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Automation of simultaneous release tests of two substances by sequential injection chromatography coupled with Franz cell

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Abstract

This presented paper deals with a methodology for the separation and simultaneous determination of two active substances in topical pharmaceutical formulation composed of lidocaine (L) and prilocaine (P). The methodology described is based on the sequential injection chromatography (SIC) with UV detection. Monolithic Column Chromolith Flash RP-18, 25 mm × 4.6 mm (Merck, Germany) was used. Separation was performed using elution with binary mobile phase composed of acetonitrile–phosphate buffer 0.05 M (40:80 (v/v)) + 0.01% triethylamine (adjusted to pH 7.1 with H_3PO_4) at a flow rate of 0.6 ml min^{-1}. The analysis duration was <7 min. The method was linear over the range of 2.5–200 mg l^{-1} with a detection limit of 0.25 mg l^{-1} for both substances.

The system was then coupled with Franz cell. Fully automated system for the in vitro release testing of semisolid dosage forms based on sequential injection analysis (SIA) was developed. Simultaneous measurement of L and P release was done by this system. Samples were taken in 10.5 min intervals during 4 h of the release test. Each test was followed by calibration with five standard solutions. Receiving medium was replenished automatically by the system.

Keywords: Sequential injection chromatography; Local anaesthetics; In vitro release studies; Automation

1. Introduction

The key parameter for any drug product is its efficacy. The time and expense associated with clinical trials make them unsuitable as routine quality control methods. Therefore, in vitro surrogate tests are often used to assure that product quality and performance are maintained over time and in the presence of change. A variety of physical and chemical tests have historically been provided. More recently, in vitro release testing has been used. An in vitro release rate can reflect the combined effect of several physical and chemical parameters, including solubility and particle size of the active ingredient and rheological properties of the dosage form. In most cases, in vitro release rate is a useful test to assess product correspondence between prechange and postchange products [1].

Although official methods have been developed for dissolution test studies of solid dosage forms, which serve as a routine indicator of batch-to-batch uniformity or another method in the USP 28 [2] to study active substance release from transdermal delivery patch systems, no official rule for the performance of release testing of semisolid dosage forms is given. The only existing recommendations are guidelines provided from OECD [3] and FDA [1].

Device for release measurement recommended by FDA and OECD is so called Franz diffusion cell. Usually six cells are recommended, but systems with three or only one cell are mentioned as well. The Franz diffusion cell (Fig. 1) consists of two parts – donor part and acceptor part – those are separated by a membrane. The donor compartment holds the drug preparation and the acceptor compartment the receiving medium. For release experiments, normally artificial membranes are used to separate the donor and acceptor compartment physically. The membrane should allow the active ingredient readily diffuse to receiving medium as it is "released" from the dosage form and is not rate limiting for the diffusion. The acceptor medium should be mixed to ensure the uniformity of diffusion and the concentration homogeneity. Diffusion is temperature dependent, thus the acceptor fluid must be maintained at a constant temperature. While carrying out the experiment, samples of acceptor medium are drawn off through the sampling port of the Franz

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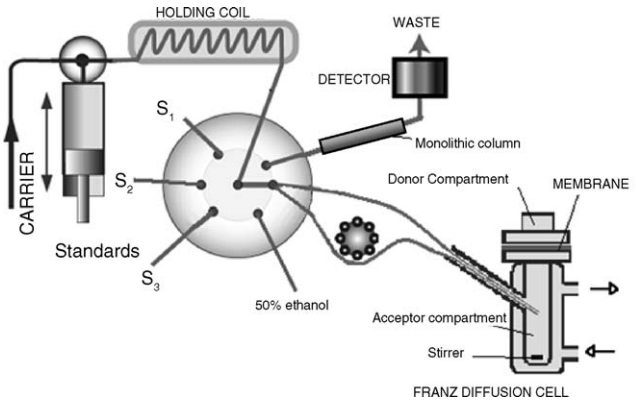

Fig. 1. The scheme of SIC system with Franz cell for release test measurement.

cell. Manually provided tests, where samples are taken by pipette and subsequent analysis by e.g. HPLC is followed, are time and labour consuming.

