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Combining restricted access material (RAM) and turbulent flow for the rapid on-line extraction of the cyclooxygenase-2 inhibitor rofecoxib in plasma samples

Anda Vintiloiu [a], Wayne M. Mullett [a,*], Robert Papp [a], Dieter Lubda [b], Elizabeth Kwong [a]

[a] *Pharmaceutical Research and Development, Merck Frosst Center for Therapeutic Research, 16711 Trans-Canada Highway, Kirkland, Que., Canada H9H 3L1*
[b] *Department of Life Science and Analytics, Merck KGaA, Frankfurter Strasse 250, 64293 Darmstadt, Germany*

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Abstract

Restricted access material (RAM) has been used in the packing of a solid-phase extraction (SPE) column for on-line extractions under turbulent flow conditions. The bio-compatible RAM material works by the principle of size exclusion in addition to conventional reversed-phase chromatography, thereby allowing the extraction and preconcentration of small analyte molecules from biological samples such as plasma. Using small column dimensions (0.76 mm × 50 mm) and a consequently high linear velocity, turbulent flow was achieved during online sample extractions. The improved mass-transfer rate characteristic of turbulent flow allows fast sample cleanup without decreased extraction efficiency. The novel use of the RAM column, connected upstream to a C_{18} monolithic column, allowed the direct injection, extraction, separation, and MS/MS detection of plasma samples spiked with rofecoxib in a span of 5 min. Calibration curves obtained using this RAM turbulent flow coupled column method showed good linearity ($R^2 > 0.99$) and reproducibility (%RSD ≤ 7%). The lower limit of quantitation of rofecoxib in plasma samples was found to be 40 ng/ml. The extraction method showed good recovery of rofecoxib from a plasma matrix with minimal signal loss and robustness after more than 200 plasma injections.

Keywords: Restricted access material; Turbulent flow; On-line extraction; Direct injection

1. Introduction

The direct injection of biological samples onto an HPLC analytical column is problematic due to the irreversible adsorption of proteins onto the stationary phase, resulting in substantial loss of column efficiency and an increase in back pressure [1]. Consequently lengthy, labor-intensive and error-prone sample preparation is often necessary to eliminate the biological matrix and to pre-concentrate the analyte. Alternatively, shorter analysis times can be achieved by performing online solid-phase extraction (SPE) prior to its transfer to the analytical column. For this purpose, restricted access material (RAM) columns have become more predominant in recent years [1,2–5]. Such extraction materials work by principle of size exclusion in addition to conventional reversed-phase chromatography. The pore diameter measures approximately 6 nm and as a result prevents large matrix components (>15 kDa) from accessing the hydrophobic pores [4]. Small organic analytes easily diffuse into the pores where they are retained by the extraction phase and pre-concentrated, while proteins wash away with the aqueous mobile phase. Also the surface of RAM-ADS particles is made with an alkyl diol bonded phase outside the pores. The external polar groups increase the material's biocompatibility since proteins and

* Corresponding author. Tel.: +1 514 428 3088; fax: +1 514 428 2855.
E-mail address: wayne_mullett@merck.com (W.M. Mullett).

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hydrophobic plasma components do not adsorb to this surface [1].

On-line extractions under turbulent flow have further improved and accelerated automated sample preparation for various classes of compounds such as pesticides [6], lipid lowering agents [7], tranquilizers [8] and has been the subject of several articles [9–16] and a patent [17]. In the case of online extractions, the first separation step to be performed is much less complex, most often consisting in the isolation of small organic molecules from a biological matrix. The high separation capacity can be traded for higher flow rates and therefore shorter run times. Traditional laminar flow chromatography aims at maximizing the separation efficiency of the chromatographic system by minimizing particle size, increasing column length, and limiting the flow rate to a corresponding van Deemter optimum. As the linear velocity is increased beyond the optimum, the separation efficiency rapidly degrades until turbulence is reached at a reduced velocity of approximately 10^3 to 10^4, whereby the separation efficiency starts to improve again [14]. The phenomenon responsible for the improving separation efficiency at the onset of turbulence is an increase of the mass transfer rate in the mobile phase [10]. Turbulent flow in a packed column depends on particle size as well as on the nature and linear velocity of the mobile phase as defined by the Reynolds number. Critical Reynolds numbers, at which the flow changes from laminar to turbulent in a packed column, can vary depending on the literature source between 10 [11] and 180 [18,19]. There is a very large transition zone between a purely laminar and a purely turbulent regime. Critical Reynolds numbers as low as 3–7 were obtained in several articles [6,10,13,14] claiming turbulent flow. At these conditions, the flow profile is not purely turbulent, however it is chaotic enough to deviate from laminar conditions and therefore increase mass transfer of the analyte for access to the extraction phase. Similarly, a second parameter indicative of flow characteristics is the dimensionless reduced velocity (ν), given by the equation:

