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Determination of rofecoxib in human plasma and breast milk by high-performance liquid chromatographic assay

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Abstract

A rapid and simple HPLC assay was developed for the determination of rofecoxib in human plasma and breast milk. After solid-phase extraction, rofecoxib was resolved on a C18 column and detected by UV detection at 272 nm. Standard curves were linear over the concentration range 10–2000 μg/L ($r^2 > 0.99$). Intra- and inter-day coefficients of variation for both matrices were <10% and the limit of quantification was around 10 μg/L.

Keyword: Rofecoxib

1. Introduction

Breast milk is the optimal food for babies, but the breast-fed infant may be exposed to drugs during maternal pharmacotherapy. It is important to know the extent of drug transfer into human breast milk in order to assess the likely 'dose' received by the infant during the lactation period.

Rofecoxib (Fig. 1) is a new member of a subgroup of nonsteroidal anti-inflammatory drugs (NSAIDs) known as selective cyclooxygenase-2 (COX-2) inhibitors [1–3]. It is approved by the US Food and Drug Administration (FDA) for the following indications: treatment of primary dysmenorrhea, management of acute pain in adults, and relief of the signs and symptoms of osteoarthritis [3]. Its selective inhibition of COX-2 means that rofecoxib has a significantly lower incidence of gastrointestinal adverse events compared with traditional NSAID therapy [2,4]. Rofecoxib has been widely prescribed since its launch.

The pharmacokinetic properties of rofecoxib in healthy volunteers and patients have been described previously [2,5,6]. However, nothing is known about the pharmacokinetics of rofecoxib in the plasma and breast milk of lactating mothers or their infants and no assay has been reported for measuring refecoxib in human milk. In order to provide useful information regarding the transfer of rofecoxib into breast milk a sensitive assay for rofecoxib in human plasma and breast milk is required.

Several high-performance liquid chromatographic (HPLC) methods for measuring rofecoxib in human plasma have been developed [7–13]. Most of these methods require a mass spectrometer [7–9] or post-column photochemical derivatization with fluorescence detection [10,11] and therefore are expensive, complex and time-consuming. HPLC methods using ultraviolet (UV) detection with liquid–liquid extraction were reported by Sattari and Jamali [12] and Aravind et al. [13]. The aim of this work was to develop a simple, sensitive and accurate HPLC method for measuring rofecoxib in human plasma and breast milk that could be used in the study of rofecoxib distribution into human milk.

2. Experimental

2.1. Materials

Rofecoxib (100% purity) was kindly donated by Merck (Rahway, NJ, USA). HPLC grade methanol was purchased

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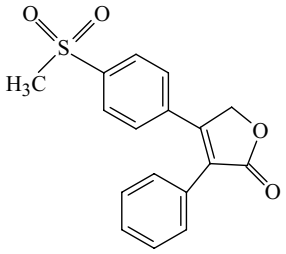

Rofecoxib

Fig. 1. Chemical structure of rofecoxib.

from BDH (Poole, UK). Distilled, deionised water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA). The human plasma used as the assay blank and for the preparation of standards was obtained from New Zealand Blood Services (Christchurch, New Zealand). The human breast milk used as the assay blank and for the preparation of standards was kindly donated by healthy volunteers. Solid-phase extraction cartridges (Strata-X, 30 mg adsorbent, 1 ml reservoir volume) were purchased from Phenomenex (Torrance, CA, USA).

2.2. Chromatography

HPLC analysis was performed on the Agilent 1100 series system equipped with a quaternary pump, a variable wavelength detector set at 272 nm and an autosampler (Hewlett-Packard, Waldbronn, Germany). An Aqua C18 5 μm, 4 mm × 3.0 mm internal diameter guard column and an Aqua C18 5 μm, 75 mm × 4.6 mm internal diameter analytical column (Phenomenex, Torrance, CA, USA) were used for separation. Data were collected and analysed using the Agilent ChemStation (Hewlett-Packard, Waldbronn, Germany). The mobile phase was a mixture of methanol and water (50:50, v/v). The mobile phase was filtered through a 0.45 μm filter and degassed under vacuum before use. The flow rate was 1.0 ml/min and the system was operated at ambient temperature.

