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Simultaneous determination of celecoxib, hydroxycelecoxib, and carboxycelecoxib in human plasma using gradient reversed-phase liquid chromatography with ultraviolet absorbance detection

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Abstract

A new HPLC method for the simultaneous determination of celecoxib, carboxycelecoxib and hydroxycelecoxib in human plasma samples has been developed. Following a solid-phase extraction procedure, the samples were separated by gradient reversed-phase HLPC (C_{18}) and quantified using UV detection at 254 nm. The method was linear over the concentration range 10–500 ng/ml. The intra-assay variability for the three analytes ranged from 4.0 to 12.6% and the inter-assay variability from 4.9 to 14.2%. The achieved limits of quantitation (LOQ) of 10 ng/ml for each analyte allowed the determination of the pharmacokinetic parameters of the analytes after administration of 100 mg celecoxib.

Keywords: Celecoxib; Hydroxycelecoxib; Carboxycelecoxib

1. Introduction

Celecoxib (Celebrex, CXB) is a non-steroidal anti-inflammatory drug approved for the treatment of rheumatic pain and ostheoarthritis. CXB is the first selective COX2 inhibitor, developed to reduce the serious side-effects of non-steroidal anti-inflammatory drugs associated with the inhibition of COX1 seen with non-selective COX inhibitors. CXB is actively metabolized to its hydroxylated (hydroxycelecoxib, OH-CXB) and carboxylated (carboxycelecoxib, COOH-CXB) metabolites (Fig. 1), and less than 2% of the parent compound is eliminated unchanged [1]. Previous reports indicate that the major enzyme involved in the hepatic biotransformation of CXB is CYP2C9, a CYP isoform that is known to exist as several genetic variants [2]. Although CXB generally causes fewer side-effects than previous drugs, the risk for gastric/duodenal ulcers may be increased in patients with reduced CYP2C9 activity [3]. To facilitate a clinical study investigating the impact of CYP2C9 genetic polymorphisms, an HPLC assay for the simultaneous determination of CXB and its metabolites in human plasma was required. A literature search revealed that CXB pharmacokinetic analysis in rats and humans had previously focused on the quantitation of the parent drug [4–8], or relied on the use of

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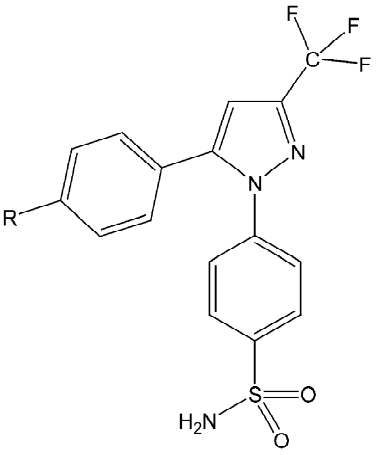

R=CH_3: Celecoxib
R=CH_2OH: Hydroxycelecoxib
R=COOH: Carboxycelecoxib

Fig. 1. Chemical structures of celecoxib, hydroxycelecoxib, and carboxycelecoxib.

radiolabeled compounds for the determination of metabolites [9,10]. In connection with an in vitro study, a method for the quantification of CXB and OH-CXB was described, but the secondary metabolite, COOH-CXB, was not measured [2]. Recently, an LC–MS method for the quantitation of CXB in human plasma was published [5], however mass-selective detection is cost-intensive and often not available for routine analysis. We therefore developed and validated a new method for the simultaneous quantitation of CXB, OH-CXB, and COOH-CXB in human plasma using solid-phase extraction combined with gradient elution reversed-phase HPLC and UV detection.

2. Experimental

2.1. Chemicals and reagents

4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]-benzenesulfonamide (celecoxib), hydroxycelecoxib, and carboxycelecoxib were kindly provided by Pharmacia Upjohn (St. Louis, MO, USA). Sodium acetate, potassium hydrogenphosphate, methanol, and acetonitrile (gradient grade) were purchased from Merck (Darmstadt, Germany). Phenacetin was purchased from Sigma (Deisenhofen, Germany). Blank plasma was obtained from the Department of Hematology, University Medical Center Charité, Humboldt University, Berlin.

2.2. Chromatography

CXB, OH-CXB, and COOH-CXB were quantified using a Shimadzu HPLC system (Duisburg, Germany) consisting of two LC-6A pumps, a SIL-6B automatic sampler, and a SPD-6AV spectrophotometric detector. Class LC10 software Version 1.6 (Shimadzu) was used for data acquisition and processing. The compounds were separated at 60 °C on a Phenomenex Luna C_{18} column (5 μm, 150×4.6 mm I.D.) (Phenomenex, Aschaffenburg, Germany) with a guard column and quantified by ultraviolet detection at 254 nm.

