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Improved procedure for the the determination of rofecoxib in human plasma involving 96-well solid-phase extraction and fluorescence detection

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Abstract

An improved assay for the determination of rofecoxib in human plasma samples is described. The analyte and an internal standard were extracted from the plasma matrix using solid-phase extraction in the 96-well format with an Empore C_8-SD extraction plate. The analytes are chromatographed on a Waters Symmetry C_{18} analytical column (3.5 μm, 50×4.6 mm) with a mobile phase consisting of acetonitrile–water (35:65, v/v). Analyte detection was via fluorescence following post-column photochemical derivatization. Eight point calibration curves over the concentration range of 0.5–80 ng/ml yielded a linear response when a $1/y$ weighted linear regression model was employed. Based on the replicate analyses ($n=5$) of spiked standards, the within-day assay precision was better than 8% RSD at all points on the calibration curve, within-day accuracy was within 6% of nominal at all standard concentrations. The between-run precision and accuracy of the assay, as calculated from the results of the analysis of quality control samples, was better than 7% RSD and within 5% of nominal. Assay throughput was improved by a factor of three as compared to previously described methods. The method was partially automated using a combination of a Packard Multi-Probe liquid handling system and a TomTec Quadra 96 workstation.

Keywords: Solid-phase extraction; Rofecoxib; Enzyme inhibitors

1. Introduction

Selective inhibitors of cyclooxygenase 2 (COX-2) have the potential to displace traditional, non-selective, nonsteroidal antiinflammatory drugs (NSAIDs) as the treatment of choice for the relief of inflammation and pain. This potential is based on their improved side effect profile in comparison to that observed for NSAIDs, especially during chronic use [1]. Rofecoxib [4-(4-methanesulfonylphenyl)-3-phenyl-5H-furan-2-one, compound **I**, Fig. 1] has been found to be a COX-2 selective inhibitor at the recommended clinical doses for osteoarthritis and pain relief [2,3]. The compound has recently received marketing approval in the USA (under the registered trade name VIOXX) and many other countries around the world.

Methods for the determination of rofecoxib in human plasma were required in order to support human clinical pharmacokinetic studies of the compound. Previously, we have described a method for the determination of rofecoxib in plasma that used

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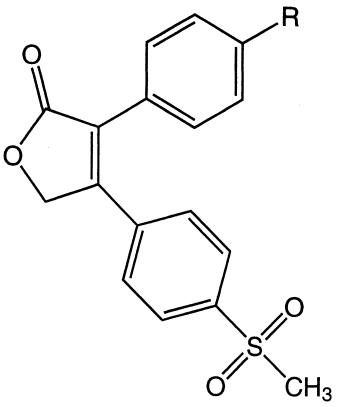

Compound I: R = H
Compound II: R = CH_3

Fig. 1. Structures of rofecoxib (**I**) and internal standard (**II**).

liquid–liquid extraction with a mixture of hexane–methylene chloride (50:50, v/v) to isolate the analyte followed by HPLC with postcolumn photochemical derivatization and fluorescence detection [4]. Additional methods for the determination of rofecoxib in human plasma that utilize liquid–liquid extraction for analyte isolation together with HPLC with UV [5] or tandem mass spectrometric detection [6] have recently been published.

A major drawback of each of the published methods is low sample throughput. These methods all use liquid–liquid extraction for analyte isolation. This technique is labor intensive, thus limiting the number of samples that can be processed per day. To support a large scale clinical program, a method with a higher throughput was required to analyze rofecoxib in human plasma. In order to overcome the limitations of liquid–liquid extraction, our studies focused on developing methodology to extract the analyte from plasma using 96-well solid-phase extraction [7]. A method for the determination of rofecoxib in human plasma using sample preparation via 96-well solid-phase extraction is the subject of this publication. Sample throughput of the described assay is almost three times higher than previously published methods.

