre-concentration, physical-chemical conversion of analytes into detectable species, dialysis, stopped flow technique, etc.). All of the mentioned SIA features have been the subject of several studies aimed to establish its theory and particularities and it has been widely reviewed [2].

Sequential injection chromatography (SIC) is a new area of analysis within the sequential injection technique (SIA). A simple SIC system could be developed by incorporating reversed phase monolithic columns into the commercially available SIA manifold.

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Monolithic supports have become the subject of extensive study in recent years and they were developed on the basis of a new sol gel process, which includes the hydrolysis and polycondensation of alkoxysilanes. The different procedures for the preparation of monolithic porous silica or other polymer rods were described in the literature [3–7].

In contrast to conventional HPLC columns, monolithic columns are formed from a single piece of porous silica gel (monolith). The performance of the reversed-phase monoliths is equivalent to a typical C-18 5 µm particulate HPLC column. Due to the presence of large through-pores and mesopores, the monoliths possess a much higher porosity than conventional particulate HPLC columns. The resulting column back-pressure is therefore much lower and allows operation in a SIA system. Thus, it can be incorporated into an SIA system regardless of the limitations of the syringe pump.

Paracetamol and acetylsalicylic acid are components of multidrug pharmaceutical preparations for the therapy of pain and they are widely used analgesic and antipyretic drugs, while caffeine is a well-known stimulant. Concerning the different mechanism of action, they sometimes act as synergists, which leads to higher efficacy.

For the assay of acetylsalicylic acid, paracetamol, and caffeine in such mixtures, different methods have been reported, including spectrophotometry [8], derivative

spectrophotometry [9], flow injection partial least-squares UV spectrophotometry [10, 11], stopped-flow Fourier-transform infra-red spectrometry [12], planar chromatography [13, 14], solid phase spectroscopy [15], and micellar electrokinetic chromatography [16].

Considering the properties of the compounds investigated, such as mid-polarity, as well as thermolability and low volatility, the use of HPLC methods has been mostly explored [17–20].

The present paper describes a new, simple, and fully automated set-up consisting of an SIA analyser incorporating a monolithic column and presents its functionality in the simultaneous determination of 4 different compounds paracetamol (PAR), caffeine (CAF), acetylsalicylic acid (ASA), and internal standard benzoic acid (BA). This method can be used to accomplish the determination PAR, CAF, and ASA as a drug substances and also for their determination in different tablet formulations.

2 Experimental

2.1 Reagents

The caffeine standard was obtained from Sigma-Aldrich (Prague, Czech Republic), paracetamol and acetylsalicylic acid were from Balex a.s. (Pardubice, Czech Republic), and benzoic acid used as internal standard was from Galena (Opava, Czech Republic). Stock standard solutions were prepared every day in methanol in a concentration of $1000 \mu\text{g mL}^{-1}$. The final concentrations of the sample, working standard solutions, or reference standards for pharmaceutical tablet analysis were obtained by diluting the stock solution in the mobile phase.

Methanol and acetonitrile (Chromasolv, for LC) were obtained from Sigma-Aldrich, orthophosphoric acid (85%) was from Merck (Darmstadt, Germany). All other chemicals used were of analytical grade quality. ACIFEIN® and

ACYLCOFFIN® were supplied by SLOVAKOFARMA (Hlohovec, Slovak Republic), PANADOL EXTRA® was supplied by SmithKline Beecham Consumer Healthcare (Brentford, Great Britain). The deionised water was purified by a Milli-Q system (Millipore Corp., Bedford, MA).

