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Chromatographic techniques in analysis of cyclooxygenase-2 inhibitors in drugs and biological samples

Review Article

Małgorzata Starek*, Monika Dąbrowska

Department of Inorganic and Analytical Chemistry, Faculty of Pharmacy, Collegium Medicum, Jagiellonian University, 30-688 Kraków, Poland

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Abstract: Non-steroidal anti-inflammatory drugs, as a therapeutic class, are among the most often used active pharmaceutical ingredients in heath care in the world. They are mostly available without prescription and often used for treatment of fever and pain. An extensive research of the literature published in analytical and pharmaceutical chemistry journals has been conducted and the chromatographic methods which were used for the purity, stability and pharmacokinetic studies of the cyclooxygenase-2 inhibitors, in formulations and biological materials have been reviewed. The methodology for the analysis of selected drugs is very well documented and many examples are available in the literature. The common use of chromatographic techniques with various detection attachments provide possibility for monitoring of drugs in therapy.

Keywords: *COX-2 inhibitors • Analysis • Chromatography • Stability • Pharmacokinetic*

1. Introduction

Chromatography is a simpler and more accurate technique to perform qualitative and quantitative developments of the individual components than separated directly from the mixture. The basics of chromatography were defined by Tswett [1] over 100 years ago. The great progress in its use was directly linked to the development and improvement of technologies that promote chemical industry which comprises pharmaceutical, biotechnology, clinical and many other. The essential technology changes include higher pressure liquid systems, more regular and smaller particle stationary phases, modified media, more repeatable sample introduction systems, and a wide range of detectors much more sophisticated than the visual detection. However, these innovations developed the chromatography systems needed significant technology infrastructure to preserve them, including part inventories and skilled technicians to perform repairs, preventive maintenance, and system updates. These more advanced systems in many instances have introduced significant development [2].

Because of the very nature of requirement of separation of multiple components during analysis of stability samples, chromatographic methods have taken precedence over the conventional methods of analysis. Apart from separation of multiple components, the advantage of chromatographic methods is that these possess greater accuracy and sensitivity for even small quantities of degradation products produced. Most viewed chromatographic methods that have been used are thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), high-performance liquid chromatography (HPLC), and gas chromatography (GC). A large number of publications have appeared in the last decade on the use of LC for stability-indicating method development.

Non-steroidal anti-inflammatory drugs (NSAIDs) are pharmaceuticals which have been used in treatment with analgesic, antipyretic and anti-inflammatory effects, and which are relatively safe. One of the causes for their popularity is that, they do not give sedation, respiratory depression or addiction. Although their chemical structure is variable all of them share the acidic nature. Recently, new drugs have been introduced, such as the second-generation cyclooxygenase (COX) inhibitors or coxibs (*e.g.* celecoxib).

Selective inhibitors of the cyclooxygenase-2 (COX-2), also are mentioned to as coxibs, have been developed

* *E-mail: mstarek@interia.pl*

as substances with therapeutic actions suchlike to those of NSAIDs, but without the gastrointestinal side effects. Long-term studies tried to explore the efficiency of coxibs in preventing creation of adenomatous colon polyps or Alzheimer's disease have shown that selective and nonselective COX inhibitors may increase the frequency of cardiac infarctions and other cardiovascular reactions [3].

Because of the elevated polarity and acidic nature of NSAIDs (pKa values 4 - 4.5), LC-MS and LC tandem MS have experienced a leap during the last years, both in the field of technological development and application, avoiding the derivatization step demanded by GC-MS methods, particularly for acidic drugs.

Chromatographic separation is another important process in reducing ion suppression. Complete chromatographic separation enhances detectability and reproducibility despite the fact that the separation is not necessary using MS/MS detection. Due to the acidic character of NSAIDs the use of buffer or acidic solutions (such as ammonium formiate or formic acid) in the eluent is preferred. It causes reduction of signal intensities due to suppressing effects in the MS interface [4].

Several chromatographic methods have been set for the studies of NSAIDs and their metabolites in miscellaneous samples. Impurity profiling is a general term including structure elucidation/identification as well as determination of the impurities of a chemical substance. The significance of this process in researches has been emphasized multiple times with chromatographic techniques always being mentioned as a widely used and extremely valuable analytical tool in this field. Besides efficacy and quality, safety of the drug substances and finished drug products is the most important prerequisite in the pharmaceutical industry. Patients of different age and different stage of illness may need to take drugs for long times: therefore these products must comply with maximal standards of safety and quality. Therefore, analytical methods have to be able to conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, *etc*. and separation of drug from degradation products. In available literature there are several review articles about the determination of COX-2 inhibitors in various samples [5-8]. These present all accessible analytical methods (chromatographic, spectrophotometric, electrochemical and other) that were used to determination of the active contents of selected drugs in pharmaceutical and biological samples, and were validated according to the current procedures. Sample preparation, method's conditions and detections were discussed.

The aim of this review is to present published work on the application only of chromatographic procedures for the analysis of coxibs (Fig. 1), covering an extended period from 2000 to present. The procedures are reviewed in terms of sampling strategies, stability, determination of metabolites, and pharmacokinetic studies. The article provides a complementary approach for pharmacokinetic studies that demand a chromatographic method of analysis for the temporal and quantitative study of the parent drug, as well as the metabolic species in a biological matrix. The analytical parameters and methods of sample preparation used in the discussed LC methods (such as sample type, stationary phase, mobile phase, detection) are presented in Table 1.

2. Chromatographic techniques in coxibs researches

2.1. Purity and stability studies

Purity and stability testing is an important part of the process of drug product development. Chromatographic methods were very often developed to separate impurities and degradates from the drug substance to monitor its quality and establish a stability profile. Stress testing of the drug substance, which is carried out under thermal, humid, oxidative, acidic, alkaline, and photolytic conditions, helps to determine the intrinsic stability of the compound.

2.1.1. Celecoxib

The identification of the five impurities present in crude celecoxib drug was described. Impurities I (4-methylacetophenone) and II (methyl-4-methylbenzoate) were characterized by LC-MS data and further confirmed by synthesis. Impurities III (5-(4-methylphenyl)-3-trifluoromethyl-1H-pyrazole), IV (4-[5-(2'-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl] benzenesulfonamide) and V (4-[4-(4'-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]-benzenesulfonamide) were isolated by preparative reversed phase high-performance liquid chromatography (RP-HPLC) method and were characterized using spectral data [9].

A RP-HPLC method was developed and validated for forced-degradation study. Separation was carried out on RP C-18 column with buffer: acetonitrile (40:60, v/v) as mobile phase, and monitored on photo-diode array detector at a wavelength of 254 nm. It will be useful to determine assay and known impurity of celecoxib [10].

A TLC method for quantitation of doxazosin mezylate and celecoxib in capsules, in the presence of their degradation products was described. TLC silica gel GF254 and cyclohexane: dichloromethane : diethylamine (50:40:10, *v/v/v*) were used as a stationary and mobile

