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Evaluation of monolithic HPLC columns for various pharmaceutical separations: Method transfer from conventional phases and batch to batch repeatability

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Abstract

Methods developed on conventional particle-packed C18 columns for pilocarpine, propranolol, glibenclamide, glimepiride, insulin and their respective degradation products or related compounds were transferred from the conventional Superspher 100 RP-18e column to Chromolith Performance RP-18e columns. All transfers were successful applying the same chromatographic conditions, except for insulin where the acetonitrile content of the mobile phase was reduced by 0.5%. The intraday and interday precisions for both retention time and peak area were evaluated over a wide concentration range. Results were found to be equal, or slightly better on Chromolith Performance with RSD% < 1.1% in all cases. Monolithic batch to batch repeatability of both retention time and peak area, compared for monolithic columns from different batches gave an RSD% of less than 1.3%. The separation of each drug and its related products was investigated on monolithic columns at flow rates from 1 to 9 ml/min, and superior resolution was always obtained using monolithic over conventional columns at the same flow rate. A total of seven monolithic columns from four different batches were used in this study.
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Keywords: Monolithic columns; Chromolith performance; Repeatability; Method transfer; Propranolol; Glibenclamide; Glimepiride; Pilocarpine; Insulin

1. Introduction

Monolithic silica rod columns were first introduced in 1991 by Nakanishi and Soga [1]. The preparation of these silica rod materials involved a sol–gel process using highly pure silica. The formed silica rod is then encased in polyetherether ketone (PEEK) shrink-warp tubing, which prevents void formation. The obtained highly porous skeleton is characterized by a bimodal pore structure consisting of large macropores (diameter 2 μm), and mesopores (13 nm in diameter). Monolithic columns also have a significantly higher total porosity after octadecylsilylation than conventional particulate columns, over 80% versus ca. 65%, respectively [2]. However, due to the much lower density of monolithic columns, the loadability of a conventional column of the same size is much higher. Till now the commercially availability of monolithic stationary phases is limited to normal silica, C8 and C18 only. Furthermore, because of the significant shrinkage during the formation of the skeleton, it is difficult to prepare straight rods longer than about 15 cm, which limits the length of the final columns. However, it is possible to enhance the separation efficiency by coupling several monolithic columns together. The use of coupled monolithic columns and its effect on increasing the injection volume loading has been reported [3].

Nowadays about 450 papers were published describing the use of monolithic columns in various fields. This includes drug analysis [4–7], food and environmental analysis [8,9] and bioanalysis [10–12]. However, the number of developed methods using monolithic columns is much smaller than that using particle packed columns.

There have already been investigations comparing the feasibility and parameters during method transfer from traditional LC columns to monolithic columns [5,13–16].

Good repeatability of monolithic C18 columns has been reported. However, most of the studies performed so far were either done by monoliths-distributing companies or the number of data presented was too low to reliably estimate precision. In

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other works, the test mixtures were not directly related to a practical application [17,18]. Therefore, people are still not certain about the quality of monolithic phases. The goal of our study is to investigate monolithic columns with real method transfers and real separation challenges.

In this study, we shall concentrate on investigations about batch to batch repeatability of retention time and peak area using a sufficient number of data, when these columns are applied for the separation of small pharmaceutical compounds and polypeptide drug molecules from their side components.

In order to evaluate the performance of monolithic columns, the five pharmaceutical substances pilocarpine, propranolol, glibenclamide, glimepiride and insulin were chosen. The compounds differ in their physicochemical properties. Official HPLC monographs for all these substances are available, which facilitates the comparison of these results to other separations. The chromatographic behavior of commercially available Chromolith Performance RP-18 HPLC columns for the rapid analysis of the above-mentioned compounds will also be investigated. This includes separation from their degradation products or related compounds. Furthermore, the performance of these columns will be compared to that of a conventional particle-packed C18 column.

Pilocarpine hydrochloride (3S,4R)-3-ethyl-4-[(1-methyl-1*H*-imidazol-5-yl) methyl] dihydrofuran-2(3*H*)-one hydrochloride, is a parasympathomimetic compound, which is widely used in ophthalmic solutions for the treatment of glaucoma. Deprotonation and reprotonation processes cause the racemisation of this drug. Pilocarpine can also hydrolyze under basic conditions to form pilocarpic acid. Pilocarpine can epimerize at the α-position to form isopilocarpine. Since only the configuration on one of the two chiral centers of pilocarpine changes the compounds are diastereomers. Isopilocarpine can then hydrolyse to form isopilocarpic acid [19].

Propranolol hydrochloride (2RS)-1-[(1-methylethyl) amino]-3-(naphthalene-1-yloxy)propane-2-ol, is a non-selective beta-adrenergic blocking agent widely used in the treatment of hypertension, angina pectoris and cardiac arrhythmias. The main degradation products of this drug are 3-(naphthalene-1-yloxy)propane-1,2-diol and 1,1′-[(1-methylethylimino]bis[3-naphthaline-1-yloxy)propane-2-ol [20].

Glibenclamide (second generation sulfonylurea) and *glimepiride* (third generation sulfonylurea) are oral blood sugar lowering drugs. Sulphonylureas are used to treat Type II diabetes [21].

Human insulin is a polypeptide hormone consisting of two peptide chains (A and B), which contains one intrasubunit and two intersubunit disulfide bonds. The major degradation product formed during mild acidic hydrolysis of insulin is the deamidated hydrolysis product A-21 desamido insulin [22].

