Monolithic silica liquid chromatography columns for the determination of cyclooxygenase II inhibitors in human plasma

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Abstract

Methods employing monolithic HPLC columns for the determination of the cyclooxygenase II inhibitors rofecoxib (**I**) and 3-isopropoxy-4-(4-methanesulfonylphenyl)-5,5′-dimethyl-5H-furan-2-one (DFP, **III**) in human plasma are described. Each analyte, together with an internal standard was extracted from the plasma matrix using solid-phase extraction in the 96-well format. The analytes were chromatographed on a Chromolith Speed Rod monolithic HPLC column (4.6×50 mm). Analyte detection for rofecoxib was via fluorescence following post-column photochemical derivatization. Detection for **III** was based on the native fluorescence of the compound. The precision, accuracy, and linearity of the methods were found to be comparable to those obtained using methods employing conventional packed HPLC columns. Use of the monolithic column permitted mobile phase flow-rates of up to 6.5 ml/min to be employed in the assays. The use of elevated flow-rates enabled the per sample analysis time to be reduced by up to a factor of 5 compared with assays based on packed HPLC columns. The results of experiments aimed at evaluating the ruggedness and reproducibility of monolithic columns employed in bioanalytical methods are presented.
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1. Introduction

An ever-increasing need exists within the pharmaceutical industry to shorten the drug development timeline. Maximizing the throughput of assays that support clinical studies is, therefore, a key concern in a bioanalytical laboratory. For HPLC based assays, the process of reducing analysis time while adequately resolving analytes from endogenous components and metabolites is often accomplished with short columns packed with small particles (e.g., $L \leq 50$ mm, $d_p = 3$ μm). The theoretical advantages of small packing particles include higher optimum linear velocities as well as shallower slopes in the high velocity region of plate height versus linear velocity curves [1]. In principle, high speed, high resolution separations can be obtained by operating small particle packed columns at high flow-rates. Unfortunately, the high back pressure associated with these columns effectively limits their operation to mobile phase flow-rates of less than about 2 ml/min.

The recent introduction of commercially available silica-based monolithic HPLC columns [2] presents an alternative to conventionally packed columns.

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Produced via sol–gel chemistry, using technology developed by Nakanishi et al. [3], these porous monoliths are designed such that a tightly controlled bimodal pore size distribution is obtained. Bulk mobile phase flow occurs through macropores, nominally 2 μm in diameter. The structure and geometry of the macropores impart to the monolith a lower flow resistance relative to a packed bed column. Mesopores having a nominal diameter of 130 Å provide the surface area needed for stationary phase coverage and analyte partitioning. The ability during synthesis to control the size of the macropores relative to the silica skeleton is the key to the improved chromatographic performance of these columns. The enhanced permeability of these columns allows separations to be conducted at high flow-rates (>5 ml/min) without excessive backpressure. In addition, the monoliths are claimed to provide reduced band broadening, arising from stagnant mobile phase mass transfer, thereby minimizing efficiency loss at high linear velocities [4]. The main advantage of monolithic columns, therefore, is that analysis time can be decreased without compromising resolution.

A limited number of bioanalytical applications of monolithic columns have appeared in the literature [5,6]. Each of the published applications have used tandem mass spectrometry (MS) as the chromatographic detection method. Tandem MS detection requires that the monolithic column effluent flow be split prior to the MS interface, as currently available interfaces cannot tolerate flow-rates in excess of 2 ml/min. Flow splitting potentially limits assay sensitivity as a portion of the injected sample is diverted to waste.

In our laboratories we are evaluating the use of monolithic columns in quantitative bioanalytical applications that use detection methods other than tandem mass spectrometry. Use of high mobile phase flow-rates with monolithic columns has the potential of markedly increasing the throughput of bioanalytical assays based on conventional HPLC detection methodologies, thus opening the possibility of shattering the commonly accepted perception that such assays require lengthy analysis times.