2.2 Apparatus

An overall schematic view of the sequential injection chromatography system with the monolithic column is depicted in **Figure 1**. A FIALab® 3000 system (FIALab® Instruments, USA) is a commercially produced instrument consisting of a syringe pump (syringe reservoir 5 mL) and an 8-port selection Cheminert valve (Valco Instrument Co., USA). The FIALab® 3000 was equipped with an S2000 fiber-optic UV-VIS diode array detector (Ocean Optics, Inc., USA) with an LS-1 UV-VIS tungsten lamp (Ocean Optics, Inc., USA). The solarization resistant optic fibers and 10 mm Z-flow cell were from Avantes Inc. (Colorado, USA). The whole SIA system was controlled by the latest version of the program FIALab for Windows 5.0. Flow connecting lines were made of 0.75 mm ID PTFE tubing. Mobile phases and samples were aspirated through the selection valve and then delivered to the monolithic column and to the detector. Sample compound separation was performed on a Chromolith® Flash RP-18e, 25–4.6 mm column (Merck, Germany). The monolithic column was placed between the selection valve and flow cell of the detector. The mobile phase was aspirated through the filter ending (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.

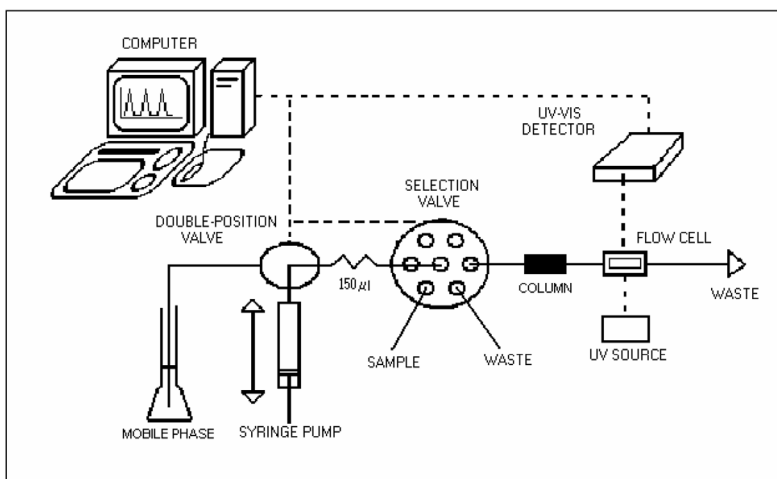


Figure 1. Scheme of SIC set-up for the chromatographic separation.

2.3 Method and sample preparation

2.3.1 Mobile phase

The optimal mobile phase for separation of PAR, CAF, ASA, and internal standard BA was acetonitrile:0.01 M phosphate buffer (10:90, v/v) whose pH was adjusted to 4.05 by means of orthophosphoric acid (8.5%). The mobile phase was degassed before application by means of helium.

The finally selected optimised conditions were as follows: injection volume 25 μL for standard solutions or sample of tablets, the isocratic mobile phase was pumped at flow rate 0.6 mL min^{-1} at ambient temperature, and the detection wavelength was 210 and 230 nm.

2.3.2 Solutions and sample preparation

Different commercial pharmaceutical formulations were analysed such as ACIFEIN® tablet (containing 250 mg of ASA, 200 mg of PAR, and 50 mg of CAF), ACYLCOFFIN® tablet (450 mg of ASA and 50 mg of CAF) and PANADOL EXTRA® tablet (500 mg of PAR and 65 mg of CAF). Determination of the active substances in the tablets was performed by weighing 10 tablets (calculation of average weight of one tablet), crushing the tablet mass, using an average weight of one tablet and dissolving it in 100 mL of methanol solution containing internal standard BA at a concentration of 2500 $\mu\text{g mL}^{-1}$. For the sample dissolution, 10 min of ultrasonication was used and a volume of 0.5 mL of this methanol sample suspension was then transferred to the 50.00 mL calibrated flask, diluted with mobile phase to the mark, and mixed. A volume of 20.00 mL was placed in a centrifugation tube and centrifuged at 2000 g for 5 min. A volume of 25 μL of supernatant was analysed by the SIA-chromatography system. The comparative standard solutions were prepared for each type of tablet in the same concentration according to the content of the analyte in the tablet. Identification of peaks in the pharmaceutical tablet samples was based on comparison of the retention times of the compounds in standard solutions. Peak identity was confirmed by UV-VIS spectra by means of a diode array detector (scanning the whole spectrum during the sample position in the flow cell of the UV-VIS detector). Standards and samples were measured in triplicate and the mean peak height values were used for data acquisition.