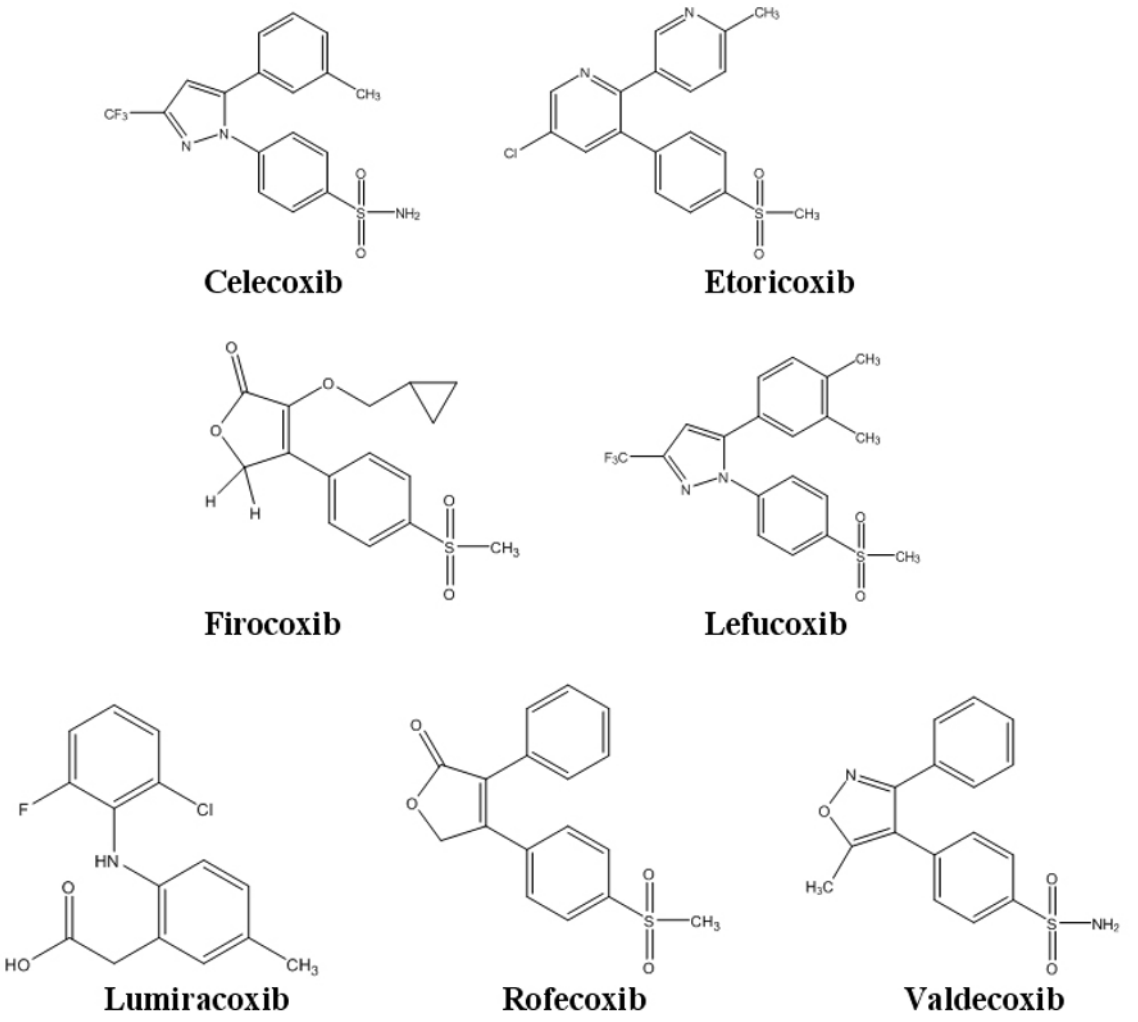

Figure 1. Chemical structures of COX-2 inhibitors (coxibs).

phase, respectively. Detection was performed at 253 nm. R_F value was 0.23 for celecoxib. The degradation product of celecoxib, 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl, was prepared in the laboratory by hydrolysis with 2 M hydrochloric acid. Its structure was confirmed by IR spectrum [11].

Forced degradation studies of celecoxib were performed by LC method at room temperature for 8hours using acid (1 M HCl), base (1 M NaOH) and 30% hydrogen peroxide, to provide an indication of the stability-indicating properties and specificity of the method. Separation was carried out on HiQSil RP C-18 column with mixture of methanol: water (85:15, v/v) as a mobile phase, and spectrophotometric detection at 251 nm [12].

The development and validation of a stability-indicating LC method for the analysis of celecoxib in tablets on a calixarene column was reported. Forced degradation studies were performed using acid (1 M HCl), base (1 M NaOH), hydrogen peroxide (30%) and UV-radiation (for 4 h). No interference from degradates was noticed [13].

The stability of celecoxib in the injection solvent was determined periodically by injecting replicate preparations of processed samples for up to 24 h (in the auto sampler at 5°C) after the initial injection. Stability of each analyte in the biomatrix during 6 h (bench-top) was determined at ambient temperature (25°C) at four concentrations. Freezer stability of drug in human plasma was assessed by analyzing the samples stored at −20°C for 21 days [14].

Stability-indicating HPLC method was performed for analysis of celecoxib in bulk drug and in micro-emulsions, after dilution with mobile phase to furnish working solutions. The stability was determined by repeated analysis of samples during the course of experimentation on the same day and after storage of the solutions. Celecoxib was stable when stored for 48 h

Table 1. Summary of published LC methods.

Compound	Sample type	Extraction method	Detection	Stationary phase	Mobile phase	Ref.
celecoxib	substance	extraction to mobile phase	252 nm	Hichrom C18 (250×4.6 mm, 5 μm)	0.01 M KH_2PO_4 in water : ACN (45:55)	[9]
	substance	extraction to mobile phase	252 nm	Hyperprep-C18 (250×10 mm, 8 μm)	ACN : water (50:50)	[9]
	substance	extraction to mobile phase	254 nm m/z 381	Zorbax C18 (250×4.6 mm)	0.01 M CH_3COONH_4 : ACN (40:60)	[9]
	tablets	extraction to ACN	254 nm	Caltrex AIII (125×4 mm, 10 μm)	ACN + water (55+45)	[9]
	human plasma	1 μg of I.S. (DRF-4367) was added to 500 μL of plasma, mixed, 3 mL of ACN was added and vortexed. After centrifugation organic layser was separated and evaporated. Residue was reconstituted in mobile phase	235 nm	Kromasil KR 100-5C18 (250×4.6 mm, 5 μm)	sol.A: 0.05 M formic acid (pH 3.0), sol.B: Milli Q water : ACN (5:95), sol.C: Milli Q water : MeOH (10:90), gradient elution	[14]
	substance, micro-emulsion	extraction to mobile phase	250 nm	Supelco 516 C18 DB RP (250×4.6 mm, 5 μm)	MeOH : water (75:25)	[15]
	substance, pharma-ceuticals	extraction to ACN	242 nm	Intensil ODS-3 (250×4.6 mm, 5 μm)	0.1 M hexamethyl-disilazane in ACN : water (55:45)	[16]
	skin	extraction from skin samples by evaluating different volumes of extractor solvent (mobile phase), ultrasound bath time, different centrifugation times and influence of Ultra Turrax tissue cutters	251 nm	RP-C18 idLiChrospher (100×4 mm, 5 μm)	MeOH : water (72;28)	[17]
	human plasma	50 μL of I.S. (mefenamic acid), 500 μL of ACN and 100 mg NaCl were added to 450 μL of plasma. Solutions were mixed and centrifuged	254 nm	Chromolith Performance RP-18e (100×4.6 mm)	ACN : MeOH : water (45:10:45) containing 0.2% acetic acid (pH 3.5)	[18]
	substance	extraction to MeOH, buffer and cyclodextrin solutions	250 nm	Hypersil BDS C18 (250×4.6 mm)	ACN : MeOH : phosphate buffer (5:1:4)	[19]
	substance, mice tissues	extraction to absolute ethanol; after reaction of celecoxib with $Na^{125}I$ in presence of chloramines-T as oxidizing reagent at 60°C	—	RP nucleosil phenyl (250×4.5 mm, 5 μm)	ACN : water in 0.1% acetic acid (pH 3.16), gradient elution	[20]
	male adults plasma, urine, feces	plasma was extracted using SPE C18 Bond Elut columns. Radioactive compounds retained on column were eluted with ACN and MeOH. Extracts were evaporated and dryness. Residues were reconstituted in ACN in sodium phosphate buffer	exc 240 nm em 380 nm	C-18 Nova Pak (150×3.9 mm, 4 μm)	ACN : 0.025 M sodium acetate buffer (pH 4.5), gradient elution	[45]
	rat urine, fecal	urine was extracted using C18 Bond Elut SPE columns with water, ACN and MeOH. Extracts were evaporated and residues were reconstituted in mobile phase. Fecal homogenates were extracted with MeOH. Extracts were centrifuged. Pellet was resuspended in MeOH, vortexed and extracted to MeOH. After evaporating, residues were reconstituted in mobile phase	exc 240 nm em 380 nm	Novapak TM C18 (150×3.9 mm, 4 μm)	ACN : 0.01 M sodium phosphate buffer (pH 7.4), gradient elution	[46]
	rat bile	bile was extracted using C18 Bond Elut SPE columns with water, ACN and MeOH. Extracts were evaporated and residues were reconstituted in mobile phase	exc 240 nm em 380 nm	Novapak TM C18 (150×3.9 mm, 4 μm)	ACN : 0.025M ammonium acetate buffer (pH 4.5), gradient elution	[46]

Continued **Table 1.** Summary of published LC methods.

