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Automated sample preparation coupled to sequential injection chromatography: On-line filtration and dilution protocols prior to separation

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ABSTRACT

Sequential injection chromatography (SIC) is a valuable tool in analytical chemistry as it can combine the automation capabilities of low pressure continuous flow techniques and the separation power of HPLC into a single instrumental configuration. The present study reports an automated SI setup allowing on-line filtration and dilution of the samples before separation through a short monolithic column. The applicability of the procedure was evaluated by studying the behavior of acyclovir formulations under forced degradation conditions. Minimal sample preparation is required prior to analysis. Thorough validation of the on-line dilution SIC assay was carried out and proved its validity in terms of critical parameters such as precision, accuracy and robustness. The results were evaluated by parallel experiments and analysis using the procedure recommended by the USP based on conventional HPLC using particulate-based column.

1. Introduction

Flow and sequential injection analysis (FI and SI) are mature analytical techniques that have offered thousands of useful methods and approaches to the analytical community in all fields of applications. Their principles are well established [1,2], while major advantages include automation of sample preparation, increased throughput and precision, less consumption of reagents and generation of waste. On the other hand, until now, one of the drawbacks compared to high pressure separation techniques was the difficulty to perform simultaneous determinations of more than one analyte. The attempts towards this goal, although in many cases ingenious, were rather complicated or required strictly controlled conditions and were typically based on kinetic discrimination [3], on-line sample splitting [4] or usage of different detection systems [5]. The breakthrough in multi-species determinations by FI or SI was made very recently by the incorporation of short monolithic columns to low pressure continuous flow manifolds, introducing the concept of flow or sequential injection chromatography (SIC) [6].

Merck has recently commercialized silica-based reversed phase monolithic columns (Chromolith®) suitable for HPLC applications in many analytical fields [7]. The structural properties of monolithic stationary phases allow efficient separations typically comparable to 3–5 μm particulate columns at usually 10-times lower back pressures. This can be achieved by controlling independently the permeability (μm-sized throughpores) and efficiency of the analytical columns (nm-sized mesopores) [8]. The majority of the so far reported applications of SIC are oriented to pharmaceutical analysis, where SI is mainly used for sample introduction and propulsion through the column towards the detector [9]. Other fields of applications of SIC include environmental [10] and food analysis [11]. However, in our opinion the real potentials of the incorporation of monolithic columns to low pressure FI and SI systems have not yet been fully explored and demonstrated. The "power" of SIC lies on performing automated sample preparation steps (derivatization, solid phase extraction, dilution) prior to separation using the same low pressure experimental setup. Very limited reports towards this direction include the determination of amino acids after automated derivatization with o-phthalaldehyde [12], on-line solid phase extraction of pharmaceuticals from environmental samples [13] and generation of solvent gradients using programmed operation of two low pressure pumps with potentials to perform separation of more complex mixtures [14].

Sample dilution is an important pretreatment step in separation methods [15] as it (i) reduces the signals produced by endogenous components of the matrix, (ii) reduces the viscosity or ionic strength of the sample, (iii) ensures the compatibility of the sample with the mobile phase, (iv) adjusts the concentration of the sample to the range of the calibration graph [16], (v) modifies the matrix of the sample according to the demands of the following pretreatment steps [17] and (vi) protects the short analytical column from

