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Evaluation of monolithic C18 HPLC columns for the fast analysis of pilocarpine hydrochloride in the presence of its degradation products

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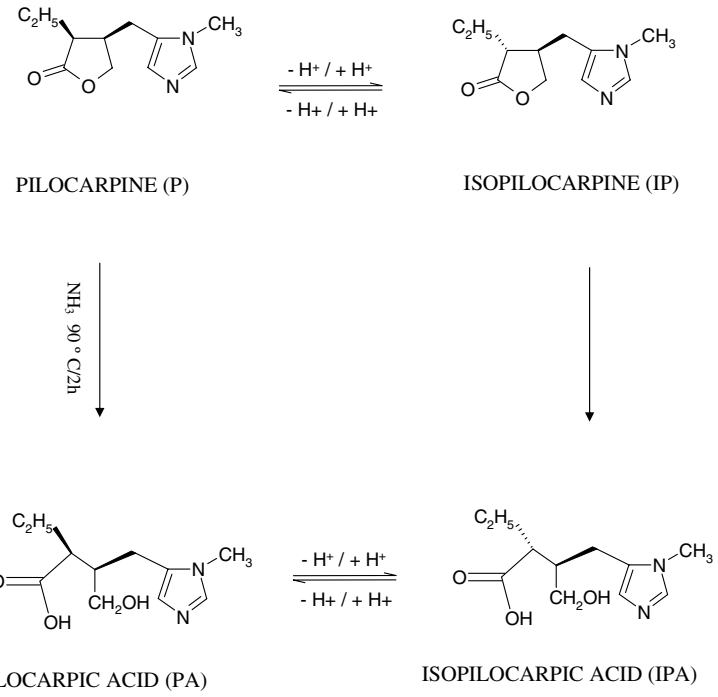
Monolithic Performance C18 HPLC columns (Chromolith Performance RP-18e, Merck) were applied for the determination of pilocarpine hydrochloride in the presence of its degradation products isopilocarpine, pilocarpic acid and isopilocarpic acid. The method was validated using a set of six monolithic columns and compared to a conventional C18 column. The separation of pilocarpine from its degradation products was investigated on monolithic columns at different flow rates from 1 to 9 ml/min. Superior resolution was obtained using monolithic columns over the conventional C18 column at the same flow rate of 1 ml/min with a total run time of 9 min compared to 13.5 min for the conventional C18 column. Comparable resolution to that obtained in the C18 column (but with better peak symmetry) was obtained at a flow rate of 4 ml/min, although the total run time was reduced to 3.5 min. The precision for both retention time and peak area was investigated over a wide concentration range and found to be equal, or slightly better on Chromolith Performance compared to the conventional column. The overall RSDs% ranged from 0.5% to 1.16% for the conventional column, while for monolithic columns ranged from 0.38% to 0.87% at a flow rate of 1 ml/min and from 0.38% to 0.89% at a flow rate of 4 ml/min. Monolithic column to column reproducibility ($n = 6$) was measured. The RSDs% ranged from 0.34% to 0.68% for retention time and from 0.3% to 0.94% for peak areas. The detection and quantitation limits on monolithic columns at both flow rates (1 and 4 ml/min) were found to be 0.17 μg/ml and 0.5 μg/ml, compared to 0.31 μg/ml and 1 μg/ml on the conventional column. Monolithic silica rods have also shown the advantage of reducing the time to wash and to re-equilibrate the column. The method showed good linearity and recovery and was found to be suitable for the analysis of pilocarpine hydrochloride formulations.

1. Introduction

During the last years, one of the most interesting occurrences in liquid chromatography was the introduction of monolithic silica columns which enable the use of highly porous materials. These columns are formed of a single rod of silica based material. Based on the work carried by Minakuchi and Soga (1991), monolithic silica material has been manufactured using a sol-gel process which includes the hydrolysis and polycondensation of alkoxysilanes in the presence of water soluble polymers. After ageing, the phase dried to form a rod with a bimodal pore structure consisting of large macropores (diameter 2 μm), and mesopores (13 nm in diameter). The large macropores are responsible for a low flow resistance and therefore allow the application of high eluent flow-rates, while the small pores ensure sufficient surface area (300 m^2/g approximately) for separation efficiency. Monolithic columns also have a significantly higher total porosity after octadecylsilylation than conventional particulate columns, over 80% vs. ca 65%, respectively (Nakanishi et al. 1997). In spite of this promising characteristics few analytical applications have been developed using monolithic columns. Up to now they are not mentioned as an official method in any pharmacopoeia. Furthermore, the transferability of analytical methods from the conventional C18 columns to monolithic columns is still under discussion.

