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Original Paper

Fast HPLC method for the determination of glimepiride, glibenclamide, and related substances using monolithic column and flow program

This work presents a fast method for the simultaneous separation and determination of glimepiride, glibenclamide, and two related substances by RP LC. The separation was performed on a Chromolith Performance (RP-18e, 100 mm × 4.6 mm) column. As mobile phase, a mixture of phosphate buffer pH 3, 7.4 mM, and ACN (55 : 45 v/v) was used. Column oven temperature was set to 30°C. The total chromatographic run time was 80 s. This was achieved using a flow program from 5 to 9.9 mL/min. Precisions of the interday and the intraday assay for both retention times and peak areas for the four analyzed compounds were less than 1.2%. The method showed good linearity and recovery. The short analysis time makes the method very valuable for quality control and stability testing of drugs and their pharmaceutical preparations.

Keywords: Fast HPLC / Flow programming / Glibenclamide / Glimepiride / Monolithic columns

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1 Introduction

Glibenclamide (second generation sulfonylurea) and glimepiride (third generation sulfonylurea) are oral blood sugar-lowering drugs. Sulfonylureas are used to treat Type II diabetes. They inhibit ATPase-dependent sodium channels in pancreatic beta cells [1]. The structure of glimepiride, glibenclamide, and two related substances are shown in Fig. 1.

An important parameter to be considered in a chromatographic separation is the duration of the analysis. Accordingly, fast HPLC methods are important to improve productivity in pharmaceutical analysis. Researchers are trying to develop fast LC methods to reduce the analysis time without compromising the quality of the results. A possible tool for converting a standard LC method to a fast LC method is the use of monolithic silica columns [2 – 5]. Due to the high permeability of monolithic columns which is provided by the bimodal pore structure, high flow rates can be used with acceptable backpressure [6, 7]. Thus, high velocity isocratic elution should be applicable to accelerate the elu-

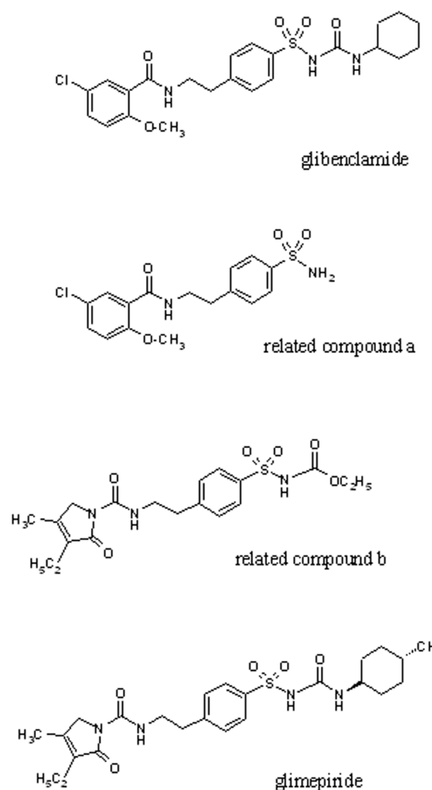

Figure 1. Structures of glibenclamide, glimepiride, and two related substances.

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Abbreviations: HETP, height equivalent to theoretical plates

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tion of the analyzed compounds and hence reduce the total run time. In monolithic columns, the increase in flow rate does not lead to substantial losses in resolution because of the low mass transfer resistance compared to conventional particle-packed columns [8, 9]. However, when some compounds of the analyzed mixture have close retention time values, while others have not, loss of resolution between the closely related peaks limits the ability for further reduction in analysis time. In such cases, further reduction in chromatographic run time could be achieved by the application of gradient elution of mobile phase or flow-rate programming. In gradient elution, the solvent polarity (composition) is continuously varied or stepped. Two high pressure liquid pumps and a system for mixing and degassing the mobile phase must be used. Furthermore, the HPLC gradient mixers must provide a very precise control of solvent composition to maintain a reproducible gradient profile. The use of flow-rate gradient (flow-rate programming) in HPLC separations involves a stepwise increase in the flow rate using one pump according to a defined flow program [10 – 18]. As an important advantage of flow-rate program over gradient elution of mobile phase, equilibration of the system is not required after each separation. This is important to achieve fast analysis of a series of samples. Flow-rate programming is more suitable for monolithic than conventional particle-packed columns due to higher permeability and lower backpressure. Instrumentation failure due to high column backpressure usually occurs when flow programming is applied on conventional particle-packed columns [19, 20]. Furthermore, the increase in column temperature in RP HPLC usually leads to a decrease in peak broadening and retention time [21 – 23]. Accordingly, high column temperature in combination with a flow program seems to be promising for significant reduction in analysis time.