2. Experimental

2.1. Materials

Rofecoxib (purity >99%) was obtained from the Chemical Data Department of Merck Research Labs (Rahway, NJ, USA). The internal standard, 4-(4-(methylsulfonyl)phenyl-3-(4-tolyl)-2(5H)-furanone (compound **II**, Fig. 1) was supplied by S. Leger of the Medicinal Chemistry Department of Merck Frosst Canada (Kirkland, Canada). Acetonitrile (ACN) and methanol (Omnisolve HPLC grade) were purchased from EM Science (Gibbstown, NJ, USA). All other reagents were ACS grade from Fisher Scientific (Fair Lawn, NJ, USA). Drug-free human plasma was purchased from Sera-Tech Biologicals (New Brunswick, NJ, USA).

Empore 96-well disk plates (C_8-SD) were purchased from 3M (St. Paul, MN, USA). Polypropylene 96-well plates (2.0 ml/well) were obtained from Matrix Technologies (Lowell, MA, USA). Enzyme-linked immunosorbent assay (ELISA) plates were from Corning-Costar (Cambridge, MA, USA). Polypropylene 96-well plates (1.2 ml/well) for use in the autosampler were purchased from Microliter Analytical Supplies, (Suwanee, GA, USA). The 1.2-ml/well plates were covered with a storage mat (Marsh Biochemical Products, Rochester, NY, USA) prior to their placement in the autosampler.

2.2. Instrumentation

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) Model 410 pump, a Varian Prostar 96-well format autosampler (Varian, Walnut Creek, CA, USA), an AURA Industries (Staten Island, NY, USA) photochemical reactor consisting of a 254-nm UV lamp mounted with a 10 m×0.3 mm I.D. reaction coil and a Perkin-Elmer model LC 240 fluorescence detector. The photochemical reactor was installed between the analytical column and the fluorescence detector. The detector output was connected to a PE-Nelson (Cupertino, CA, USA) TurboChrom data system via a PE-Nelson 941 analog-to-digital interface.

A Matrix Impact^2 8-channel expandable electronic pipette was used for manual sample manipulations,

while a Packard MultiProbe 204DT liquid handling system (Downers Grover, IL, USA) and a TomTec Quadra 96 SPE workstation (Model 320, Hamden, CT, USA) were used to automate the liquid transfer procedures. The QIAvac vacuum manifold for solid-phase extraction (SPE) and the Sigma 4K15 96-well format centrifuge were obtained from Qiagen (Chatsworth, CA, USA).

2.3. Chromatographic conditions

The mobile phase consisted of a mixture of acetonitrile–water (35:65, v/v, %). A Waters Symmetry C_{18} analytical column (50×4.6 mm, 3.5 μm) preceded by a Waters Symmetry C_{18} cartridge guard column (Waters Assoc., Milford, MA, USA) was used for the separation. The mobile phase was delivered through the columns at a flow rate of 1.2 ml/min. The total run time was 10 min. The columns were operated at ambient temperature (approximately 22°C). The sample injection volume was 135 μl. The fluorescence detector was set at an excitation wavelength of 250 nm and an emission wavelength of 400 nm.

2.4. Preparation of standards

An 8-μg/ml stock solution of rofecoxib was prepared by weighing 0.8 mg of reference material into a 100-ml red volumetric flask and dissolving the compound in about 50 ml of acetonitrile before filling the flask to volume with acetonitrile. A 1.0-μg/ml stock solution was prepared by diluting 6.25 ml of the 8-μg/ml to 50 ml with acetonitrile, while a 0.1-μg/ml stock solution was prepared by diluting 5 ml of 1.0-μg/ml stock solution to 50 ml with acetonitrile.

Working standards of 1.6 and 0.8 μg/ml were prepared by dilution of the 8-μg/ml stock solution with acetonitrile whereas working standards of 0.4, 0.2, and 0.1 μg/ml were prepared by dilution of the 1-μg/ml stock solution with the same solvent. Working standards of 0.04, 0.02 and 0.01 μg/ml were prepared by dilution of the 0.1-μg/ml stock solution with acetonitrile. All standard solutions were found to be stable for at least 3 months when stored protected from light at room temperature.

Plasma standard samples were prepared by spiking 25 μl of each working standard into 0.5 ml of drug free plasma placed in a 13×85 mm polypropylene test tube (Sarstedt, Newton, NC, USA). The resulting standards were used to quantitate samples containing rofecoxib over the concentrations range of 0.5–80 ng/ml. Clinical samples containing higher concentrations of rofecoxib were diluted with control plasma prior to analysis.