$$\nu = \frac{u d_p}{D_m} \quad (1)$$

where u is the linear velocity, d_p the particle diameter, D_m the diffusion coefficient which has a value of $1 \times 10^{-5}\,cm^2\,s^{-1}$ for a small drug molecule (molecular mass of 300–500) [11]. A turbulent regime is therefore obtained at reduced velocity values above 10^3 to 10^4 [11]. Finally, an indication of change in flow characteristics from laminar to turbulent is characterized by a deviation from linearity in experimental pressure versus flow rate plots [11]. All these diagnostic indicators were used to assess the flow characteristics of our system.

Our efforts in this project consisted in performing an on-line SPE using a self-made biocompatible RAM-ADS extraction column under turbulent flow conditions. In contrast to turbulent systems previously used, which can employ short lived extraction columns [9], the extraction phase used in this work physically prevents contact of proteins with the hydrophobic extraction phase. In combination with this size exclusion process, the biocompatible surface of the particles further minimizes protein adsorption and extends column lifetime.

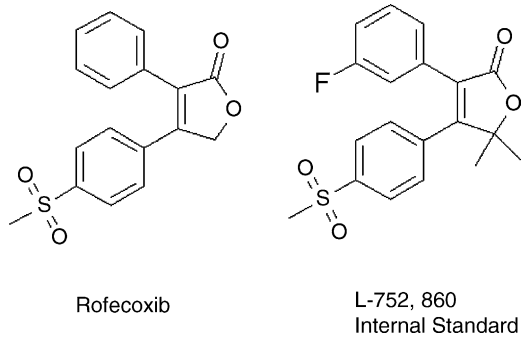

Fig. 1. Structures of rofecoxib and the internal standard.

Turbulent flow was achieved during the loading of the biological sample onto the extraction column, with the advantage of accelerating the clean-up of the sample while maintaining high extraction efficiency. In order to generate turbulent flow, the extraction column used a small internal diameter (<1 mm) packed with RAM particles of large diameter (40–63 μm). This analytical methodology was applied to the measurement of rofecoxib and an internal standard in rat plasma, to determine the feasibility of this technique to minimize sample manipulation in our lab. Usefulness of this method with other compounds is expected since RAM columns containing a C_{18} extraction phase have been previously shown to extract a broad range of drugs including benzodiazapines, beta-blockers, cocaine and barbiturates [1]. Rofecoxib is cyclooxygenase-2 (COX-2) inhibitor with low solubility and polarity, and no ionizable groups. COX-2 inhibitors have demonstrated efficacy in treating pain and inflammation, while reducing gastric side-effects common to non-steroidal anti-inflammatory drugs (NSAIDs) [20–22]. Rofecoxib is currently withdrawn from the market.

2. Experimental

2.1. Materials

HPLC-grade acetonitrile and methanol were purchased from EMD (Gibbstown, NJ, USA). Reagent A.C.S. grade acetic acid was obtained from Fisher Scientific (Nepean, Canada) and HPLC-grade tetrahydrofuran from Fisher Scientific (FairLawn, NJ, USA). Deionized water was generated using a Millipore Milli-Q system (Bedford, MA, USA). Rofecoxib and the internal standard L-752, 860 (Fig. 1) were obtained from the Chemical Data Department of Merck Research Labs (Rahway, NJ, USA).