The aim of this work was to develop and validate a fast HPLC method for the separation and simultaneous determination of glibenclamide with its degradation product (related compound a) and glimepiride with its degradation product (related compound b). The monolithic Chromolith Performance RP-18 HPLC column will be applied with a flow-rate program for reducing run time in RP HPLC without significantly affecting efficiency or resolution.

2 Experimental

2.1 Chemicals and reagents

The following substances 1-[[4-[2-[(5-chloro-2-methoxybenzoyl) amino]ethyl]phenyl] sulfonyl]-3-cyclohexylurea (glibenclamide) (purity > 99.9%), 5-chloro-2-methoxy-*N*-[2-(4-sulfamoyl-phenyl)ethyl]benzamide (related compound a) (99.0%), methyl[[4-[2-[(5-chloro-2-methoxybenzoyl)amino]ethyl]phenyl]sulfonyl]carbamate (related compound b) (95.6%), and 1-[[4-[2-[(3-ethyl-4-methyl-2-oxo-2,5-dihydro-1-*H*-pyrrol-1-yl)carbonyl]-amino]ethyl]phenyl]sulfonyl]-3-(*trans*-4-methylcyclohexyl)urea (glimepiride) (99.7%) were provided by Aventis (Frankfurt, Germany). ACN of HPLC grade was purchased from Acros Organics (Gelnhausen, Germany). Water for HPLC/LiChrosolv was purchased from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate, and phosphoric acid 85% were purchased from Riedel-de-Haën (Seelze, Germany), lactose monohydrate, polyvidone 25 000, microcrystalline cellulose, and magnesium stearate from Merck, sodium starch glycolate and ferrum oxydatum flavum from Caelo (Hilden, Germany).

2.2 Instrumentation

Analyses were performed on a Merck Hitachi HPLC system, consisting of a solvent pump (model L 6200 A), an autosampler (AS 2000A), a diode array detector (L-7450), and an interface (D-6000). The column oven (T1) was from Techlab (Erkerode, Germany). The data were collected and analyzed using the D7000 HSM software (Merck).

2.3 Chromatographic conditions

The separation was performed on a Chromolith Performance RP-18e (100 mm × 4.6 mm, Merck) and a Supershaper 100 RP-18 column (endcapped, 4 μm particle size, 125 mm × 4 mm, Merck). The mobile phase was prepared by dissolving 650 mg of sodium dihydrogen phosphate dihydrate in 550 mL of water. The pH of the resulting buffer was adjusted to 3 by 85% w/w phosphoric acid. The final mobile phase was prepared by mixing 55 volumes of the buffer with 45 volumes of HPLC grade ACN. A flow-rate program was used as shown in Table 1. The injection volume was 10 μL, the detection wavelength for glibenclamide and its two related compounds a and b was 210 nm, and for glimepiride it was 228 nm. The column oven temperature was set to 30°C for all runs.

2.4 Preparation of sample solvent

The sample solvent consists of 20 volumes of 4 mM phosphate buffer pH = 7 and 80 volumes of ACN. The buffer was prepared based on British Pharmacopoeia 1999 [24] by mixing 21.2 mg of KH_2PO_4 and 34.65 mg of Na_2HPO_4 in 100 mL of water.

2.5 Preparation of standards and synthetic mixture

Primary stock solutions of glibenclamide were prepared by dissolving the weighed amounts of glibenclamide in a 25 mL volumetric flask to obtain concentrations of

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0.001–0.240 mg/mL. A synthetic mixture of drug product compounds was prepared containing glibenclamide, glimepiride, and the related products a and b at different concentrations in the range of 0.001–0.240 mg/mL.

2.6 Preparation of excipient solution

Excipients were prepared for glibenclamide and glimepiride tablets containing the following substances: lactose monohydrate 82.80% w/w, sodium starch glycolate 4.40% w/w, magnesium stearate 0.50% w/w, and ferric oxide 0.45% w/w. The mixture was homogenized by trituration in a mortar.