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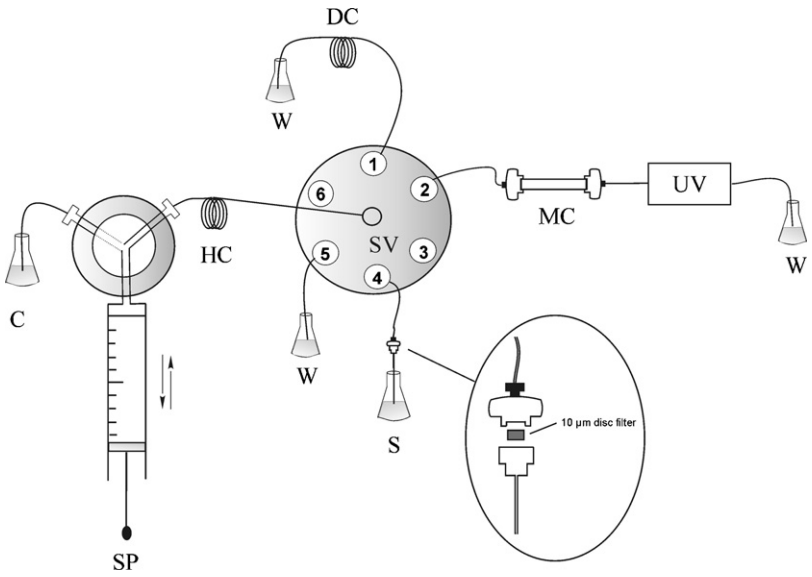

Fig. 1. Schematic representation of the SI setup: C, carrier (0.2% CH_3COOH); SP, syringe pump (V = 10,000 μl); HC, holding coil; SV ,selection valve; DC, dilution coil; MC, monolithic column (250 mm × 4.6 mm i.d.); S, sample; W, waste; UV, UV detector (254 nm).

overloading. In this context, SI offers significant possibilities and advantages over previously applied on-line dilution protocols. On-line dilution can be carried out by zone sampling in a practically single-channelled operation mode [18], without reconfiguration of the manifolds by adding extra dilution lines [19], dialysis units [20] or mixing chambers [21].

The aim of the present study was to expand the automation in sample preparation coupled to sequential injection chromatography by incorporating on-line filtration and dilution steps prior to separation, avoiding the need for any reconfiguration of the experimental setup. All necessary analytical steps (dilution, injection, separation) were programmed through suitable software. The applicability of such an approach was demonstrated by studying the stability of acyclovir formulations – an antiviral drug – under forced degradation conditions. The developed analytical scheme was fully validated, while the on-line filtration and dilution steps enabled the direct injection of the samples without any prior pretreatment by expanding the linear range of the calibration graph and protecting the short monolithic column from the highly acidic or basic matrix of the sample due to the applied stress conditions. The efficiency and reliability of the developed analytical scheme was further evaluated by independent experiments and analysis by the USP recommended HPLC procedure.

2. Experimental

2.1. Materials

All reagents were of analytical grade and provided by Merck, unless stated otherwise. Doubly deionized water was produced by a Millipore® system.

Acyclovir (lot no. ACP/WS/001/03, Assay = 99.9%) and guanine (lot no. GUN/WS/001/03, Assay = 99.6%) reference standards were provided by Matrix Laboratories Ltd. (India). Acyclovir standard stock solutions were prepared by dissolution of an accurately weighed amount in water. A 100 mg l^{-1} guanine standard stock solution was prepared by ultrasonically dissolving an accurately weighed amount (10.0 mg) in 100 ml of 0.01 M NaOH. The stock solutions were stable for at least 1 week if kept refrigerated and protected from light. It should be noted that the reported acyclovir concentrations are calculated on an anhydrous basis. Since acyclovir may contain up to 6% of water, it is necessary to determine its exact content by Karl–Fischer titration and make the appropriate corrections.

The mobile phase consisted of 0.2% (v/v) CH_3COOH (pH 3.0). It was filtered under vacuum through 0.45 μm filters and degassed ultrasonically for 30 min prior to use.

Excipients in acyclovir tablets (maize starch, microcrystalline cellulose, polyvidone, colloidal silicon dioxide and magnesium stearate) were purchased by local suppliers.

2.2. Instrumentation

A schematic diagram of the SIC setup is depicted in Fig. 1. The FIALab 3000 system (FIALab® Instruments, WA, USA) consisted of a 10000-μl syringe pump and a 6-port Cheminert® selection valve (Valco Instrument Co., Houston, USA). The whole system was controlled by the FIALab software. PTFE tubing (0.5 mm i.d.) was used throughout including connections and dilution coils. The volume of the holding coil (HC) was 700 μl, and of the dilution coil (DC) 1000 μl. A 25 mm × 4.6 mm i.d. monolithic column (Chromolith®, Merck) was used throughout this study. A Shimadzu SPD-10AV UV-Vis detector was coupled to SI, while data acquisition was carried out through the Clarity® software (DataApex, Czech Republic).