3 Results and discussion

3.1 Method development and optimisation

The main goal of the study was to propose a sequential injection cycle, which enables optimal separation conditions for determination of PAR, CAF, and ASA, and to demonstrate the possibility of using this method for the analysis of real pharmaceutical samples. The on-line cou-

pling of the SIA-monolith technique has been introduced; some problems encountered during the first development of this coupling are discussed in ref. [21].

The major criterion of the work was to develop a simple SIC assay to be used in routine control of these drugs in pharmaceutical tablets. Therefore, this work focused on optimization of the conditions for simple and fast, as well as low cost analysis, including selection of the appropriate column or mobile phase to obtain satisfactory results, free of interference from excipients, robust and straightforward enough for routine use in pharmaceutical analysis.

From the UV spectra of all the analysed compounds, the optimal detection wavelength was 210 nm, providing sufficient sensitivity without derivatization of compounds. On the other hand, this commercial SIA system permitted simultaneous measurement of four different wavelengths. The second wavelength used was 230 nm for better peak identification.

The choice of appropriate internal standard was made from several compounds (methylparaben, ethylparaben, salicylic acid, and benzoic acid), all having similar characteristics to the compounds determined. Some of these compounds showed long retention times or partial interference with the peak of analyte. Benzoic acid was sufficiently separated from other compounds of interest and prolonged the analysis time only slightly.

To obtain satisfactory resolution and to avoid peak tailing of compounds, optimization of the proposed method was carried out using different mobile phases. For successful separation of compounds, the different total column lengths 25, 35, and 50 mm were tested. The retention characteristics of the compounds were similar. The choice of the 25 mm column length was suitable for separation of compounds of interest with sufficient resolution. Use of the mobile phase methanol:0.01 M citric buffer (pH from 3.0 to 4.3) gave asymmetrical peaks with long retention times and with tailing. Mobile phases of various compositions of acetonitrile, methanol, and water were also tested. Using the system acetonitrile:methanol:0.01 M citric buffer 5:5:90 (v/v/v) (pH tested range 3.0–4.5) the peak tailing and long retention times were not suppressed. With the use of acetonitrile instead of methanol, all the compounds were well separated, reducing peak tailing. The best results were obtained using the mobile phase acetonitrile:0.01 M phosphate buffer 10:90 v/v (pH 4.05 adjusted with phosphoric acid), flow rate 0.6 mL min^{-1} . The optimisation of the pH of the mobile phase was one of the important conditions of the separation. With decreasing pH of the mobile phase the analysis time became longer due to the decreasing ionization of BA and ASA. Higher pH values resulted in low resolution between CAF and ASA, and decreasing retention time of BA. The mobile phase pH value of 4.05 was chosen from the tested range

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

Unit	Command	Parameter	Action
Loop start (#) 3			Start of analysis
Syringe pump	Valve in		Set valve position
Syringe pump	Flow rate [$\mu\text{L s}^{-1}$]	100	
Syringe pump	Aspirate [μL]	3300	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 PAR Syringe pump	Flow rate [$\mu\text{L s}^{-1}$]	10	
Syringe pump	Aspirate [μL]	25	Sample aspirated
Syringe pump	Delay until done		
Valve	Port 8		Set port position
Syringe Pump	Flow rate [$\mu\text{L s}^{-1}$]	10	
Syringe Pump	Dispense [μL]	1000	Sample propelled through the column, PAR eluted
Spectrometer	Reference scan		
Spectrometer	Absorbance scanning		Peak of PAR recorded
Syringe pump	Delay until done		
Spectrometer	Stop scanning		
Analyte name CAF Syringe pump	Flow rate [$\mu\text{L s}^{-1}$]	10	
Syringe pump	Dispense [μL]	550	CAF eluted
Spectrometer	Absorbance scanning		Peak of CAF recorded
Syringe pump	Delay until done		
Spectrometer	Stop scanning		
Analyte name ASA Syringe pump	Flow rate [$\mu\text{L s}^{-1}$]	10	
Syringe pump	Dispense [μL]	700	ASA eluted
Spectrometer	Absorbance scanning		Peak of ASA recorded
Syringe pump	Delay until done		
Spectrometer	Stop scanning		
Analyte name BA Syringe pump	Flow rate ($\mu\text{L s}^{-1}$)	10	
Syringe pump	Empty		BA eluted
Spectrometer	Absorbance scanning		Peak of BA recorded
Syringe pump	Delay until done		
Spectrometer	Stop scanning		
Loop end	End of analysis		

of 3.0–4.5 as a compromise between peak resolution and time of analysis. The total mobile phase volume for one analysis was 3.3 mL and the time required less than 6 min under optimal conditions.