Mobile phase A consisted of 10% acetonitrile and 90% 0.01 *M* sodium hydrogen phosphate buffer (pH 5.4), and mobile phase B consisted of 80% acetonitrile and 20% 0.01 *M* sodium hydrogen phosphate buffer (pH 5.4). Gradient elution started at 10% B for 10 min, was increased to 38% at 47 min and to 73% at 63 min, kept constant at 73% until 68 min and was reduced to 10% at 68.1 min (Fig. 2). Total analysis time was 75 min at a flow-rate of 1.5

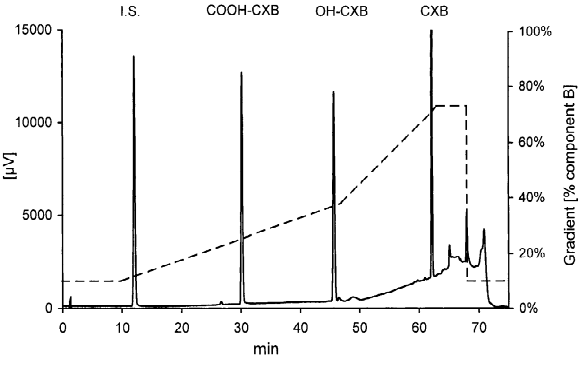

Fig. 2. HPLC trace of celecoxib (CXB), hydroxycelecoxib (OH-CXB), carboxycelecoxib (COOH-CXB), and phenacetin (I.S.) in methanolic solution using UV detection at 254 nm. The broken line indicates the mobile phase gradient used to achieve the separation.

ml/min. The compounds were quantified using their peak height ratio to an internal standard (phenacetin).

2.3. Sample preparation

In a 10 ml glass tube, 0.5 ml plasma was mixed with 15 μl internal standard solution (1 mg phenacetin/100 ml methanol) and 300 μl 0.2 *M* sodium acetate buffer (pH 5.0). Subsequently, 1.8 ml acetonitrile was added to precipitate plasma protein, samples were vortexed and centrifuged for 5 min at 15 000 *g*. Supernatants were transferred to a new tube and the organic phase was evaporated at 50 °C under nitrogen. The remaining aqueous phase was diluted with 2 ml distilled water and subjected to solid-phase extraction on a Benchmate II sample processor (Zymark, Hopkinton, MA, USA). Waters Oasis HLB 1 cc solid-phase extraction cartridges (Waters, Milford, MA, USA) were conditioned with 1 ml methanol and 1 ml water, and the sample was loaded onto the column at a flow-rate of 0.07 ml/min and rinsed twice with 1.5 ml of 5% methanol in water. Compounds were eluted with 2 ml methanol, which was evaporated to dryness at 50 °C under nitrogen. Samples were reconstituted in 75 μl methanol and a volume of 50 μl was subjected to HPLC analysis.

2.4. Preparation of stock solutions, calibration standards and quality control samples

A stock solution was prepared by dissolving 10 mg of each compound in methanol in a 100 ml volumetric flask. The solution was stored at −20 °C. For preparation of calibration standards and quality control samples, appropriate aliquots of the stock solutions were evaporated to dryness and reconstituted in blank human plasma. The final concentrations were 0, 10, 25, 50, 250, and 500 ng/ml for the calibration standards and 0, 20, 80, and 400 ng/ml for quality control samples. Standards were stored in aliquots of 600 μl until analysis. Although stability during repeated freeze–thaw cycles has been demonstrated [6], samples were stored at −20 °C out of caution, but also for lack of storage space at 4 °C or at room temperature.

3. Results

3.1. Separation and impact of pH on retention times

CXB, OH-CXB, COOH-CXB, and the internal standard phenacetin were sufficiently separated under the HPLC conditions applied. Retention times were 12 min for the internal standard, 30 min for COOH-CXB, 45 min for OH-CXB, and 61 min for CXB (Fig. 2). While the retention times of phenacetin, OH-CXB, and CXB remained largely unaffected by changes in the pH of the mobile phase, the retention time of the more hydrophilic metabolite COOH-CXB changed substantially with pH modulation in the range pH 4–6 (Fig. 3).

3.2. Specificity

No interferences were observed in blank plasma samples. Blank plasma samples for specificity testing were prepared in the same way as study samples. Three different blank plasma samples were used in the pre-validation process, and a blank plasma sample of each individual study subject was included in the analysis. Fig. 4 shows the chromatograms of a blank plasma sample (a), a quality control sample (b) and a plasma sample after administration of 100 mg celecoxib (c). Commonly used pain medications were assessed for potential interferences. Phenacetin was