2.2. Equipment

All analyses were performed using an Agilent HP1100 (Palo Alto, CA, USA) equipped with a column switching valve and a TSP UV6000LP diode-array detector (San Jose, CA, USA). Mass spectrometry measurements were obtained using a Thermo-Electron LCQ Deca ion-trap mass spectrometer (San Jose, CA, USA) equipped with an APCI source. Instrument control, sample scheduling and data processing was accomplished using XCalibur™ software version 1.2, Thermo-Electron Corporation (San Jose, CA, USA). The extraction was performed on a self-made column (0.76 mm × 50 mm, 1/8 inch. o.d. stainless steel) which was slurry-packed using RAM particles (Lichrospher® 60, RP-18 ADS, 40–63 μm diameter) obtained from Merck KGaA (Darmstadt, Germany). The column was sealed at each end using a stainless steel zero volume internal reducing union (1/8 inch. to 1/16 inch.) containing a 2 μm frit, all purchased from Supelco (Bellefonte, PA, USA). The analytical column used was a Chromolith SpeedROD (RP-18, 50 mm × 4.6 mm) purchased from Merck KGaA (Darmstadt, Germany). An ADS inline filter with a stainless steel frit from Merck KGaA was installed between the autosampler and the RAM column, to trap any particulate materials.

2.3. HPLC conditions

A typical two-column switching setup was used and the instrumental method is outlined in Table 1. Two pumps were used in the method to allow simultaneous reconditioning of the RAM with separation of the analyte. Fifty microliters of either aqueous matrix or rat plasma samples were injected onto the RAM column using a loading mobile phase (water–acetonitrile 95:5, v/v) at a flow rate of 5 ml/min. Plasma proteins were washed to waste under these conditions (t = 0–1 min) after which the solvent delivered by the secondary pump (water–methanol 50:50, v/v, with 1% acetic acid) was back-flushed through the RAM column (t = 1–2 min). The analyte and internal standard were eluted from the RAM onto the C_{18} monolithic column where they were separated isocratically (2–5 min). The RAM column was then simultaneously washed (t = 2–3 min) using a tetrahydrofuran-acetonitrile (90:10, v/v) mixture delivered by the primary pump to wash away any hydrophobic plasma residues and subsequently reconditioned (t = 3–5 min) back to the initial loading mobile phase. The analytical column was continuously reconditioned with mobile phase delivered by the secondary pump.

2.4. MS conditions

The APCI source was operated at 450 °C in positive ion mode. High purity nitrogen (>99.0%) was used as the sheath and auxiliary gas at instrumental settings of 80 and 20 units, respectively. The discharge current was set to 5 μA. The capillary temperature and voltage were set to 180 °C and 46 V, respectively. The tube lens offset was set at 15 V. Selected-reaction monitoring (SRM) mode was used to quantify rofecoxib (315/297 m/z) and the internal standard (361/343 m/z), with collision energy settings of 25 and 26%, respectively, applied to the ring electrode. High purity helium (99.999%) was used as the collision gas.

2.5. Sample preparation

Stock solutions of rofecoxib (299 μg/ml) and the internal standard (30 μg/ml) were individually prepared in ACN–H_2O (50:50, v/v) and stored up to 1 week. The rofecoxib stock was further diluted in ACN–H_2O (50:50, v/v). From these solutions, each working standard level was prepared by adding eight parts water or plasma, one part internal standard, and 1 part of appropriately diluted rofecoxib stock solution. Prior to its loading onto the column, the plasma was centrifuged at 1000 × g for 5 min to remove any suspended particles. The final concentrations of the working standards were 0.3, 1.2, 5.0, 14.9 and 29.9 μg/ml for rofecoxib with 3 μg/ml internal standard. Samples were kept refrigerated between analyses for up to 2 days.

Table 1
HPLC gradient program extraction and separation method

Step	Main pump			Secondary pump		
	Time (min)	%B	Flow rate (ml/min)	%B	Flow rate (ml/min)	Switching valve position
Loading	0.00	0	5.00	100	1.00	2
	1.00	0	5.00	100	1.00	2
Analyte elution from RAM	1.01	–	0.00	100	1.00	1
	2.00	–	0.00	100	1.00	1
Analyte separation/detection (secondary pump)	2.01	100	2.00	100	1.00	2
and RAM column reconditioning (main pump)	3.00	100	2.00	100	1.00	2
	3.01	0	2.00	100	1.00	2
	5.00	0	2.00	100	1.00	2

Mobile phase used: main pump solvent A → water–acetonitrile (95:5, v/v); solvent B → acetonitrile–tetrahydrofuran (90:10, v/v). For the secondary pump, the mobile phase B was water–methanol (50:50, v/v) with 1% acetic acid.