Peak height evaluation was performed using the FIALab® software. The typical sequence of individual steps of the programme is given in **Table 1**.

Table 2. Parameters of SIC process.

	Paracetamol	Caffeine	Acetylsalicylic acid	Benzoic acid
Retention time [s]	112.3	160.0	238.5	331.5
Peak resolution	$R_{\text{PAR,CAF}} = 7.7$	$R_{\text{CAF,ASA}} = 7.5$	$R_{\text{ASA,BA}} = 5.6$	
Peak symmetry	2.75	2.25	1.41	1.30
Number of theoretical plates	3302	2392	1836	1522

Table 3. Analytical parameters and method validation results.

	Paracetamol	Caffeine	Acetylsalicylic acid	Benzoic acid
Calibration range [$\mu\text{g mL}^{-1}$] ^{a)}	0.5–75	0.5–50	0.5–50	0.5–100
Correlation coefficient	0.9997	0.9999	0.9993	0.9995
Limit of detection [$\mu\text{g mL}^{-1}$]	0.5	0.3	0.3	0.8
Limit of quantification [$\mu\text{g mL}^{-1}$]	1.6	1.0	1.0	2.7
System precision (%) ^{b)} 20 $\mu\text{g mL}^{-1}$	1.82	1.25	1.13	1.76
5 $\mu\text{g mL}^{-1}$	1.25	1.10	1.21	2.63
LOQ	2.43	2.87	3.60	4.30
Repeatability of T_r [%] ^{c)}	0.28	0.29	0.37	0.43

a) Each concentration was measured in triplicate.

b) Relative standard deviation (RSD) values were calculated for intra-day repeated standard injections at three concentration levels $c = 20$ [$\mu\text{g mL}^{-1}$], $c = 5$ [$\mu\text{g mL}^{-1}$], and $c = \text{LOQ}$; $n = 10$.

c) Repeatability of T_r – RSD of retention times for intra-day repeated standard injections, $n = 10$.

3.2 Parameters of sequential injection chromatography process

The basic chromatographic parameters of the sequential injection chromatography system were calculated from experimental data, such as peak symmetry factor, resolution factor, number of theoretical plates and intra-day repeatability of the retention times and they are given in **Table 2** and **Table 3**.

The parameters characterising the robustness of the method (its ability to remain unaffected by small changes in the parameters such as organic modifier content and pH of the mobile phase, buffer concentration, temperature, and injection volume) were discussed. Due to the short length of the column the maximum content of organic modifier was 12% (v/v) of acetonitrile in the mobile phase. Contents of acetonitrile higher than 12% (v/v) resulted in peak resolutions lower than 1.5, mainly for PAR and CAF. Lower contents of acetonitrile (<10%) (v/v) caused undesirable peak tailing and increased analysis time. Due to the different acid-base characteristics of the compounds, optimisation of the mobile phase pH was one of the important conditions for separation. The opti-

imum pH for successful separation was in the range 3.95–4.15. At pH <3.95, the resolution between CAF and PAR decreased (less than 1.5) and the retention time of ASA and BA was prolonged. At pH >4.15, low resolution (less than 1.5) was observed between the peaks of ASA and CAF, and the retention time of BA was shortened. The influence of temperature on the separation was also studied. At mobile phase and column temperatures above 25°C an undesirable decrease of peak resolution was observed. Concentration of the phosphate buffer did not influence the shape of the peak and resolution in the tested range 0.1–0.01 M.

