Simultaneous Determination of Rofecoxib and Celecoxib in Human Plasma by High-Performance Liquid Chromatography

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Abstract

An innovative reversed-phase high-performance liquid chromatographic method is validated for the simultaneous determination of rofecoxib and celecoxib in human plasma. The internal standard is 4-*n*-pentyl-phenyl-acetic acid. Good chromatographic separation is achieved using a Zorbax SB-CN (5 µm) analytical column operated at room temperature and mobile phase consisting of acetonitrile and water containing 0.1M potassium dihydrogen orthophosphate buffer adjusted to pH 2.4 with 85% orthophosphoric acid (42:58, v/v). UV detection is performed at 254 nm, and the flow rate is maintained at 1.0 mL/min. Plasma samples are extracted into an organic solvent (1-chlorobutane) and evaporated under an air flow. The calibration curve for rofecoxib is linear over the range of 10 to 500 µg/L, and the celecoxib calibration curve is linear over the range of 20 to 2000 µg/L. The lower limit of quantitation for rofecoxib and celecoxib is 10 and 20 µg/L, respectively, using 1.0 mL of human plasma. The validation data show that the assay is sensitive, accurate, specific, and reproducible for the determination of rofecoxib and celecoxib. This method is therefore appropriate for pharmacokinetic studies to quantitate these therapeutic agents in patients with arthritis conditions.

Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used to treat pain and chronic arthritis conditions such as osteoarthritis (OA) and rheumatoid arthritis (RA). These agents are thought to act via the inhibition of the enzyme cyclooxygenase (COX). Two isoforms of COX have been identified (1). COX-1, the constitutive form, is expressed in many tissues (including the stomach, intestine, kidneys, and platelets) and mediates routine homeostatic actions of prostaglandins, including gastric mucosal protection and platelet function (2). COX-2, the inducible form, is expressed constitutively in a few organs (particularly the brain and kidney); induced by growth factors, cytokines, and mitogens; and primarily responsible for the production of prostaglandins that mediate inflammation, pain, and fever (3).

Currently available NSAIDs (including aspirin, ibuprofen, diclofenac, and naproxen) inhibit both COX-1 and COX-2 (4–6). The pattern of expression of the two COX isoforms indicates that the anti-inflammatory and analgesic effects of NSAIDs result principally from COX-2 inhibition, whereas unwanted side effects such as gastric erosion, ulceration, and bleeding result from inhibition of COX-1 (7). It has been hypothesized that a drug that specifically inhibits COX-2 (i.e, has no effect on COX-1 at clinical doses in humans) would reduce pain, inflammation, and fever with a substantially reduced risk of serious gastrointestinal complications such as perforations, ulcers, and bleeding (8,9). Rofecoxib (ROF) [Vioxx, (4-[4-(methylsulfonyl)phenyl]-3-phenyl-2(*5H*)-furanone] and celecoxib (CEL) [Celebrex, (4-[5-(4-methylphenyl)-3-(trifluoro-methyl)-1H-pyrazol-1-yl] benzenesulfonamide] (10,11) are selective COX-2 inhibitors that have originally been approved for the treatment of acute pain, OA, and RA (19,20).

It is important to note here that on September 30, 2004, Merck & Co., Inc., the manufacturer of ROF (Vioxx), announced a voluntary worldwide withdrawal of the drug, following the release of preliminary data from a prospective, randomized, and placebo-controlled study called APPROVe (Adenomatous Polyp Prevention on Vioxx) (25), which showed that ROF (Vioxx) increased the risk of cardiovascular events. In April 2005, the U.S. Food and Drug Administration (FDA) has required the manufacturers of all COX-2 inhibitors, including CEL (Celebrex), to revise the labeling for their products to include a boxed warning highlighting the potential for increased risk of cardiovascular events and the well-described, serious, and potentially life-threatening gastrointestinal bleeding associated with their use.