Pilocarpine hydrochloride, (3*S*,4*R*)-3-ethyl-4-[(1-methyl-1*H*-imidazol-5-yl) methyl]dihydrofuran-2(3*H*)-one hydrochloride, is a parasympathicomimetic compound, which is widely used in ophthalmic solutions for the treatment of glaucoma. It may also be orally administered for the treatment of patients with impaired secretion of the salivary glands, resulting from drug therapy or cancer radiation (Greenspan and Daniels 1987). Pilocarpine becomes pharmaceutically inactive due to either racemisation and/or hydrolysis of the lactone. Pilocarpine is prone to racemisation as the α-position adjacent to a carbonyl group is mildly acidic and can undergo keto/enol tautomerism. Deprotonation and reprotonation processes cause the racemisation of this drug. Pilocarpine can also hydrolyse under basic conditions to form pilocarpic acid. Since pilocarpine can epimerize at the α-position to form isopilocarpine this can then hydrolyse to form isopilocarpic acid (Scheme). Many methods have been reported for the determination

Scheme: Hydrolysis and racemization of pilocarpine



of pilocarpine as such or with its degradation products. Most methods employ HPLC, including both normal and reversed phase chromatography (Kennedy et al. 1981; Noordam et al. 1981; Vespalec et al. 1988; Gomez-Gomar et al. 1989; Sternitzke et al. 1992; Matsuura et al. 1993). Typically reported chromatographic run times for the reported HPLC methods are in the range of 13–25 min, and in many of these methods peak symmetry and resolution is still problematic. Gas chromatographic methods have also been reported, but these require precolumn derivatization of the analyte (Dziedzic et al. 1976; Bayne et al. 1976). A capillary electrophoresis method has also been reported for the analysis of pilocarpine and its degradation products with a total run time of about 16 min (Charman et al. 1992).

The aim of this work was to evaluate the chromatographic behavior of commercial available Monolithic Performance RP-18e HPLC columns for the rapid analysis of pilocarpine, including separation from its three degradation products. Furthermore, the performance of this columns should be compared to that of a conventional C18 (Superspher) stationary phase. 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 (Fan et al. 1996).

2. Investigations and results

2.1. Method validation

Monolithic Performance RP-18e columns and a Superspher RP-18 conventional column have been applied for the determination of pilocarpine hydrochloride including

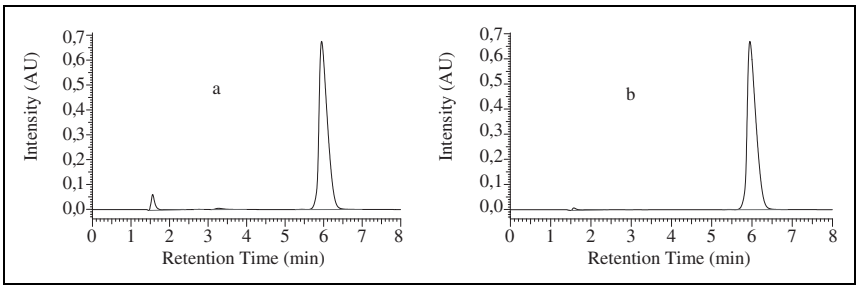


Fig. 1:
Representative chromatograms of pilocarpine – excipient (a) and pilocarpine standard (b)

Table 1: Recovery results (n = 10) of pilocarpine hydrochloride from ophthalmic solution at three concentration levels