Sequential injection analysis (SIA) has been proposed by Růžička and Marshall as an efficient tool for automated liquid handling [4]. The technique is based on forward and reverse movements of a piston of a syringe pump, which together with a multi-position selection valve enables precise sampling of chemicals into the system and propelling of the sequenced zones to the reactors and detector [5]. Automation, velocity of the analysis and low consumption of sample and reagents are the most important features that favour the SIA technique for application in many fields of analysis, primarily for monitoring long term processes. SIA was used for dissolution tests of various pharmaceutical formulations, e.g. ibuprofen tablets [6], analgetic tablets with aspirin, phenacetin and caffeine [7], and tablets with ergotamine tartrate [8]. The utilization of SIA in pharmacy was summarized in review [9].

Sequential injection chromatography (SIC) system was created by coupling a monolithic chromatography column into a common sequential injection analysis (SIA) manifold [10]. The monolithic columns possess a much higher porosity than conventional particulate high pressure liquid chromatography (HPLC) columns and can be coupled to the SIA system without loss of performance or limitations due to the very low column back-pressure. The performance of the reverse-phase monoliths is equivalent to a typical C18 5 μm particulate HPLC column [11].

The main objective of this work was to develop a new method for simultaneous determination of two compounds in a topical pharmaceutical formulation. Then application of the method to release tests by coupling SIC system and Franz diffusion cell to automate simultaneous release study of two local anaesthetics was carried out.

Systems for release study of one compound without monolithic column based on SIA apparatus were suggested by our group for indomethacin [12] and salicylic acid [13] formulations evaluation.

Lidocaine (2-diethylamino-*N*-(2,6-dimethylphenyl)acetamide) and prilocaine (*N*-(2-methylphenyl)-2-(propylamino)-propanamide) (see Fig. 2) are topical anaesthetics, frequently used to avoid pain during venipuncture and superficial surgery.

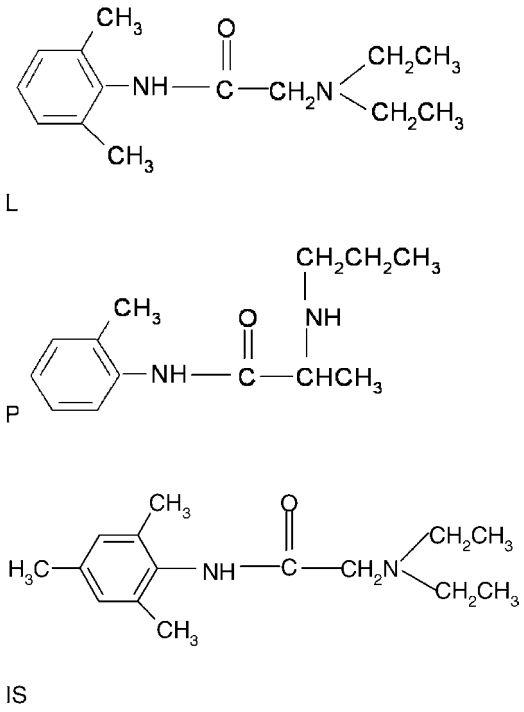

Fig. 2. The chemical structures of lidocaine (L), prilocaine (P) and trimecaine (IS).

Some papers dealing with lidocain and prilocain analyses are listed below: a set of 17 samples containing a constant amount of lidocaine (667 μM) and a decreasing amount of prilocaine (667–0.3 μM) was analysed by LC-DAD at three different levels of separation, followed by parallel factor analysis (PARAFAC) of the obtained data [14]. Solid phase microextraction and GC–MS was used for monitoring in blood [15]. HPLC-UV was also used for analysis in blood [16]. Octanol/water partition coefficients of local anaesthetics were determined by HPLC [17]. Lidocaine was analysed in human serum by HPLC-UV [18], and GC with SPE [19]. Also short-capillary electrophoresis with electrochemiluminescence detection was used for lidocaine determination [20]. No work has described automation of liberation profiles of L and P.

2. Experimental

2.1. Reagents

All solutions were prepared from analytical grade chemicals and Millipore (Bredford, MA, USA) Milli-QRG ultra pure water. Acetonitrile, supragradient, was obtained from Biotech (Scherlau Chemie, Germany). HPLC grade methanol was provided by Sigma–Aldrich (Prague, Czech Republic). Phosphoric acid, 85% p.a. and sodium hydrogen phosphate were purchased from Merck (Darmstadt, Germany). Triethylamine was obtained from Fluka.