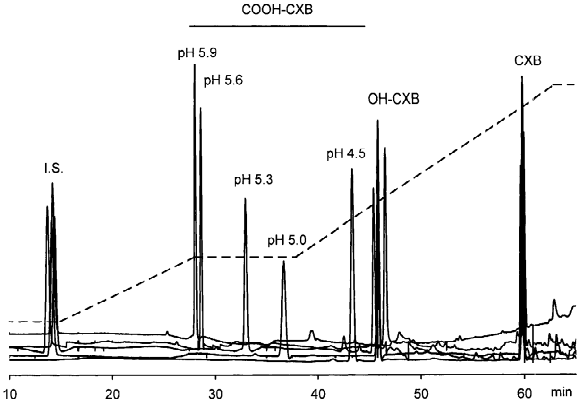

Fig. 3. Effect of mobile phase pH on the retention times of celecoxib (CXB), hydroxycelecoxib (OH-CXB), carboxycelecoxib (COOH-CXB), and phenacetin (I.S.).

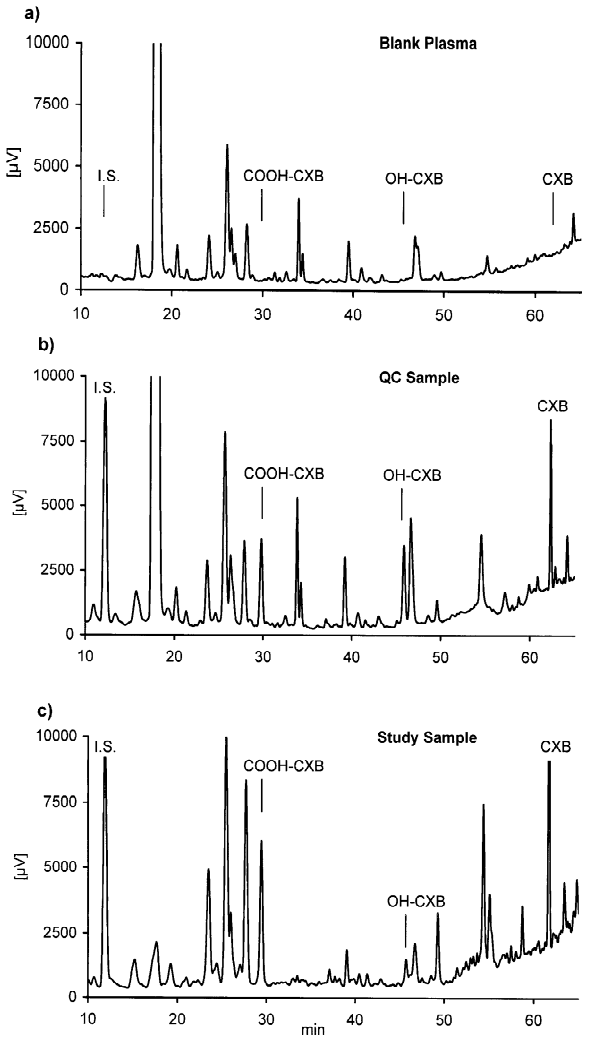

Fig. 4. HPLC traces for celecoxib (CXB), hydroxycelecoxib (OH-CXB), carboxycelecoxib (COOH-CXB), and phenacetin (I.S.). (a) Blank plasma sample, (b) quality control sample (80 ng/ml), (c) plasma sample 1.5 h post-administration of 100 mg celecoxib (152 ng/ml COOH-CXB, 18.9 ng/ml OH-CXB, 269 ng/ml CXB).

used as an internal standard, and its more hydrophilic metabolite paracetamol (acetaminophen) elutes at much shorter retention times and therefore cannot cause interference. Diclofenac eluted at 11 min, ibuprofen was not detectable under the extraction and HPLC methods applied, and caffeine eluted at 2.5 min. Additionally, CXB is typically not monitored in a clinical setting with uncontrolled comedication. Under the conditions of this clinical study, comedication was not permitted.

Table 1
Limits of detection (LOD) and limits of quantitation (LOQ) for celecoxib, hydroxycelecoxib, and carboxycelecoxib

	LOD		LOQ (n=11)	
	(ng)	(ng/ml)	Inter-assay RSD (%)	Accuracy (%)
Celecoxib	0.5	10	13.9	107.2
Hydroxycelecoxib	0.5	10	13.4	102.5
Carboxycelecoxib	0.25	10	11.4	100.4

3.3. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD was determined as the amount of drug corresponding to a signal-to-noise ratio of 3:1. LOQ values were determined as the lowest concentration of the analyte in plasma that could be quantified with an inter-assay RSD of <20% and an accuracy between 80 and 120%. LOD and LOQ values for the three analytes are reported in Table 1.

3.4. Linearity

Assay linearity was evaluated over the concentration range 10–500 ng/ml. The mean slopes and r^2 values are reported in Table 2.