2.3. Standards

A standard stock solution of rofecoxib (0.4 mg/ml) was prepared by dissolving 10 mg of rofecoxib in 25 ml of methanol. The plasma and milk calibration curves of rofecoxib were constructed by spiking drug-free human plasma or breast milk with rofecoxib stock solution at concentrations of 10, 25, 100, 400 and 2000 μg/L.

Bulk rofecoxib plasma and milk standards for determination of freeze–thaw stability were prepared in single 10 ml aliquots in the following concentrations: 25, 400, and 2000 μg/L. Rofecoxib plasma and milk quality control (QC) standards were prepared in the same concentrations as the bulk standards and stored in multiple 0.6 ml aliquots for assay with each analytical run. Both bulk standards and QC standards were stored at −30 °C until analysed. QC standards were discarded once thawed and analysed.

2.4. Sample preparation

Plasma and milk samples were cleaned up by a solid-phase extraction procedure. For the plasma samples, Strata-X cartridges (30 mg/ml) were preconditioned by washing with 1 ml of methanol followed by 1 ml of water. Plasma (0.5 ml) samples were applied to the preconditioned cartridges. The cartridges were washed with 3 × 1 ml of 5% methanol in water. After drying, rofecoxib was eluted from the cartridges with 1 ml of methanol. The eluates were evaporated to dryness in a Speed Vac Concentrator with a −106 °C trap (SVC 200H, Savant Instruments, Farmingdale, NY, USA) under reduced pressure and at 1725 rpm. Residues were dissolved in 250 μl of mobile phase and 40 μl aliquots were injected into the HPLC system. For the breast milk samples, 0.5 ml of acetonitrile was added to 0.5 ml of milk to precipitate the proteins. The mixture was vortexed for 30 s and centrifuged at 15,000 × *g* for 5 min. The clear supernatant was transferred to a clean tube and evaporated to dryness. Residues were dissolved in 0.5 ml of water and applied to the preconditioned cartridges for extraction in the same way as the plasma samples.

2.5. Validation

The standard curve was a plot of the peak area of rofecoxib versus the corresponding concentrations of rofecoxib in the standard curve samples. The linearity of the standard curve was evaluated using least-squares linear regression analysis. To determine recovery of rofecoxib at concentrations of 25, 400 and 2000 μg/L from plasma or milk, an identical set of standards prepared in the mobile phase was analysed. Absolute recoveries at each concentration were measured by comparing the response of extracted standards with the response of standards which had not been subjected to sample extraction. Intra- and inter-day coefficients of variation of the assay were determined by the analysis of six QC samples at each concentration on the same day and of one QC sample at each concentration on six different days, respectively. The limit of quantification for this assay was defined as the lowest concentration of rofecoxib that could be detected with intra- and inter-day coefficients of variation <20% ($n = 5$) and a mean value <20% deviation from the spiked value.

3. Results and discussion

3.1. Chromatography

Under the chromatographic conditions employed, the retention time of rofecoxib was 4.1 min and the peak of rofecoxib was free of interference from any peaks present in the plasma or milk blank (Fig. 2).