Quality control (QC) samples containing rofecoxib at concentrations of 60 ng/ml (high QC), 30 ng/ml (medium QC) and 1.5 ng/ml (low QC) were prepared by diluting 1-ml aliquots of solutions of rofecoxib in acetonitrile at concentrations of 6, 3 and 0.15 μg/ml to 100 ml with control plasma. The QC samples were then stored in 1-ml aliquots at −20°C.

2.5. Plasma extraction procedure

Aliquots (0.5 ml) of clinical samples or quality control samples were pipetted into 13×85 mm polypropylene tubes. A 25-μl aliquot of acetonitrile was added to each of the samples to make their volume equal to those of the standards. A 25-μl volume of internal standard (II) solution (0.2 μg/ml in acetonitrile) was added to the samples and standards and the tube contents were vortex mixed. A 0.5-ml volume of water was added to each tube and the resulting solutions were vortex mixed. A 96-well disk SPE plate (C_8, standard density) was conditioned by passing 0.3 ml of methanol followed by 0.6 ml of water through each of the wells. Low negative pressure was applied (<2 in.Hg; 1 in.Hg = 338.638 Pa) during plate conditioning to prevent drying of the wells. Aliquots (0.75 ml) of the diluted plasma solutions were transfered into the conditioned SPE plate. The solutions were drawn through the plate wells using negative pressure (10–15 in.Hg). A 1-ml volume of 10% acetonitrile in water was passed through each of the extraction wells. The plate was then positioned on the top of an ELISA plate and the extraction plate/ELISA plate assembly was centrifuged for 5 min at 1500 rpm (514 *g*) to remove residual solvent. The extraction plate was then placed on top of a 96-deep well plate (well volume = 1.2 ml). Each well in this plate contained 300 μl of water. Acetonitrile (150 μl) was added to each of the extraction wells. The SPE disk plate/deep round well plate assembly was centrifuged for 5 min at

1500 rpm (514 *g*) to elute the analytes. The resulting solutions in each of the wells of the plate were mixed. The plate was sealed with a 96-well storage mat and placed on the 96-well plate compatible autosampler.

3. Results and discussion

3.1. Chromatographic system development

In our previously described liquid–liquid extraction–HPLC method [4] the total run time using a BDS-Hypersil C_{18} analytical column (100×4.6 mm) was 20 min. In order to improve sample throughput a shorter run time was desirable. Therefore, alternative HPLC columns were evaluated to separate rofecoxib and internal standard from co-extracted endogenous components. Best results were obtained using a Waters Symmetry C_{18} analytical column (50×4.6 mm, 3.5 μm); the run-time could be reduced to 10 min using this column. The higher efficiency provided by the use of the smaller packing material compensates for the reduced length of this column, thus resulting in a significantly reduced analysis time.

Based on its highly specific and sensitive nature, the previously described [4] post-column photochemical derivatization/fluorescence HPLC detection system was used in the present assay. Details of the photochemistry of rofecoxib and the characterization of the fluorescent species formed during the post-column photochemical derivatization have been described previously [4].

3.2. Development of sample preparation procedure

All assays for the determination of rofecoxib in human plasma that have been described to date use liquid–liquid extraction for analyte isolation [4–6]. In order to support large scale clinical studies, the development of a high sample throughput procedure that was significantly less labor intensive was highly desirable. SPE in the 96-well format has, in recent years, become a method of choice in many laboratories to prepare biological fluid samples for analysis [7]. Advantages of this methodology include high throughput and the potential for automation. Therefore, we focused on developing a 96-well SPE method to prepare human plasma samples for rofecoxib analysis. The use of 96-well extraction plates containing several different stationary phases was investigated. Best results, in terms of recovery and fewest endogenous peaks, were achieved using a plate containing 3M Empore C_8-SD extraction disks in each of the wells. Sample cleanliness and analyte recovery were optimized by investigation of different wash solvents. Washing each of the plate wells with a 10% solution of acetonitrile in water prior to analyte elution with 100% acetonitrile was found to maximize the removal of endogenous species without a significant loss in analyte recovery. Processing the wash and elution steps via centrifugation as opposed to vacuum was found to improve assay consistency and eliminate cross sample contamination.