Column type	Theoretical value (mg/ml)	Mean recovery (mg/ml)	Recovery (%)	RSD (%)
Superspher RP-18	0.008	0.00762	95.36%	0.84%
	0.2	0.1920	96.00%	0.65%
	0.5	0.4960	99.20%	0.70%
Chromolith Performance RP-18	0.008	0.0077	96.50%	0.81%
	0.2	0.1960	98.23%	0.66%
	0.5	0.4990	99.80%	0.30%

separation from its degradation products. The method was found to be transferable from the conventional to the monolithic columns. It has been demonstrated, that the same elution order of the used mixture (isopilocarpine, pilocarpine, pilocarpic acid and isopilocarpic acid) was obtained in monolithic and conventional columns. This indicates that the selectivity of the two column types is equivalent. The specificity of the method was also examined by observing if there was any interference of the inactive ingredients of pilocarpine hydrochloride eye drops. The HPLC chromatograms recorded for pilocarpine inactive ingredients showed no peaks at the retention times of pilocarpine hydrochloride and its degradation products. Fig. 1 shows representative chromatograms for extracted pilocarpine from drug matrix (chromatogram a) and pilocarpine standard (chromatogram b). The specificity was also demonstrated by the good separation of the products obtained by induced degradation of pilocarpine hydrochloride sample (isopilocarpine, pilocarpic acid and isopilocarpic acid). The accuracy of the method was tested by determination of the recovery using the excipients used in a pilocarpine hydrochloride eye drop formulation. The recovery was investigated by spiking pilocarpine hydrochloride solution to a blank matrix (see 4.6). The mixture was evaluated on both conventional and monolithic columns. Accepted recovery percentage was obtained. Results are summarized in Table 1. To ensure assay precision within day ($n = 5$) and between days ($n = 5$) precisions were assessed at 3 concentration levels for a conventional (Superspher) as well as a set of six monolithic (Chromolith Performance) columns. The tested Chromolithic columns originated from three batches each containing two columns. Results are summarized in Table 2. Column to column repeatability for Chromolith Performance was measured. The RSDs% ranged from 0.34% to 0.68% for retention time (t_R) and from 0.3% to 0.94% for peak area (AUC). The calibration curves (peak area vs. concentration) for pilocarpine hydrochloride in sample diluent were investigated over the concentration range of 0.08–1 μg/ml and found to be linear. Residual plot did not show any trend, correlation coefficients were about 0.9996 and 0.9998 using conventional and monolithic columns, respectively. Limit of detection (LOD, $S/N = 3$) and an estimate for the limit of quantitation (LOQ, $S/N = 10$) on monolithic columns at flow rates of 1 as well as 4 ml/min were found to be 0.17 μg/ml and 0.5 μg/ml,

Table 2: Precision on conventional as well as monolithic columns over a concentration range of 0.008–0.5 mg/min pilocarpine HCL using n = 5 for both within day and between days repeatabilities*

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
		0.008 mg/min	0.2 mg/min	0.5 mg/min	(n = 15)	0.008 mg/min	0.2 mg/min	0.5 mg/min	(n = 15)
Superspher 100 RP-18 column		0.84%	0.7%	0.88%	0.52%	1.16%	0.9%	0.84%	0.66%
Chromolith Performance RP-18e	1 min/min	0.85%	0.6%	0.54%	0.45%	0.87%	0.67%	0.84%	0.65%
Batch No. Um 1042	4 min/min	0.7%	0.52%	0.4%	0.66%	0.85%	0.76%	0.87%	0.65%
Rod No. 1042/020									
Chromolith Performance RP-18e	1 min/min	0.50%	0.77%	0.44%	0.44%	0.51%	0.72%	0.4%	0.53%
Batch No. Um 1043	4 min/min	0.69%	0.49%	0.68%	0.49%	0.59%	0.81%	0.89%	0.52%
Rod No. 1043/041									
Chromolith Performance RP-18e	1 min/min	0.68%	0.51%	0.34%	0.42%	0.73%	0.53%	0.38%	0.42%
Batch No. Um 1045	4 min/min	0.74%	0.77%	0.87%	0.45%	0.88%	0.43%	0.85%	0.54%
Rod No. 1045/036									
Chromolith Performance RP-18e	1 min/min	0.61%	0.73%	0.75%	0.52%	0.53%	0.54%	0.79%	0.51%
Batch No. Um 1042	4 min/min	0.64%	0.47%	0.31%	0.38%	0.62%	0.74%	0.7%	0.51%
Rod No. 1042/012									
Chromolith Performance RP-18e	1 min/min	0.66%	0.54%	0.61%	0.38%	0.69%	0.78%	0.87%	0.58%
Batch No. Um 1043	4 min/min	0.67%	0.68%	0.67%	0.45%	0.57%	0.66%	0.71%	0.53%
Rod No. 1043/032									
Chromolith Performance RP-18e	1 min/min	0.73%	0.69%	0.44%	0.51%	0.73%	0.58%	0.53%	0.68%
Batch No. Um 1045	4 min/min	0.58%	0.62%	0.49%	0.48%	0.50%	0.8%	0.71%	0.76%
Rod No. 1045/023									