Compound	Sample type	Extraction method	Detection	Stationary phase	Mobile phase	Ref.
celecoxib	rat plasma	plasma with I.S. was treated with H_3PO_4. Sample was extracted with cation exchange/ hydrophobic mixed mode SPE column, and eluted with ammonium hydroxide in MeOH. Extract was evaporated and residue was dissolved into mobile phase	exc 240 nm em 380 nm	Novapak TM C18 (150×3.9 mm, 4 μm)	ACN : 0.01 M sodium phosphate buffer (pH 9) (50:50)	[46]
	human plasma, breast milk	0.2 mL of ACN was added to plasma or milk sample. After vortexing, solutions was centrifuged	—	Aqua C18 (75×4.6 mm, 5 μm)	ACN : 0.01 M phosphate buffer (pH 3.5) (50:50) containing 0.1% TEA	[47]
	human liver microsome	incubation mixture (potassium phosphate buffer pH 7.4, $MgCl_2$, EDTA, $NADP^+$, D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase, microsomal protein, celecoxib) was dissolved in ACN. After incubation, I.S. (diclofenac) was added. Sample was centrifuged. Supernatant was evaporated and residue was reconstituted in 30% ACN in water	254 nm	RP C18	sol.A: 0.05% aqueous H_3PO_4, sol.B: ACN : H_2O (90:10) in 0.05% H_3PO_4, gradient elution	[48]
	rabbit plasma, urine, fecal	1 mL of plasma was acidified with H_3PO_4 and extracted by C18 Bond Elut SPE columns with ACN. Eluent was dried and reconstituted in mobile phase. To urine sample (pH 5 with sodium acetate buffer and glacial acetic acid), liquid scintillation cocktail was added (metabolite [^{14}C]celecoxib). Fecal homogenate was extracted with 15 mL of MeOH and mixed. Extracts were centrifuged and decanted. Residues were extracted twice with MeOH. Extracts were dried and reconstituted in mobile phase	m/z 380 316, 296, 276, 256, 247, 225, 221, 198, 179, 159, 139, 80, 69	Waters NovaPak C18 HPLC (150×3.9 mm, 4 μm)	ACN : 0.025M ammonium acetate (pH 4.5), gradient elution	[49]
	human plasma	100 μL of I.S. (sulindac) was added to 1 mL of plasma, mixed with acetate buffer (pH 5) and extracted with ethyl acetate. After centrifugation, solvent was evaporated and residue was reconstituted in mobile phase	—	Shim Pack GLC-CN C (150×4.6 mm, 5 μm)	ACN : 1% acetic acid solution (4:1)	[50]
	human serum	25 μL of I.S. (celecoxib derivative) was added to 0.5 mL serum, mixed with 500 μL of saturated NaCl and 1 mL of ACN. Samples were extracted with chloroform and centrifuged. Extract was evaporated and residue was dissolved in mobile phase	exc 240 nm em 380 nm	Prontosil C18 AQ (150×3 mm, 3 μm)	water : ACN (40:60)	[51]
	substance, rat plasma	0.1 mL of plasma was spiked with celecoxib and I.S. (ibuprofen). After acidifing (H_2SO_4) samples were extracted with isooctane + isopropanol (95+5) and mixed. Solvent was separated, evaporated and residue was reconstituted with mobile phase	254 nm	RP C18 (100×4.6 mm, 5 μm)	ACN : water : acetic acid : TEA (47:53:0.1:0.03)	[52]

Continued **Table 1.** Summary of published LC methods.

Compound	Sample type	Extraction method	Detection	Stationary phase	Mobile phase	Ref.
celecoxib	human plasma	100 μL of ACN + water (50+50) and 150 μL of I.S. (rofecoxib) were added to 1 mL of plasma. After vortexing, sodium acetate buffer (pH 5) and DCM + hexane (50+50) were added. Sample was shaked, centrifuged and solvent was evaporated. Residue was reconstituted in mobile phase	m/z 382.4 362	Nucleosil C8 (120-5, 11×2 mm)	MeOH : water (50:50) and 1% acetic acid	[53]
	human plasma	0.5 mL of plasma was mixed with 15 μL of I.S. (phenacetin), 300 μL of sodium acetate buffer (pH 5) and 1.8 mL of ACN, vortexed and centrifuged. Organic phase was evaporated and aqueous phase was diluted with distilled water and extracted by SPE columns with MeOH. After evaporating, residues were reconstituted in MeOH	254 nm	Phenomenex Luna C18 (150×4.6 mm, 5 μm)	sol.A: ACN : 0.01 M NaH_2PO_4 (pH 5.4) (10:90), sol.B: ACN : 0.01 M NaH_2PO_4 (pH 5.4) (80:20), gradient elution	[54]
	microbial cultures	cultures, after 10 days of incubation with celecoxib were extracted with ethyl acetate. extracts were evaporated and dried samples were reconstituted in MeOH	251 nm	Luna C18 (250×4.6 mm, 5 μm)	ACN : water (pH 3.2 with H_3PO_4) (60:40)	[55]
	microbial cultures	cultures, after 10 days of incubation with celecoxib were extracted with ethyl acetate. extracts were evaporated and dried samples were reconstituted in MeOH	m/z 100 500	X-terra MS C-18 (50×4.6 mm, 5 μm)	ACN : 0.05 M formic acid (50:50)	[55]
	human plasma	50 μL of I.S. (flutamide) and 50 μL of phosphate buffer (pH 5) were added to 0.5 mL of plasma. After vortexing, 5 mL of n-hexane + isoamyl alcohol (97+3) was added, shaken and centrifuged. Supernatant was combined with mobile phase	260 nm	C18 μ-Bondapak (250×3.9 mm)	0.01 M H_3PO_4 : ACN (40:60) containing 20 μL of TEA : H_3PO_4 to obtain pH 4	[56]
	human plasma	1 μg of I.S. (DRF-4367) was added to 500 μL of plasma, mixed, 3 mL of ACN was added and vortexed. After centrifugation organic layser was separated and evaporated. Residue was reconstituted in mobile phase	235 nm	Kromasil KR 100-5C18 (250×4.6 mm, 5 μm)	sol.A: 0.05 M formic acid (pH 3.0), sol.B: Milli Q water : ACN (5:95), sol.C: Milli Q water : MeOH (10:90), gradient elution	[14]
etoricoxib	human plasma	10 μL of I.S. (valdecoxib) was added to 1 mL of plasma spiked with etoricoxib and mixed with borate solution. mixture was extracted with ethyl acetate, centrifuged and collected with mobile phase	235 nm	BDS Hypersil C18 (150×4.6 mm, 5 μm)	10 mM ammonium acetate buffer : ACN (65:35)	[24]
	human plasma	50 μL of I.S. (rofecoxib) was added to 950 μL of plasma. After vortexing, diethylether + dichloromethane (6+4) was added, vortexed and centrifuged. Supernatant was combined with mobile phase	284 nm	BDS Hypersil C-18 (250×4.6 mm, 5 μm)	aqueous buffer: 0.3 mL TEA and 0.4 mL H_3PO_4 per L (pH 2.95) : ACN (62:38)	[25]
	substance	extraction to MeOH	235 nm	UPLC™ BEH C18 (100×2.1 mm, 1.7 μm)	buffer: 0.01 M ammonium acetate solution and glacial acetic acid (pH 5.0) : ACN (60:40)	[26]

Continued **Table 1.** Summary of published LC methods.

Compound	Sample type	Extraction method	Detection	Stationary phase	Mobile phase	Ref.
etoricoxib	human plasma	50 μL of I.S. (zaleplon) and diethylether + DCM (7+3) were added to 1 mL of plasma. Sample was mixed, centrifuged and evaporated. Dried extract was reconstituted in water + MeOH (50+50)	284 nm	Waters Symmetry® C18 (250×4.6 mm, 5 μm)	water : ACN (58:42)	[28]
	human plasma	25 μL of I.S. (etoricoxib derivative) and ACN + water (50+50) were added to 0.5 mL of plasma and vortexed. Ammonium carbonate buffer (pH 9.5) was added and vortexed. Next, samples were mixed with buffer in 96-well C8 extraction plate	m/z 359→280	BDS Hypersil C18 (50×3.0 mm, 3 μm)	ACN : 10 mM ammonium acetate (pH 4) (35:65)	[57]
	human blood	recombinant human COX-1 and COX-2 were expressed in baculovirus-Sf9 cells and enzymes	275 nm	Novapak C18 (150×3.9 mm)	ACN : water : acetic acid (50:50:0.1)	[58]
firocoxib	horse, dog plasma	1 mL of plasma was extracted using SPE columns with ACN in water. Solution was evaporated and residue was reconstituted in 0.25 mL of 40% ACN + water	290 nm	InertsilTM ODS-3 (150×4.6 mm, 5 mm)	ACN : water : TFAA (45:55:0.025)	[29]
lefucoxib	rats plasma, urine, fecal	50 μL of NaOH and 500 μL of MtBE were added to 200 μL of sample and homogenized with water. Solution was vortexed and centrifuged. Supernatant was extracted with MtBE, dried and collected with MeOH + water (80+20)	exc 254 nm em 430 nm m/z 395 375 296	Kromasil C18 (250×4.6 mm)	MeOH : water, gradient elution	[63]
	tablets	extraction to ACN	272 nm	Phenomenex Synergi fusion C18 (150×4.6 mm, 4 μm)	H_3PO_4 (pH 3.0; 25 mM) : ACN (40:60)	[30]
	human plasma	acidified plasma (0.5 mL) was extracted using pentane + methyl chloride (2+1). Extract was dried and reconstituted in water + MeOH (80+20)	270 nm	SEP RP octyl (150×3.2 mm)	ACN : 0.05% H_3PO_4, gradient elution	[59]
lumiracoxib	human plasma, urine, fecal	plasma sample was extracted twice with ACN, mixed, evaporated and reconstituted in water + MeOH + ACN (8+1+1). Urine sample was filtered and lyophilized. Residues were reconstituted in MeOH + water (2+8). Fecal sample was extracted three times with MeOH. Extracts were combined and portioned with hexane. After evaporating, residues were reconstituted in MeOH	280 nm	ODS-2 (250×4.6 mm, 5 μm)	sol.A: 0.02 M ammonium acetate (pH 6) sol.B: ACN, gradient elution	[59]
	animal skin	skin samples were mounted on diffusion system with SC facing donor compartment and dermis facing acceptor solution (phosphate buffer pH 7.4 with 3% polysorbate20). Acceptor solution was withdrawn at predetermined time points. SC samples were tape-stripped ten times and immersed in ACN. After shaken, remaining skin was cut into small pieces, mixed with ACN	—	C8 RP Schinpack (150×4.6 mm, 5 μm)	ACN : 0.01 M sodium phosphate buffer (pH 2.5) (50:50)	[61]

Continued **Table 1.** Summary of published LC methods.