The existing liquid–liquid extraction methods [4–6] all require the extraction solvent to be evaporated and the samples reconstituted prior to injection into HPLC systems. This step is labor intensive and time consuming. A major advantage of the use of the Empore plates is that the analyte and internal standard could be eluted in a small volume (150 μl) of acetonitrile. The elution solvent could thus be directly diluted with water to approximate the solvent composition of the mobile phase and the samples could be directly injected into the HPLC system without the need for an evaporation/reconstitution step. The elimination of this step resulted in significant time saving during sample preparation.

3.3. Assay automation

The initial assay development and validation studies were performed manually using an adjustable 8-channel pipette. Following this work, several manual assay steps were automated using a combination of a Packard Multi-Probe liquid handling system and TomTec Quadra 96 SPE workstation.

The Packard Multi-Probe liquid handling system was used to transfer samples from individual test tubes into a 96-deep well plate. The probes used for samples transfer were washed with 1.2 ml of a solution of acetonitrile–water (50:50) between samples in order to eliminate cross sample contamination.

The TomTec Quadra 96 SPE workstation was used to perform the plate conditioning and sample load steps of SPE procedure. In addition, the system was used to load the wash and elution solvent onto the SPE plate, prior to the centrifugation steps.

During automation of the assays using TomTec 96 SPE Quadra workstation we found that it was important to adjust the height of tips so as to avoid sample splash; improper tip height adjustment was found to cause cross-sample comtamination that was attributed to the splashing of samples into adjacent wells of the plate.

3.4. Assay throughput

The combination of a reduced sample run time together with the use of automated 96-well SPE resulted in approximately a 3-fold increase in the number of samples that could be analyzed by a single analyst in a day as compared to the liquid–liquid extraction procedure. The increased sample throughput was absolutely critical to meet the demands of a drug development program with an aggressive timeline.

3.5. Assay selectivity

Fig. 2 shows chromatograms of extracted control plasma (A), a plasma standard containing rofecoxib (B) and an internal standard and a plasma sample taken from a subject 48 h after receiving a 50-mg oral dose of rofecoxib (C). A comparison of Fig. 2A with Fig. 2B illustrates that no endogenous peaks co-eluted with either the analyte or internal standard. The selectivity of the assay was further confirmed by analysis of pre-dose plasma samples from subjects involved in clinical trials; all of the samples were free of interfering peaks.

3.6. Assay linearity

Weighted (weighting factor $= 1/y$ where $y =$ peak height ratio) least-squares regression calibration curves, constructed by plotting the peak height ratio of analyte to internal standard vs. standard concentration yielded coefficients of regression typically greater than 0.999 over the concentration range of the assay. The use of the weighted least-squares regression resulted in less than a 7% deviation between the nominal standard concentrations and the experimentally determined standard concentrations calculated from the regression equation.