The HP 1100 HPLC instrument (Agilent Technologies, CA, USA) comprised a quaternary pump, a vacuum degasser, a column thermostat, an autosampler and a DAD spectrophotometric detector. Chromatograms and chromatographic parameters (peak areas, retention times, theoretical plates etc) were recorded and calculated respectively via the Chem Station® software.

The UV-irradiation unit was constructed by placing a 20 W mercury lamp in a cylindrical-shaped metallic box. A ventilator was adjusted at the bottom of the box to avoid overheating of the unit. The design of the unit allowed eight quartz tubes containing the samples to be placed around the lamp, enabling multi-samples process.

A model DL-18 Karl Fischer titrator (Mettler-Toledo) was used for the determination of the water content of acyclovir. The sampling probe and the disc filters (10 μm) for the on-line filtration protocol were provided by Distek.

Table 1
SIC sequence.

a/a	Time (s)	Valve position	Pump action	Flow rate (ml min^{-1})	Volume (μl)	Action description
1	194	N/A	Aspirate	3.0	9700	Filling of the syringe pump
2	1	4	Off	–	–	Selection of sample port
3	2	4	Aspirate	0.6	20	Aspiration of sample in the HC
4	1	1	Off	–	–	Selection of DC port
5	10	1	Deliver	0.6	100	Propulsion of sample to DC
6	1	5	Off	–	–	Selection of waste port
7	10	5	Deliver	1.8	300	Flushing of the HC
8	1	1	Off	–	–	Selection of DC port
9	2	1	Aspirate	0.6	20	Aspiration of sample in the HC
10	1	2	Off	–	–	Selection of monolithic column port
11	900	2	Deliver	0.6	9000	Chromatographic analysis
12	1	1	Off	–	–	Selection of DC port
13	10	1	Deliver	1.8	300	Flushing of the DC

2.3. On-line dilution SIC procedure

The SIC sequence is included in detail in Table 1. It consisted of three general steps: (i) on-line dilution (steps 2–7), (ii) sampling (steps 8–9) and (iii) separation – detection (steps 10–11). Each SI cycle was initiated by filling the syringe with 9700 μl of mobile phase (step 1) and ended by washing the dilution coil (steps 12–13).

At the beginning and end of a working day all ports and lines of the SI manifold were flushed with 3 ml of deionized water. The monolithic column was equilibrated by passing 2 × 10 ml of mobile phase at a flow rate of 0.6 ml min^{-1} and was stored in acetonitrile when not in use. It should be noted that when changing between samples, an additional washing step was performed in order to avoid carryover effects; 2 × 200 μl of the new sample/standard were aspirated in the HC, and then flushed through port 5 to waste (W).

2.4. HPLC reference procedure

HPLC experiments were carried out using the procedure recommended by the US Pharmacopoeia [22]. In brief, 20 μl of the samples were injected in a Hypersil® C_{18} analytical column (250 mm × 4.6 mm i.d., 5 μm, MZ AnalysenTechnik, Germany). The mobile phase (0.02 M CH_3COOH) was delivered at a flow rate of 1.5 ml min^{-1}, while UV detection was carried out at 254 nm. The system suitability tests described in the USP were run prior to samples analyses.

2.5. Forced degradation conditions

In order to examine the behavior of acyclovir under stress conditions, 1 mg ml^{-1} solutions were used in all cases. These solutions were subjected to aqueous, acidic, basic and oxidative hydrolysis and UV-digestion according to Table 2. The resulting samples were introduced to the on-line dilution SIC system without any other pretreatment.

Table 2
Design of experiments for studying the degradation of acyclovir.