In this paper, the use of commercially available monolithic HPLC columns in assays for the determination of rofecoxib (Compound **I**, Fig. 1) and an experimental cyclooxygenase II (COX-II) inhib-
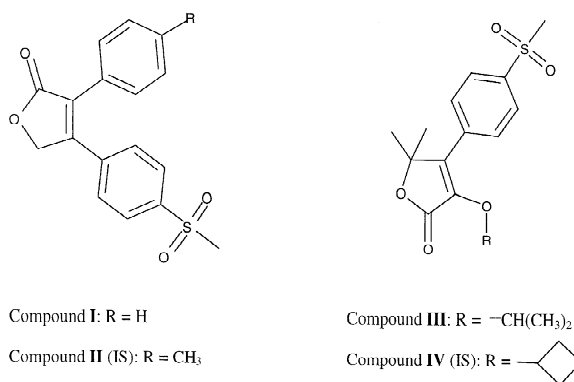

Fig. 1. Chemical structures of compounds **I** and **III** and the respective internal standards (**II** and **IV**).

itor, 3-isopropoxy-4-(4-methanesulfonylphenyl)-5,5′-dimethyl-5H-furan-2-one (DFP, Compound **III**, Fig. 1), in human plasma is described. The assay for **I** utilizes fluorescence detection following post-column photochemical derivatization, while the assay for **III** uses detection based on the native fluorescence of this compound. The methods are applied to the analysis of human clinical samples and the results, with respect to both analyte quantitation and assay throughput, are compared to those obtained with methods that employed conventional packed columns. The operation of the monolithic columns is further evaluated with respect to column to column reproducibility and efficiency, following the analysis of a large number (>1500) of injected plasma extracts.

2. Experimental

2.1. Materials

Compounds **I**, **III**, and **IV** were obtained from the Chemical Data Department of Merck Research Laboratories (Rahway, NJ, USA). Compound **II** was synthesized by S. Leger of the Medicinal Chemistry Department of Merck Frosst Canada (Kirkland, Quebec, Canada). Solvents (OmniSolv, HPLC grade) were obtained from EM Science (Gibbstown, NJ, USA). Drug free (control) human plasma was purchased from Sera-Tec Biologicals (New Brunswick, NJ, USA). All other reagents were of ACS grade and were used as received. Empore™ 96-well solid-phase extraction plates (C-8 standard density) were ob-

tained from 3 *M* (St. Paul, MN, USA). SPEC® 96-well solid-phase extraction plates (C-8) were obtained from Ansys Technologies, Inc. (Lake Forest, CA, USA).

2.2. Instrumentation

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) Series 200 pump, a Varian (Walnut Creek, CA, USA) ProStar 430 autosampler, and a Perkin-Elmer Series 200 fluorescence detector. The detector output was connected to a PE Nelson (San Jose, CA, USA) NCI-900 network chromatography interface. Chromatographic data was acquired and processed using Perkin-Elmer TurboChrom software. A sampling rate of 5 points/s was used. In the assay of **I**, the HPLC system included an Astec (Whippany, NJ, USA) Beam Boost photochemical reactor equipped with a 254 nm lamp and a 5 m reaction coil (0.3 mm I.D.).

2.3. Chromatographic conditions

Conditions for the analysis of rofecoxib with a conventional HPLC column have been described previously [7]. For the analysis of rofecoxib (**I**) with the monolithic column, the mobile phase consisted of a 65:35 v/v% mixture of H_2O:acetonitrile that was filtered through a 0.45 μm nylon membrane filter prior to use. A Chromolith (Merck KGaA, Darmstadt, Germany) Speed Rod RP-18e analytical column (4.6×50 mm) and a Keystone (Bellefonte, PA, USA) Javelin Beta Basic C_{18} (4×10 mm; 5 μm) guard column were operated at ambient temperature (approximately 21 °C). The mobile phase flow-rate was 3.0 ml/min. Injection volume was 50 μl. Excitation and emission wavelengths were 250 and 400 nm, respectively, and the detector time constant was 0.1 min. Total run time was 2.3 min.