2. Experimental

2.1. Chemicals and reagents

Acetonitrile HPLC grade, pilocarpine hydrochloride 99% and propranolol hydrochloride 99% were purchased from Acros Organics (Belgium). Methanol HPLC grade was purchased from Fisher Scientific (UK). Phosphoric acid 85%, triethylamine, sulfuric acid, sodium lauryl sulphate, ammonia, anhydrous sodium sulphate, lactose monohydrate, polyvidone 25000, microcrystalline cellulose and magnesium stearate were purchased from Merck (Darmstadt, Germany). Human insulin synthetic 95–98% (HPLC) approximately 24 IU/mg and tetrabutylammonium dihydrogen phosphat were purchased from Sigma–Aldrich (Switzerland). The following substances 1-[[4-[2-[(5-chloro-2-methoxy-benzoyl) amino]ethyl]phenyl] sulphonyl]-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%), 1-[[4-[2-[(3-ethyl-4-methyl-2-oxo-2,5dihydro-1*H*-pyrrol-1-yl)carbonyl]-amino]ethyl]phenyl] sulphonyl]-3-(*trans*-4-methylcyclohexyl)urea (*glimepiride*) (99.7%) and Insuman ^{R} Basal 100 international unit/ml suspension for injection were provided by Aventis (Frankfurt, Germany). Potassium dihydrogen phosphate, sodium dihydrogen phosphate dihydrate, disodium hydrogen phosphate, and phosphoric acid 85% were purchased from Riedel-de-Haën (Seelze, Germany). Sodium starch glycolate and ferric oxide were purchased from Caelo (Hilden, Germany), respectively. Bi-distilled water was used throughout. All used chemicals were at least of analytical grade.

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 UV–vis detector (L-4250), 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). The separation was performed on a Superspher 100 RP-18e column (endcapped, 4 μm particle size, 125 mm × 4 mm, Merck) and a set of seven Chromolith Performance RP-18e (100 mm × 4.6 mm, Merck).

2.3. Separation conditions

2.3.1. Pilocarpine

2.3.1.1. Chromatographic conditions for pilocarpine. The mobile phase was prepared according to a previously recommended method [23] by mixing 980 ml buffer solution with 20 ml methanol. The buffer was prepared by mixing 13.5 ml of 85% phosphoric acid, 3 ml of triethylamine and water to a total volume of 1000 ml. The injection volume was 20 μl and the detection wavelength was 214 nm. All separations were performed at ambient temperature.

2.3.1.2. Preparation of standard. The buffer described in Section 2.3.1.1 was used as sample diluent. Dilutions were carried out using the sample diluent, to obtain solutions of known concentrations to be used for the standard preparation and the assay purposes. The concentration levels described in European Pharmacopoeia 2005 were used.

2.3.1.3. Preparation of mixtures. *Solution a* (*Isopilocarpine*): Isopilocarpine was obtained from pilocarpine by racemization. One millilitre of 0.1 M NaOH was added to a 5 ml solution of 0.5 mg/ml pilocarpine hydrochloride in a 25 ml volumetric flask to allow deprotonation followed by the addition of 1 ml 0.1 M HCl to allow reprotonation. The volume of the finally resulting solution was completed to 25 ml by sample diluents.

Solution b (*Pilocarpic acid and isopilocarpic acid*): Pilocarpic acid and isopilocarpic acid which are not commercially available were generated by base catalyzed hydrolysis in a way similar to [19]. To 5 ml of 1 mg/ml pilocarpine aqueous solution in a 25 ml volumetric flask, 100 μl of concentrated ammonia was added and the mixture was heated in an oven to 90 °C for about 2 h. The mixture was allowed to cool to room temperature and then diluted to 25 ml with sample diluent.

Pilocarpine/degradation products mixture: The final mixture that contains pilocarpine with its three degradation products was prepared by mixing 8 ml of solution a, 8 ml of solution b and 5 ml of 0.5 mg/ml pilocarpine hydrochloride solution. The solution was completed to a total volume of 25 ml using sample diluent.

2.3.1.4. Addition of excipient. Excipient for pilocarpine hydrochloride ophthalmic solution was prepared containing the inactive ingredients disodium edetate 4 mg, polyvinylpyrrolidone 1.7 mg, sodium dihydrogen phosphate 0.8 mg, disodium hydrogen phosphate 0.94 mg, sodium chloride 0.9 mg and benzalkonium chloride 0.13 mg in 100 ml of bi-distilled water.

2.3.2. Propranolol

2.3.2.1. Chromatographic conditions for propranolol. The mobile phase was prepared according to the method described in the European Pharmacopoeia 1997 [20], by mixing 1.15 g sodium lauryl sulphate, 10 ml of a mixture of 1 volume of sulfuric acid and 9 volume of water, 20 ml of 17 g/L solution of tetrabutylammonium dihydrogen phosphate, 370 ml of water and 600 ml of acetonitrile. The pH of the finally resulting solution was adjusted to 3.3 using diluted sodium hydroxide solution. The injection volume was 20 μl and the detection wavelength was 292 nm. All separations were performed at ambient temperature.

2.3.2.2. Preparation of standard. The primary stock solution of propranolol hydrochloride was prepared in the mobile phase to obtain solutions of known concentrations to be used for the standard preparation and the assay purposes in the range of 0.002–1 mg/ml.

2.3.2.3. Preparation of degradation products. The two main degradation products of propranolol hydrochloride are 3-(naphthalene-1-yloxy)propane-1,2-diol and 1,1′-[(1-methylethylimino]bis[3-naphthaline-1-yloxy)propane-2-ol. They were generated by the addition of a 1 ml of 0.1 M NaOH to a 5 ml solution of 1 mg/ml propranolol hydrochloride in a 25 ml volumetric flask. The solution was left for 20 min to allow hydrolysis. Then 1 ml 0.1 M HCl was added to neutralize the solution. The volume of the finally resulting solution was completed to 25 ml with mobile phase.

2.3.2.4. Addition of excipient. Excipient for propranolol hydrochloride tablets was prepared containing the following substances specified as a percentage of tablet weight, propranolol hydrochloride 26.7% (w/w), lactose monohydrate 51.3% (w/w), microcrystalline cellulose 20% (w/w), and magnesium stearate 2% (w/w) [24].

2.3.3. Glibenclamide and glimepiride

2.3.3.1. Chromatographic conditions for glibenclamide, glimepiride and related substances. The mobile phase was prepared by dissolving 650 mg sodium dihydrogen phosphate dihydrate in 550 g water. The pH of the resulting solution was adjusted to 3 by adding two drops of phosphoric acid 85% and 351.5 g acetonitrile to 1000 ml. The injection volume was 10 μl and the detection wavelength for glibenclamide, related compounds *A* and *B* was 210 nm and for glimepiride was 228 nm. The column oven temperature was set at 35 °C for all runs.

2.3.3.2. Preparation of sample solvent. The sample solvent consists of 20 volumes of 4 mM phosphate buffer (pH 7) and 80 volumes acetonitrile. The buffer was prepared based on the British Pharmacopoeia 1999 [25].