Compound	Sample type	Extraction method	Detection	Stationary phase	Mobile phase	Ref.
parecoxib	tablets	extraction to MeOH	210 nm	Phenomenex Synergi fusion (150×4.6 mm, 4 μm)	water, pH 7 adjusted with NaOH (0.1 M) : ACN (52:48)	[44]
lumiracoxib	canine plasma	48 μL of DFU and 120 μL of TFAA were added to 100 μL of plasma. After vortexing, 4 mg KCl was added, mixed, 600 μL of cyclohexane + diethyl ether (3+2) was added, shaken and centrifuged. Supernatant was combined with ACN	exc 265 nm em 375 nm	Luna C18 ODS2 (150×4.6 mm, 3&5 μm)	ACN : buffer (10 mM $AcONH_4$, adjusted to pH 5 with AcOH) (55:45)	[74]
	substance	extraction to mobile phase	225 nm	Hichrom RPB (250×4.6 mm)	water : ACN (50:50)	[31]
	substance	extraction to mobile phase	225 nm	Hyperprep-HS-C18 (250×10 mm, 10 μm)	water : ACN (65:35)	[31]
	substance	extraction to ACN	220 nm	Shimpak VP-ODS C18 (150×4.6 mm, 5 μm)	ACN : 0.05% H_3PO_4 (pH 2.6) (35:65)	[32]
	human plasma, breast milk	to milk samples ACN was added and vortexed. Supernatant was evaporated and residue was dissolved in water. Then, plasma or milk samples were extracted using SPE columns by MeOH. Eluates were evaporated and residues were dissolved in mobile phase	—	Aqua C18 (75×4.6 mm, 5 μm)	MeOH : water (50:50)	[34]
	human serum	20 μL of I.S. (diazepam) and 20 μL of working solution of rofecoxib in ACN were added to 1 mL of serum. After mixing, 5 mL of DCM was added, mixed and centrifuged. DCM was dried by adding anhydrous sodium sulfate and evaporating. Residue was reconstituted in ACN	254 nm	Novapak-C18 (150×4.6 mm, 5 μL)	ACN : water (37.5:62.5)	[35]
	substance	extraction to water + ACN (50+50)	220 nm	Waters Symmetry C8 (250×4.6 mm, 5 μm)	0.1% H_3PO_4 : ACN, gradient elution	[35]
rofecoxib	human plasma	frozen plasma was thawed at room temperature and vortexed. 25 μL of I.S. (rofecoxib derivative) was added. Sample was diluted with water, mixed and extracted using SPEC C8 96-well SPE plates. Plate was centrifuged after water + ACN wash. Extracts were filtered and centrifuged	exc 250 nm em 400 nm	Chromolith Speed Rod RP-18e (50×4.6 mm)	water : ACN (65:35)	[65]
	human plasma	100 μL of ACN and 75 μL of I.S. (celecoxib) were added to 1 mL of plasma. After mixing, sodium acetate buffer (pH 5) and DCM + hexane (50+50) were added. Samples were shaken, centrifuged and evaporated. Residue was reconstituted in mobile phase	m/z 315.4 297	Nucleosil C (120-5, 11×2 mm)	MeOH : water (50:50)	[66]
	rat, dog plasma	0.85 mL of sodium acetate buffer (pH 5) and 25 μL of I.S. (4'-methylphenyl analog of rofecoxib) were added to 0.15 mL of plasma. after extraction with methylene chloride + hexane (50+50), samples were centrifuged and placed in acetone/dry ice bath to freeze aqueous layer. Organic phase was isolated, evaporated and residue was reconstituted in mobile phase	exc 250 nm em 375 nm	Base Deactivated Silica Hypersil (100×4.6 mm, 5 μm)	35% aqueous CH_3CN	[67]

Continued **Table 1.** Summary of published LC methods.

Compound	Sample type	Extraction method	Detection	Stationary phase	Mobile phase	Ref.
	human plasma	1 μg of I.S. (DRF-4367) was added to 500 μL of plasma, mixed, 3 mL of ACN was added and vortexed. After centrifugation organic layser was separated and evaporated. Residue was reconstituted in mobile phase	235 nm	Kromasil KR 100-5C18 (250×4.6 mm, 5 μm)	sol.A: 0.05 M formic acid (pH 3.0), sol.B: Milli Q water : ACN (5:95), sol.C: Milli Q water : MeOH (10:90), gradient elution	[14]
	substance	extraction to diluent	240 nm	Agilent Zorbax SB-CN (250×4.6 mm, 5 μm)	sol.A: 0.01 M KH_2PO_4, 0.01 M 1-octane sulfonic acid sidium salt (pH 3.0 with H_3PO_4) : ACN (80:20), sol.B: water : ACN (30:70), gradient elution	[39]
	human plasma	1mL of I.S. (rofecoxib) was added to sample. After vortexing, 0.2 mL of $HCLO_4$ was added and vortexed. Next, mixture was shaken with 10 mL of diethyl ether and centrifuged	239 nm	Cosmosil C18 (150×4.6 mm, 5 μm)	ammonium acetate buffer : ACN (60:40), containing 0.1% TEA (pH 6.5 with glacial acetic acid)	[41]
	human plasma	50 μL of rofecoxib workin solution and diethylether + DCM (7+3) were added to 1 mL of plasma. Sample was vortexed and centrifuged. Organic layer was was evaporated and extract was reconstituted in water + MeOH (80+20)	210 nm	YMC ODS-AQ (250×4.6 mm, 5 μm)	water : MeOH (47:53)	[42]
	substance, tablets	extraction to MeOH	220 nm	Waters Chromolith Performance RP-18e (100×4.6 mm, 2 μm)	1% aqueous TEA (pH 7.4) : MeOH (64:36)	[43]
valdecoxib	human plasma	400 μL of I.S. (valdecoxib derivative) was added to 400 μL of sample, vortexed, loaded onto C18 Bond Elut SPE cartridges and eluted with ACN. Solvents were removed and residues were reconstituted in mobile phase	m/z 313 118	Zorbax XDB-C8 (50×2.1 mm, 5 μm)	ACN : water (50:50) containing 10 mM ammonium acetate	[69]
	human urine	frozen urine samples were thawed, vortexed and centrifuged. 500 μL of supernatants were mixed with 500 μL of I.S. (structural analog of valdecoxib) in ammonium acetate buffer (pH 6.8). Samples were vortexe and extracted by RapidTrace automatic SPE system with ACN. Solvent was evaporated and residues were reconstituted in mobile phase	m/z 313 118	RP Keystone Prism RP (50×2 mm, 5 μm)	ACN : water (50:50) (pH 6.0) containing 10 mM 4-methylmorpholine	[70]
	substance	mixtures of unlabeled and $^{13}C_6$-labeled metabolites were obtained from dog urine after oral dosing with 1:1 ratio of valdecoxib and [$^{13}C_6$]valdecoxib. Deuterated standards of metabolites were dissolved in deuterated water	m/z 329 196, 180, 132, 117, 78	Novapak C-8 (150×3.9 mm, 5 μm)	sol.A: ACN : MeOH : 25 mM ammonium acetate buffer (pH 4) (1:2:27), sol.B: ACN : MeOH : 25 mM ammonium acetate buffer (pH 4) (1:2:3), gradient elution	[71]
	canine plasma	48 μL of DFU and 120 μL of TFAA were added to 100 μL of plasma. After vortexing, 4 mg KCl was added, mixed, 600 μL of cyclohexane + diethyl ether (3+2) was added, shaken and centrifuged. Supernatant was combined with ACN	exc 265 nm em 375 nm	Luna C18 ODS2 (150×4.6 mm, 3&5 μm)	ACN : buffer (10 mM $AcONH_4$, adjusted to pH 5 with AcOH) (55:45)	[74]

Continued Table 1. Summary of published LC methods.