Medium[a]	Exposure time (h)		
	R.T.[b]	80 °C	UV
Water	8/24	1/8/24	1/8
HCl (0.1 M)	8/24	1/8/24	1/8
NaOH (0.1 M)	8/24	1/8/24	1/8
H_2O_2 (1%, v/v)	1/8/24	–	–
H_2O_2 (3%, v/v)	1/8/24	–	–

[a] 1 mg ml^{-1} acyclovir in all cases.
[b] Room temperature.

Parallel stability experiments were also conducted at Cosmopharm's facilities. The obtained samples were filtered through 0.45 μm disposable syringe filters, diluted manually 1:10 and injected in the HPLC system via the autosampler.

Thermal stability studies of the acyclovir in solid state were carried out at 70 °C for a period of 15 and 30 days. Accurately weighed amounts of the samples were dissolved in water and analyzed by the SIC and HPLC procedures as mentioned above.

2.6. Sample preparation

Not less than 10 tablets were weighed and ground to a fine powder. Accurately weighed amounts were dispersed in the suitable solvent depending on the experiment, to a nominal acyclovir concentration of 1 mg ml^{-1}. Following the degradation experiments, the samples were either introduced directly to the SIC system or manually filtered and diluted 1:10 prior to HPLC analysis.

2.7. Calculation of dilution factors

The achieved dilution factors were calculated based on dispersion coefficient measurements. If D_o is the dispersion coefficient of the sample during the analysis cycle without the application of the on-line dilution steps and D_T is the total dispersion coefficient including the dilution step, the effective dilution factor (D_{EF}) can be derived by the equation:

$$D_T = D_o \times D_{EF}$$

The dispersion coefficients were determined experimentally using a SI setup similar to that depicted in Fig. 1, but without the monolithic column and the sampling probe [18]. Caffeine aqueous solution was used as a model system (λ_{max} = 274 nm) at a concentration of 20 mg l^{-1}. It should be noted that caffeine is often used as a model compound by HPLC instrumentation manufacturers during operational or performance qualification tests of the detector linearity [23].

3. Results and discussion

3.1. Sequential injection chromatographic system

Previous studies by high pressure liquid chromatography have reported that aqueous acetic acid solutions are efficient eluents for the determination of acyclovir using reversed phase stationary phases and UV detection [22,24]. Preliminary experiments were carried out in order to examine the applicability of such a system under SIC conditions. The setup shown in Fig. 1 was used for this purpose. The experiments confirmed that a short monolithic

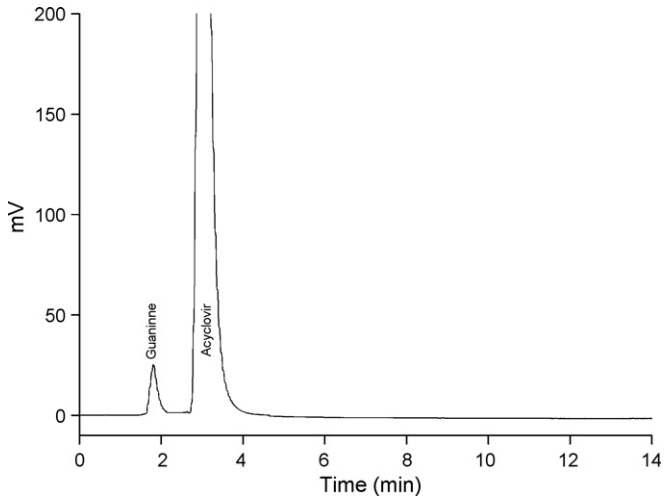

Fig. 2. Sequential injection chromatogram of a standard mixture of guanine (2% level) and acyclovir (100% level).

column (25 mm × 4.6 mm i.d.) enabled sufficient separation of acyclovir and its major impurity guanine. A sample injection volume of 20 μl was used throughout. The two system suitability criteria (SSC) established by the USP – SSC (1) resolution of not less than 2.0 between guanine and acyclovir (at 100 mg l^{-1} each) and a relative standard deviation of acyclovir area of not more than 2.0% (n = 6) and SSC (2) relative standard deviation of guanine area (at 2.0 mg l^{-1}) of not more than 2.0% (n = 6) – were fulfilled in all cases [22]. A typical chromatogram of a standard mixture of acyclovir (100% level) and guanine (2% level) is depicted in Fig. 2. As can clearly be seen, under the selected experimental conditions, the discontinuous operation mode of the syringe pump of the SIC system did not cause any disturbances of the base-line prior to the elution of the analytes.