2.3.3.3. Preparation of standard. 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.

2.3.3.4. Addition of excipient. Inactive ingredients for glibenclamide and glimepiride tablets were prepared containing the following substances: 74.6 mg lactose monohydrate, 4.0 mg sodium starch glycolate, 0.5 mg polyvidone 25000, 10.0 mg microcrystalline cellulose, 0.5 mg magnesium stearate and 0.4 mg ferric oxide. The mixture was homogenized by trituration in a mortar.

2.3.4. Insulin

2.3.4.1. Chromatographic conditions for insulin. The aqueous part of the mobile phase was prepared by dissolving 28.4 g anhydrous Na_2SO_4 in 1000 ml water followed by the addition of 2.7 ml of concentrated phosphoric acid 85% (buffer pH 2.3). Mobile phase consists of buffer pH 2.3: acetonitrile in a ratio of (74:26, v/v) and (74.5:25.5, v/v) for conventional and monolithic columns, respectively.

The injection volume was 10 μl and the detection wavelength was 214 nm. All separations were performed at ambient temperature.

2.3.4.2. Preparation of standard. The standard solution of human insulin was prepared in 0.9% (w/v) sodium chloride solution with the addition of few drops of 0.1 M hydrochloric acid to improve solubility. Solutions of known concentrations to be used for the standard preparation and the assay purposes were prepared in the range of 0.03–3.00 mg/ml.

2.3.4.3. Preparation of mixture. A Pharmaceutical preparation that contains human insulin commercially referred to as Insuman

Basal™ 100 international unit/ml (Suspension for injection) was used. The other ingredients of Insuman Basal ™ are: protamine sulphate, *m*-cresol, phenol, zinc chloride, sodium dihydrogen phosphate dihydrate, glycerol, sodium hydroxide and hydrochloric acid.

3. Results and discussion

3.1. Optimization of chromatographic parameters

HPLC methods previously described for the determination of pilocarpine, propranolol, glibenclamide, glimepiride and insulin using conventional particle-packed C18 columns have been adapted to our available conventional column Supershper 100 RP-18 column (endcapped, 4 μm particle size, 125 mm × 4 mm, Merck). Adaptation only included changes in column length, particle size or flow rate. The methods were then validated and transferred without further modification to the monolithic Chromolith Performance RP-18e (100 mm × 4.6 mm, Merck). For pilocarpine a method described in ref. [23] was taken. The used method has been previously tested on a set of conventional C18 columns, however was not tested on Superspher commercial type column, which was used in this study. For propranolol the method used based on European Pharmacopoeia 1997 [20], however some parameters were changed including the flow rate and the properties of the octadecylsilyl silica column used. The conventional Superspher 100 RP-18e column (endcapped, 4 μm particle size, 125 mm × 4 mm, Merck) was used with a flow rate of 1 ml/min, instead of a 5 μm particle size, 200 mm × 5 mm octadecylsilyl silica column at a flow rate of 1.8 ml/min as specified in European Pharmacopoeia 1997. The conventional C18 based method used for glibenclamide, glimepiride and the two related substances was formerly developed in our laboratories [26]. For insulin the method was adapted from the European Pharmacopoeia 2005 [27], isocratic elution with a 26% acetonitrile concentration was chosen and the Superspher 12.5 cm column packed with 4 μm particles instead of 25 cm column packed with 5 μm particles specified in the European Pharmacopoeia 5th Edition was used.

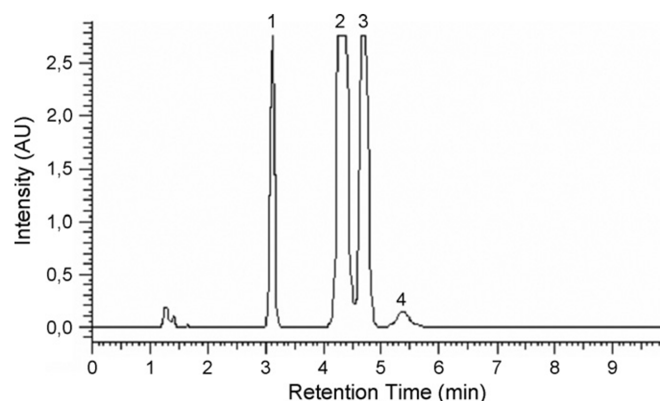

Fig. 1. Representative chromatogram for phenol (1), *m*-cresol (2), human insulin (3) and 21-desamido human insulin (4) on monolithic (Chromolith Performance RP-18e) column at a flow rate of 1 ml/min. Mobile phase consists of buffer pH 2.3: acetonitrile (74:26, v/v).

3.2. Method validation

Methods for pilocarpine, propranolol, glibenclamide and glimepiride were found to be successfully transferable from the conventional particle-packed to the monolithic rod columns without any modification. For the relatively larger molecule insulin, the method was not successfully transferred at the first go. The peak of insulin was eluted too early when the method was firstly transferred to the monolithic column, so it overlapped with the peak of *m*-cresol. The amount of the organic modifier (acetonitrile) in the mobile phase was decreased from 26 to 25.5% in order to obtain good resolution (Fig. 1). This tiny decrease of organic modifier has a strong effect on the retention times of insulin and desamido-insulin whereas the retention times of the small molecules phenol and cresol were kept nearly unchanged. It is not clear why the insulin method was not successfully transferred from the conventional particle-packed to the monolithic column under the same chromatographic conditions. However, one should keep in mind that the mechanism by which polypeptides interact with the reversed-phase surface is a bit different from that of small drug molecules. The retention time of insulin should be mainly governed by adsorption which is highly sensitive to alterations of the mobile phase. The retention time of

Table 1
Resolution values of the critical peak pairs on conventional and monolithic columns