The conventional HPLC system for the analysis of **III** employed a Waters Symmetry C_{18} column (50× 4.6 mm, 3.5 μm) preceded by a 0.2 μm in-line prefilter. The mobile phase was prepared by mixing 420 ml acetonitrile, 580 ml water and 0.7 ml of 85% phosphoric acid. The apparent pH of the resulting mixture was adjusted to 3 via dropwise addition of 10 N sodium hydroxide. The mobile phase was

passed through a 0.2 micron nylon filter prior to use. The mobile phase flow-rate was 1.4 ml/min and the injection volume was 30 μl. Detection was via fluorescence (λ_{ex}=300 nm, λ_{em}=360 nm). Total run time was 6 min.

Compound **III** was analyzed using the monolithic column with a mobile phase consisting of a 65:35 v/v% mixture of 15 m*M* NaH_2PO_4:acetonitrile. The mobile phase was filtered through a 0.45 μm nylon membrane filter prior to use. The apparent pH of the mobile phase was adjusted to 3.0 with 85% H_3PO_4. The guard column and monolithic analytical column used for the analysis of rofecoxib described above were also used for the analysis of **III**. The mobile phase flow-rate was 6.5 ml/min. Injection volume was 30 μl. Excitation and emission wavelengths were 300 and 360 nm, respectively, and the detector time constant was 0.1 min. Assay run time was 1.2 min.

2.4. Preparation of standards

Standard solutions of **I** were prepared as described previously [7]. All standard solutions were prepared in volumetric flasks made of low actinic glass.

Plasma standards were prepared by spiking 25 μl of each working standard into 0.5 ml human control plasma and were subsequently used to quantitate **I** in plasma at concentrations between 0.5 and 80 ng/ml. Clinical samples found to contain **I** at concentrations above 80 ng/ml were diluted in control plasma and re-analyzed.

In order to prepare standards of **III**, a 60 μg/ml stock solution was prepared by weighing 3.0 mg of reference material into a 50 ml volumetric flask and diluting to volume with acetonitrile. The solution was sonicated to ensure complete dissolution of the solid. Working standards at the appropriate concentrations were prepared by dilution of the 60 μg/ml stock standard. All standard solutions were prepared in low actinic volumetric flasks.

Plasma standards were prepared by spiking 25 μl of each working standard into 0.5 ml human control plasma and subsequently used to quantitate **III** in plasma at concentrations between 5 and 400 ng/ml. Clinical samples found to contain **III** at concentrations above 400 ng/ml were diluted in control plasma and re-analyzed.

Table 1
Solid phase extraction procedure for the determination of **III** in human plasma

	Condition	Wash	Elution	Dilution[a]
Compound **III**	0.5 ml CH_3OH	0.5 ml H_2O	250 µl (40:60)	200 µl H_2O
	1 ml H_2O	1 ml (85:15)	$H_2O:CH_3CN$	
		$H_2O:CH_3CN$		

[a] To ensure compatibility with HPLC mobile phase.

2.5. Plasma extraction procedures

Following the addition of internal standard (**II**), samples containing **I** were extracted using a 96 well solid-phase extraction (SPE) method with an Empore C8 SPE plate, that has been described previously [7].

Frozen plasma samples containing **III** were thawed at room temperature and vortex mixed for approximately 1 min after which a 0.5 ml aliquot was pipetted into a 5 ml polypropylene tube. A 25 µl aliquot of acetonitrile was added to compensate for the volume of spiking solution added to the plasma standards followed by the addition of 25 µl internal standard solution (2 µg/ml **IV** in acetonitrile). The sample was then diluted with 0.5 ml H_2O.

Samples were extracted using SPEC C-8 96-well SPE plates according to the procedure in Table 1. The SPE plate was centrifuged at 644 _g_ for 5 min after the water/acetonitrile wash and elution steps to remove residual liquid from the sorbent. The final extract was filtered through a 96-well filter plate (0.45 µm nylon filter, Spe-ed 96, Applied Separations, Allentown, PA, USA) by centrifugation at 644 _g_ for 2 min and a 30 µl aliquot of the extract was injected onto the HPLC system.