Preliminary validation experiments on the precision, linearity and sensitivity of the SIC system proved that the relative standard deviation of the area and retention times of the main peak was less than 2% and 1% within the same working day respectively. Satisfactory day-to-day reproducibility in terms of retention time (R.S.D. < 3%) was achieved provided that the monolithic column was flushed with at least 20 ml of acetonitrile at the end of the working day and stored overnight filled with the same solvent. Linearity was found to be satisfactory within the range of 5–120% (5–120 mg l^{-1}) acyclovir with a regression coefficient of 0.998. The detection and quantitation limits were 0.1 and 0.3 mg l^{-1} respectively based on the S/N approach [25].

3.2. SIC coupled to on-line filtration and dilution

As mentioned in the previous section, the preliminary validation experiments confirmed linearity of the assay between 5 and 120 mg l^{-1} acyclovir. Provided that the degradation experiments were carried out at an acyclovir concentration of 1 mg ml^{-1} at least 10-fold dilution of the samples is necessary prior to injection in the SIC system, while undiluted samples in highly acidic or alkaline medium could deteriorate the short monolithic analytical column. Additionally, filtration is required in order to remove un-dissolved excipients and protect the multi-position valve and analytical column. To minimize the need for time-consuming manual pretreatment of the samples and develop a fully automated setup, an on-line filtration and dilution step was adopted prior to separation.

The on-line filtration step was performed by adjusting a suitable sampling probe (Distek) at the respective port of the multi-position valve, as can be seen in Fig. 1. 10 μm pores polyethylene filter discs were used in all cases. When changing samples, the sampling probe was back-flushed with 1 ml of mobile phase at a flow rate of 2 ml min^{-1} to minimize the risk of carry-over effects. To avoid increased back-pressure due to filter clogging, the filter discs were replaced every 10 injections.

Table 3
On-line dilution experiments.

V_S (μl)	V_{DC} (μl)	V_A (μl)[a]	D_{EF} [b]
20	50	20	4.81
20	75	20	7.06
20	100	20	10.6
20	125	20	17.3

[a] Fixed at 20 μl according to the experiments of Section 3.1.
[b] Mean of five analyses.

The on-line dilution protocol was based on zone sampling and included three steps: (i) aspiration of a defined sample volume (V_S) in the holding coil (HC), (ii) propulsion of an equal or larger volume (V_{DC}) to the dilution coil and (iii) aspiration of a fraction of the zone back in the HC for subsequent analysis (V_A). In this way, a well-defined concentration gradient is formed due to dispersion effects and the achieved dilution factor depends on the three volumes mentioned above [18]. In this case, the analysis volume (V_A) was fixed at 20 μl (see Section 3.1) in all subsequent experiments.

The results are shown in Table 3. As can be seen, by suitable volume combinations dilution factors in the range of ca. 4.8–17.3 could be achieved. Five injections were made in all instances, while the relative standard deviations were less than 1% in all cases. The most suitable dilution factor for the proposed method is ca. 10. In this way the calibration curve can be expanded and the sample matrix be sufficiently diluted to avoid possible deterioration of the short monolithic column due to the high acidity or alkalinity. According to Table 3 this dilution factor can be achieved by the volume combination of V_S = 20 μl/V_{DC} = 100 μl/V_A = 20 μl.

3.3. Validation of the on-line dilution SIC assay

The developed on-line filtration and dilution SIC assay was validated according to parameters proposed by the ICH guidelines [25], namely linearity, detection and quantitation limits, precision, accuracy, selectivity and robustness.