The FDA has also required Pfizer, the manufacturer of another selective COX-2 inhibitor, valdecoxib (Bextra), to suspend its sales following concerns over cardiovascular and skin problems. Further evaluation of the pharmacokinetics and pharmacodynamics of ROF and CEL after single and multiple doses requires development of analytical techniques to quantitate these two agents in biological samples.

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Most of the published methods quantitate either ROF (12–16) or CEL (17,18) using expensive liquid chromatograph–mass spectrometry technology (12,13,23,24), or high-performance liquid chromatography (HPLC) with post-column photochemical derivatization with fluorescence detection (13), or solid-phase extraction (16) and with evaporation under nitrogen gas (12,13,15). In this paper, a simple, accurate, isocratic, and sensitive HPLC method for the simultaneous quantitation of both ROF and CEL following liquid–liquid extraction of 1.0 mL of plasma is described.

Experimental

Chemicals

Merck Frosst (Quebec, Canada) kindly supplied ROF pure substance and Pharmacia Corporation (North Peapack, NJ) kindly supplied CEL pure substance. The chemical structures of these compounds were published previously (10,11). The internal standard 4-*n*-pentyl-phenyl-acetic acid (4-*n*-PPAA) was obtained from the Clinical Trials Centre of St. Vincent's Hospital (Sydney, Australia). Acetonitrile and methanol were HPLC grade and were purchased from Mallinckrodt Baker Inc. (Phillipsburg, NJ). 1-Chlorobutane, HiperSolv HPLC, grade was purchased from BDH Laboratories (Poole, U.K.). Orthophosphoric acid (85%, Unilab grade) was purchased from Ajax Chemicals (Sydney, Australia) and potassium dihydrogen orthophosphate (AnalaR grade) was purchased from BDH Chemicals (Victoria, Australia). Distilled water was used after recirculation through a Milli-Q water purification system (Millipore, Billerica, MA). Blank human plasma was supplied from the Australian Red Cross Blood Bank (Sydney, Australia).

Equipment

The HPLC system consisted of the following components: a Varian 9010-Model pump, Varian 9050-Model UV detector, ETP Kortec (K65B) autosampler (Varian, Walnut Creek, CA), and Zorbax SB-CN analytical column (4.6- × 150-mm, 5 µm) (Agilent Technologies, Palo Alto, CA). Varian star 5.3 chromatographic software was used for peak detection and integration.

Chromatography

The chromatographic analysis was performed at room temperature on a Zorbax SB-CN (5 µm) column with a mobile phase of acetonitrile and water, containing 0.1M potassium dihydrogen orthophosphate buffer adjusted to pH 2.4 with 85% orthophosphoric acid (42:58, v/v). UV detection was performed at 254 nm and the flow rate was maintained at 1.0 mL/min. Aliquots of 50 µL were injected onto the HPLC column.

Preparation of standards

Stock solutions of ROF and CEL were prepared by dissolving the appropriate amount of pure drug substance in methanol to yield a final drug concentration of 0.1 g/L ROF and 0.1 g/L CEL. Working solutions were prepared from a 1/10 ROF or 1/10 CEL dilution of the stock. The working solution of the internal standard (4-*n*-PPAA) was prepared from a 1/10 dilution of a stock solution (1 g/L in methanol). The standard solutions were stored at –20°C.

Preparation of calibration standards and quality control samples

Calibration standards were prepared in plasma covering the concentration range between 10 and 500 µg/L ROF and 20 to 2000 µg/L CEL by adding appropriate volumes of the working solutions to drug-free plasma. The volume of methanolic working solution never exceeded 50 µL/mL of plasma. Six calibration concentrations were used to define the standard curve (10, 25, 50, 100, 200, and 500 µg/L for ROF and 20, 50, 100, 200, 500, and 2000 µg/L for CEL). These calibration samples were divided into 5-mL glass extraction tubes as 1-mL aliquots, and stored frozen at –20°C away from direct light until assay. Quality control samples were prepared by dilution of an independently prepared stock solution of ROF (0.1 g/L) and CEL (0.1 g/L). The quality control samples were prepared in drug-free human plasma to final concentrations of 20, 150, and 300 µg/L for ROF; and 30, 300, and 1000 µg/L for CEL.