3. Results and discussion

3.1. Determination of **I** in human plasma

Previously, a sensitive and selective HPLC method for the determination of **I** in human plasma using post column photochemical derivatization and fluorescence detection has been reported by our laboratory [7]. This procedure utilized 96 well SPE for sample preparation followed by HPLC with postcolumn photochemical derivatization and fluorescence detection. Although a high throughput sample

preparation procedure was employed, the method utilized standard reversed-phase HPLC methodology, which resulted in the need for a run time of 10 min. Thus, there was a significant disconnect between the throughput of the sample preparation procedure and that of the chromatographic method; more samples could be extracted in a day than could be chromatographed in a 24 h period. The mismatch between sample preparation throughput and sample analysis throughput of this method served as an impetus for investigating the use of a monolithic column for this assay.

As mentioned previously, the assay for **I** utilized post column photochemical derivatization followed by fluorescence detection. Using a monolithic column at accelerated flow-rates potentially could result in a decrease in detection sensitivity, as the residence time for the analyte in the photochemical reactor would be reduced, potentially leading to a decreased yield of the fluorescent product. To evaluate this point, injections of a 20 ng/ml solution of **I** were made onto the monolithic column operating at flowrates between 1 and 3 ml/min. The signal-to-noise (s/n) ratio of the resulting peak of **I** then was calculated and is shown in Fig. 2. The results indicate that the s/n ratio of the rofecoxib peak
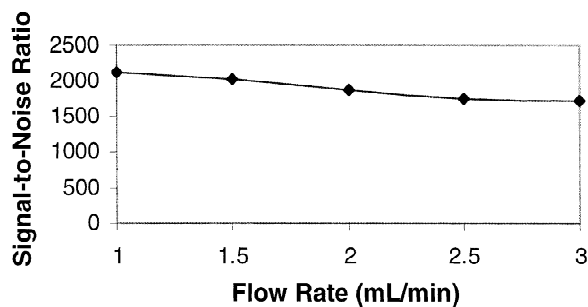
Fig. 2. Effect of mobile phase flow-rate on the signal-to-noise ratio (s/n) of the peak resulting from the injection of a 20 ng/ml solution of **I** onto the monolithic column.
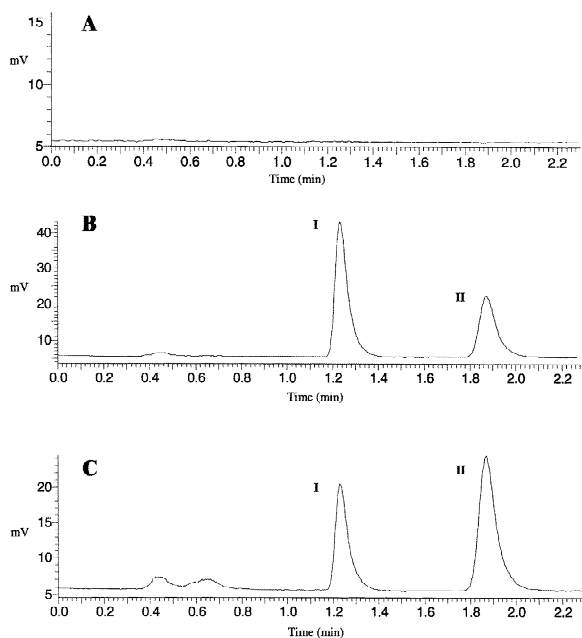
Fig. 3. Representative chromatograms from the assay of **I** in human plasma (A) human control plasma, (B) plasma spiked with 20 ng/ml **I** and 10 ng/ml **II** (IS), and (C) a human clinical sample collected 0.5 h after dosing with **I**. The concentration of **I** is equivalent to 7.1 ng/ml.

decreases by about 20% on increasing flow-rate from 1 to 3 ml/min; this slight decrease in signal was not found to significantly affect the sensitivity of the assay.

In that assay sensitivity was not adversely affected at higher flow-rates, the assay for **I** was successfully transferred directly from the packed HPLC column

with a mobile phase flow-rate of 1.2 ml/min to the monolithic column with a mobile phase flow-rate of 3.0 ml/min; no changes in mobile phase composition or sample preparation were required.