3.3.1. Linearity, detection and quantitation limits

The plot of the peak area of acyclovir versus its mass concentration was found to be linear (r > 0.997) in the range of 50–1200 mg l^{-1}. This range is in agreement with the preliminary findings without the on-line dilution step, as described in Section 3.1. The regression equation is

$$A = 9.903(\pm 0.394) \times \gamma(\text{acyclovir}) + 503.1(\pm 192.6)$$

where A is the peak area and γ(acyclovir) is the mass concentration of the analyte in mg l^{-1}. Eight calibration points were used in all instances. The percent residuals were in the range of −5.2 to +4.6% and were distributed randomly around the theoretical "zero" value, confirming the validity of the regression line. The detection (LOD) and quantitation (LOQ) limits were estimated to be 1 (S/N = 3) and 3 mg l^{-1} (S/N = 10) respectively.

3.3.2. Within and day-to-day precision

Adaptation of the extra on-line dilution step is expected decreasing the precision of the assay. The within day precision was evaluated by preparing six independent acyclovir solutions at the 100% level (1000 mg l^{-1}). Each sample was injected in triplicate

Table 4
Stability of acyclovir.

Medium	Temperature (°C)	Exposure time (h)	Acyclovir recovery[a] (%)
Neutral (water)	R.T.[b]	8	99.7
	R.T.	24	101.8
	80	1	98.9
	80	8	99.8
	80	24	97.6
Acidic (0.1 M HCl)	R.T.	8	97.3
	R.T.	24	98.1
	80	1	81.2
	80	8	58.3
	80	24	6.2
Alkaline (0.1 M NaOH)	R.T.	8	98.8
	R.T.	24	96.4
	80	1	97.1
	80	8	94.3
	80	24	82.3
Oxidative (1% H_2O_2)	R.T.	1	99.2
	R.T.	8	101.1
	R.T.	24	95.8
Oxidative (3% H_2O_2)	R.T.	1	99.7
	R.T.	8	98.1
	R.T.	24	90.4
UV (neutral)	R.T.	1	47.1
	R.T.	8	7.9
UV (acidic)	R.T.	1	2.5
	R.T.	8	N.D.[c]
UV (alkaline)	R.T.	1	45.5
	R.T.	8	N.D.

[a] Experiments carried out at 1 mg ml^{-1} acyclovir.
[b] Room temperature.
[c] Not detected.

within a working day. The relative standard deviation of the peak areas of acyclovir was 2.6% ($n = 6 \times 3$).

The day-to-day precision was evaluated by constructing calibration curves for six consecutive days. The studied range was 50–1200 mg l^{-1} using six calibration points (50, 200, 500, 750, 1000 and 1200 mg l^{-1}). Each standard was injected in triplicate. The relative standard deviation of the slopes of the six calibration curves was 7.2%.

3.3.3. Selectivity

The selectivity of the assay was evaluated by analyzing synthetic samples containing placebo at the expected concentration in the pharmaceutical formulations. The placebo mixture consisted of maize starch (15 mg/tab), microcrystalline cellulose (77 mg/tab), polyvidone (10 mg/tab), colloidal silicon dioxide (3 mg/tab) and magnesium stearate (2 mg/tab). Accurately weighed amounts of the mixture were dispersed in water and ultrasonicated for 15 min prior to analysis using the on-line filtration/dilution SIC system. No interfering peaks were observed. In order to further evaluate the selectivity of the assay, placebo suspensions were subjected to the forced degradation experiments according to Table 2. Similar results were obtained verifying the selectivity of the analytical procedure.

3.3.4. Accuracy

The accuracy of the SIC assay was validated by analyzing synthetic placebo samples spiked with acyclovir reference material at concentration levels of 100, 500, 1000 and 1200 mg l^{-1}. Three independent samples were prepared at each concentration level and each sample was analyzed in triplicate. The percent recoveries were satisfactory in all cases, ranging between 99.0 and 102.3%.