* results on monolithic columns include precision at flow rates of 1 and 4 min/min

Table 3: Performance parameters on conventional and monolithic columns*

Column type	Theoretical plate N (Plate per column for pilocarpine)	Asymmetry factor for Pilocarpine Peak	Resolution (Rs) Pilocarpine/Pilocarpic acid
Conventional C18 (flow rate 1ml/min)	1783	1.7	2.10
Monolithic C18 (flow rate 1ml/min)	3648	1.26	2.78
Monolithic C18 (flow rate 2ml/min)	3074	1.28	2.33
Monolithic C18 (flow rate 3 ml/min)	2559	1.23	2.23
Monolithic C18 (flow rate 4 ml/min)	2488	1.22	2.13
Monolithic C18 (flow rate 5 ml/min)	2415	1.21	1.92
Monolithic C18 (flow rate 6 ml/min)	1950	1.35	1.8
Monolithic C18 (flow rate 7 ml/min)	1777	1.4	1.58
Monolithic C18 (flow rate 8 ml/min)	1430	1.32	1.43
Monolithic C18 (flow rate 9 ml/min)	1336	1.3	1.32

* The following equations were used to calculate the above mentioned chromatographic parameters according to USP method ($N = 16(t_R/w)^2$, asymmetry factor (AF) = B/A at 10% of peak height (A & B are the two half width of the peak center at each side at 10% height from the peak base) and Resolution $Rs = 2(t_{R2} - t_{R1}/w_2 + w_1)$

respectively, compared to 0.31 μg/ml and 1 μg/ml on the conventional C18 column. The lower limits of detection and quantitation obtained by the monolithic columns are partly due to the lower background noise obtained with these columns.

2.2. Performance parameters

Peak performance parameters were also calculated according to USP equations (Table 3). Compared to a traditional particulate column (Superspher RP-18), Chromolith Per-