Column type	Resolution (R_s)[a] Pilocarpine/pilocarpic acid	Resolution (R_s) propranolol impurity A/propranolol	Resolution (R_s) glibenclamide related compounds a/b	Resolution (R_s) human insulin/21-desamido insulin
Superspher RP-18e (flow rate 1 ml/min)	2.10	4.39	3.40[b]	2.60
Chromolith RP-18e (flow rate 1 ml/min)	2.78	4.40	4.10[b]	4.50
Chromolith RP-18e (flow rate 2 ml/min)	2.33	4.18	3.86	4.10
Chromolith RP-18e (flow rate 3 ml/min)	2.23	3.26	3.20	3.95
Chromolith RP-18e (flow rate 4 ml/min)	2.13	2.80	2.80	3.74
Chromolith RP-18e (flow rate 5 ml/min)	1.92	2.00	2.40	3.49
Chromolith RP-18e (flow rate 6 ml/min)	1.80	1.94	1.90	3.35
Chromolith RP-18e (flow rate 7 ml/min)	1.58	1.80	1.70	3.32
Chromolith RP-18e (flow rate 8 ml/min)	1.43	1.66	1.40	3.27
Chromolith RP-18e (flow rate 9 ml/min)	1.32	1.58	1.13	3.20

[a] Resolution was calculated according to the fundamental equation $R_s = 2(t_{R2} - t_{R1}/w_2 + w_1)$.
[b] Values at a flow rate of 1.25 ml/min.

the small molecules is much more controlled by the transport process through the mesopores.

It has been demonstrated, that the same elution orders and patterns of the used mixtures were obtained in monolithic and conventional columns. This indicates that the selectivity of the two column types is equivalent.

At a flow rate of 1 ml/min, monolithic columns were found to produce a similar or better resolution as conventional ones, within a shorter run time using the same mobile phase (taking in account the small difference in column length). With the application of higher flow rates on Chromolith Performance columns a small reduction in resolution was observed. However at a flow rate of 4 ml/min the resolution obtained with the monolithic columns was still satisfactory ($R_s \geq 2$), with the advantage of reducing the total run time. This flow rate (4 ml/min) was selected for precision studies, as it provides the shortest analy-

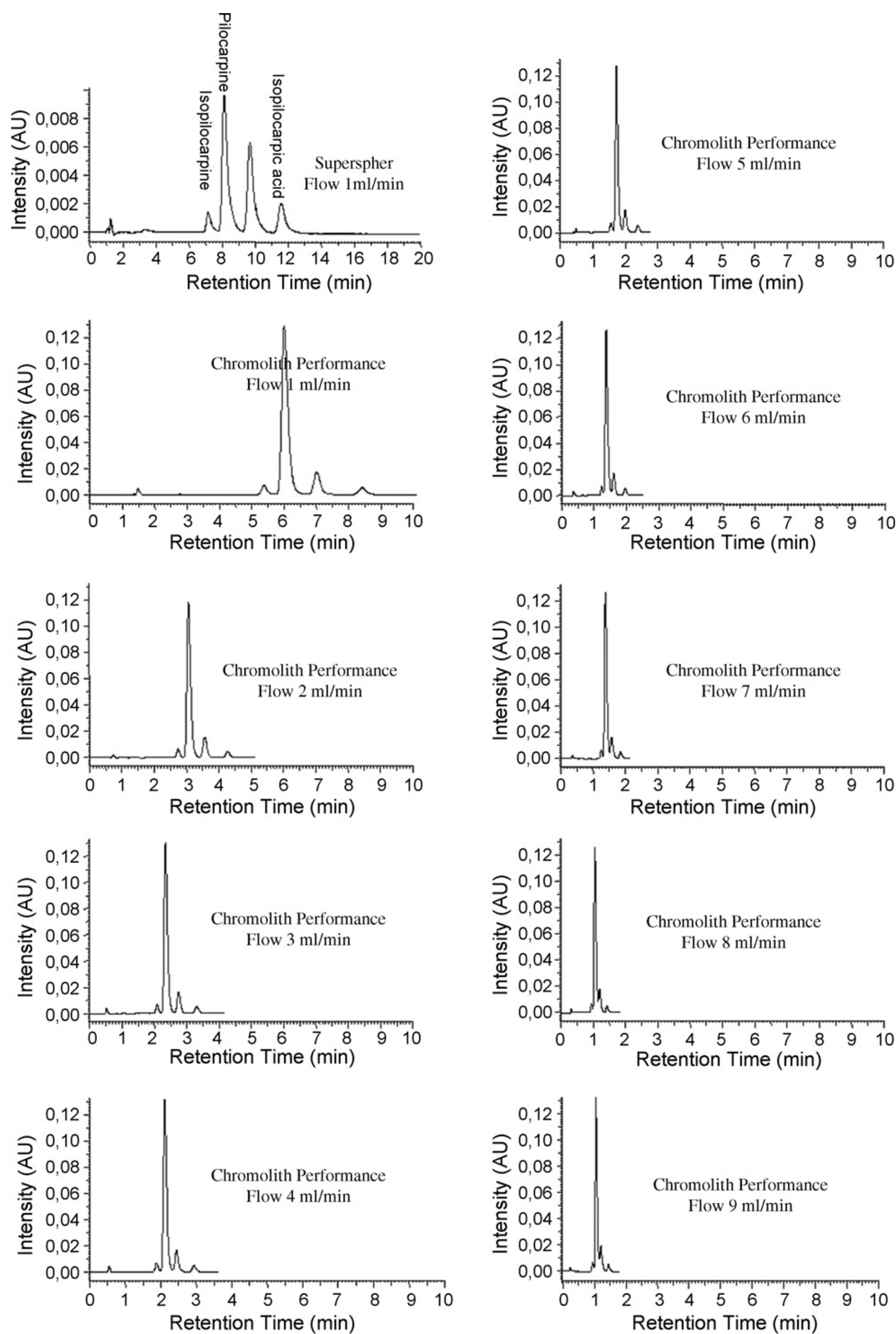

Fig. 2. Representative chromatograms for pilocarpine hydrochloride and its degradation products on conventional (Superspher RP-18e) column, and on monolithic (Chromolith Performance RP-18e) column at different flow rates from 1 to 9 ml/min. Mobile phase consists of a buffer pH 3: methanol (98:2, v/v). Difference in peak intensity between conventional and monolithic columns is due to difference in concentration of pilocarpine and its degradation products.