Standards of prilocaine and lidocaine were obtained from MP Biomedicals (Eschwege, Germany). Internal standard stock solution was prepared by weighing 0.05 g and dissolving in

50 ml of 50% ethanol or methanol. Stock solution of the standards was prepared dissolving 0.05 g of prilocaine and 0.05 g of lidocaine in 50 ml methanol or 50% ethanol, respectively. Working solutions were prepared by appropriate dilution of stock solution with methanol or 50% ethanol. An amount of 2.5 ml of stock solution of internal standard was added to each 50.0 ml standard solution.

Internal standards tested: benzocaine, trimecaine and procaine, were obtained from Kulich (Hradec Kralove, Czech Republic). The working concentration of internal standards was 50 mg l^{-1}.

The tested topical preparations: EMLA cream (AstraZeneca AB, Södertälje, Sweden) contained eutectic mixture of local anaesthetic (25 mg of lidocaine and 25 mg of prilocaine per 1 g).

The ointment with content of 2.5% of lidocaine and 2.5% of prilocaine was tested. Composition of ointment base was following: vaselinum album 42.33 g, adeps lanae 23.00 g, cera alba 2.00 g, paraffinum liquidum 25.42 g, paraffinum solidum 1.83 g, monoglycerida adipis suilli 0.17 g, ceresinum 5.25 g/100 g of ointment base.

Membranes tested were obtained from MILLIPORE (Bredford, MA, USA): polycarbonate HTTP, pore size 0.45 μm; polycarbonate GTTP, pore size 0.2 μm and mixed cellulose esters HAWP, pore size 0.45 μm.

2.2. Apparatus

The connection of SIC system and Franz cell for liberation tests measurements is depicted in Fig. 1. It was built of the FIAlab 3500 (FIAlab Instruments, USA) commercial system comprising of 5.0 ml piston pump, peristaltic pump and six-port selection valve. The whole tubing of the SIA system was made of PTFE, i.d. 0.5 mm.

Monolithic column Chromolith Flash RP-18, 25 mm × 4.6 mm (Merck, Germany) was used in the system. It was placed between UV detector and selection valve. UV detection was set up at 212 nm (near absorption maxima for both substances).

Franz cell (release unit) is a double-wall thermostated vessel with a precise inert volume of 15.00 ml. Its parameters of acceptor compartment are: width 3.6 cm, height 6.0 cm, inner diameter 2.0 cm; donor compartment: cap width 3.6 cm, cap height 2.0 cm. These compartments are separated by membrane. The acceptor compartment of the Franz cell was filled up to the label on the side inlet. The side inlet was used for insertion of loading and filling tubes (both parts of loading circuit, which was 60 cm long). The inside solution was mixed (400 rpm) by an electromagnetic stirrer (IKA Labortechnik, Stauffen, Germany). The temperature was maintained at 32 °C by thermostat (Medingen, Dresden, Germany) by inlet and outlet of the double-walled Franz cell.

The comparative HPLC system consisted of a binary pump LCP 4100 (Ecom, Prague), Waters autosampler 717 plus, UV Detector Waters 486 (Waters, Milford, MA, USA) and chromatographic software CSW v.1.7 for Windows (Data Apex s.r.o., Prague, Czech Republic). Analyses were performed on the same above mentioned column.

2.3. Sample preparation, determination in the pharmaceutical preparation

About 0.1 g of EMLA cream was weighted and transferred into 50.0 ml volumetric flask. An amount of 2.5 ml of internal standard (1000 mg l^{-1}) was added, filled up by 50% ethanol and sonicated for 5 min. A volume of 10 μl was analysed by the SIC system. Identification of peaks in the pharmaceutical cream samples was based on the comparison of the retention times of compounds in the standard solutions. Quantification of the analysed compounds was performed using the method of the internal standard and comparison of the particular peaks in the record of the standard and sample solutions.

2.4. Release test method

For release system testing ointment with contents of 2.5% of each anaesthetic (lidocaine and prilocaine) was used. As acceptor solution widely used 50% ethanol was chosen. Firstly the membrane was soaked by the acceptor medium for 1 h. The acceptor solution was mixed (400 rpm) and thermostated at ± 32 °C. After that appropriate amount (to have thickness about 1 mm) of the tested ointment was placed on the membrane. The precise amount of applied drug is not known, but for kinetic reasons it is essential to have a reservoir of the drug always available to diffuse through. This corresponds to an infinite dosing.