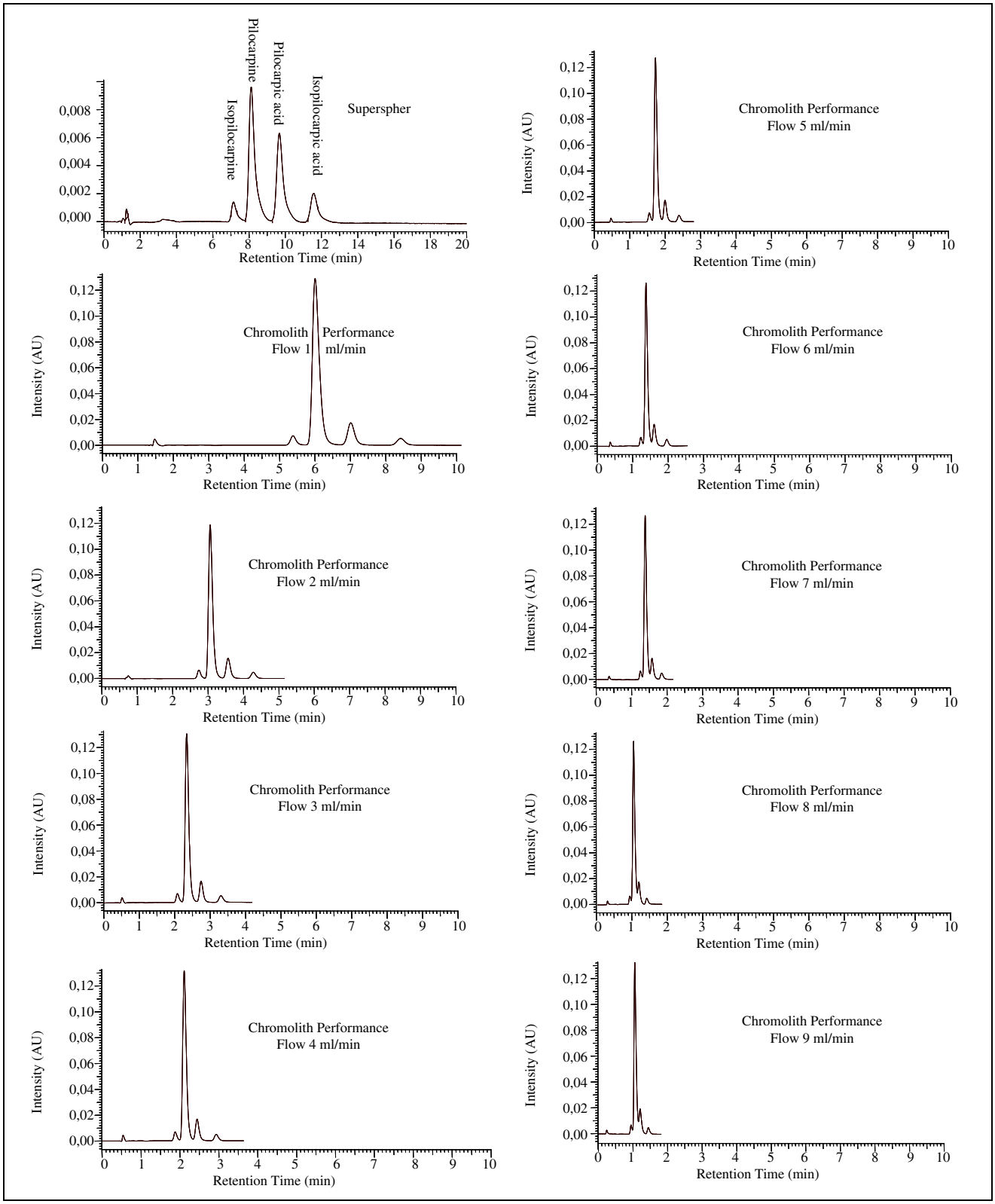


Fig. 2: Representative chromatograms for pilocarpine hydrochloride and its degradation products on conventional (Superspher RP-18) column, and on monolithic (Chromolith Performance RP-18e) column at different flow rates from 1 to 9 ml/min. Mobile phase consists of a buffer pH = 3: methanol (980 : 20, v/v). Difference in peak intensity between conventional and monolithic columns is due to difference in concentration of pilocarpine and its degradation products

formance RP-18e columns were found to produce better resolution and peak symmetry in a shorter run time (taking in account the small difference in column length) at the same flow rate of 1 ml/min. 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 acceptable and comparable to that obtained with the conventional column operated at a flow rate of 1 ml/min, with the advantage of reducing the total run time from 13 min to about 3.5 min. Representative chromatograms for pilocarpine hydrochloride and its three degradation products on conventional and monolithic columns are shown in Fig. 2. The high permeability of the monolithic columns were evidenced by a flow rate of 9 ml/min generating a total system back pressure of only 137 bar. 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 4 ml/min. At a flow rate of 1 ml/min backpressure is about six times smaller on a monolithic column than on a Superspher RP 18 column. Backpressures at different flow rates on conventional Superspher and chromolith Performance RP-18e columns are shown in Fig. 3. Column efficiency was measured by plotting the height equivalent to theoretical plates (HETP) against the flow rates of the mobile phase (Fig. 4). The height equivalent to theoretical plate H was calculated from the column length L and theoretical plate N according to H=L/N. A flat curve was obtained, indicating that monolithic columns can operate at high flow rate with only small decrease in efficiency.
In a conventional Superspher column more time was required to re-equilibrate (about 30 min) or to wash the stationary phase. In contrast, the time required to re-equilibrate or wash the monolithic columns was markedly shorter (re-equilibrium time was about 5 min), probably due to the rigidity of the monolithic columns.

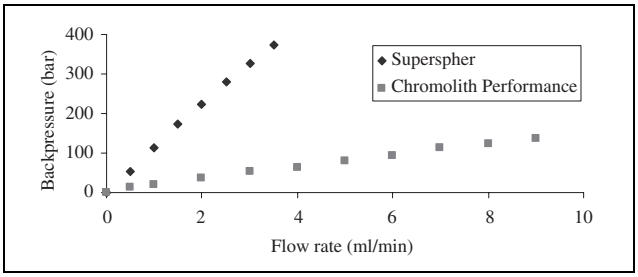


Fig. 3: Plot of column backpressure against flow rate of mobile phase for conventional (Superspher) and monolithic (Chromolith Performance) C18 columns. Mobile phase consists of a buffer pH = 3: methanol (980 : 20, v/v)