sis times with baseline resolution (R_s values) higher than 2. As expected, the total analysis time was reduced to approximately a quarter at a flow rate of 4 ml/min using monolithic columns. Resolution values on conventional and monolithic column at different flow rates are shown in Table 1. Resolution values are shown for the critical peak pairs that were most sensitively affected by changes in column type. The specificity of the methods was examined by observing if there was any interference of the inactive ingredients of the pharmaceutical preparations in each drug case. The HPLC chromatograms recorded for the inactive ingredients of the analyzed compounds showed no peaks at the retention times of the active drugs and their degradation products or related compounds. The specificity was also demonstrated by the good separation of the degradation products and/or related compounds from the main compound peak in each method (Figs. 2–5). The accuracy of the methods was tested by determination of the recovery using the excipients used in drug formulation of each of the used drugs. Recovery values are listed in Table 2. Precision which was an important aim of this study was carefully tested. To ensure assay precision within day (five injections were performed each day) and between days (determined at 5 days) repeatabilities of retention time and peak area were assessed at three concentration levels on the conventional (Superspher) and the monolithic columns for each separation. Repeatability results for pilocarpine as an example for small drug molecules, and insulin as an example

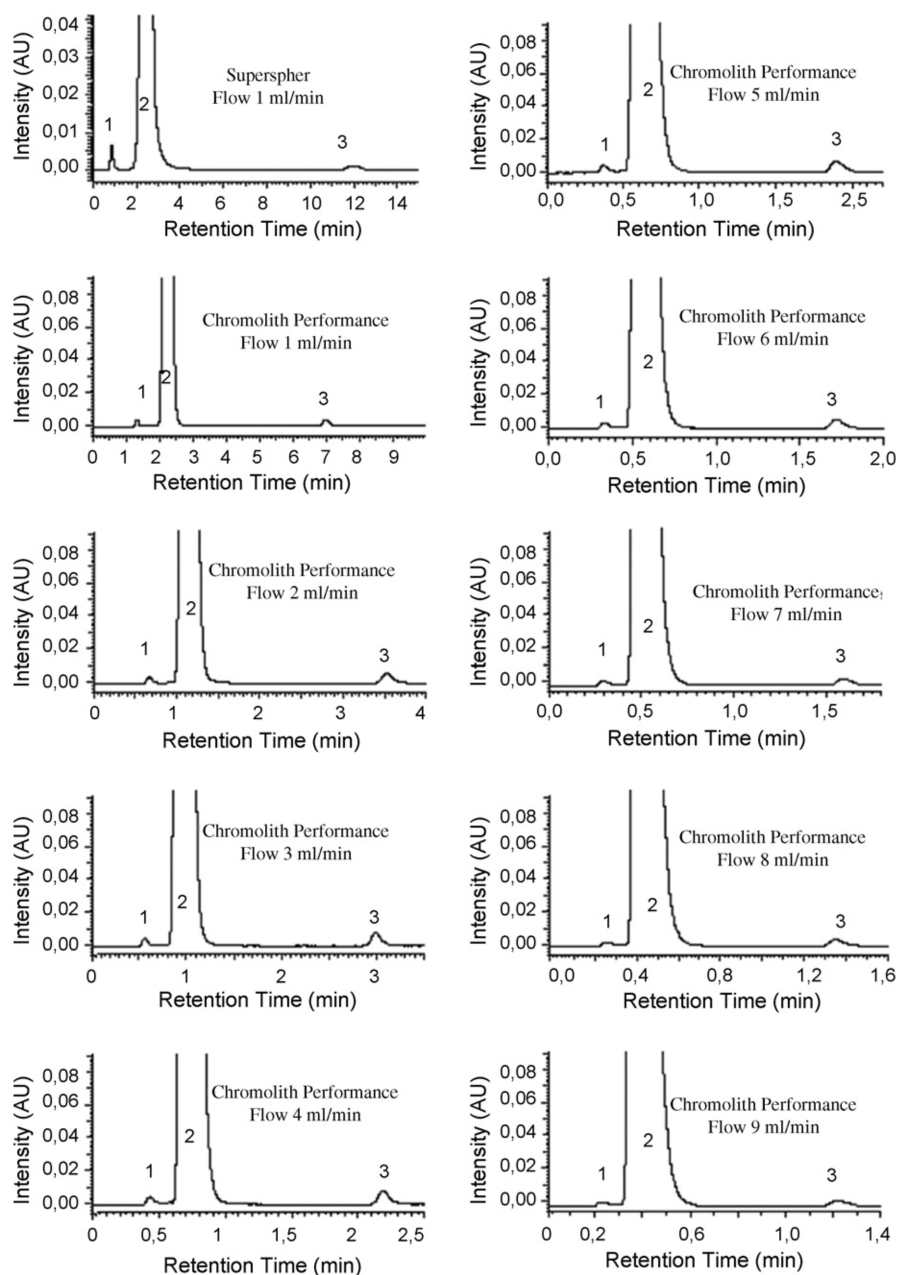

Fig. 3. Representative chromatograms for propranolol hydrochloride (peak 2) and its two degradation products a and b (peaks 1 and 3, respectively) on conventional (Superspher RP-18e) column and on monolithic (Chromolith Performance RP-18e) column. Mobile phase consists of buffer pH 3.3: acetonitrile (40:60, v/v).