3.3.5. Robustness

The flow rate of the mobile phase was varied within the range of 0.54–0.66 ml min^{-1} (variation of ±10%) and the volume fraction of acetic acid in the range of 0.18–0.22% (variation of ±10%). In both cases the percent recoveries of a standard solution of 1000 mg l^{-1} acyclovir were acceptable, being between 95.7–103.9% and 98.7–99.8% respectively. On the other hand, the retention time of the API was varied in the range of ±0.3 min in the case of the flow rate and in the range of ±0.15 min in the case of the mobile phase.

3.4. Stability of acyclovir formulations under stress conditions

Acyclovir is an antiviral active pharmaceutical compound (API) that is highly active against simple herpes virus of 1 and 2 type, and surrounding depriving virus. It was developed in 1976 by the English researcher Gertrude Elion, who received the Nobel Prize partially for working on this drug.

Stability testing is a critical parameter in the quality control of pharmaceutical formulations. Its purpose is to provide evidence on how the quality of a drug substance or product varies with time under the influence of a variety of environmental factors and to establish the shelf-life and recommended storage conditions. It is therefore important to develop stability-indicating analytical assays capable to detect possible degradation of the formulations [26]. On this basis, the behavior of the drug under forced degradation conditions may help to identify the degradation pathways and the intrinsic stability of the molecule and validate the stability-indicating power of the analytical procedures used. Results from these studies will form an integral part of the information provided to regulatory authorities.

Table 5
Comparative characteristics of the SIC and the reference HPLC methods.

Instrumental parameters	On-line dilution SIC method	USP HPLC method [22]
Type of column	L1 monolithic - Chromolith® (25 mm × 4.6 mm i.d.)	L1 - Hypersil® C_{18} (250 mm × 4.6 mm i.d., 5 μm)
Mobile phase	0.2% (v/v) CH_3COOH (pH 3.0)	0.02 mol l^{-1} CH_3COOH
Flow rate	0.6 ml min^{-1}	1.5 ml min^{-1}
Injection volume	20 μl	20 μl
Detection	UV @ 254 nm	UV @ 254 nm

Chromatographic figures of merit	On-line dilution SIC method	USP HPLC method [22]
t_R (acyclovir)/t_R (guanine) (min)	3.28/1.91	6.12/3.30
R.R.T. (guanine/acyclovir)[a]	0.58	0.54
Resolution factor/R_s	2.21	3.91
N[b]	466	3520
N per meter[c]	18,640	14,080
Precision (area)/R.S.D.	2.6%	<2.0%
Precision (t_R)/R.S.D.	<1.0%	<1.0%

[a] R.R.T.: relative retention time.
[b] Number of theoretical plates.
[c] Number of theoretical plates per meter.

The developed SIC approach was applied to the study of the stability of acyclovir formulations under forced degradation conditions. The experimental details and timetable of the experimental study can be found in Section 2.5, while the experimental results are included in Table 4. Acyclovir was found to be stable in solid state, since more than 98% was recovered after the period of 30 days. The same behavior was observed for suspensions of acyclovir formulations in water. Quantitative recoveries were observed at either room temperature or 80 °C over a period of 24 h. On the other hand, acyclovir was found to be more stable in alkaline conditions compared to acidic. At 80 °C only ca. 6% of the API was recovered after 24 h in 0.1 M HCl (see Fig. 3), while the respective value in 0.1 M NaOH was more than 80%. Spiking experiments and peak purity tests using the PDA detector of the HPLC instrument confirmed guanine as the degradation product under acidic hydrolysis of acyclovir. The drug proved to be relatively stable under oxidative conditions, since only ca. 10% degradation was observed in 3% H_2O_2 within 24 h, although in a previous study a comparable extent of degradation was observed within 3 h [27]. UV radiation decomposed acyclovir under all conditions tested. After a period of 8 h, only ca. 8% of the drug was recovered in water (see Fig. 4), while no acyclovir was detected in acidic and alkaline medium. Similar behavior (within ±5%) was confirmed in all cases by applying the USP method using high pressure liquid chromatography with a particulate-based column.