3. Results and discussion

3.1. Performance of the packed RAM/SPE column

3.1.1. Pressure profile and experimentally obtained Reynolds numbers

It is known that column backpressure varies linearly with flow rate under laminar conditions and quadratically under turbulence, as described by Ergun's equation (see Eq. (2)) [23].

$$\Delta p = \frac{150\bar{V}_o\eta(1-\varepsilon)^2 L}{\Phi_s{}^2 d_p{}^2 \varepsilon^3} + \frac{1.75\rho\bar{V}_o^2(1-\varepsilon)L}{\Phi_s d_p \varepsilon^3} \quad (2)$$

where d_p is the particle diameter (in m), L the column length (in m), Δp the column backpressure (in Pa), $\bar{V}_o$ the superficial velocity (can be assumed to be same as linear velocity) (in m/s), ε the porosity of packing (values vary between 0 and 1, dimensionless), Φ_s the particle sphericity (equal to 1 in case of perfectly spherical particles, dimensionless), η the dynamic viscosity (in cP) and ρ the density (in kg/m^3).

Therefore, a simple procedure to confirm the transition from laminar to turbulent flow is to evaluate the backpressure over several mobile phase flow rates. The relation between pressure and flow rate for a newly packed RAM column was found to be linear at low flow rates, while showing a deviation from linearity at flow rates above approximately 2.5 ml/min as shown by Fig. 2. This deviation suggests a change in the flow regime from laminar to partially turbulent. If one applies Ergun's equation to a transitional flow regime, as is the case at flow rates above 2.5 ml/min using our column, a mathematical combination of the linear and the quadratic term is obtained, with their relative contributions depending on the Reynolds number achieved. This explains why deviations from linearity are observed in Fig. 2, yet a perfect quadratic relationship is not reached.

The Reynolds numbers have been calculated for the system operating at different flow rates (Table 2). Previous reports of turbulent flow chromatography experiments achieved Reynolds numbers between 3 and 10 marking the onset of turbulence [6,10,11,14]. We have obtained values as high as 14, confirming the creation of turbulent flow in our system at flow rates higher than ~2.5 ml/min.

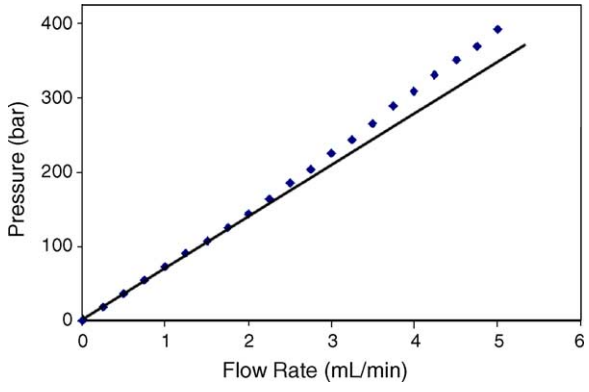

Fig. 2. Pressure profile for the packed RAM column showing a deviation from linearity at high flow rates. Runs were conduced at room temperature using water–acetonitrile (95:5, v/v) mobile phase.

Table 2
Calculated Reynolds numbers and reduced velocity at various mobile phase flow rates

Flow rate (ml/min)	Reynolds numbers[a]	Reduced velocity[b]
1	1.7–2.7	1.5–2.3 × 10^3
2	3.4–5.4	2.9–4.6 × 10^3
3	5.2–8.1	4.4–6.9 × 10^3
4	6.9–10.9	5.9–9.3 × 10^3
5	8.6–13.6	7.3–11.6 × 10^3

[a] Reynolds number = $u\rho d_p/\mu$; u = linear velocity of fluid; ρ = density; d_p = particle diameter and μ = dynamic viscosity of mobile phase based on water–acetonitrile (95:5, v/v). Dynamic viscosity was calculated as being 95% that of water and 5% that of acetonitrile. Particle size range 40–63 μm.
[b] See Eq. (1) for calculation.