Representative chromatograms of blank human control plasma, control plasma spiked with 20 ng/ml **I** and 10 ng/ml **II** (internal standard), and a 0.5 h post dose clinical sample from a subject receiving **I** are shown in Fig. 3. Utilization of the monolithic column together with the increased mobile phase flow-rate permitted the assay run time to be reduced by a factor of 4, from 10 min to 2.5 min.

The intra day accuracy and precision of the modified assay were evaluated by preparing and analyzing a set of five standard curves, each prepared in a unique lot of control human control plasma. Results from the analysis of the replicate standard curves are presented in Table 2. Accuracy of the calibration standards ranged from 96.7 to 107.1% of nominal. Intra day precision, expressed as the coefficient of variation (%C.V.), was better than 5% for each point on the standard curve. These results were comparable to those obtained with the conventional packed HPLC column [7].

To further assess the performance of the assay, a cross validation was conducted in which a set of clinical samples was extracted using the established 96 well SPE procedure. Following extraction, the samples were divided into two aliquots, one of which was analyzed using the Chromolith HPLC method (comparator) and the other by the previously validated (reference) method which used a conventional packed HPLC column. A plot of plasma concen-

Table 2
Intra day accuracy and precision for the assay of **I** in human plasma

(**I**) Standard concentration (ng/ml)					Mean[a] ($n=5$)	Accuracy[b] (%)	C.V. (%)
Nominal	Assayed						
0.5	0.52	0.55	0.51	0.55	0.56	0.54	107.1	4.4
1	1.03	1.01	0.96	0.97	0.96	0.99	98.7	3.2
2	1.99	1.94	2.02	1.93	1.99	1.97	98.7	2.0
5	5.00	4.87	4.50	4.94	5.07	4.87	97.5	4.6
10	9.73	9.99	9.58	10.35	10.80	10.09	100.9	4.9
20	19.20	19.04	18.24	20.05	20.21	19.35	96.7	4.2
40	40.06	40.75	40.65	39.48	42.25	40.64	101.6	2.5
80	83.84	79.88	78.84	78.04	80.52	80.22	100.3	2.8

[a] $n=5$ from unique lots of control plasma.
[b] Expressed as [(mean assayed concentration)/(nominal concentration)] × 100.
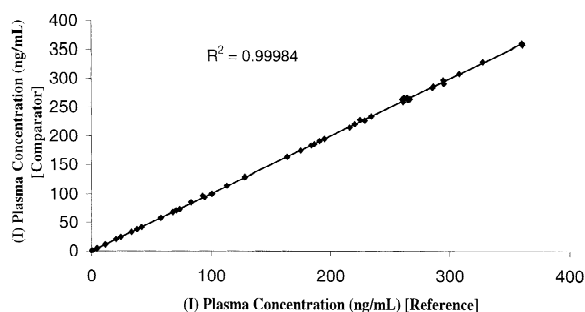
Fig. 4. Cross validation of method for the determination of **I** using the monolithic column. Plot of plasma concentrations of **I** in human clinical samples assayed with the monolithic column (comparator) versus the original (reference) method.

trations of **I** determined by the Chromolith method versus the concentration determined by the reference method is presented in Fig. 4. The results show excellent correlation ($R^2 > 0.999$) between the methods, indicating that comparable results are obtained using either a packed column or the monolithic column.

The post column photochemical derivatization step limited the maximum flow-rate that could be used. The pressure drop generated across the Teflon reaction coil at flow-rates above 3 ml/min was significant, exceeding the manufacturer's limit of 800 p.s.i. A flow-rate of 3 ml/min required a double sided PEEK ferrule to maintain a seal between the column outlet and reaction coil. In spite of these limitations, the analysis time at 3 ml/min was under 2.5 min, over four times faster than the original packed column method.