After drug application the release test was started. The peristaltic pump was switched on and maintained at a constant flow (2.5 ml min^{-1}). The receiving medium constantly flowed through the sampling circuit to ensure that the concentration of the taken sample was the same as in the donor compartment of the Franz cell. The peristaltic pump was switched off after 300 s and 10 μl of the sample was aspirated by piston pump from sampling circuit. Peristaltic pump was switched on and analysis with monolithic column and UV detection took place. The acceptor compartment side of the cell was filled up with 10 μl of fresh receiving medium by aspirating stock solution from respective port of the selection valve by the syringe pump. Whole cycle was repeated for period of 4 h.

The sample uptake and its compensation by fresh acceptor medium caused modification in the active substance concentration. Recalculation of the measured concentration to the real values followed this equation:

$$C_{n,\text{corrected}} = C_{n,\text{measured}} + \text{volume}_{\text{sample}}/\text{volume}_{\text{acceptor,FDC}}\,\Sigma C_{n-1,\text{measured}}$$

where C_n is the concentration of n-sample, and FDC is the Franz diffusion cell.

The cumulative drug amount (Q_n) permeated at each time point related to the area of tested membrane was obtained as follows: $Q_n = C_{n,\text{corrected}}\text{volume}_{\text{acceptor,FDC}}/\text{diffusion area}$

The measurement was repeated in six replicates. The data were summarized in graph—linear plots were obtained by plotting the cumulative amounts released (μg/cm^{-2}) per square root

of time (h^{-1}). This is according to Higuchi's [21] models for release of both completely dissolved (I) or suspended (II) drug from semisolid preparation:

$$\text{model I}: Q = A2c_0\sqrt{Dt/\pi}$$

$$\text{model II}: Q = A\sqrt{[Dtc_s(2c_0 - c_s)]}$$

where Q is the amount released at time t, A the diffusion area (cm^2), D the diffusion coefficient ($cm\,h^{-1}$), t the time (h), c_s the saturation concentration, and c_0 is the concentration in the semisolid preparation at $t = 0$.

From these equations emerge that the release should be linear with the square root of time (irrespective if the drug is suspended or dissolved), if the release is governed via a matrix controlled diffusion process. The slope of the regression line represents the release rate of the product. This release rate is formulation-specific and can be used to monitor product quality.

3. Results and discussion

3.1. Method optimisation

Firstly the method for application of RP-18 monolithic column to the SIA system for lidocaine and prilocaine determination was developed. The different composition of mobile phase (mixture of methanol, acetonitrile, water and phosphate buffer in different ratios) for successful separation of lidocaine, prilocaine and IS (internal standard-trimecaine) was tested. Different pH of mobile phase was studied, to obtain good separation within relatively short analysis time. The volume of the mobile phase per one analysis should be <5 ml (the volume of the piston pump). The flow rate was limited by the use of low pressure syringe pump. With higher flow rates, the pressure increased. Thus the flow rate was set at 0.6 ml/min. The optimal mobile phase was acetonitrile–phosphate buffer 0.05 M (40:80 (v/v)) + 0.01% triethylamine, pH 7.1 adjusted with phosphoric acid. Mobile phase was degassed before application by means of helium. The addition of triethylamine to the mobile phase resulted in decreasing of peak tailing and better peak asymmetry. Several internal standards were tested and trimecaine (2-(diethylamino)-*N*-(2,4,6trimethylfenyl)acetamid) was found as optimal. The peak of trimecaine was well separated. The retention time was longer than of lidocaine and prilocaine, but the total time of the analysis was <7 min. The total volume for one analysis was 3.8 ml of mobile phase. The volume of taken sample was 10 μl.

The obtained SIA chromatogram of lidocaine, prilocaine and internal standard (trimecaine) is depicted in Fig. 3. All compounds presented in the sample (prilocaine, lidocaine) and internal standard were clearly separated. The optimised method was validated by a standard procedure. All validation procedure was calculated on ratios between peak height of the tested substance and peak height of internal standard. It was counted on peak heights, not areas, because the software is not able to detect peak areas.