Sample pretreatment

Internal standard (100 µL) was added to 1 mL of standard and quality control samples. The drugs were extracted from plasma using 8 mL of 1-chlorobutane and mixed on a rotating shaker for 10 min. After the samples were centrifuged at 3000 rpm for 10 min, the organic layer was transferred to a tapered tube and evaporated under air at 50°C in a water bath. The extract was reconstituted in 200 µL mobile phase and briefly vortexed. The aqueous layer was transferred to an autosampler vial, and aliquots of 50 µL were injected into the HPLC for analysis.

Assay validation

A 4-day validation of the analysis of ROF and CEL was performed. The percent deviation (%DEV) and the relative standard deviation (%RSD) were calculated using Microsoft Excel 97 (Microsoft Corp., Redmond, WA). The acceptance criteria used to validate the assay have been published or are available as guidelines (21,22).

Linearity

Duplicate calibration curves were generated each day for three consecutive days. One reagent blank, plasma blank, and control zero (blank plasma with internal standard added) were analyzed in each run. The linear regression of the ratio of peak height of ROF and CEL and the internal standard versus the concentration were weighted by $1/x$ (reciprocal of the concentration).

Accuracy and precision

The accuracy and intra- and interday precision of the method were estimated using duplicate standard curves and by assaying three replicate quality control samples at three different concentrations (low, mid, and high, $n = 6$) for each drug, in three analytical runs ($n = 18$). The accuracy was determined by comparing the means of the measured concentrations with the nominal (theoretical) concentrations expressed as %DEV. The overall mean precision was defined by the %RSD of three quality control samples at three different concentrations (low, mid, and high) analyzed over 3 days ($n = 18$).

Specificity and selectivity

Interference from endogenous compounds was investigated by

the analysis of six different blank plasma matrices. Potentially coadministered drugs used in combination with ROF or CEL [including aspirin, paracetamol (acetaminophen), fenoprofen, naproxen, diclofenac, diflunisal, piroxicam, and ibuprofen] were also analyzed for interference under the HPLC conditions.

Lower limit of quantitation

The lower limit of quantitation (LLOQ) was investigated in plasma using six replicates of the following calibration standards: 10 µg/L ROF and 20 µg/L CEL. The concentration that was chosen as the LLOQ was accepted if the %DEV from the nominal value (measure of accuracy) and the RSD (measure of precision) was less than 15% (21).

Recovery

The recovery of ROF and CEL was determined on duplicate standards at six concentrations by comparing the peak height of extracted standards with the peak height of recovery standards prepared in mobile phase and injected directly onto the column to produce the identical "on-column" concentrations. The recovery of the internal standard was calculated from the average peak height of extracted replicates with the peak height of replicates directly injected onto the HPLC column.

Stability

Duplicate quality control samples at two concentrations were used to assess the stability of ROF, CEL, and internal standard in mobile phase at room temperature for 24 h after extraction. The stability of the drugs and internal standard during sample handling was also verified by subjecting the samples to two and three freeze–thaw cycles.

Results and Discussion

Specificity and selectivity

Blank plasma samples from six different individuals showed no interfering endogenous substances in the analysis of ROF and CEL (Figure 1). This assay has been performed for approximately 2 years and, although there appears to be a small peak before the ROF peak on visual inspection, this peak was considered insignificant because it has not interfered with the ROF peak.

Potentially coadministered drugs [including aspirin, paracetamol (acetaminophen), fenoprofen, naproxen, diclofenac, diflunisal, piroxicam, and ibuprofen] were tested and had retention times that were different from those of ROF (5.5 min), CEL (14.6 min), and internal standard (8.2 min).