3.5. Intra- and inter-assay variability

The intra-assay RSD for the three analytes ranged from 4.0 to 12.6% and the inter-assay RSD from 4.9

Table 2
Assay linearity for the quantitation of celecoxib, hydroxycelecoxib, and carboxycelecoxib (n=11)

		Mean	SD	RSD (%)
Celecoxib	Slope	0.007	0.0010	13.5
	r^2	0.9965	0.0055	0.6
Hydroxycelecoxib	Slope	0.004	0.0003	7.5
	r^2	0.9987	0.0009	0.1
Carboxycelecoxib	Slope	0.004	0.0003	7.9
	r^2	0.9987	0.0020	0.2

Table 3
Intra- and inter-assay variability, accuracy, and recovery for the quantitation of celecoxib, hydroxycelecoxib, and carboxycelecoxib

Intra-assay variability ($n=6$)			Inter-assay variability				Recovery ($n=6$)		
(ng/ml)	RSD (%)	Accuracy (%)	(ng/ml)	n	RSD (%)	Accuracy (%)	(ng/ml)	Recovery (%)	RSD (%)
Celecoxib									
25	12.6	99	20	11	14.2	102.1	20	84	11.0
120	9.1	102	80	10	11.9	93.4	120	70	9.6
600	6.3	107	400	11	10.9	91.0	600	66	8.9
Hydroxycelecoxib									
25	9.8	109	20	11	12.8	98.1	20	89	13.0
120	7.7	108	80	10	6.2	95.5	120	75	8.2
600	7.0	105	400	11	4.9	91.5	600	65	8.8
Carboxycelecoxib									
25	6.6	93	20	11	13.2	97.4	20	99	6.8
120	5.0	97	80	10	7.4	95.9	120	90	5.5
600	4.5	93	400	11	8.7	96.3	600	73	4.8

to 14.2%. The individual values are reported in Table 3.

3.6. Accuracy and recovery

The accuracy of the measurements was determined using three quality control samples for each compound in each run and the results are reported in Table 3.

The recovery of CXB, OH-CXB, and COOH-CXB from spiked human plasma was compared with the directly injected analyte at concentrations of 25, 120 and 600 ng/ml. Values ranged from 66 to 84% for CXB, from 65 to 89% for OH-CXB, and from 73 to 99% for COOH-CXB (Table 3). Recovery values tended to be lower with higher analyte concentrations, possibly due to the beginning of saturation of the SPE material. However, the effect was too small to affect assay linearity or intra- and inter-assay variability.

4. Discussion

Celecoxib and its metabolites hydroxycelecoxib and carboxycelecoxib were quantified in human plasma by combining solid-phase extraction with gradient reversed-phase HPLC analysis. Unlike previously described methods [4–8], CXB and its metabolites were measured simultaneously, and the use of UV detection eliminated the need for the radiolabeled drugs required in CXB pharmacokinetic studies in the past [9,10]. The solid-phase extraction procedure allowed simple and automated sample preparation. Several SPE column materials were tested, but it became apparent that reversed-phase materials (C_4, C_8, C_{18}, phenyl) were not able to extract the hydrophilic metabolites, particularly COOH-CXB. Therefore, Waters Oasis HLB columns were tested and proved to be suitable for the application. However, associated with the ability of this SPE material to bind both polar and non-polar compounds was the extraction of multiple endogenous compounds from plasma, causing interfering peaks that could not be resolved from the analytes with isocratic HPLC. Accordingly, we opted for gradient elution, and with variations in pH (Fig. 3) and solvent gradient satisfactory separation was achieved.

In conclusion, the newly developed method allowed the simultaneous analysis of CXB, OH-CXB, and COOH-CXB with LOQ values (10 ng/ml) that were lower or comparable to those previously reported with UV or fluorescence detection for the analysis of the parent compound alone [4,6,7]. The method was found to be suitable for the generation of plasma concentration–time curves and the subsequent determination of pharmacokinetic parameters for the three analytes (Fig. 5) after administration of a low dose (100 mg) of CXB.

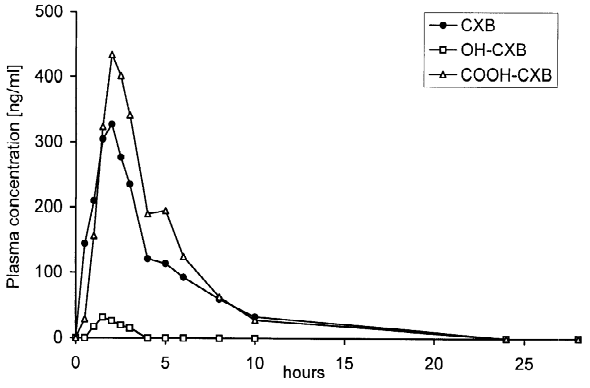

Fig. 5. Plasma concentration–time curves for CXB, OH-CXB, and COOH-CXB following administration of 100 mg celecoxib to a healthy volunteer.

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