Linearity was established with a series of working solutions prepared by diluting the stock solution with 50%ethanol

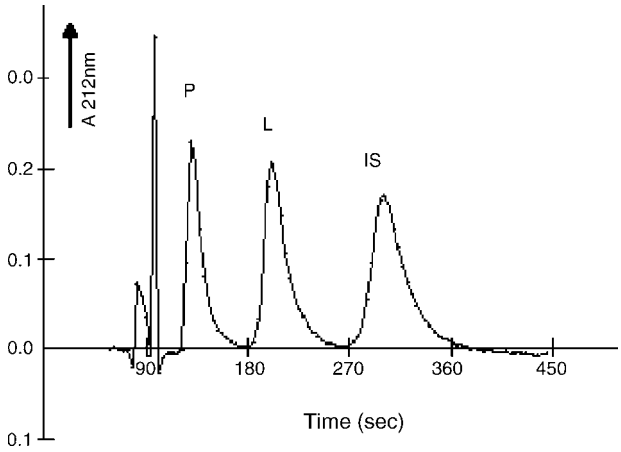

Fig. 3. SIA chromatogram of EMLA cream analysis. P, prilocaine; L, lidocaine; IS, internal standard (trimecaine).

to the final concentrations. Concentrations used for each compound were as follows: 2.5, 5, 10, 50, 100, 150 and 200 mg l^{-1}. Each concentration was injected in triplicate. The calibration curve was calculated from the mean value of the ratios between peak height of the known concentration and height of the internal standard for each concentration level. The calibration graphs are described by the following equations: for P: $A = (0.0636 \pm 0.0026)c - (0.36 \pm 0.27)$ (where A is the absorbance and c is the analyte concentration), the correlation coefficient was 0.9957; for L: $A = (0.03171 \pm 0.00081)c - (0 \pm 0.084)$, the correlation coefficient was 0.9984.

The limit of detection (LOD) was calculated by comparison of the three-fold variation of signal to noise ratio (3S/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 (10S/N).

Repeatability was calculated as R.S.D. for six injections of the standard with concentration 50 mg l^{-1}. It was calculated on peak heights only. The results of R.S.D. counted on peak ratios for prilocaine and lidocaine were 2.62 and 2.10, respectively.

Visual inspection of chromatograms of standards and placebo solutions was conducted to ensure selectivity. The SIA chromatogram of placebo of the ointment is shown in the Fig. 4. None interference peak was found in the retention time of all compounds after the placebo sample injection.

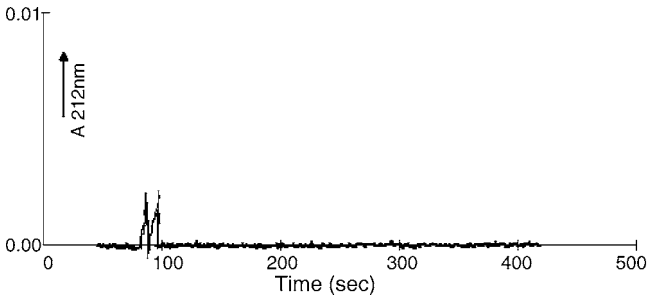

Fig. 4. SIA chromatogram of placebo.

Table 1
Summary of SIC analysis parameters

	Prilocaine	Lidocaine	IS trimecaine
Calibration range (μg ml^{-1})	2.5–200	2.5–200	
Correlation coefficient	0.9957	0.9984	
Limit of detection (μg ml^{-1})	0.25	0.25	
Limit of quantification (μg ml^{-1})	0.75	0.75	
Repeatability (% R.S.D.) (concentration 50 μg ml^{-1}, $n=6$)	2.44	1.89	1.16
Peak asymmetry	2.0	1.86	1.44
Peak resolution	$R_{P,L}=2.8$	$R_{L,IS}=3.6$	
Retention time (s)	146	207	297
Repeatability of retention time (%) (concentration 50 μg ml^{-1}, $n=6$)	0.54	1.73	1.72
Accuracy (% recovery of spiked cream)	103.96	102.40	
Selectivity	No interferences	No interferences	No interferences
SIA cream analysis (recovery, % of labelled amount)	97.25	100.57	
HPLC cream analysis (recovery, % of labelled amount)	98.11	100.23	

Intermediate precision was set by comparing the results of assays in the SIC with the conventional HPLC determination. It was carried out under the same conditions as well as the SIC system. The results for three injections for average contents of P and L were 98.15% and 100.23% of the labeled amount, respectively. There was no statistical difference between these two methods.