Compound	Sample type	Extraction method	Detection	Stationary phase	Mobile phase	Ref.
valdecoxib	mouse plasma, urine, feces	plasma samples were extracted three times with ACN. After centrifuging, extracts were collected, dried and residues were reconstituted in mobile phase. Urine samples were centrifuged. Homogenized fecal samples were extracted three times with ACN and twice with mobile phase (sol.A). Extracts were dried and reconstituted in mobile phase	—	Novapak C-8 (150×3.9 mm, 4 μm)	sol.A: ACN : MeOH : 25 mM ammonium acetate buffer (pH 4) (1:2:27), sol.B: ACN : MeOH : 25 mM ammonium acetate buffer (pH 4) (1:2:3), gradient elution	[75]

ACN – acetonitrile; DCM – dichloromethane; DFU – 5,5-dimethyl-3-(3-fluuorophenyl)-4-(4-methylsulpholyl)phenyl-2(5H)-furanone; exc – excitation; em – emission; I.S. – internal standard; MeOH – methanol; MtBE – methyl tert-buthyl ether; SC – stratum corneum; sol. – solution; TEA – triethylamine; TFAA – trifluoroacetic acid; "—"– no data

at laboratory temperature (32°C) and under refrigeration (8°C) in mixture of methanol: water (75:25, v/v) [15].

A RP-HPLC method for separation and determination of process-related impurities of celecoxib in bulk drugs and pharmaceuticals was developed. The stability of celecoxib and its impurities in the mobile phase was evaluated by analyzing solutions with impurities at 0.1% of the specification level, after 48 h at room temperature, and after stored for 24 h in a refrigerator at 4°C [16].

Praça *et al.* [17] developed the analytical HPLC method with UV detection to determinate celecoxib extracted from different skin layers. The skin extraction procedure was carried out using porcine ear skin. The stability of the skin samples containing celecoxib was also evaluated, showing little variation in the concentrations of the drug at room temperature and under refrigeration after different exposure times (12 h at 25°C and 24 h at 4°C).

The stability of celecoxib in human plasma was assessed for spiked plasma samples stored at −20°C for up to 2 months, +4°C for at least 1 month and at ambient temperature for 12 h. Authors also demonstrated the applicability of the HPLC method for pharmacokinetic studies in humans. Plasma concentrations were determined in 12 healthy volunteers, who received 200 mg of celecoxib [18].

The variation of the complex formation constant (1:1 or 1:2) with cyclodextrins type (α-CD, β-CD, γ-CD, HP-β-CD), pH (2.4 – 12.3), buffer concentration and type (citrate, phosphate), and temperature (25°C, 30°C, 37°C) were investigated. In addition, the drug hydrophobicity and the thermodynamic parameters were investigated to estimate their contribution to complex stability. The HPLC method was used for samples in the absence of CDs at pH 2.4 and 6.0, where the inherent solubility becomes too small to reliably estimate through first derivative spectrophotometry. The obtained results were: celecoxib forms 1:1 inclusion complexes with all investigated CDs. The relatively high K_{11} values for HP-β-CD and β-CD indicate the existence of an effective geometric fit. Drug ionization, media composition and hydrophobic effects play an important role in the complex formation. The tendency of celecoxib to complex with β-CD in aqueous solution was mainly driven by the hydrophobic effect to within 77% of the complex stability. Neutral celecoxib/β-CD complex formation was found to be driven both by enthalpy and entropy changes [19].

El-Azony [20] discussed the labeling of celecoxib by iodine-125 depending on the significance of the celecoxib as chemotherapeutic agent beside its expected significance for the ^{125}Celecoxib as radiotherapy. The factors which effect the radiochemical yield of ^{125}Celecoxib such as celecoxib concentration (from 1.3 to 5.2 M), temperature (25-80°C), pH and oxidizing agent (chloramines-T and N-bromosuccinimide) have been studied, and the labeled compound (^{125}Celecoxib) has been purified by using HPLC for studying its distribution in infected mice. The radiochemical yield and purity of the product were determined by TLC and HPLC. For TLC study a TLC SG-60 F254 plates as a stationary phase and mixture: methylene chloride: ethyl acetate (2:1, *v/v*), as a mobile phase were used. The R_F values for celecoxib were 0.8-1. The obtained results indicate that ^{125}Celecoxib was completely separated from celecoxib and it was stable up to 24 h at room temperature. The obtained results clarify that the uptake of 125Celecoxib after 0.5 h post injection in the kidneys, blood stomach and liver was the highest. By increasing the time post injection from 1 to 24 h, its uptake in other organs decreased gradually.

2.1.2. Etoricoxib

A LC method has been showed to monitor the formation of an enolate intermediate in a synthetic route to

etoricoxib. The derivatization of the enolate with methyl iodide to form a stable methylketosulfone derivate is required [21].

Hartman *et al.* [22] described a HPLC method for the quantitative analysis of etoricoxib and its impurity. A method development included the optimization of stationary phase, pH, temperature and composition of mobile phase for the separation of thirteen process impurities and three major degradation products. The stability-indicating ability of the method was tested by the identification of photolytic and oxidative decomposition products, and those were confirmed by UV, LC/MS and NMR spectra.

Using a HPTLC silica gel 60F254 plates, as a stationary phase, and toluene : 1,4-dioxane : methanol (8.5:1:0.5, *v/v/v*) as a mobile phase, analysis of etoricoxib in bulk drug and formulations were provided. Wavelength at 235 nm was used for the detection. R_F value for etoricoxib was 0.24. The drug undergoes degradation when subjected to neutral (distilled water), acidic (0.1 M HCl), basic (0.1 M NaOH) hydrolysis, oxidation (1, 3, 6% hydrogen peroxide) and dry heat (at 80°C) treatment. Solutions for hydrolysis and oxidation were stored for 8 h at room temperature in the dark [23].

The stability of etoricoxib in plasma was evaluated with four studies: a short-term stability study (at room temperature for 12 h), a long-term stability study (at -20°C for 2 months), a freeze-thaw study (analysis immediately after preparation and on a daily basis after repeated freeze-thaw cycles at -20°C on three consecutive days) and stability in processed sample (at three levels of concentration; samples ready for injection were kept for 8 h). No significant decrease of etoricoxib concentration in plasma samples was detected. The method was used to study relative bioavailability of etoricoxib in twelve healthy male volunteers following single dose administration of etoricoxib [24].

Shakya and Khalaf [25] used a HPLC technique for the determination and stability studies of etoricoxib in human plasma. The short term stability was examined by keeping replicates of the plasma samples at room temperature for ca. 24 h. Stability of etoricoxib in plasma was > 89 %, with no evidence of degradation during sample processing and 45 days storage at -70°C.

Vora and Kadav [26] reported an ultra-performance liquid chromatography (UPLC) method for separation of etoricoxib and its degradation products which were formed by forced degradation under acidic (1 M HCl), alkaline (1 M NaOH), strong oxidizing (50% H_2O_2), thermal and photolytic (105°C for 4 days; 254 nm) conditions. Etoricoxib degraded under strong oxidative conditions resulting in formation of 3 major degradation products. The drug substance degraded with time on heating at 80°C in acidic and basic hydrolysis to 5 and 2 major degradation products, respectively. No degradates after exposure to photolytic and thermal stress conditions was observed.

During the development Matthews *et al.* [27] found that highly fluorescent products were formed when etoricoxib was exposed to UV light (254 nm). The formation of highly fluorescent products was the basis for the development of a highly sensitive HPLC/fluorescent assay for the indirect determination of etoricoxib in human plasma. When etoricoxib was irradiated online in a photochemical reactor, three products were detected in a gradient HPLC-UV system, on a Thermo Hypersil Hypurity C18 column using acetonitrile: water as a mobile phase. These products were characterized by HPLC-MS/MS analysis (m/z 357, 359→278, 280), on Hypersil HyPurity C18 column with mixture, acetonitrile : ammonium acetate buffer (pH 4) (35:65, v/v) as a mobile phase. The major photolysis products of etoricoxib were further isolated and their structures were elucidated using nuclear magnetic resonance (NMR) and HPLC-NMR.

Ramakrishna *et al.* [28] described HPLC/UV method for quantitation of etoricoxib in plasma samples using liquid–liquid extraction. This method provides information about the stability of etoricoxib in plasma and during sample processing. It has been used for clinical pharmacokinetic studies of etoricoxib. Stability was examined by replicates of the low and high plasma quality control samples at room temperature for approximately 24 h. Freeze–thaw stability of the samples was obtained over three freeze-thaw cycles, by thawing at room temperature for 2–3 h and refrozen for 12–24 h for each cycle. Stability of etoricoxib in human plasma was tested after storage at approximately −70°C for 30 days.

2.1.3. Firocoxib

Stability of firocoxib in plasma was also evaluated during storage at −20°C for more than 2 years and after three, six, or eight freeze–thaw cycles at −20°C. Post-preparation (extract) storage stability was assessed at room temperature after approximately 16 h. The results indicated that firocoxib is a very stable compound for extended periods of time [29].