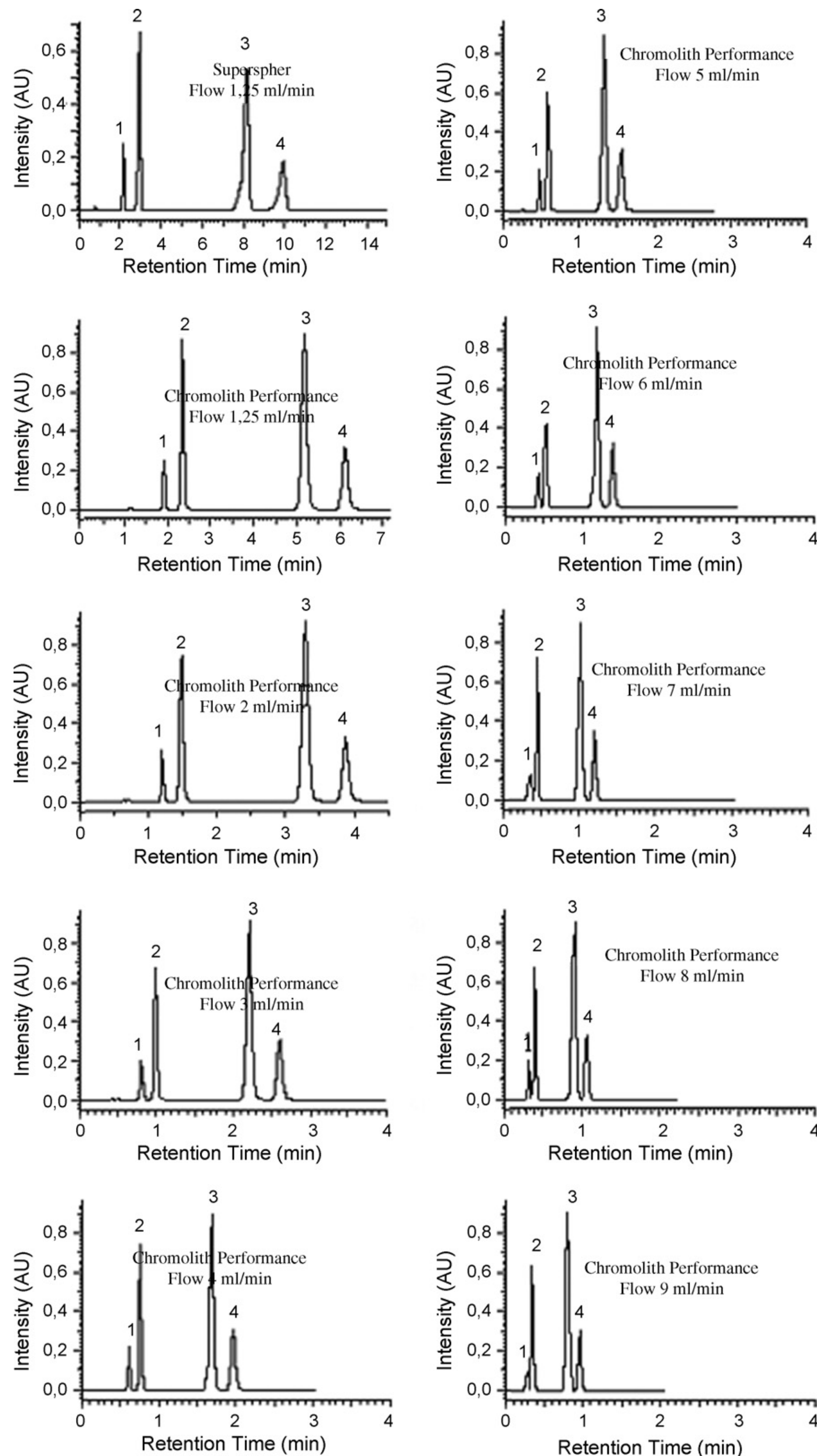

Fig. 4. Representative chromatograms for related compound A (1) related compound B (2), glibenclamide (3) and glimepiride (4) on conventional (Superspher RP-18e) and on monolithic (Chromolith Performance RP-18e) columns at different flow rates from 1.25 to 9 ml/min. Mobile phase consist of buffer pH 3: acetonitrile (55:45, v/v).

for polypeptide drugs are shown in Tables 3 and 4, respectively. Similar results were obtained for propranolol and glibenclamide. The highest RSD% value for the between days repeatability of peak area for propranolol and glibenclamide on the conventional column were 1.17 and 1.25%, respectively. In comparison, the highest RSD% values for between days repeatability of peak area for propranolol and glibenclamide on monolithic columns were 1.00 and 0.93%, respectively. Compared to earlier