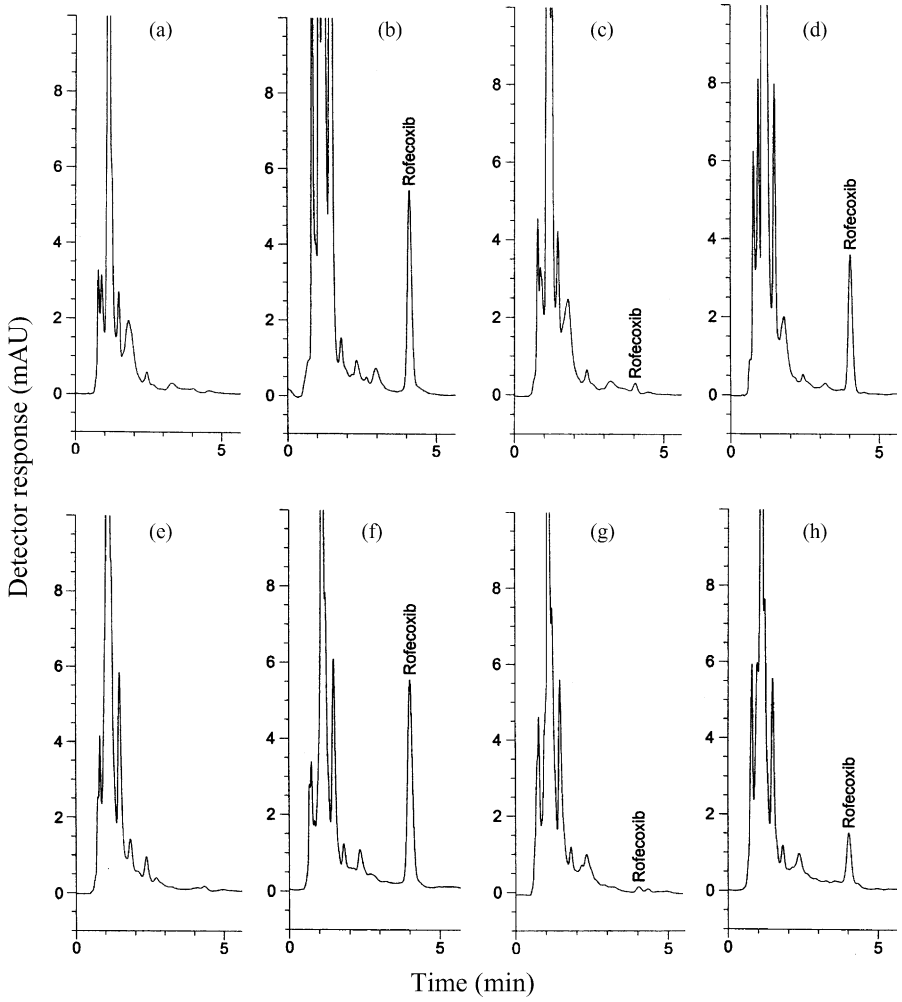

Fig. 2. HPLC chromatograms obtained from (a) blank plasma sample; (b) plasma sample spiked with rofecoxib to give a concentration of 400 μg/L; (c) plasma sample spiked with rofecoxib to give a concentration of 10 μg/L; (d) plasma sample taken from a healthy lactating volunteer 5 h after administration of a single oral dose of rofecoxib 25 mg (rofecoxib concentration = 216 μg/L); (e) blank breast milk sample; (f) breast milk sample spiked with rofecoxib to give a concentration of 400 μg/L; (g) breast milk sample spiked with rofecoxib to give a concentration of 10 μg/L; and (h) breast milk sample from a healthy volunteer taken 4.5 h after administration of a single oral dose of rofecoxib 25 mg (rofecoxib concentration = 99 μg/L).

3.2. Sample preparation

Solid-phase extraction (SPE) is widely used for biological sample preparation before analysis. Compared to the classic liquid–liquid extraction, SPE is faster and easier to perform, uses less solvent and provides clean extracts and high recoveries. Strata-X was chosen for solid-phase extraction in this study because it is a new, patent-pending SPE cartridge that is engineered to simplify sample preparation. Milk is a complex biological matrix that contains nearly as many different components as plasma [14]. When milk samples were applied directly to the preconditioned cartridges, the SPE cartridges were blocked. This is likely to be related to the high and variable lipid and protein content of milk. Hence, a protein precipitation step prior to SPE was applied to the milk sample cleanup.

It is usually considered desirable to use an internal standard when performing HPLC quantitation. An appropriate internal standard can control for extraction and HPLC injection variability. Our method did not utilise an internal standard as we have used a simple solid-phase extraction step that is not suspected to induce a large variability in the extraction recoveries. In addition, the use of an autosampler improves the injection-volume precision and provides low HPLC injection variability [15]. Intra- and inter-day coefficients of variation for this method (all <10%) showed that our procedure was acceptable without an internal standard.

3.3. Linearity and limit of quantification

Both plasma and milk standard curves of rofecoxib were linear ($r^2 > 0.99$) over the concentration ranges 10–2000 μg/L. The intercept with the *y*-axis was not significantly different from zero. The limit of quantification of rofecoxib was around 10 μg/L in both plasma and milk.