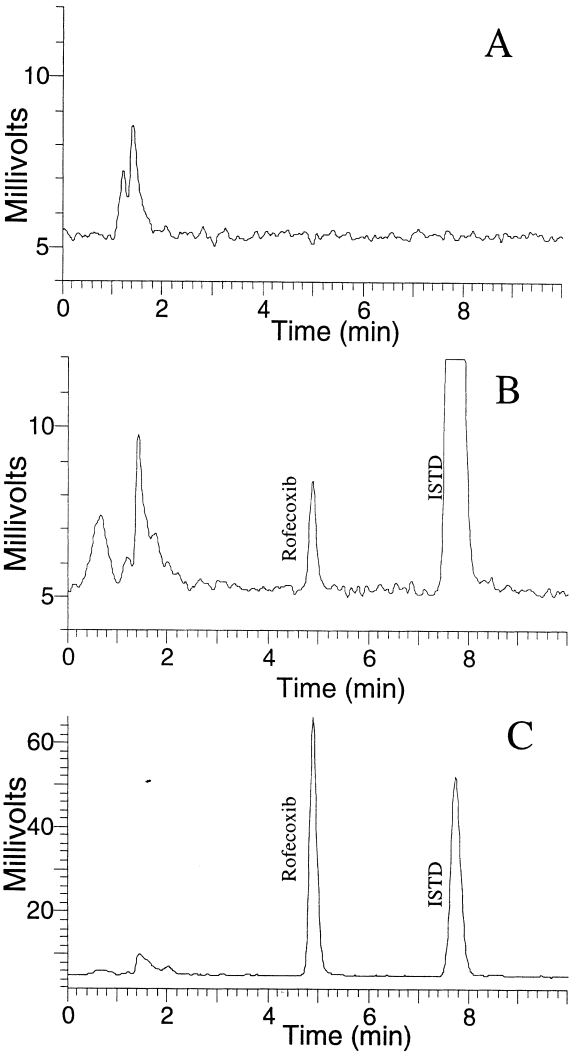

Fig. 2. Representative chromatograms of human plasma. (A) Control plasma, (B) plasma standard containing 0.5 ng/ml rofecoxib and internal standard (ISTD; 10 ng/ml) and (C) plasma sample from a human subject 48 h after oral administration of 50 mg rofecoxib. The concentration of rofecoxib was equivalent to 12.4 ng/ml.

3.7. Assay precision and accuracy

Replicate standards ($n = 5$) were analyzed to assess

the within-day variability of the assays. The mean assayed concentrations as well as the mean accuracy and relative standard deviations (RSDs) of the analyses are shown in Table 1.

3.8. Limit of quantification

The limit of quantification of the assay, defined as the lowest concentration that yielded a within-day RSD of less than 10% and a within-day accuracy between 90 and 110% of nominal concentration, was 0.5 ng/ml in human plasma using 0.5 ml of plasma.

3.9. Extraction recovery

The recovery of the extraction procedure was determined by comparing the responses (peak area) of working standards of rofecoxib injected directly into the HPLC system with those of extracted plasma standards. The results (Table 2) indicate that the recovery of rofecoxib from human plasma was greater than 98% at all concentrations tested. Recovery of the internal standard from human plasma was found to be greater than 95%.

3.10. Quality control samples

QC samples, containing concentrations of 1.5, 30 and 60 ng/ml of rofecoxib in plasma were prepared and frozen (−20°C) in 1-ml volumes. It was found that rofecoxib in quality control human plasma samples was stable through three freeze–thaw cycles. Quality control samples were analyzed each day along with human clinical samples to assess the inter-day variability of the assay. Representative plasma quality control sample data acquired over a

Table 2
Recovery of rofecoxib from human plasma

Concentration (ng/ml)	Recovery (%)
1	98.1
40	102.7
80	98.7

Table 3
Inter-day variability of the assays for determination of rofecoxib as assessed by the relative standard deviation of low, medium, and high quality control (QC) samples

Nominal QC concentration (ng/ml)	Mean analyzed concentration (ng/ml)	RSD (%)
1.5	1.5	6.7[a]
30	28.9	4.8[a]
60	57.4	5.1[a]

[a] $n=7$ runs over a 3-week period.

Table 1
Intraday precision and accuracy of the assay as assessed by the replicate ($n=5$) analysis of standards

Rofecoxib nominal concentration (ng/ml)	Rofecoxib mean[a] concentration (ng/ml)	Precision[b], RSD(%)	Accuracy[c] (%)
0.5	0.5	1.6	93.6
1.0	1.1	7.5	105.8
2.0	2.0	3.6	99.3
5.0	5.0	1.6	100.4
10.0	10.2	4.2	102.2
20.0	19.8	0.8	99.0
40.0	40.6	4.7	101.4
80.0	79.5	2.0	99.4

[a] Mean concentrations calculated from the weighted linear least-squares regression curve constructed using all five replicate values at each concentration.

[b] Relative standard deviation calculated using peak height ratios (rofecoxib/internal standard) for the five replicate values at each concentration.

[c] Expressed as [(mean calculated concentration)/(nominal concentration) · 100].

3-week period during the analysis of clinical samples is shown in Table 3.

4. Conclusions

A high throughput semi-automated assay has been developed for the determination of rofecoxib in human plasma. Application of 96-well SPE technology together with improved chromatography has led to significant improvements in sample throughput. The assay has been found to be precise, accurate and suitable for the analysis of plasma samples collected during large scale clinical studies. The ruggedness of the assay has been demonstrated by the successful analysis of several thousand clinical samples by multiple analysts.

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