Accuracy of the method was set by spiking of the EMLA cream with the solution of known concentration of 50 mg l^{-1}. The percentage of recovery is shown in the Table 1.

To demonstrate the efficiency of the SIC technique, the relative standard deviation of the retention times for the intra-day repeated standard injections ($n=6$), the peak resolution and peak asymmetry were calculated.

Results obtained under the final conditions are shown in Table 1.

3.2. Release test results

The drug release studies were carried out using polycarbonate membrane with pore size 0.2 μm. The differences in release rate and lag time between other tested membranes were negligible in preliminary testing. No inhibition in drug release as well as a proper separation of the preparation in donor part and acceptor phase was found with polycarbonate membrane. The release medium was chosen 50% ethanol because of good solubility of tested substances. Also methanol was tested, but the evaporation was quite high and the results were not so reproducible.

The method without internal standard was used. The volume of mobile phase needed per one analysis was only 2.8 ml. Concentration data were evaluated for 4 h. The amount of sample taken was small and the receiving medium was compensated, thus, the sampling frequency could be expanded without spoiling of the release process. The number of taken samples was enough for linear graph construction (22 points versus recommended six points). After each measurement calibration curve was generated. (standard solutions 2.5; 5; 10; 50; 100 and 150 μg ml^{-1} were utilized).

An x-intercept, typically corresponding to a small fraction of an hour, is normal characteristic of such plots and is called

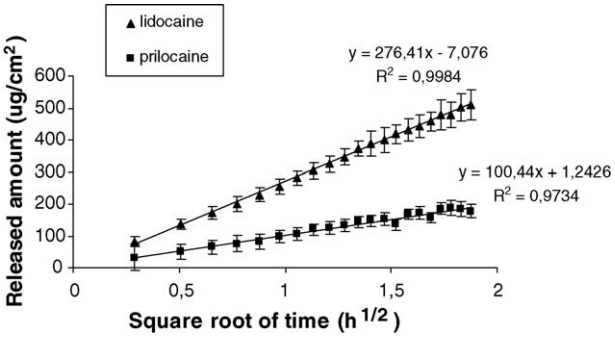

Fig. 5. Linearized release profiles of lidocaine and prilocaine.

"lag time". The lag time corresponds to the time of delay during the first contact of the drug with the membrane surface until steady state flux conditions are reached [22]. The lag time was 0.025 h$^{1/2}$ for lidocaine and −0.012 h$^{1/2}$ for prilocaine.

One batch of the ointment formulation was measured six-times for 4 h. It means the whole procedure from spreading of the ointment was repeated six-times. The average values of release profiles (± R.S.D.) of prilocaine and lidocaine, obtained in simultaneous measurement, are shown in Fig. 5. The release rate was 276.4 μg cm^{-2} h$^{-1/2}$ for lidocaine and 100.4 μg cm^{-2} h$^{-1/2}$ for prilocaine. The correlation coefficient of the plotted line was 0.99 for lidocaine and 0.97 for prilocaine. The R.S.D. in these six measurements was from 7.1 to 10.0%.

4. Conclusion

The sequential injection chromatography (SIC) system with implemented separation component monolithic column was successfully used for lidocaine and prilocaine simultaneous determination in pharmaceutical preparation. The results were compared with HPLC analysis. The advantages were lower organic solutions consumption, because of non continual flow in the SIC system, and less expensive instrumentation.

The proposed methodology based on the SIC with UV detection was successfully applied for the release testing of semisolid preparations. Simultaneous release profiles of two above men-

tioned local anaesthetics were obtained. The combination of the SIC with the Franz cell gave a unique automated system for in vitro release testing. Automated system for simultaneous release study of two substances was presented for the first time.

During all procedures no human control was needed. The volume of taken sample was small (10 μl) and the replenishing of acceptor medium was automated. Another advantage was that the samples were measured on-line, and thus results can be easily monitored. There was no delay between sample collection and sample measurement. Such a system could be favourably used for manufacturing process control, for monitoring of pre- and post-changes of product properties, batch uniformity monitoring, etc. The SIA is a relatively simple and adapted method and could be also used as a screening device in pre-formulation and product development.

Acknowledgements

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