The SIC chromatogram obtained on analysis of ACIFEIN® tablets (containing PAR, CAF, ASA, and BA as internal standard) is depicted in **Figure 2**. For sake of comparison an HPLC chromatogram obtained under the same conditions as in the SIC system (identical mobile phase and same flow rate) is depicted in **Figure 3**. Differences in retention times between the SIC system and HPLC are caused by different dead volumes of the systems and by different inner diameters of flow lines.

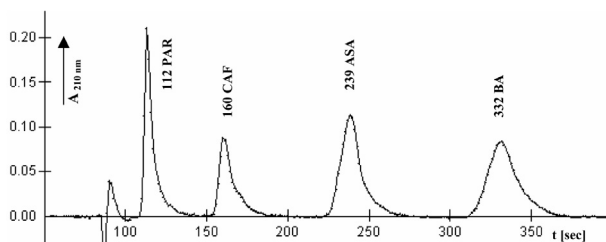


Figure 2. SI chromatogram of the separation of the compounds in ACIFEIN® tablets. Mobile phase: acetonitrile-(0.01 M) phosphate buffer (10:90, v/v) pH 4.05, flow rate 0.6 mL min⁻¹, UV detection at 210 nm.

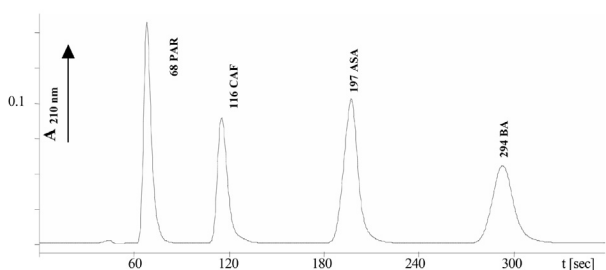


Figure 3. HPLC chromatogram of the separation of the standard solutions under the same conditions as well as the SIC system.

3.3 Analytical parameters and validation

The validity of the SIC assay was established through a study of linearity, sensitivity, repeatability, and recovery.

Linearity was established with a series of working solutions prepared by diluting the stock solution with mobile phase to the final concentrations. Each concentration was injected in triplicate and the mean value of peak height was taken for the calibration curve. The calibration graphs involved at least 8 experimental points for each compound and they are described by the following equations: for PAR: $A = (0.015447 \pm 0.000188)c - (0.002578 \pm 0.004049)$, where A is the absorbance and c the analyte concentration, the correlation coefficient was 0.9997; for CAF: $A = (0.027808 \pm 0.000116)c + (0.001192 \pm 0.001308)$, the correlation coefficient was 0.9999; for ASA: $A = (0.007280 \pm 0.000121)c + (0.002731 \pm 0.002620)$, the correlation coefficient was 0.9993; for internal standard BA: $A = (0.028537 \pm 0.000367)c + (0.001420 \pm 0.007912)$, the correlation coefficient was 0.9995.

The limit of detection (LOD) was calculated by comparison of the three-fold variation of signal to noise ratio ($3 S/N$) obtained from analysis of the standards, and the limit of quantification (LOQ) was defined as the lowest measured quantity above which the analyte can be quantified at a given statistical level of $10 S/N$.

The intra-day precision of the method was determined by preparing the standards of PAR, CAF, ASA, and BA at

three concentration levels and peak heights for each compound were determined after processing each standard 10 times.

The method validation results obtained under the final conditions are shown in Table 3. The method was found to fulfil common requirements of accuracy, precision, and linearity (calibration range with correlation >0.999 , RSD for repeated standard injections at three concentration levels ($n = 10$) less than 4.3%, correlation with the HPLC determination (t -test)).

To validate the precision of the method, 5 different sample solutions were used, which were prepared from the same batch and analysed consecutively. This approach provides a means of covering the precision of the entire method, from sample preparation to data handling. The accuracy of the method ascertained by measuring the samples fortified with known quantity of the analytes. The procedure was performed under the following conditions: dissolution of one average tablet mass (ACIFEIN®) in 100 mL of methanol solution containing internal standard BA at a concentration of 2500 $\mu\text{g mL}^{-1}$, ASA at a concentration of 2000 $\mu\text{g mL}^{-1}$, PAR at a concentration of 2000 $\mu\text{g mL}^{-1}$, and CAF at a concentration 500 $\mu\text{g mL}^{-1}$. Comparing the results of the samples with and without standards addition gave values of the recoveries $>96.2\%$.