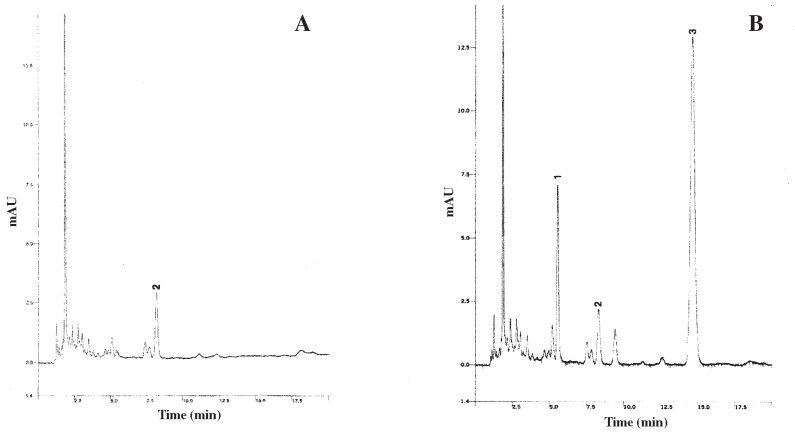

Figure 1. Chromatograms of blank human plasma (A) and a standard extracted from human plasma (B): ROF (200 µg/L) (1), internal standard (2), and CEL (500 µg/L) (3).

Validation

Calibration curves in human plasma were linear over the concentration range 10–500 µg/L for ROF and 20–2000 µg/L for CEL. The slope, intercept, and correlation coefficients of the calibration curves were: $0.12 \times 10^{-2} \pm 0.02 \times 10^{-3}$, 0.13×10^{-1}, and 0.999 (mean ± SD; $n = 6$); and $0.06 \times 10^{-3} \pm 0.01 \times 10^{-4}$, 0.09×10^{-2}, and 0.999 for ROF and CEL, respectively. The LLOQ of 10 µg/L for ROF and 20 µg/L for CEL was chosen. The accuracy and precision of the LLOQ was 6.7% DEV and 10.1% RSD (measure of precision) for ROF and 0.8% DEV and 10.7% RSD for CEL.

Table I shows the accuracy and precision of the quality control samples prepared in human plasma. The results indicate that the method is accurate and precise. Mean accuracy data over

Table I. Accuracy and Precision of ROF and CEL in Human Plasma*

Concentration (µg/L)	Accuracy† (%DEV)	Precision (%RSD) Within-day (N_w = 6)	Between-day (N_b = 6)	N
ROF 20 (low)	–7.3	6.2	12.1	18
ROF 150 (mid)	1.1	2.3	2.1	18
ROF 300 (high)	–9.8	1.6	2.0	18
CEL 30 (low)	–6.2	1.3	8.3	18
CEL 300 (mid)	1.1	2.2	3.4	18
CEL 1000 (high)	–9.3	1.5	1.6	18

* Abbreviations: DEV = deviation from the nominal value, N_w = number of replicates per run, N_b = number of different runs, and N = total number of replicates.
† Mean over 3 days.

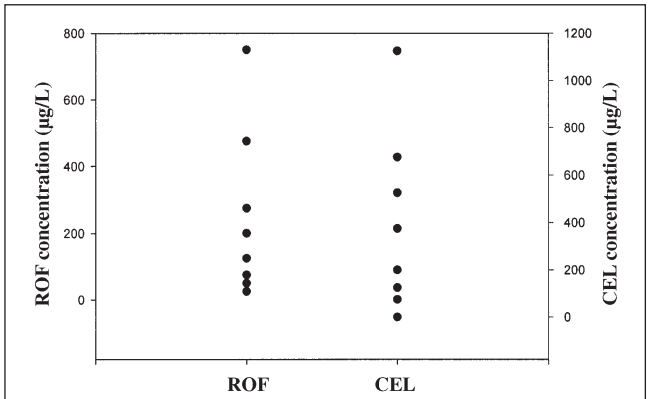

Figure 2. Trough plasma concentrations of ROF (n = 8) and CEL (n = 8) in patients who received the same dose of each drug (ROF 25 mg and CEL 200 mg).