2.7 Recovery

Recovery for glinbenclamide and glimepiride was investigated on three concentration levels, 0.24 mg/mL (120%), 0.2 mg/mL (100%), and 0.16 mg/mL (80%). A set of six standards 100% and six recovery samples were prepared for each concentration level. A 90 mg matrix was added to each recovery sample. The two sets were placed in an ultrasonic bath for 15 min, centrifuged for 3 min at 14000 rpm in closed vials, and transferred for injection.

3 Results and discussion

3.1 Optimization of the chromatographic separation

A mobile phase developed in our laboratory [25] containing a phosphate buffer (pH 3)/ACN (55:45 v/v) was used during the development procedures. In order to decrease the chromatographic run time as much as possible, an increase in the mobile phase flow rate was tried in successive runs from 1.25 up to 9 mL/min (Fig. 2). The four compounds were eluted within 66 s at a flow rate of 9 mL/min; however, there was a loss of resolution between the peaks of related compounds a and b. With the aim of improving the resolution between compound a and compound b at a flow rate of 9 mL/min, the elution strength of the mobile phase was modified by decreasing the ACN percentage from 45 to 42%. The resolution was improved but the chromatographic run time was increased to about 2.2 min. Thus the ACN content was kept at 45%. In order to obtain a quantitative separation between the four compounds at the shortest analysis time, a flow program was used ranging from 5.0 to 9.9 mL/min to allow a rapid elution of the four compounds without sacrificing resolution. A stepwise increase of the flow rate from 5 to 6 mL/min was applied at the first 35 s to avoid the loss of resolution between the closely related compounds a and b. After that, the flow rate was increased gradually to 9.9 mL/min to accelerate the elution of the late eluting glibenclamide and glimepiride. The separation was accomplished within 80 s. The four compounds were well separated from each other. A representative chromatogram for the fast separation of glibenclamide, glimepiride, and their two related compounds is shown in Fig. 3. The temperature of the column oven was set to 30°C because no further improvement in peak broadening or decrease in retention times was observed at higher temperatures, even when a mobile phase water bath was used in combination with the column oven. Temperatures were tested up to 45°C using this flow-rate program. The effect of temperature was only minor but measurable at the low flow rate of 1.25 mL/min at which the retention times for all the four peaks was reduced using higher temperature. Representative chromatograms of the analyzed mixture at a flow rate of 1.25 mL/min using a column oven set to 30 and 45°C are shown in Fig. 4. No change in the result or precision was observed with a slight variation in buffer pH in the range 2.5–3.5. To ensure assay precision, within-day repeatability ($n = 5$) and between-days repeatability ($n = 5$) were assessed at three concentration levels for each of the four compounds. The RSDs% was found to be <1.0% for retention time and <1.2% for peak area (Table 2).

The specificity of the method was examined by observing whether there was any interference from the inactive ingredients. The HPLC chromatograms recorded for the inactive ingredients showed no peaks at the retention times of the four compounds.

Calibration curves (peak area *vs.* concentration) for the four analyzed compounds in sample solvent were investigated over a wide concentration range. Results are summarized in Table 3. The LOD (S/N = 3) and an estimate for the LOQ (S/N = 10) for the four analyzed compounds were also summarized in Table 3. The accuracy of the method was tested by determination of the recovery using the inactive ingredient used in glibenclamide and glimepiride tablet formulations. Good recovery percentages were obtained for both glibenclamide and glimepiride. The recovery results for both glibenclamide and glimepiride at three concentration levels are summarized in Table 4.

3.2 Performance parameters

Peak performance parameters were calculated according to fundamental equations (Table 5). A slow decrease in resolution was observed by applying high flow rates in a monolithic column. Column efficiency was measured by plotting the height equivalent to theoretical plates (HETP) against the flow rates of the mobile phase. The HETP H was calculated from the column length L and theoretical plate N according to $H = L/N$. A flat HETP *versus* linear velocity curves were obtained for the four analyzed compounds (Fig. 5). This indicates that monolithic columns can operate at high flow rates with only small decrease in efficiency.