2.1.4. Lumiracoxib

The stability-indicating capability of the method was determined by subjecting a sample solution of lumiracoxib to accelerated degradation by acidic (0.05 M HCl, at 80°C for 1.5 h), basic (2 M NaOH, at 80°C for 4 h), neutral (water, at 80°C for 8 h), oxidative (5% H_2O_2, at ambient temperature for 20 h, protected

from light), and photolytic conditions (exposing the samples in a photostability chamber to 200 Wh m^2 of near ultraviolet light for 3 h [30].

2.1.5. Rofecoxib

Two unknown impurities of rofecoxib bulk drug at levels below 0.1% were detected by isocratic HPLC method. These impurities were isolated from crude sample of rofecoxib using RP-HPLC. Mass spectra were run with ionization electron beam energy of 70 eV. ^{1}H NMR and ^{13}C NMR measurements were performed in chloroform and dimethyl sulfoxide (DMSO). IR spectra for rofecoxib and impurities were recorded in the solid state as KBr dispersion. Synthetic impurities were obtained through aerial oxidation of rofecoxib, after modification by stirring a solution of rofecoxib in ethyl acetate in the presence of activated charcoal under 2 kg of air pressure at 90°C for 72 h. The structures of impurities were characterized as 4-[4-(methylsulphonyl)phenyl]-3-phenyl-5-hydroxofuran-2-one and 4-[4-(methylsulphonyl)phenyl]-3-phenyl-2,5-furandione [31].

Shehata *et al.* [32] developed the method for simultaneous determination of rofecoxib in presence of both its photodegraded and alkaline degradation products. It was achieved by developing an HPLC and two chemometric methods. The photodegradation was performed using a UV lamp. Kinetic of the base (in 0.25, 0.5 and 1 M NaOH) promoted hydrolysis of rofecoxib was studied by following the concentration of the remaining drug within 30 min at 5-min interval at 25°C.

A TLC chromatographic-densitometric method for simultaneous determination of rofecoxib and its degradation products in tablets and solutions was developed. Separation was provided with TLC F254 plates as a stationary phase, and mixture: chloroform: acetone : toluene : glacial acetic acid (12:5:2:0.1, *v/v/v/v*), as a mobile phase. Densitometric detection at 256 nm was used. R_F values for rofecoxib were about 0.69. The method was used for the analysis of stability of this compound in solutions, depending on pH (water or hydrochloric acid or sodium hydroxide solutions at a concentration ranging from 0.1 to 0.01 M), temperature (37, 65, 90°C) and variable time of incubation [33].

Zhang *et al.* [34] presented a HPLC method for measuring rofecoxib content in human plasma and breast milk. The plasma and milk concentration–time profiles for a healthy volunteer after a single 25 mg oral dose of rofecoxib were shown. 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. The standard stock solution of rofecoxib was shown to remain stable for at least 6 months at 4°C. The assay is currently being used in a clinical study to investigate the distribution of rofecoxib into human breast milk.

A HPLC method for determination of rofecoxib in human serum was presented. Upon exposure to UV light, the drug was found to undergo a photocyclization reaction, giving a species with high absorbance. The photocyclization reaction was optimized by varying the solvent mixture, pH and time of the reaction [35].

Photodegradate solution of rofecoxib was placed in a flask for 1 h under room light. One major photodegradate was detected, eluting at 14.5 min, as a substituted phenanthrene. After degradation in 1 M NaOH, three major degradates were observed at 8.3, 11.6 and 17.1 min and determined to be a ring opened dicarboxylate, a hydroxyfuranone and an anhydride of rofecoxib, respectively. The structures of all degradates were established by the NMR analyses of the isolated compounds from a photo stressed rofecoxib solutions or from samples prepared by independent synthesis. A HPLC method was showed to separate impurities and degradates from the rofecoxib drug substance in order to monitor its quality and stability. Two major degradates were observed. The first reaction was the base-promoted hydrolysis of the lactone moiety followed by oxidation, which yields the dicarboxylate. The other reaction was a photocyclization of the cis-stilbene moiety, which yields a phenanthrene derivative [36].

Simultaneous determination of a rofecoxib and [$^{13}C_7$] rofecoxib in human plasma to support the clinical oral bioavailability (BA) study of rofecoxib was described. The method was based on HPLC with atmospheric pressure chemical ionization tandem mass spectrometric (APCI-MS/MS) detection in the negative ionization mode using a heated nebulizer interface. Separation was carried out on YMC ODS AQ column with mixture of acetonitrile: water (50:50, v/v) as a mobile phase. Stability of $^{13}CD_3$- and $^{13}C_7$-labeled rofecoxib were analyzed in different solutions and after addition of [$^{13}CD_3$]- or [$^{13}C_7$] rofecoxib to control human plasma and extraction. The HPLC–MS/MS assay was utilized to support a clinical study to determine BA of 12.5-and 25-mg oral doses of rofecoxib, using I.V.. doses of $^{13}C_7$-labeled rofecoxib. It was shown that [$^{13}CD_3$] rofecoxib was not suitable for BA studies due to an efficient deuterium exchange in water containing solvents and in plasma. Instead, [$^{13}C_7$] rofecoxib was demonstrated to meet all the analytical criteria necessary for conducting studies *in vivo* [37].

2.1.6. Valdecoxib and parecoxib

A RP-LC method was developed for the separation of valdecoxib and its impurity SC-77852. The mixture of methanol: 1% aqueous solution TEA (pH 7.35, adjusted

with 85% H_3PO_4) (52:48, v/v) as a mobile phase, and XTerra RP18 column were used to the separation. Detection was carried out at 220 nm. Described method was used for assay of valdecoxib and SC-77852 in Bextra film-coated tablets [38].

Forced degradation study of valdecoxib was based on the use of UV light (254 nm), heat (60°C), acid (2 M HCl), base (2 M NaOH), water and oxidation (6% H_2O_2). A ten days exposure was chosen for heat and light studies, whereas 48 h were chosen for acid, base and oxidative degradation. Valdecoxib was found to be stable when exposed to UV light, heat, acid and base hydrolysis. Its degradation yielded formation of impurity A (t = 4.5 min) during oxidation with H_2O_2 solution [39].

Ravi *et al.* [40] reported a TLC method for analysis of valdecoxib. A silica gel aluminum HPTLC 60 GF254 plates, and mixture: toluene: acetone : 5% ammonia (7:5:1, *v/v/v*), were used as a stationary and mobile phases, respectively. Detection was run at 236 nm. R_F value for valdecoxib was 0.56. The chromatograms of acid (0.1 M HCl for 48 h), hydrogen peroxide (6% for 48 h) and photodegraded (UV light for 8 h) samples of valdecoxib showed only the spots of the pure drug. Chromatograms of a base (0.1 M NaOH for 48 h) degraded sample showed an additional peak at R_F 0.76.

Low values of percentage differences between area ratios for stability-test samples and fresh QC samples confirm the stability of valdecoxib on the bench top for 4 h at room temperature, in an autosampler for 12 h, and inside the freezer for 14 days at -20°C. The method was used to determine pharmacokinetic data for valdecoxib in human volunteers [41].

Ramakrishna *et al.* [42] described a HPLC–UV determination method for valdecoxib in plasma using liquid/liquid extraction. Further, method supplied an information about the stability of valdecoxib in plasma and during sample processing. The short-term stability was tested by keeping replicates of the low and high plasma quality control samples at room temperature for approximately 24 h. Freeze-thaw stability of the samples was received over three freeze–thaw cycles, by defrosting at room temperature for 2–3 h, refrozen for 12–24 h. Autosampler stability was tested by analysis the plasma samples, which were stored in the autosampler tray for 24 h. Stability in human plasma was tested after storage at approximately −70°C for 30 days.

The optimal HPLC conditions for separation and determination of valdecoxib and its degradation product (SC-77852) were established. The goal of this investigation was to set up the optimal chromatographic conditions for the separation of both α- and β-anomers in dosage forms, using a monolithic HPLC column. This was of special importance since the requirement was only to investigate the presence of the β-anomer (SC-77852) in the final product [43].

Vadlamudi *et al.* [44] presented a HPLC procedure for the analysis and dissolution rate studies of parecoxib in tablets, contributing therefore for the quality control and safety of the drug. The studies were performed by a semi-automated system. Drug tests were carried out in different media of HCl, sodium lauryl sulphate in water and distilled water, for 120 min at 37°C.

2.2. Drug metabolites and pharmacokinetic studies

The pharmacokinetics and drug interactions of COX-2 inhibitors were studied by various chromatographic techniques. The study of drug metabolism and toxicity of its metabolites are very important in the process of drug discovery. Miscellaneous chromatographic methods used in the stability researches were also used in studies of the metabolites detection.