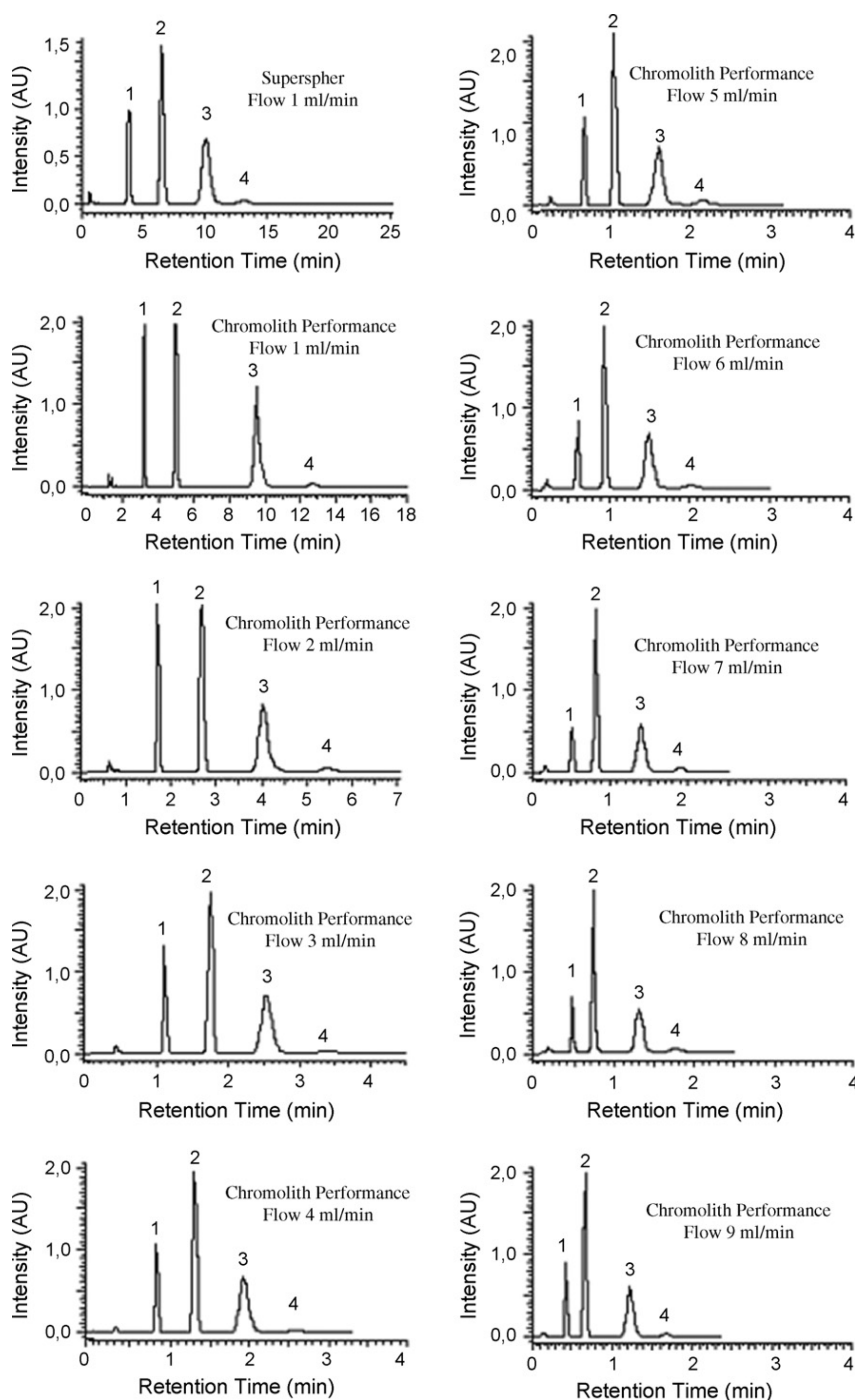

Fig. 5. Representative chromatograms for phenol (1), *m*-cresol (2), human insulin (3) and 21-desamido human insulin (4) on conventional (Superspher RP-18e) and on monolithic (Chromolith Performance RP-18e) columns at different flow rates from 1 to 9 ml/min. Mobile phase consist of buffer pH 2.3: acetonitrile in a ratio of (74:26, v/v) and (74.5:25.5, v/v) for conventional and monolithic columns, respectively.

works, the number of data is now sufficient to reliably estimate precision.

Precision of peak area and retention time was found to be slightly better on monolithic columns than on the conventional columns possibly due to the favorable accessibility of the pores and thus the faster mass transfer. This reduced baseline noise is probably due to the higher rigidity of the monolithic silica network skeleton. The selected methods include different ratios of organic modifier in the mobile phase: 2% in the pilocarpine method, 26% in the insulin method, 45% in the glibenclamide method and 60% in the propranolol method. This will probably give a representative evaluation of precision and method transfer success. Column to column repeatability for Chromolith Performance was measured in each method. A set of six monolithic columns originating from three different batches was tested for the pilocarpine method. For propranolol, glibenclamide

Table 2
List of recovery results for pilocarpine, propranolol, glibenclamide, and glimepiride at three concentration levels

Drug	Column type	Theoretical value (mg/ml)	Mean recovery[a] (mg/ml)	Recovery (%)	RSD (%)
Pilocarpine hydrochloride	Superspher RP-18e	0.008	0.00762	95.36	0.84
		0.200	0.1920	96.00	0.65
		0.500	0.4960	99.20	0.70
	Chromolith Performance RP-18e	0.008	0.0077	96.50	0.81
		0.200	0.1960	98.23	0.66
		0.500	0.4990	99.80	0.30
Propranolol hydrochloride	Superspher RP-18e	0.020	0.0197	98.95	1.97
		0.040	0.0395	98.00	1.26
		0.080	0.0794	99.27	0.91
	Chromolith Performance RP-18e	0.020	0.0198	99.00	1.91
		0.040	0.0398	99.50	1.23
		0.080	0.0796	99.60	1.00
Glibenclamide	Superspher RP-18e	0.160	0.1596	99.80	0.69
		0.200	0.2001	100.05	1.27
		0.240	0.2393	99.71	0.91
	Chromolith Performance RP-18e	0.160	0.1598	99.92	0.56
		0.200	0.1996	99.83	0.70
		0.240	0.2402	100.08	0.69
Glimepiride	Superspher RP-18e	0.160	0.1612	100.75	0.86
		0.200	0.1976	98.80	0.91
		0.240	0.2397	99.87	1.13
	Chromolith Performance RP-18e	0.160	0.1610	100.62	1.02
		0.200	0.1994	99.70%	0.86
		0.240	0.2405	100.20	0.95

[a] Mean value of 10 determinations.

Table 3
Precision on conventional and monolithic columns over a concentration range of 0.008–0.500 mg/ml pilocarpine HCl using $n = 5$ for both within day and between days repeatabilities (results on monolithic columns include precision at flow rates of 1 and 4 ml/min)

Column type and No	Within day repeatability RSD% of AUC[a]			Within day repeatability RSD% of t_R	Between day repeatability RSD% of AUC			Between day repeatability RSD% of t_R
	0.008 mg/ml	0.200 mg/ml	0.500 mg/ml	($n = 15$)	0.008 mg/ml	0.200 mg/ml	0.500 mg/ml	($n = 15$)
Superspher 100 RP-18e	0.84	0.70	0.88	0.52	1.16	0.90	0.84	0.66
Chromolith Performance RP-18e Batch No. Um 1042 Rod No. 1042/020								
1 ml/min	0.85	0.60	0.54	0.45	0.87	0.67	0.84	0.65
4 ml/min	0.70	0.52	0.40	0.66	0.85	0.76	0.87	0.65
Chromolith Performance RP-18e Batch No. Um 1043 Rod No. 1043/041								
1 ml/min	0.50	0.77	0.44	0.44	0.51	0.72	0.40	0.53
4 ml/min	0.69	0.49	0.68	0.49	0.59	0.81	0.89	0.52
Chromolith Performance RP-18e Batch No. Um 1045 Rod No. 1045/036								
1 ml/min	0.68	0.51	0.34	0.42	0.73	0.53	0.38	0.42
4 ml/min	0.74	0.77	0.87	0.45	0.88	0.43	0.85	0.54
Chromolith Performance RP-18e Batch No. Um 1042 Rod No. 1042/012								
1 ml/min	0.61	0.73	0.75	0.52	0.53	0.54	0.79	0.51
4 ml/min	0.64	0.47	0.31	0.38	0.62	0.74	0.70	0.51
Chromolith Performance RP-18e Batch No. Um 1043 Rod No. 1043/032								
1 ml/min	0.66	0.54	0.61	0.38	0.69	0.78	0.87	0.58
4 ml/min	0.67	0.68	0.67	0.45	0.57	0.66	0.71	0.53
Chromolith Performance RP-18e Batch No. Um 1045 Rod No. 1045/023								
1 ml/min	0.73	0.69	0.44	0.51	0.73	0.58	0.53	0.68
4 ml/min	0.58	0.62	0.49	0.48	0.50	0.80	0.71	0.76

[a] Area under curve.