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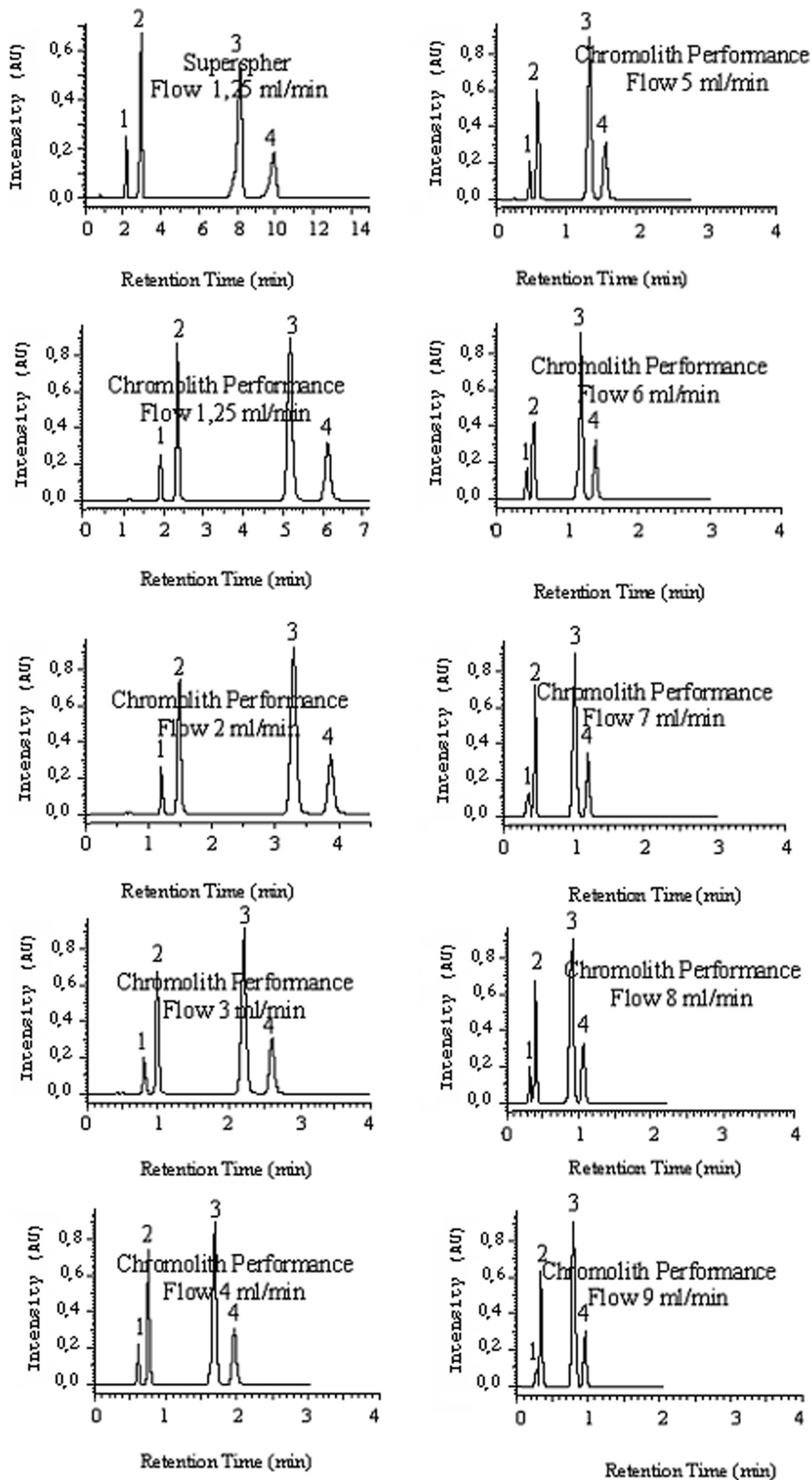

Figure 2. Representative chromatograms for (a) related compound a, (b) related compound b, (c) glibenclamide, and (d) glimepiride on conventional (Superspher RP-18) and on monolithic (Chromolith Performance RP-18) columns at different flow rates from 1.25 to 9 mL/min. Mobile phase consists of buffer (pH 3)/ACN (55 : 45).