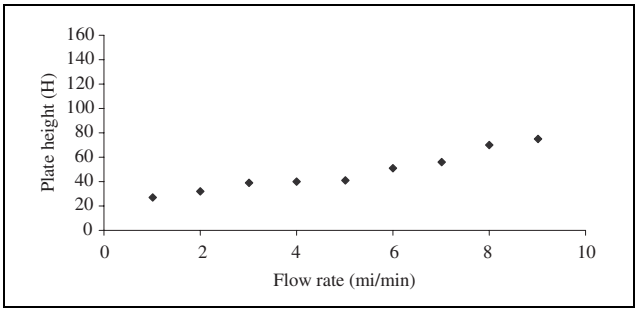


Fig. 4: Plot of height equivalent to theoretical plate against mobile phase flow rate for one of the Chromolith Performance RP-18e columns with pilocarpine hydrochloride

3. Discussion

Both types of columns were able to separate pilocarpine from its three degradation products, however monolithic columns were found to perform the separation with shorter run time and better peak symmetry and resolution compared to a conventional column, under the same chromatographic conditions. In fact, peak tailing with monolithic columns was reported in some previously mentioned studies (Kele and Guichon 2002; McCalley 2003). However in many other works with monolithic columns a minimal or no peak tailing were reported (Bidlingmaier et al. 1999; Nederkassel et al. 2003; Novakova et al. 2004). Precision was also better on the Chromolith Performance than on the conventional columns. When higher flow rates were applied on monolithic columns there was some loss in resolution. A flow rate of 4 ml/min was selected for precision studies, as it provides the smallest analysis time with a baseline resolution value higher than 2 and comparable to that obtained by conventional columns. As expected, the total analysis time was reduced to about a quarter at a flow rate of 4 ml/min using monolithic column. The same mobile phase was applied on conventional and monolithic columns, to enable the direct comparison of the two columns performances and to see if a chromatographic method could be transferred from conventional to monolithic column without further modification. This method is applicable for the rapid quantitation of pilocarpine and its degradation products. Monolithic columns have been shown as an excellent alternative to conventional silica based columns. Rapid analytical procedures could 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. Furthermore, the above described method could also be useful for stability testing of pilocarpine hydrochloride formulations.

4. Experimental

4.1. Chemicals and reagents

Acetonitrile HPLC grade was purchased from Acros Organics (Belgium). Methanol HPLC grade was obtained from Fisher Scientific (UK). Phosphoric acid 85%, triethylamine and ammonia were purchased from Merck (Darmstadt, Germany). Pilocarpine hydrochloride was obtained from Acros Organics (Belgium). All chemicals were of analytical grade, and deionized water was used throughout.

4.2. 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), and an Interface (D-6000). The data were collected and analyzed using the D7000 HSM software (Merck).

4.3. Chromatographic conditions

The separation was performed on a Supershper 100 RP-18 column (endcapped, 4 μm particle size, 125 mm × 4 mm, Merck) and a set of six Chromolith Performance RP-18e (100 × 4.6 mm, Merck) The mobile phase was prepared according to a previously recommended method (Tony et al. 1996) 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 pH was adjusted to 3 by the addition of 50% sodium hydroxide.) The mobile phase was degassed by sonication before use. The flow rate was 1 ml/min on the conventional column (except for measurement of backpressure at which flow rates up to 4 ml/min were used), while different flow rates from 1 to 9 ml/min were applied on monolithic columns. The injection volume was 20 μl and the detection wavelength was 214 nm. All separations were performed at ambient temperature.

4.4. Preparation of standards

The buffer described in 4.3 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, 2004 were used.

4.5. Preparation of degradation products

4.5.1. Solution a (isopilocarpine)

Isopilocarpine was obtained from pilocarpine by racemization. 1 ml 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.

4.5.2. 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 that described by Repta and Higuchi (1971). To a 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.

4.5.3. 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.

4.6. Preparation of excipient solution

The excipient matrix for pilocarpine hydrochloride ophthalmic solution was prepared containing the inactive ingredients disodium edetate 4% w/v, polyvinylpyrrolidone 1.7% w/v, sodium dihydrogen phosphate 0.8% w/v, disodium hydrogen phosphate 0.94% w/v, sodium chloride 0.9% w/v and benzalkonium chloride 0.13% w/v in 100 ml of deionized water.

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