The existence of turbulence in our system was also evaluated by the reduced velocity values obtained at different flow rates (Table 2). Using a narrow diameter column and large RAM particles, critical values needed for turbulence (10^3 to 10^4) were reached at flow rates as low as 1 ml/min. The calculated Reynolds numbers and reduced velocities, as well as the pressure profile deviation from linearity, all indicate the onset of turbulence. As previously mentioned, even a partial turbulent flow provides the desired property of increased mass transfer in the mobile phase. Consequently, the extraction efficiency should not decrease at higher flow rates, as was investigated below.

3.1.2. Analyte breakthrough from the RAM column

A standard solution was injected into the system at volumes ranging from 10 to 90 μl in order to test the capacity of the RAM column for analyte retention. The loading and eluting mobile phases consisted of water–acetonitrile (95:5, v/v) and water-methanol (50:50, v/v) with 1% acetic acid, respectively. The loading step was carried out at 3 ml/min for 3 min, followed by a 3 min elution step at 1 ml/min, ending with a 2 min reconditioning step using the loading mobile phase in preparation for the next injection. Linearity between the area of the eluting analyte peak and the injection volumes was compared. No evidence of analyte breakthrough was observed as indicated by UV detection and peak shape was maintained over all injection volumes. A linear increase in the peak area was observed over the entire injection volume range of 10–90 μl (R^2 = 0.9942, n = 5) confirming the ability of the RAM column to effectively preconcentrate the analyte from large volume injections.

Using a similar procedure, the analyte breakthrough from the RAM column was measured as a function of loading flow rate. The peak area of the elution peak was plotted against the increasing flow rate (1–5 ml/min) in Fig. 3. No breakthrough was observed with increasing flow rates as the peak areas were reproducible regardless of the loading flow-rate (%RSD = 1.2, n = 5). The increased mass transfer rate at higher flow-rates maintained the extraction efficiency and

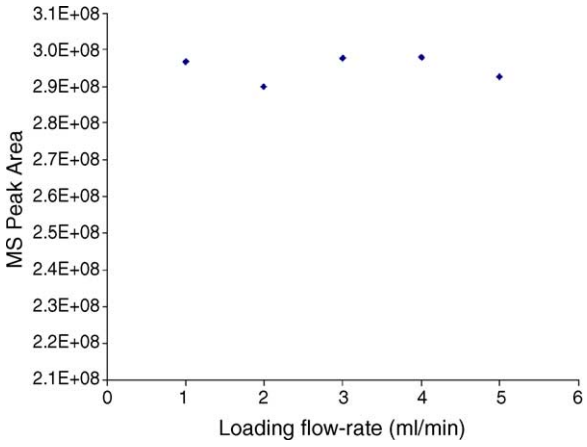

Fig. 3. Recovered peak area of rofecoxib with increasing loading flow-rates.

appeared to compensate for potential losses in efficiency due to the reduced residence time of the analyte in the column.

3.1.3. Protein elution from RAM column

Protein elution through the RAM column was demonstrated using UV detection at 280 nm. Blank rat plasma was injected using a loading mobile phase of H_2O–ACN (95:5, v/v). The high aqueous content of the mobile phase was important to prevent proteins from precipitating when performing direct injection of biological fluids. Elution profiles were collected using different injection volumes (Fig. 4) and flow rates (Fig. 5), to determine optimal values for the shortest protein elution time and valve timing. As expected, higher injection volumes required slightly longer elution times. However, the increase is not significant enough to prevent large injection volumes. An increase in loading flow rate also shows a decrease in plasma elution time, favoring the use of high flow rates to reduce run-time while maintaining extraction efficiency.