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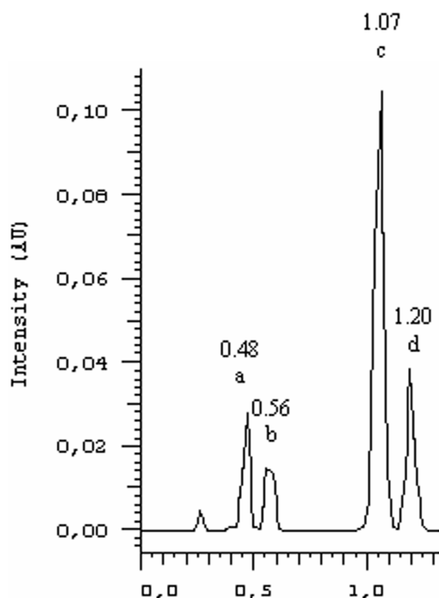

Figure 3. Representative chromatograms for (a) related compound a, (b) related compound b, (c) glibenclamide, and (d) glimepiride on a Chromolith Performance RP-18 column at a flow-rate program (5 – 9.9 mL/min) and a column temperature of 30°C. The mobile phase consists of buffer (pH 3)/ACN (55 : 45 v/v).

Table 1. Flow-rate program used during the separation

Time (min)	Flow rate (mL/min)
0.0	5.0
0.6	6.0
0.7	9.0
1.3	9.9

The high permeability of the monolithic columns was evidenced by a total system backpressure of 253 bars at a flow rate of 9.9 mL/min. This high flow rate is not applicable in the case of conventional particle-packed columns. In conventional columns, the maximum acceptable backpressure of 400 bars is reached at a flow rate of about 4 mL/min depending on the column length and the mobile phase composition. The backpressure profile during the flow program of 5 – 9.9 mL/min ranged from 144 to 250 bars.

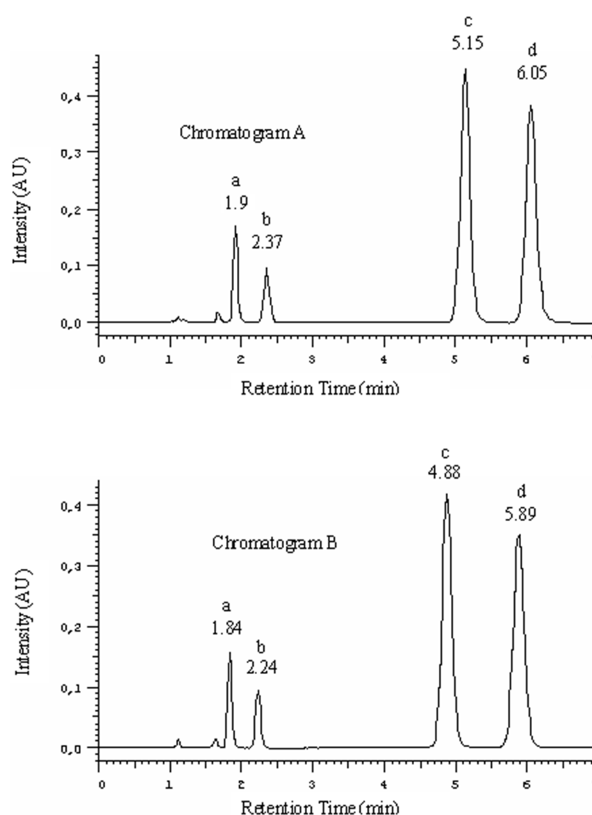

Figure 4. Representative chromatograms for (a) related compound a, (b) related compound b, (c) glibenclamide, and (d) glimepiride on a Chromolith Performance RP-18 column at a flow rate of 1.25 mL/min and a column temperature of 30°C for chromatogram a, and 45°C for chromatogram b. Mobile phase consists of buffer (pH 3)/ACN (55 : 45 v/v).

4 Concluding remarks

The data presented in this article show explicit advantages of monolithic columns in fast HPLC analysis of drugs. Application of high velocity isocratic elution with monolithic column was limited by the loss of resolution between the closely related peaks of related products a and b. Even though conventional and monolithic silica columns are stable over a similar temperature range up to 45°C an increase in temperature is problematic with monolithic columns because they are packed in insulat-

Table 2. Precision over a concentration range 0.001 – 0.100 mg/mL for related compound a and b and 0.01 – 0.24 mg/mL for glibenclamide and glimepiride using $n = 5$ for both within-day and between-days repeatabilities