3.2. Determination of **III** in human plasma

Monolithic columns have been reported to be operated successfully at flow-rates of up to 9 ml/min. In order to explore the possibility of employing a monolithic column with a mobile phase flow-rate greater than 3 ml/min for the determination of an analyte in human plasma, it was necessary to identify a compound that could be readily detected without the need for post-column photochemical derivatization. Compound **III**, a COX-2 inhibitor that has entered clinical development [8,9], was found to

exhibit significant native fluorescence and was thus utilized for this study.

A method employing 96 well SPE together with HPLC on a Waters Symmetry column (4.6×50 mm, 3.5 μm) and fluorescence detection for the determination of **III** in human plasma had been established, validated and employed for the analysis of clinical samples in our laboratory. The mobile phase flow-rate of the established assay was 1.4 ml/min and the run time of the assay was 6 min.

A flow-rate of 6.5 ml/min, limited by the capacity of the HPLC pump, could be employed when this assay was transferred from the packed column to the monolith. These conditions resulted in a significantly shortened analysis time while maintaining adequate separation of **III** from the internal standard and endogenous components. The high permeability of the monolith was evidenced by the fact that a flow-rate of 6.5 ml/min generated a total system backpressure (including the guard column) of only 2300 p.s.i. In comparison, the conventional column, packed with 3.5 μm material, exhibited a system backpressure of approximately 3500 p.s.i. when it was operated at a flow-rate of 3.5 ml/min (Fig. 5).

The phase ratio (β) of a monolith such as the Chromolith is approximately three times lower than a typical particle packed column owing to the monolith's higher total porosity [3]. Monoliths are, therefore, less retentive than packed columns. In order to compensate for the decreased retentivity of the Chromolith, it was necessary to employ a slightly weaker mobile phase (35% versus 40% acetonitrile) than that used in the original method for the determination of **III**. Under these conditions, the peaks
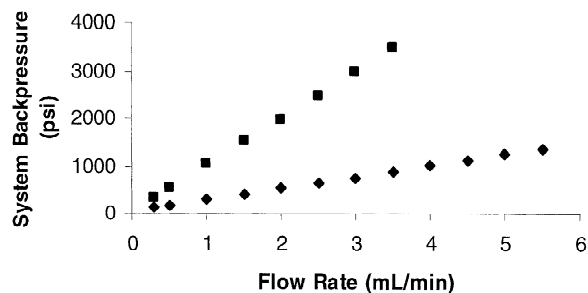

Fig. 5. Effect of mobile phase flow-rate on HPLC system backpressure. ♦=Chromolith monolithic column (4.6×50 mm), ■=Waters Symmetry Packed Column (4.6×50 mm).
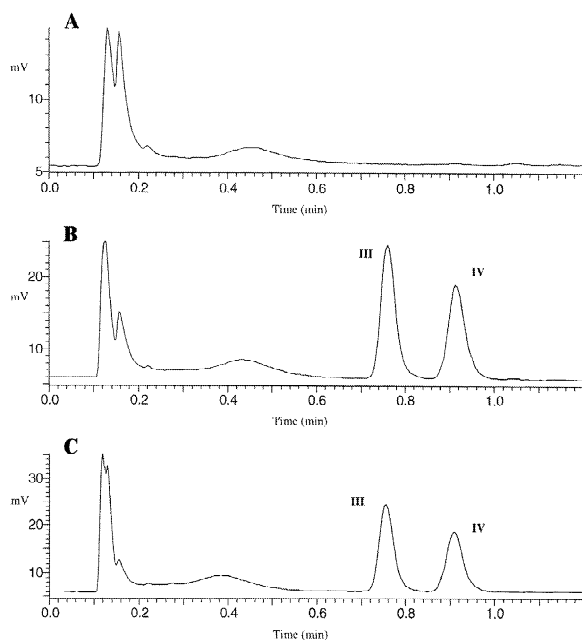
Fig. 6. Representative chromatograms from the assay of **III** in human plasma (A) control plasma, (B) plasma spiked with 100 ng/ml **III** and 100 ng/ml **IV** (IS), and a human clinical sample collected 0.5 h after dosing with **III**. The concentration of **III** is equivalent to 103.4 ng/ml.
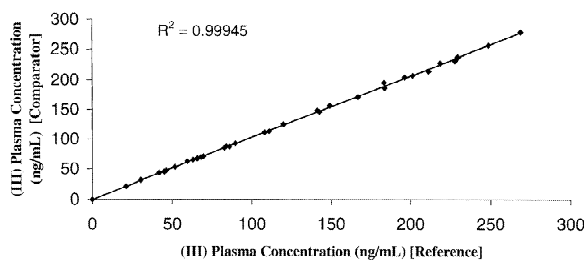