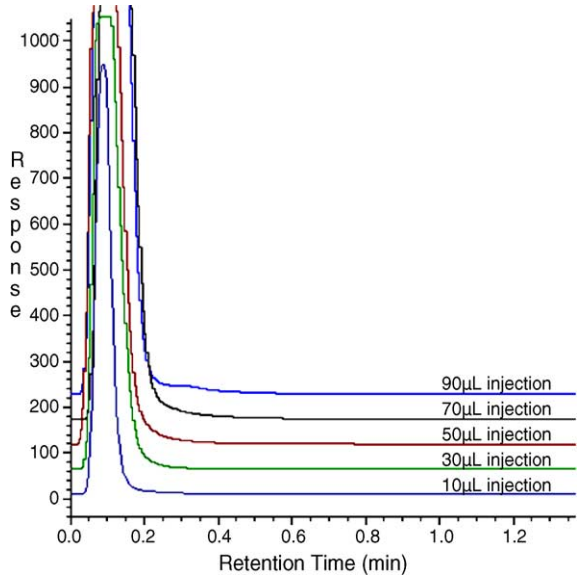

Fig. 4. Plasma elution times for different injection volumes. Runs were conducted at 3 ml/min using water–acetonitrile (95:5, v/v) mobile phase.

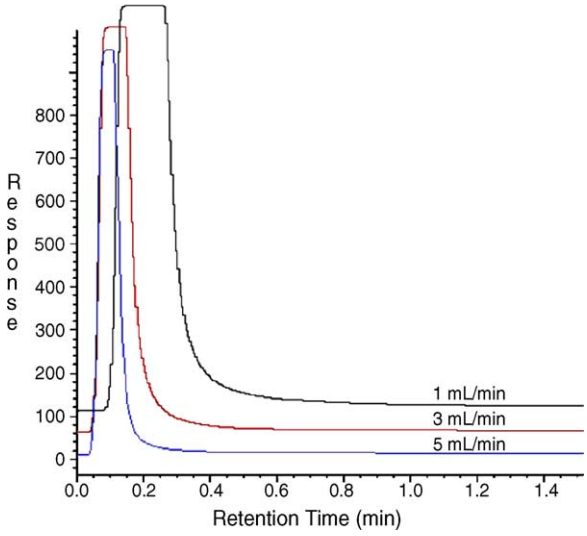

Fig. 5. Plasma elution times at different flow rates. Runs were conducted using 50 μl injection volume. Mobile phase was water–acetonitrile (95:5, v/v).

3.2. Extraction and separation of analytes

A method was designed for the complete on-line extraction and separation of the analyte (rofecoxib) and internal standard. Based on the previous protein elution experiments, 1 min was allocated to the initial loading step during which the analytes were retained by the RAM column and the proteins were washed to waste in the highly aqueous mobile phase consisting of water–acetonitrile (95:5, v/v). Switching to a highly organic mobile phase permitted elution of the extracted rofecoxib and internal standard from the RAM column and subsequent isocratic separation on the analytical column. Fig. 6a represents a typical chromatogram recorded by the online turbulent flow RAM LC–MS/MS system, showing successful extraction of the analytes from a direct injection of plasma followed by elution of the internal standard and the analyte (rofecoxib) peak in less than 5 min. It is important to realize that the composition of the elution mobile phase was carefully chosen to ensure complete removal of the extracted analytes from the RAM column while maintaining adequate peak shapes for rofecoxib and the internal standard on the C_{18} monolithic column. The retention provided by the analytical column allows an additional level of separation of any matrix components that may have co-extracted on the RAM column [3]. However, since the internal standard is separated from rofecoxib, there is potential for varying degrees of matrix effects at the different retention times. Fig. 6b shows a blank plasma injection using MS/MS detection that indicates no interferences with the peaks of interest. In addition, UV traces ($\lambda = 270$ nm) were obtained for blank plasma and extracted standard injections to further demonstrate the lack of intereferences at the retention time of the