3.4 Determination in a pharmaceutical product

The applicability of the method for the simultaneous determination of PAR, CAF, and ASA was verified by determination of these compounds in different pharmaceutical tablets with high recovery values. No interference from excipients (glucose, lactose, starch, and magnesium stearate) was observed. The results were compared with those obtained by HPLC under the same chromatographic conditions. The statistical t -test (95% level) revealed no significant difference between the average values found by both methods.

The optimal extraction medium for tablet dissolution was methanol due to the high solubility of the standards in this solvent. All compounds present in the samples of ACIFEIN®, ACYLCOFFIN®, and PANADOL EXTRA® were clearly separated. Determination of the active substance in the tablets was carried out with 5 samples having the average weight of a tablet (see sample preparation). The average labelled amounts PAR, CAF, and ASA in the ACIFEIN®, ACYLCOFFIN®, and PANADOL EXTRA® tablets are given in Table 4, where the results of assays by the SIC system are compared with those obtained by the conventional HPLC method. Both results are in good agreement with the pharmacopoeial requirements for the active compound content in pharmaceutical tablet preparations (range 85.0–115.0%) [22].

Table 4. Determination of paracetamol, caffeine, and acetylsalicylic acid in different pharmaceutical preparations.

Pharmaceutical preparation	Composition	Labelled amount [mg]	n	Recovery [%]	
				SIC	HPLC
PANADOL EXTRA®	PAR	500	5	103.0 ± 1.8	102.0 ± 1.6
	CAF	65	5	98.2 ± 0.7	98.8 ± 0.9
ACYLCOFFIN®	CAF	50	5	104.3 ± 1.9	103.1 ± 1.2
	ASA	450	5	97.4 ± 2.1	98.0 ± 2.3
ACIFEIN®	PAR	200	5	99.6 ± 1.8	98.3 ± 1.5
	CAF	50	5	104.8 ± 2.3	103.2 ± 1.4
	ASA	250	5	98.5 ± 2.6	100.9 ± 2.1

4 Concluding remarks

The SIC method with monolithic column and UV spectrophotometric detection was developed successfully for the determination of all compounds in different pharmaceutical tablet formulations, using benzoic acid as internal standard. The total analysis time was less than 6 min. The method has been validated and the results obtained were precise and accurate. The proposed method is simple and rapid and can be used for routine analysis of compounds in pharmaceutical products.

SIA is a non-separation flow analytical method. The coupling of the short monolith with the sequential injection manifold results in a new approach to implementing the separation step in a hitherto non-separation low-pressure flow method. The sequential injection chromatography system that was developed presents several clear advantages over the methodologies usually adopted. The main one is the possibility of carrying out simple separation analysis without expensive HPLC instrumentation. The low consumption of organic solvents and the low waste production due to non-continuous flow, the low cost per analysis, and the variability of the combination of a number of mobile phases by switching of an 8-port selection valve to defined positions using a simple program are by no means the least advantages of the described system. Moreover, the total price of the commercially available system (FIALab® Instruments, USA) is about one third of the equipment purchase price of commonly available HPLC instrumentation.

However, the SIC system does have some drawbacks of importance for chromatographic assays. Mention should be made of insufficient software for separation analysis evaluation (compared with the common HPLC software), limited flow rates of the syringe pump (to the maximum back pressure about 2.5–3.0 MPa, depending on the inner diameter of the syringe), limited volume of the syringe pump (commercially available maximum is 10 mL),

and limited possibility of separation due to restriction of the column length and flow rate.

In summary, due to the large number of experiments and extensive testing of the SIC system, it is possible to gain an idea of the power of the SIC technique mainly for the simple separation analysis of samples containing a maximum of 2–5 compounds. However, coupling of the monolithic columns with the sequential injection system provides another tool to solve simple separation problems rapidly and efficiently without any need for HPLC instrumentation.

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