2.2.1. Celecoxib

Characterization of the metabolism and excretion of celecoxib after the oral administration of a single dose to healthy male adults was studied. Authors determined the disposition of a single 300-mg dose of [^{14}C] celecoxib in eight healthy male subjects. The [^{14}C] celecoxib was administered as a fine suspension reconstituted in 80 mL of an apple juice/Tween 80/ethanol mixture. Blood and saliva samples were collected at selected time intervals after dosing. All urine and feces were collected on the 10 consecutive days after dose administration. Radioactivity in each sample was determined by liquid scintillation counting or complete oxidation and liquid scintillation counting. Metabolic profiles in plasma, urine, and feces were obtained by HPLC analysis. The fractions were subjected to the SPE extraction procedure. The obtained eluents were dried under a stream of nitrogen in preparation for analysis by MS/MS with electrospray ionization (ESI), and NMR using CD_3CN solution [45].

Paulson *et al.* [46] presented the metabolic disposition of celecoxib in rats. Celecoxib was metabolized extensively after I.V. administration of [^{14}C] celecoxib, and elimination of unchanged compound was minor (less than 2%) in male and female rats. Authors described that celecoxib is readily distributed to tissues. Celecoxib is eliminated by a single metabolic pathway, *i.e.* hydroxylation of the aromatic methyl group of celecoxib and additional oxidation of the hydroxymethyl metabolite to a carboxylic acid metabolite. The major route of elimination of celecoxib is metabolism followed by excretion of the metabolites in feces (90%), with the remainder of the metabolites excreted by the kidney.

A HPLC method for measuring celecoxib in human plasma and breast milk was presented. The human breast milk was donated by healthy volunteers. To investigate possible interference from commonly co-administered drugs, paracetamol, codeine, cimetidine, prednisone, sulphasalazine, omeprazole and methotrexate were analysed under their assay condition. No interference was observed. Celecoxib was found to be stable in plasma and breast milk for at least four freeze–thaw cycles when stored at −30°C. The standard stock solution of celecoxib was shown to remain stable for at least four months at 4°C [47].

Tang *et al.* [48] tried to define the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent metabolism of celecoxib in human liver microsomes and to determine which CYP protein(s) was involved, using a combination of four approaches: 1) immunoinhibition, 2) chemical inhibition, 3) correlation analysis, and 4) cDNA-expressed human CYP proteins. Celecoxib and its metabolites were separated by HPLC analysis.

Zhang *et al.* [49] described the application of LC-MS/MS for the determination of the metabolites of celecoxib in female rabbits after a single 10 mg kg^{-1} oral dose of [^{14}C]celecoxib. Urine and fecal samples were collected at predose (approximately -18 to 0 h) and every 24 h for 7 days postdose. Blood samples were collected at 0.5, 2, 8 and 24 h postdose and centrifuged to obtain plasma. The metabolic profiles of [^{14}C]celecoxib in plasma, urine and feces and the percentages of dose eliminated in urine and feces were identified. The metabolites of [^{14}C]celecoxib were separated by LC analysis. They were identified by MS/MS utilizing the parent ion scan of m/z 69 (CF_3) and the neutral loss ion scan of 176 (dehydroglucuronic acid) followed by the product ion scans of the metabolite candidates. The structures of the metabolites were discussed by interpreting their product ion spectra and comparing both, retention times and the product ion spectra with those of the standards.

Hinz *et al.* [3] conducted the experiment measuring *ex vivo* COX-2 inhibition in healthy elderly volunteers after treatment with clinically recommended antirheumatic doses of the traditional NSAID: diclofenac, and the coxibs: celecoxib and rofecoxib. As the result they found that the traditional NSAID was a more effective inhibitor of blood-derived COX-2 products than coxibs. Despite the fact that a relationship between the potency of COX-2 inhibition and changes in cardiovascular parameters remains to be established, a depth evaluation of the extent and time course of *ex vivo* COX-2 inhibition could be important for inquiry of the potential mechanisms underlying the cardiovascular side effects connected to these drugs. Blood samples, obtained predose and postdose on days 1 and 8, from volunteers receiving 75 mg diclofenac and 200 mg celecoxib twice daily, or 25 mg rofecoxib once daily for 8 days, were analyzed by HPLC for concentrations of diclofenac, and by LC-MS for celecoxib and rofecoxib.

The application of atmospheric pressure ionization (API) LC–MS using single ion monitoring (SIM) for the quantitation of celecoxib in human plasma was reported. The utility of the developed method in a preliminary clinical study to determine the pharmacokinetic parameters of celecoxib following oral administration of the drug in selected subjects was demonstrated. APCI probe was used as scanning in the positive SIM mode at m/z 382 for celecoxib [50].

The HPLC method with fluorescence detection, suitable for the analysis of celecoxib in pharmacokinetic studies in humans is described. The assay was used to investigate the steady state pharmacokinetic parameters of celecoxib in young and elderly volunteers. Celecoxib was administered twice daily for 15 days. Blood samples were withdrawn before and at different time points until 25 h after administration of the last dose. Samples were centrifuged after 30 min and serum was stored at -20°C until analyzed [51].

Developing a HPLC assay for celecoxib and delineated pharmacokinetics of the drug in the rat in the presence and absence of inflammation was discussed. The effect of inflammation was assessed by dosing animals with interferonα2a 12 and 1 h before administration of celecoxib. The lack of change in celecoxib pharmacokinetics after acute inflammation maybe due to insensitivity of its metabolic system to the acknowledged inhibitory effect of inflammation, and/or the relatively low pre-systemic metabolism of the drug [52].

The HPLC-MS/MS method was used to assay plasma specimens taken from 12 healthy volunteers after oral administration of normal daily dose of celecoxib, and the pharmacokinetic profile was described. Since celecoxib should be metabolized primarily by CYP2C9, all volunteers were genotyped for cytochrome P450 to show the role of genetic polymorphism in the pharmacokinetics of celecoxib [53].

Celecoxib and its metabolites were quantified in human plasma by combining solid-phase extraction with gradient RP-HPLC analysis. 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 after administration of a low dose of celecoxib [54].

A study about the metabolites of celecoxib formed by microorganisms and comparison them with those produced in mammals was conducted. The work helped in the production of large quantities of metabolites for

further pharmacological and toxicological evaluation apart from structure elucidation. The results indicated that a number of microorganisms metabolized celecoxib to various levels to yield eight metabolites, which were identified by HPLC analyses with UV and MS detection. HPLC analysis of biotransformed products indicated that majority of the metabolites were more polar than the substrate celecoxib [55].

Emami *et al.* [56] described an isocratic HPLC method with UV detection for determination of celecoxib in human plasma. Celecoxib was measurable at the first sampling time (0.5 h) and after 4 half-lives in all volunteers. Stability studies show that the drug was stable in the processed sample up to 72 h at room temperature and in serum samples for at least 1 year, during storage at -20°C. The proposed method was used in the pharmacokinetics and bioequivalence studies of drug preparations.

2.2.2. Etoricoxib

The determination of etoricoxib in human plasma using fluorescence detection, its and its assay in bioavailability experiments was discussed required the development of a 96-well extraction plates and HPLC-MS/MS method, which could simultaneously measure both etoricoxib, and [^{13}C] etoricoxib from the same sample [57].

Riendeau *et al.* [58] described the properties of etoricoxib, a dipyridinyl inhibitor that has been developed for high *in vitro* selectivity of COX-2 inhibition using a combination of whole blood and sensitive COX-1 inhibition assays. The preclinical pharmacological profile of etoricoxib was reported, together with comparative data on the selectivity of other agents that have been reported to selectively inhibit COX-2. Clinical studies in progress had shown that etoricoxib is well tolerated and efficacious in the treatment of the symptoms of osteoarthritis and is selective for COX-2 *in vivo* even at single doses more than 8-fold the clinically effective dose in osteoarthritis.

2.2.3. Lumiracoxib

The absorption, metabolism, disposition, and mass balance of [^{14}C] lumiracoxib were investigated in four healthy male subjects after a single 400 mg oral dose. Serial blood and complete urine and feces were collected for 168 h postdose. Major plasma metabolites (5-carboxy, 4'-hydroxy, and 4'-hydroxy-5-carboxy derivatives) were analyzed by HPLC technique. Authors observed that orally administered lumiracoxib was well tolerated and rapidly absorbed, with unchanged lumiracoxib accounting for the majority of drug present in plasma. Lumiracoxib undergoes extensive metabolism before excretion *via* urine and feces, with no evidence of formation of potentially reactive metabolites [59].

The preclinical pharmacology of lumiracoxib was reported. The pharmacokinetics of lumiracoxib was studied in rats plasma samples, after chromatographic separation with MS detection (LC-MS/MS, MS-API in the negative mode). Lumiracoxib has been found to bind and interact with the COX-2 enzyme *via* mechanisms different from other COX-2 selective inhibitors and carboxylate-containing nonselective COX inhibitors. The carboxylate group of lumiracoxib forms hydrogen bonds with the catalytic Tyr385 and with Ser530 on COX-2, rather than with the larger hydrophobic side pocket (as utilised by other COX-2 selective inhibitors developed to date, such as celecoxib) or with Arg120 (as utilised by carboxylate-containing profen-class nonselective NSAIDs, such as flurbiprofen). Lumiracoxib was highly selective for COX-2 over COX-1, and was effective in relieving pain and inflammation in a variety of rodent models. Authors established the biochemical potency and COX-2 selectivity of lumiracoxib in several assays using purified enzyme preparations, intact cells and in whole blood and tissues [60].