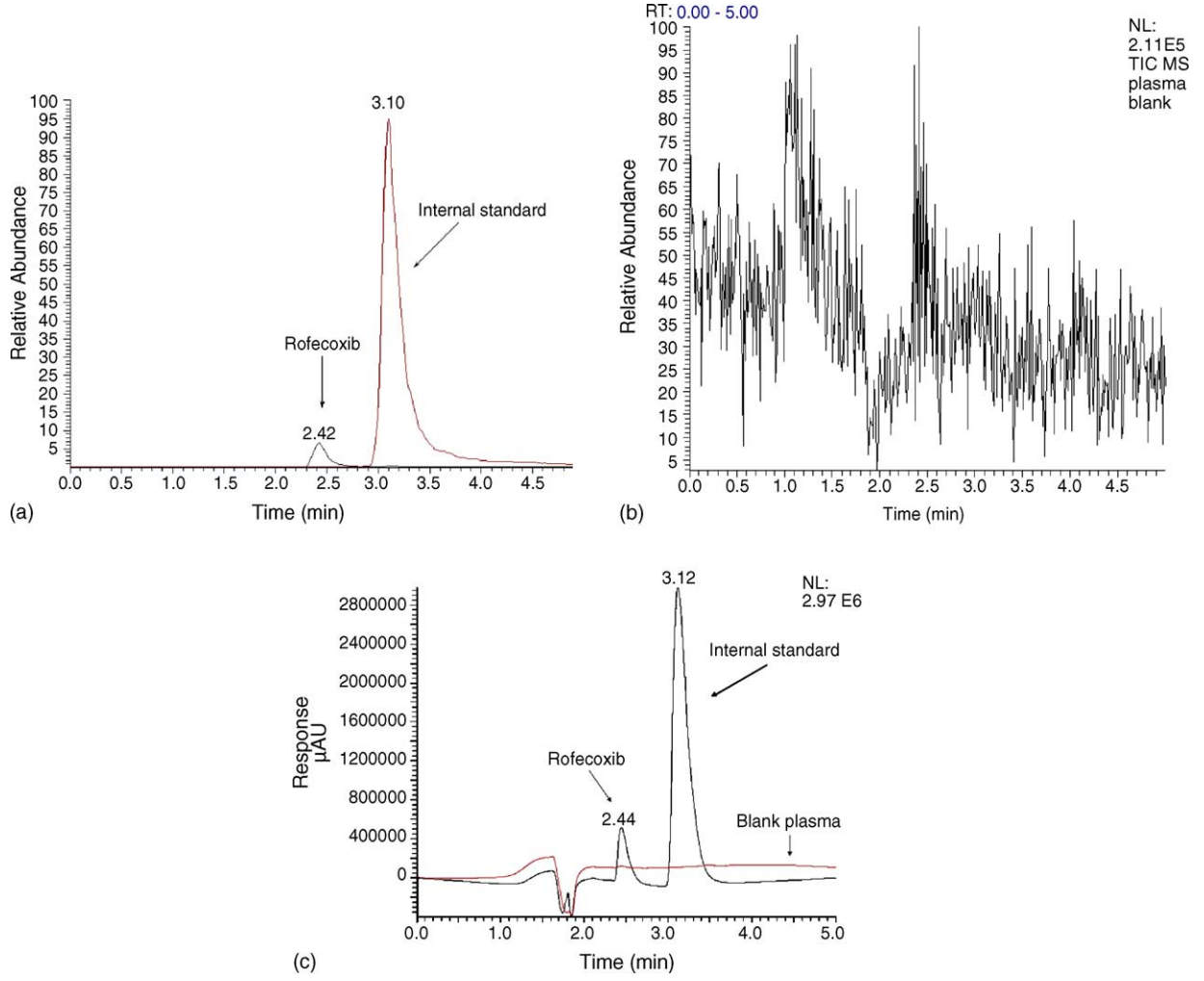

Fig. 6. (a) LC–MS/MS chromatogram showing the extracted rofecoxib (0.3 μg/ml) and internal standard peaks. See Table 1 and Section 2 for conditions. (b) Blank plasma injection using same conditions. (c) Overlayed LC–UV chromatogram of extracted rofecoxib (0.3 μg/ml) with internal standard and a blank plasma injection (λ = 270 nm).

analytes (Fig. 6c). The possibility for direct plasma injection as well as the short run time enables use in high-throughput applications.