3 days was better than 10.1% DEV for ROF and 10.7% DEV for CEL, and the between-day precision was better than 12.1% RSD for ROF and 8.3% RSD for CEL, which did not significantly differ from results reported in previously published methods (11,12,16, 23,24). The recovery was 86 ± 6.5 for ROF (mean ± SD), 85 ± 5.5 for CEL, and 65 ± 2.8 for the internal standard.

ROF, CEL, and internal standard were stable under all conditions tested, with all results falling within the acceptance criteria of ± 15% DEV from the nominal concentration.

Applicability

In a trial to prove the utility of the developed method in clinical studies, the method was used to determine the plasma concentrations of these drugs in patients receiving the same dose of each drug. As part of a population pharmacokinetics study being conducted at our center, trough samples were collected at three 24-h intervals from eight patients receiving ROF (25-mg dose) and eight patients receiving CEL (200-mg dose). Figure 2 shows significant variability in plasma concentrations of ROF and CEL in patients who received the same dose of each drug.

Conclusion

A reversed-phase HPLC assay has been validated for the simultaneous (halving labor time and running costs) determination of ROF and CEL in human plasma to serve as a tool in a population pharmacokinetics study of the two drugs. The method has been found to be accurate, precise, specific, reproducible, and economical. Therefore, it is appropriate to use pharmacokinetic studies to quantitate these therapeutic agents in patients with arthritis conditions.