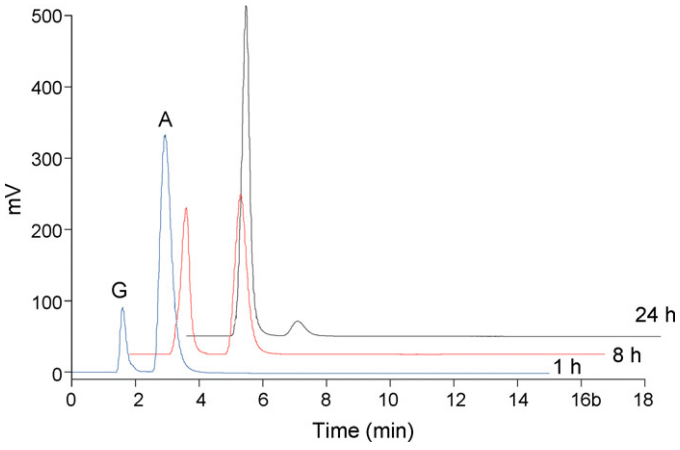

Fig. 3. Stability of acyclovir under acidic conditions within 24 h.

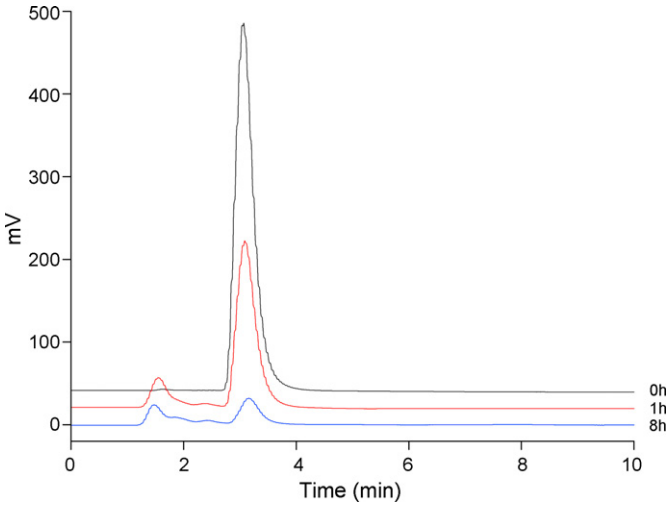

Fig. 4. UV-assisted degradation of acyclovir in neutral medium within 8 h.

3.5. Comparison of the on-line dilution SIC and the reference HPLC methods

Table 5 includes comparative instrumental and chromatographic performance data of the developed on-line dilution SIC and the reference HPLC methods. As can be seen in Table 5, the performance of the SIC approach is comparable to that of the USP proposed HPLC method. SIC offers excellent resolution between acyclovir and guanine ($R_s > 2$) and faster elution of the analytes. HPLC – as expected – offers higher resolution due to the longer column used and higher efficiency (N). However, considering the number of theoretical plates per meter, the short monolithic column seems somewhat superior to the 5 μm – particulate one. Due to the adaptation of the on-line pretreatment protocol, the SIC approach is less precise compared to the HPLC method in terms of peak area (2.6% vs. <2.0%), while both assays are precise in terms of retention times (<1.0%).

4. Conclusions

Coupling of SI to monolithic columns provides a viable solution to the major disadvantage of FI and SI, i.e. the lack of separation efficiency. However, in order to establish SIC as a valuable tool in modern analytical chemistry, SI should not be used only for sam-

ple introduction prior to separation. The ability of SIC to efficiently perform automated pretreatment was demonstrated in this work by incorporating on-line filtration and dilution steps coupled to monolithic-based separations using the same instrumental setup and a practically single-channel manifold. By simple computer-control steps, the zone sampling approach enabled a dilution factor of ca. 10, providing the capability of directly analyzing the samples without any preparation. On this basis, a stability-indicating SIC assay was developed and fully validated for the study of acyclovir formulations. It provides equally reliable results to conventional HPLC, while not requiring time-consuming manual filtration and dilution of the samples.

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