3.2.1. Recovery

Recovery studies were performed by comparing the response obtained for standards spiked in plasma to that obtained for standards prepared in an aqueous matrix (Table 3). Both sets of standards were analyzed using the same methodology. A comparison of response ratios for each standard level was made for the corresponding plasma and water injections ($n = 3$). The results obtained indicate only a slightly decreased detector response for corresponding plasma standards with recovery ranging between 76 and 86%. Based on preliminary results obtained, recovery can be improved to 89–108% by allowing longer extraction times (3 min). This two-column setup showed improved results over previous efforts where a single commercially available

Table 3
Recovery of spiked plasma versus water standards extracted using the RAM column

	Sample concentration (μg/ml)									
	0.3 μg/ml ($n = 3$)		1.2 μg/ml ($n = 3$)		5.0 μg/ml ($n = 3$)		14.9 μg/ml ($n = 3$)		29.9 μg/ml ($n = 3$)	
	Plasma	Aqueous	Plasma	Aqueous	Plasma	Aqueous	Plasma	Aqueous	Plasma	Aqueous
Mean response ratio	0.0385	0.0508	0.1842	0.2236	0.8124	0.9413	2.5003	3.0358	5.2296	6.5320
RSD (%)	6.1	1.4	2.2	5.6	1.0	4.2	4.1	6.7	4.0	1.3
Recovery (%)	75.8		82.4		86.3		82.4		80.0	

See Table 1 and Section 2 for conditions.

Table 4
Inter-day reproducibility

	Plasma sample concentration (μg/ml)				
	0.3	1.2	5.0	14.9	29.9
Mean response ratio ($n = 9$)	0.03546	0.16278	0.77136	2.42189	5.11715
%RSD	9.2	10.2	4.6	3.8	4.0

See Table 1 and Section 2 for conditions.

RAM column was employed, resulting in analyte recoveries as low as 35% [3].

3.2.2. Linearity, precision and limit of detection

A calibration curve was plotted using normalized signal for standards spiked in plasma with final concentrations in the range of 0.3–30 μg/ml. The method proved to be linear over the entire range tested with a slope of $y = (0.17539 \pm 2.37 \times 10^{-3})x$, an intercept of -0.04485 ± 0.03584, and a standard error of regression of 0.1019 ($R^2 = 0.9976$). Precision was evaluated over the same concentration range with $n = 3$ injections for each standard level. The %RSD of replicates was below 7% for all standards (Table 3). The limit of quantitation (S/N = 10) for rofecoxib was established to be 40 ng/ml based on calculations using the signal-to-noise ratio found for the lowest concentration standard. Although not the focus of this work, additional improvements in sensitivity for rofecoxib could be achieved with the selection of a quadrupole-type mass analyzer operated under optimized conditions [24]. Inter-day reproducibility was determined at each concentration using three replicate injections on three different days ($n = 9$). Samples were prepared fresh each day using blank plasma obtained from different rats. The method was shown to have acceptable precision (RSD $\leq$ 10%) at each level (Table 4). No carry-over was detected in blank samples injected immediately after the highest concentration standard. The backpressure remained constant during the column lifetime, although the in-line filter and column frits had to be changed after prolonged use due to clogging. The RAM extraction phase was found to be robust since efficiency did not deteriorate after over 200 plasma injections, highlighting the biocompatibility of the material. In comparison to existing methods for rofecoxib, including an in-house protein precipitation method, extraction and analysis time was greatly reduced, while providing adequate recovery, linearity and precision.

4. Conclusions

This paper presents the successful development of a method using solid-phase extraction from a restricted access material column under turbulent flow conditions followed by chromatographic separation. The SPE was achieved with a short extraction time without compromising the extraction efficiency. Turbulence was demonstrated during extraction according to the theoretical Reynolds numbers as well as by the pressure profile obtained for the packed column which showed a deviation from linearity at high flow rates. The turbulent flow improves mass transfer and as a result there was no increase in analyte breakthrough at high flow rates.

A method incorporating the successive extraction and separation of a test drug compound (rofecoxib) and a suitable internal standard was developed and tested on both spiked water and plasma standards. Overall, good linearity and reproducibility were obtained. Also, recovery was excellent, showing no significant signal decrease for standards spiked in plasma. Overall, the on-line extraction and separation method allows very rapid analysis (5 min) with effectively no off-line sample preparation other than centrifugation. The continual development of new restricted access materials [1], including a range of extraction phases for direct isolation of a broad range of analytes, will ensure the versatility of this approach.

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