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References

1. J.R. Vane, Y.S. Bakhle, and R.M. Botting. Cyclooxygenases 1 and 2. *Annu. Rev. Pharmacol. Toxicol.* **38:** 97–120 (1998).
2. T. Hla and K. Neilson. Human cyclooxygenase cDNA. *Proc. Natl. Acad. Sci.* **89:** 7384–88 (1992).
3. W.W. Seibert, Y. Zhang, K. Leahy, S. Hauser, J. Masferrer, and P. Isakson. Distribution of COX-1 and COX-2 in normal and inflamed tissues. *Adv. Exp. Med. Biol.* **400A:** 167–70 (1997).
4. D.J. Bjorkman. The effect of aspirin and nonsteroidal anti-inflammatory drugs on prostaglandins. *Am. J. Med.* **105(suppl. 1B):** 8S–12S (1998).
5. B. Cryer and M. Feldman. Cyclooxygenase-1 and cyclooxygenase-2 selectivity of widely used nonsteroidal anti-inflammatory drugs. *Am. J. Med.* **104(5):** 413–21 (1998).
6. H. Fenner. Deferentiating among nonsteroidal antiinflammatory drugs by pharmacokinetic and pharmacodynamic profiles. *Semin. Arthritis Rheum.* **26(6 suppl. 1):** 28–33 (1997).
7. J.R. Vane and R.M. Botting. Mechanism of action of aspirin-like drugs. *Semin. Arthritis Rheum.* **26(6 suppl. 1):** 2–10 (1997).
8. W.W. Botten. Scientific rationale for specific inhibition of COX-2. *J. Rheumatol.* **51:** 2–7 (1998).
9. J.B. Lefkowith. Cyclooxygenase-2 specificity and its clinical implications. *Am. J. Med.* **106(5B):** 43S–50S (1999).
10. Vioxx (rofecoxib tablets & oral suspension package insert). Merck & Co., Inc., Whitehouse Station, NJ, 1998.
11. M.J. Rose, E.J. Woolf, and B.K. Matuszewski. Determination of celecoxib in human plasma by normal-phase high-performance liquid chromatography with column switching and ultraviolet absorbance detection. *J. Chromatogr. B* **738:** 377–85 (2000).
12. U. Werner, D. Werner, R. Mundkowski, M. Gillich, and K. Brune. Selective and rapid liquid chromatography-mass spectrometry method for the quantification of rofecoxib in pharmacokinetic studies with humans. *J. Chromatogr. B* **760:** 83–90 (2001).
13. E. Woolf, I. Fu, and B. Matuszewski. Determination of rofecoxib, a cyclooxygenase-2 specific inhibitor, in human plasma using high-performance liquid chromatography with post-column photochemical derivatization and fluorescence detection. *J. Chromatogr. B* **730:** 221–27 (1999).
14. S. Sattari and F. Jamali. High performance liquid chromatographic determination of cyclooxygenase II inhibitor rofecoxib in rat and human plasma. *J. Pharm. Pharmaceut. Sci.* **3(3):** 312–16 (2000).
15. M.K. Aravind, R. Prescilla, and J.P. Ofenstein. A rapid and sensitive high-performance liquid chromatographic assay for rofecoxib in human plasma. *J. Chromatogr. Sci.* **40:** 26–28 (2002).
16. M. Zhang, G.A. Moore, S.J. Gardiner, and E.J. Begg. Determination of rofecoxib in human plasma and breast milk by high-performance liquid chromatographic assay. *J. Chromatogr. B* **807:** 217–21 (2004).
17. M. Abdel-Hamid, L. Novotny, and H. Hamza. Liquid chromatographic-mass spectrometric determination of celecoxib in plasma using single-ion monitoring and its use in clinical pharmacokinetics. *J. Chromatogr. B* **753:** 401–408 (2001).
18. M.S. Guirguis, S. Sattari, and F. Jamali. Pharmacokinetics of celecoxib in the presence of inteferon-induced acute inflammation in the rat: application of a novel HPLC assay. *J. Pharm. Pharmaceut. Sci.* **4(1):** 1–6 (2001).
19. L. Tive. Celecoxib clinical profile. *Rheumatology* **39(suppl. 2):** 21–28 (2000).
20. E. Tindall. Celecoxib for the treatment of pain and inflammation: the preclinical and clinical results. *JAOA* **99(11):** S13–S17 (1999).
21. L.L. Ng. Reviewer *Guidance: Validation of Chromatographic Methods from the Centre for Drug Evaluation and Research (CDER).* U.S. FDA, November, 1994, http://www.fda.gov/cder/guidance/cmc3.pdf.
22. V.P. Shah, K.K. Midha, J.W. Findlay, H.M. Hill, J.D. Hulse, I.J. McGilveray, G. McKay, K.J. Miller, R.N. Patnaik, M.L. Powell, A. Tonelli, C.T. Viswanatha, and A. Yacobi. Bioanalytical method validation—a revisit with a decade of progress. *Pharm. Res.* **17(12):** 1551–57 (2000).
23. C.M. Chavez-Eng, M.L. Constanzer, and B.K. Matuszewski. Determination of rofecoxib (MK-0966), a cyclooxygenase-2 inhibitor, in human plasma by high-performance liquid chromatography with tandem mass spectrometric detection. *J. Chromatogr. B* **748:** 31–39 (2000).
24. C.M. Chavez-Eng, M.L. Constanzer, and B.K. Matuszewski. High-performance liquid chromatographic-tandem mass spectrometric evaluation and determination of stable isotope labeled analogs of rofecoxib in human plasma samples from oral bioavailability studies. *J. Chromatogr. B* **767:** 117–129 (2002).
25. R.S. Bresalier, R.S. Sandler, H. Quan, J.A. Bolognese, B. Oxenius, K. Horgan, C. Lines, R. Riddell, D. Morton, A. Lanas, M.A. Konstam, and J.A. Baron. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *N. Engl. J. Med.* **352(11):** 1092–1102 (2005).

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