Table 4
Precision of human insulin on conventional as well as monolithic columns over a concentration range 0.03–0.3 mg/ml using $n = 5$ for both within day and between days repeatabilities (results on monolithic columns include precision at flow rates of 1 and 4 ml/min)

Column type and No.	Within day repeatability RSD (%) of AUC			Within day repeatability RSD (%) of t_R	Between day repeatability RSD (%) of AUC			Between day repeatability RSD (%) of t_R
	0.03 (mg/ml)	0.30 (mg/ml)	3.00 (mg/ml)	($n = 15$)	0.03 (mg/ml)	0.30 (mg/ml)	3.00 (mg/ml)	($n = 15$)
Superspher 100 RP-18e	1.08	0.68	0.74	0.67	1.20	0.96	0.90	0.98
Chromolith Performance RP-18e Batch No. UM1042 Rod No. 1042/020								
1 ml/min	0.84	0.61	0.73	0.54	0.79	0.95	0.70	0.75
4 ml/min	0.96	0.78	0.50	0.58	1.03	0.91	0.75	0.66
Chromolith Performance RP-18e Batch No. UM1043 Rod No. 1043/041								
1 ml/min	0.89	0.42	0.31	0.57	0.97	0.75	0.87	0.70
4 ml/min	0.67	0.84	0.59	0.45	0.93	0.73	0.93	0.80
Chromolith Performance RP-18e Batch No. UM5020 Rod No. 5020/028								
1 ml/min	0.87	0.57	0.45	0.35	0.97	0.93	0.50	0.82
4 ml/min	0.75	0.55	0.62	0.43	0.97	0.85	0.90	0.60

and insulin methods column to column reproducibility was tested using three monolithic columns from different batches. Results are summarized in Table 5. A total of seven monolithic columns from four different batches were used in this study.

The calibration curves (peak area versus concentration) for the used drugs were investigated over a wide concentration range. Residual plots did not show any trends. Results were found to be linear with high correlation coefficients. The limit of detection (LOD, S/N = 3) and an estimate for the limit of quantitation (LOQ, S/N = 10) for all the tested drugs on monolithic columns at flow rates of 1 as well as 4 ml/min were found to be lower on the monolithic than on the conventional column. Results of linearity, detection and quantitation limits are summarized in Table 6. The lower limits of detection and quantitation obtained by the monolithic columns are partly due to the lower background noise obtained with these columns probably because of the better skeleton rigidity of the monolithic network.

Column efficiency was measured by plotting the height equivalent to theoretical plates (HETP) against the flow rates of the mobile phase. As expected, flat curves were obtained for the four tested methods. This indicates that monolithic columns can operate at high flow rate with only small decrease in efficiency. The high permeability of the monolithic columns was evidenced by a flow rate of 9 ml/min generating a total system back pressure of less than 140 bar in all of the tested methods. In comparison, the conventional column packed with 4 μm particles, reached a maximum backpressure of about 400 bar when it was operated at a flow-rate of 3.5 or 4 ml/min depending on the composition of the used mobile phase.

In a conventional Superspher column more time was required to re-equilibrate or to wash the stationary phase at a flow of 1 ml/min (approximately 30 min). In contrast, the time required to re-equilibrate or wash the monolithic columns was six times shorter (re-equilibrium time was about 5 min at a flow of 6 ml/min) due to the higher flow rates which have been used

Table 5
Monolithic column to column reproducibility calculated for each method

Drug method	The used Chromolith Performance RP-18e columns	RSD (%) of AUC[a]	RSD (%) of t_R
Pilocarpine hydrochloride	Rod No. 1042/020 Rod No. 1042/012 Rod No. 1043/032 Rod No. 1043/041 Rod No. 1045/036 Rod No. 1045/023	0.3–0.94	0.68
Propranolol hydrochloride	Rod No. 1042/020 Rod No. 1043/041 Rod No. 1045/036	0.36–1.25	0.66
Glibenclamide and glimepiride	Rod No. 1042/020 Rod No. 1043/041 Rod No. 1045/036	0.33–1.09	0.63
Insulin	Rod No. 1042/020 Rod No. 1043/041 Rod No. 5020/028	0.1–0.7	0.60

[a] RSD% range is due to determination at different concentration levels.

Table 6
Linearity, detection and quantitation limits of the five analyzed compounds

Compound	Column type	Concentration range (mg/ml)	R^2	Detection limit (μg/ml)	Quantitation limit (μg/ml)
Pilocarpine	Monolithic	0.008–0.500	0.9996	0.1700	0.500
	Conventional	0.008–0.500	0.9998	0.3100	1.000
Propranolol	Monolithic	0.002–1.00	0.9988	0.0120	0.040
	Conventional	0.002–1.00	0.9992	0.0610	0.200
Glibenclamide	Monolithic	0.010–0.240	0.9998	0.1220	0.400
	Conventional	0.010–0.240	0.9996	0.1240	0.410
Glimepiride	Monolithic	0.010–0.240	0.9999	0.1520	0.506
	Conventional	0.010–0.240	0.9999	0.1570	0.523
Insulin	Monolithic	0.030–3.00	0.9998	0.0008	0.002
	Conventional	0.030–3.00	0.9998	0.0013	0.004

for equilibrium. These favorable flow rates are possible using monolithic silica with its typical structure and distribution of mesopores.

In this evaluation for monolithic Chromolith Performance columns the polypeptide Insulin was also investigated. Larger molecular weight proteins were not investigated because the applicability of reversed phase silica for the quantitation of large molecular weight proteins suffers from the problem of adsorption and the loss of repeatability of results. Few papers were found in the literature suggesting reversed phase chromatography for the quantitation of proteins [28–30]. Furthermore, silica-based monolithic columns are particularly suited for the separation of small molecules, such as drug candidates and peptides, while the polymer monolith is generally preferable for larger molecules such as proteins, nucleic acid, and synthetic polymers.

Rapid analytical procedures can be obtained when replacing the existing HPLC applications by equivalent ones using monolithic columns instead of conventional particulate columns. This new trend will be highly important in the quality control of drugs. It may be applied for processing a large number of samples in a short time, thus being a practical choice for routine quality control studies.

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