3.4. Recoveries

The absolute recoveries of rofecoxib from plasma and breast milk determined at concentrations of 25, 400 and 2000 μg/L were similar and consistent. The mean ± S.D. absolute recoveries of rofecoxib were 87.6 ± 2.8% from plasma ($n = 6$ plasma extracts at each concentration) and 84.7 ± 4.1% from breast milk ($n = 6$ breast milk extracts at each concentration).

3.5. Stability of rofecoxib

In order to assess the effects of freezing and thawing on rofecoxib concentrations bulk rofecoxib plasma and breast milk standards at 25, 400 and 2000 μg/L were subjected to four freeze–thaw cycles before analysis. The mean values ($n = 4$) measured at each concentration deviated ≤5.0% from the spiked values for both plasma and breast milk samples. Rofecoxib was found to be stable in plasma and breast milk for at least four freeze–thaw cycles when stored at −30 °C. In addition, QC samples of plasma and breast milk were stored at −30 °C for 5 months. After 5 months of storage, samples at each concentration were analysed and the rofecoxib values remained stable (<7.0% deviation from the spiked values). The standard stock solution of rofecoxib was shown to remain stable for at least 6 months at 4 °C.

3.6. Accuracy and precision

The precision and accuracy of the method were determined by intra- and inter-day assay variance (Tables 1 and 2). The intra-day coefficients of variation were less than 7.0% and the inter-day coefficients of variation were less than 10.0% for both plasma and breast milk assays at all concentrations of the QC samples.

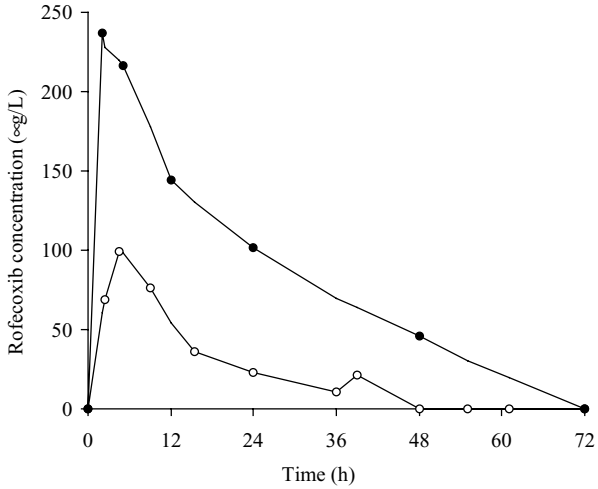

Fig. 3. Rofecoxib concentrations in plasma and breast milk over 72 h, following administration of a single oral dose of rofecoxib 25 mg to a healthy lactating volunteer: concentration of rofecoxib in (●) plasma; (○) breast milk.

3.7. Application of the assay

The assay is currently being used in a clinical study to investigate the distribution of rofecoxib into human breast milk. The plasma and milk concentration–time profiles for a healthy volunteer after a single 25 mg oral dose of rofecoxib are shown in Fig. 3.

Table 1
Intra- and inter-day assay variance of the determination of plasma rofecoxib

Type of variance	Sample	Concentration spiked (μg/L)	Concentration found (μg/L) (mean ± S.D.)	CV (%)
Intra-day ($n = 6$)	QC1	25.0	24.8 ± 1.2	4.8
	QC2	400	399 ± 12.4	3.1
	QC3	2000	1984 ± 57.5	2.9
Inter-day ($n = 6$)	QC1	25.0	21.0 ± 1.9	9.1
	QC2	400	403 ± 21.8	5.4
	QC3	2000	2050 ± 91.4	4.5

Table 2
Intra- and inter-day assay variance of the determination of milk rofecoxib

Type of variance	Sample	Concentration spiked (μg/L)	Concentration found (μg/L) (mean ± S.D.)	CV (%)
Intra-day ($n = 6$)	QC1	25.0	25.0 ± 1.5	6.1
	QC2	400	390 ± 17.8	4.6
	QC3	2000	2020 ± 32.7	1.6
Inter-day ($n = 6$)	QC1	25.0	21 ± 2.0	9.5
	QC2	400	399 ± 26.6	6.7
	QC3	2000	2050 ± 102.9	5.0

4. Conclusions

A validated HPLC method has been described and is currently being used to analyse rofecoxib concentrations in human plasma and breast milk after oral administration of rofecoxib in lactating mothers. The method has proven to be simple, rapid, sensitive and specific.

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