Fig. 7. Cross validation of method for the determination of **III** using the monolithic column. Plot of plasma concentrations of **III** in human clinical samples assayed with the monolithic column (comparator) versus the original (reference) method.

control plasma (A), control plasma spiked with 50 ng/ml **III** and 100 ng/ml **IV** (internal standard) (B), and a 0.5 h post dose clinical sample from a subject receiving **III** (C) are shown in Fig. 6.

The intra day accuracy and precision of the assay was evaluated by preparing and analyzing a series of five standard curves, each prepared in a unique lot of control human plasma. The results of the analysis of the standard curves are presented in Table 3. Accuracy of the calibration standards ranged from 98.8% to 101.6% of nominal. Intra day precision, expressed as the coefficient of variation (C.V.), was better than 2% for each point on the standard curve.

Additionally, a cross validation was performed against a reference method, as described previously for **I**. The plasma concentrations of **III** determined with the Chromolith method versus the concentrations determined with reference method are presented in Fig. 7. Again, the results indicate excellent correlation ($R^2 > 0.999$) between the methods.

of interest eluted in under 1 min, representing a significant increase in assay throughput as compared to the original method. At 6.5 ml/min (corresponding to a linear velocity of approximately 7.5 mm/s) the system generated 2200 theoretical plates for **III**.

Representative chromatograms of blank human

Table 3
Intra day accuracy and precision for the assay of **III** in human plasma

(**III**) Standard concentration (ng/ml)					Mean[a]	Accuracy[b] (%)	C.V. (%)
Nominal	Assayed						
5	5.0	5.1	5.0	5.0	4.9	5.0	100.3	1.0
10	10.1	10.0	10.0	9.6	9.7	9.9	98.8	1.8
20	20.3	19.9	19.9	19.9	19.6	19.9	99.5	1.3
50	52.2	51.1	50.6	50.2	50.0	50.8	101.6	1.7
100	101.8	101.1	100.8	98.8	97.8	100.1	100.1	1.7
200	201.0	200.6	201.6	196.9	198.9	199.8	99.9	1.0
400	402.0	407.2	398.7	401.9	388.7	399.7	99.9	1.7

[a] $n = 5$ from unique lots of control plasma.

[b] Expressed as [(mean assayed concentration)/(nominal concentration)] × 100.
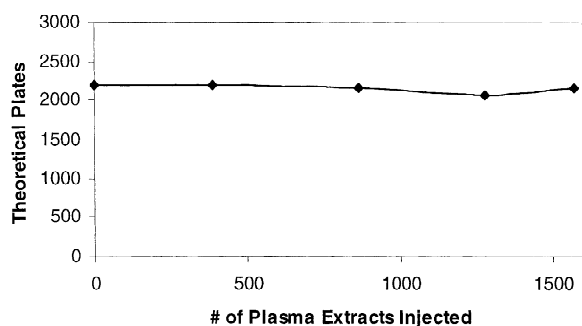
Fig. 8. Evaluation of column efficiency, as represented by the theoretical plates, N calculated from the peak for compound **III** following the injection of replicate plasma extracts. The peak resulting from the injection of 50 ng/ml standard of **III** obtained following the injection of the indicated number of plasma extracts was used for the calculation. The guard column was replaced approximately every 325 injections.

A two-part assessment of the ruggedness of the Chromolith was conducted using the assay conditions for **III**. These experiments addressed column to column reproducibility as well as the performance of a single column over the course of numerous ($N >$ 1500) injections of plasma extracts.