Compound	Within-day repeatability RSD range of AUC (%)	Within-day repeatability RSD of t_R (%)	Between-day repeatability RSD range of AUC (%)	Between-day repeatability RSD of t_R (%)
Related compound a	0.40 – 0.62	0.37	0.58 – 0.79	0.93
Related compound b	0.50 – 0.62	0.80	0.68 – 1.14	0.98
Glibenclamide	0.45 – 0.60	0.41	0.76 – 0.85	0.58
Glimepiride	0.36 – 0.56	0.58	0.63 – 1.03	0.94

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Table 3. Linearity range, detection, and quantitation limits of the four analyzed compounds

Compound	Concentration range (mg/mL)	R_2	Detection limit (μg/mL)	Quantitation limit (μg/mL)
Related compound a	0.0002 – 0.1	0.9981	0.048	0.16
Related compound b	0.0002 – 0.1	0.9998	0.050	0.18
Glibenclamide	0.001 – 0.24	0.9998	0.120	0.41
Glimepiride	0.001 – 0.24	0.9981	0.150	0.50

Table 4. List of recovery results ($n = 10$) for glibenclamide and glimepiride from a synthetic mixture of drug product compounds at three concentration levels

Compound	Theoretical value (mg/mL)	Mean recovery (mg/mL)	Recovery (%)	RSD (%)
Glibenclamide	0.16	0.1590	99.34	0.85
	0.20	0.1990	99.95	0.56
	0.24	0.2409	100.4	0.79
Glimepiride	0.16	0.1603	100.2	0.92
	0.20	0.1988	99.89	0.78
	0.24	0.2370	99.10	1.00

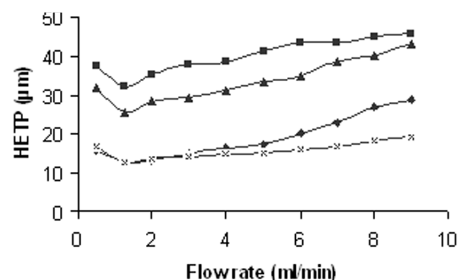

Figure 5. Van Deemter plot for the four analyzed compounds on Chromolith Performance RP-18e column: (◆) glibenclamide, (■) related compound a, (▲) related compound b, and (x) glimepiride.

Table 5. Performance parameters for glibenclamide and the two related compounds on conventional and monolithic columns

Column type	Flow rate (mL/min)	Theoretical plate (N)[a] (plate *per* column for glibenclamide)	Asymmetry factor (AF)[a] for glibenclamide peak	Resolution (R_s)[a] compound a/compound b	Total run time (min)
Conventional C18	1.25 mL/min	4435	1.8	3.4	11
Monolithic C18	1.25 mL/min	7964	1	4.1	6.5
Monolithic C18	2 mL/min	7540	1.1	3.86	4.5
Monolithic C18	3 mL/min	6725	1.1	3.2	3
Monolithic C18	4 mL/min	6151	1	2.8	2.5
Monolithic C18	5 mL/min	5807	1	2.4	1.8
Monolithic C18	6 mL/min	5003	1.1	1.9	1.6
Monolithic C18	7 mL/min	4328	1	1.7	1.4
Monolithic C18	8 mL/min	3755	1	1.4	1.3
Monolithic C18	9 mL/min	2822	1.1	1.13	1.2
Monolithic C18	5 – 9.9 mL/min	5288	1	2.2	1.3

a) The following equations were used to calculate the above-mentioned chromatographic parameters: Theoretical plate (N) = 16 $(t_R/w)^2$, asymmetry factor (AF) = B/A at 10% of peak height, and resolution (R_s) = $2((t_{R2} - t_{R1})/(w_2 + w_1))$.

ing polyetherether ketone (PEEK), indicating the importance of applying other types of heating in the column oven. The use of monolithic column with a flow-rate program was evaluated as a means of reducing the total analysis time. Flow programming would require large inlet pressure in order to achieve a meaningful reduction in the analysis time of strongly retained compounds. This high pressure is applicable in monolithic column due to its high permeability. A marked decrease in analysis time was obtained without sacrificing resolution or run to run reproducibility. The use of flow programming eliminates the need of re-equilibrium time between successive runs which is required in the case of a mobile phase gradient. The four peaks are eluted within 80 s showing that the method can be used as an efficient rapid method for series drug analysis for quality control purposes or stability testing.

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