Theoretical plate numbers of **III** were used as a metric to evaluate column performance after injection of plasma extracts. Relatively constant column performance was observed over the course of nearly 1600 injections of plasma extracts (Fig. 8). The ruggedness of the Chromolith is further evidenced by injections of 50 ng/ml neat standards made prior to and following 1574 injections of

plasma extracts. No deterioration of column performance is evident from the chromatograms of these standards (Fig. 9).

To ensure assay ruggedness, intra day assay accuracy and precision (replicate standard curves ($n = 5$) prepared in five unique lots of human control plasma) were assessed on three Chromolith Speed Rod columns. Similar results were obtained on each column (Table 4). It should be noted that the test columns originated from two different batches of sorbent material. As illustrated in Fig. 10, no significant difference in the chromatography was observed across the columns tested.

4. Conclusions

The utility of monolithic silica HPLC columns for the determination of COX-II inhibitors in human plasma has been demonstrated. Use of the Chromolith Speed Rod at high flow-rates resulted in significant reductions in analysis time (up to five-fold) relative to the original methods. In addition, the Chromolith exhibited excellent column to column reproducibility and ruggedness after nearly 1600 injections of plasma extracts. Revision of existing HPLC methods to make use of monolithic column technology represents a straightforward, facile route to shorter analysis times, with a subsequent increase in assay throughput. This technology opens the possibility of reducing the run times of selected bioanalytical methods based on conventional detec-

Table 4

Intra day accuracy and precision data for the assay of **III** obtained on three Chromolith columns

Nominal concentration (mg/ml)	Mean assayed concentration (ng/ml)[a]			Accuracy (%)[b]			C.V. (%)		
	Column 1	Column 2	Column 3	Column 1	Column 2	Column 3	Column 1	Column 2	Column 3
5	5.0	5.0	4.9	100.3	99.7	98.5	1.0	2.6	4.4
10	9.9	9.9	10.0	98.8	98.8	100.1	1.8	1.5	2.9
20	19.9	19.9	20.3	99.5	99.6	101.7	1.3	1.6	3.2
50	50.8	51.0	51.3	101.6	101.9	102.5	1.7	0.6	1.0
100	100.1	100.3	96.7	100.1	100.3	96.7	1.7	2.1	3.3
200	199.8	200.1	202.7	99.9	100.0	101.3	1.0	1.2	4.8
400	399.7	399.0	400.2	99.9	99.8	100.0	1.7	1.6	3.6

Column 1–Chromolith Batch # UM0089; Columns 2 and 3–Chromolith Batch # UM0087

[a] $n = 5$ from different lots of control plasma.

[b] Expressed as [(mean assayed concentration)/(nominal concentration)] $\times$ 100.
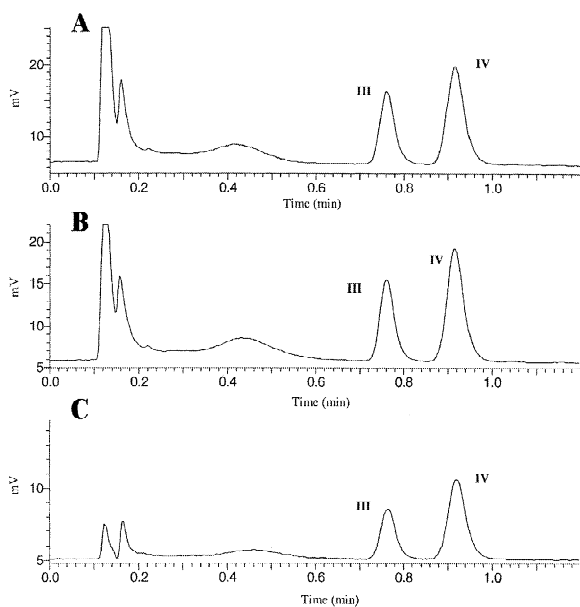
Fig. 10. Chromatograms of 50 ng/ml extracted plasma standards obtained on different Chromolith columns. (A) Column 1, (B) Column 2, (C) Column 3.

tion methods to those commonly associated with methods based on tandem mass spectrometric detection.

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