Moreira *et al.* [61] investigated whether poloxamerbased delivery systems (PBDS) containing the skin penetration enhancer (OA) could promote the *in vitro* percutaneous absorption of lumiracoxib using porcine ear skin. The potential of lumiracoxib transdermal delivery to cause primary and cumulative dermal irritations in an animal model *in vivo* was also investigated. Quantification of lumiracoxib in samples from partition coefficient ($K_{octanol/buffer}$) studies was provided by HPLC on propylsulphonic column with mobile phase: phosphate buffer 0.1 M (pH 7.4) : water : acetonitrile. Quantification of lumiracoxib in samples of *in vitro* skin permeation and retention studies was performed by HPLC on RP C8 column with acetonitrile : 0.01 M sodium phosphate buffer (pH 2.5). The obtained results shown that 25% PBDS containing 10% OA optimizes cutaneous delivery of lumiracoxib to the skin, significantly increasing drug retention of both epidermis and dermis which act as a depot for continuous and gradually percutaneous absorption of the drug. Furthermore, skin irritation tests in rabbits demonstrated that single or multiple applications of the PBDS associated with OA did not cause any visual changes in the skin, like erythema or edema.

The evaluation of the steady-state pharmacokinetics of lumiracoxib in both plasma and synovial fluid from patients with rheumatoid arthritis was described. The possible influence of lumiracoxib metabolites on efficacy was also assessed by analysis of principal metabolites

in plasma and synovial fluid. Two methods were used to derive lumiracoxib pharmacokinetic parameters: a non-compartmental method and, to further characterize the distribution constants, a population pharmacokinetic model (due to the sparse sampling nature of the synovial fluid). Lumiracoxib and its metabolites (4′-hydroxy-lumiracoxib and 5-carboxy-4′-hydroxy-lumiracoxib) were analyzed by HPLC-MS methods [62].

2.2.4. Lefucoxib

The *in vivo* and *in vitro* biotransformation study of lefucoxib was investigated in rats. Through HPLC coupled with fluorescence detector and LC–MS^n analysis, hydroxylation was found to be the primary metabolism pathway of lefucoxib in rats. The chemical structure of the dihydroxy metabolite was identified by MS^n spectra (with ESI in positive ion mode), and it was detected in rat plasma, urine and feces after an oral dose. *In vitro* metabolism studies, carried out by liver microsome incubation helped to accumulate enough metabolites for ^1H NMR analysis (using deuterated methanol solution), which was employed and proved to be successful. Through further analysis of ^1H–^1H correlated spectroscopy, chemical structures of two isomeric metabolites (monohydroxy metabolites) which had the same retention time in chromatograms were identified. The study illustrated the co-application of *in vivo* and *in vitro* metabolism on drug metabolite identification [63].

2.2.5. Rofecoxib

Endoscopy and blood and fecal analysis were used to examine and compare the gastroduodenal safety profile of two antiarthritic drugs, licofelone and rofecoxib, in dogs under long-term treatment. Blood samples were taken by venipuncture 2 h after dosing on days 2, 14, 28, 42 and 56. The plasma was harvested and stored at -20°C until it was processed for analysis. For investigations, a triple-stage quadrupole mass spectrometer arrangement was applied, together with a LC device for chromatographic separation of the analyte. A reference compound for rofecoxib was isolated from tablets [64].

The use of commercially available monolithic HPLC columns in assays for the determination of rofecoxib and an experimental cyclooxygenase II inhibitor, 3-isopropoxy-4-(4-methanesulfonylphenyl)-5,5'-dimethyl-5H-furan-2-one, in human clinical plasma samples was described [65].

Werner *et al.* [66] described an easy and selective LC-MS method for the quantification of rofecoxib in human plasma. After validation the method was used to study the pharmacokinetic profile of rofecoxib in healthy volunteers after single oral dose.

The objectives of the investigation were to determine the absorption, distribution, metabolism, and excretion (ADME) of rofecoxib in rats and dogs. The two species were used in the toxicological evaluation of this compound. Concentrations of rofecoxib in rat and dog plasma from all the kinetics studies were determined by HPLC with fluorescence detection of a product resulting from postcolumn photochemical activation. The obtained results showed that, rofecoxib displayed notable species differences in pharmacokinetic and metabolic behavior in laboratory animals. The pharmacokinetics and metabolism of rofecoxib displayed distinct species differences in rats and dogs. After oral administration, the drug was well absorbed in rats, but less so in dogs [67].

Backman *et al.* [68] assessed the effect of rofecoxib on CYPIA2 activity in humans, using tizanidine and caffeine as probe substrates, and investigation of the mechanism and consequences of a possible rofecoxib-tizanidine interaction. Plasma concentrations and the urinary excretion of tizanidine, its metabolites and rofecoxib, and pharmacodynamics variables were measured up to 24 h. The plasma concentrations of rofecoxib were determined by HPLC method. The obtained results indicate that rofecoxib is a potent inhibitor of CYPIA2 and it greatly increases the plasma concentrations and adverse effects of tizanidine.

2.2.6. Valdecoxib and parecoxib

A highly sensitive LC-MS/MS method for the simultaneous quantitation of valdecoxib and its hydroxylated metabolite in human plasma was described. The analytes were detected by MS using negative ion electrospray ionization using multiple reaction monitoring (MRM) mode. The freeze-thaw stability was determined by measuring the assay for the samples which underwent three freeze-thaw cycles. The frozen plasma samples were thawed at room temperature for 2-3 h, refrozen for minimum of 1 day, thawed for 2-3 h, refrozen for minimum 1 day, thawed and then analyzed. Extracted validation samples at low, middle and high concentrations were kept standing at room temperature for over 24 h and after standing at room temperature for at least 24 h. *In vitro* studies were performed to determine whether valdecoxib and metabolite were lost and/or degraded in human blood during sample collection and processing. In the first experiment, valdecoxib and metabolite were separately incubated with freshly collected human blood for 15 min on ice followed by centrifugation to separate the plasma from the blood. In the second experiment, they were separately incubated with freshly collected human blood for 60 min on ice followed by centrifugation to separate the plasma from the blood. In the third

experiment, they were separately incubated with freshly collected human blood for 60 min at room temperature followed by centrifugation and leaving the plasma fraction over packed erythrocytes for an additional 60 min at room temperature. Then, the plasma was separated by centrifuge and stored in a -20°C freezer until analysis. The sample long-term storage stability was analyzed after storage at -20°C for 70 or 75 days [69].

Zhang *et al.* [70] reported the development and validation of a LC–MS/MS method for the simultaneous quantitation of valdecoxib (I) and its two metabolites (II and III) in human urine. After extraction, the samples were injected onto RP-HPLC column for separation. The analytes were detected by tandem mass spectrometry using negative electrospray ionization with MRM mode. The freeze–thaw stabilities of I, II and III were determined for the samples which underwent three freeze–thaw cycles. The frozen urine samples were thawed at room temperature for 2–3 h, refrozen for minimum of 1 day, thawed for 2–3 h, refrozen for minimum 1 day, thawed and then analyzed. *In vitro* studies were performed to determine whether I, II and III were lost and/or degraded in human urine during sample collection and processing. In one experiment, I, II and III were separately incubated in freshly collected human urine for 15 min on ice then stored in a -20°C freezer. In another experiment, compounds were separately incubated in freshly collected human urine for 120 min at room temperature followed by storage in a 4°C refrigerator for an additional 24 h, then stored in a -20°C freezer. The results showed that I, II and III were stable in human urine for at least 120 min at room temperature and an additional 24 h at 4°C.

Zhang *et al*. [71] described a two-step rearrangement related to an isoxazole ring in the CID spectra of the valdecoxib metabolites. The first step composed of an intramolecular *S*N2 reaction with a five-membered ring rearrangement to form an intermediate, and the second concerned a four-membered ring intramolecular rearrangement followed by a cleavage of the N—O bond on the isoxazole ring to form a fragment ion at m/z 196. Mass measurement and stable isotope-labeled analogues were applied to help resolve the fragmentation mechanism in their mass spectra. The unique rearrangement was observed for a group of structurally related metabolites of valdecoxib that contain 5-hydroxymethyl or 5-carboxylic acid moieties.

Determination of valdecoxib in human serum using a HPLC with diode array detector at 240 nm was presented. Separation was carried out on RP C-18 column with acetonitrile : water (pH 3.2, with H_3PO_4) (60:40, v/v) as a mobile phase. The method was used to study the pharmacokinetics of valdecoxib after a single dose oral administration to human volunteers [72].

A study carried out by Karim *et al.* [73] was conducted to determine the pharmacokinetics, safety, and tolerability of single, rising intramuscular doses and the single maximum tolerated dose of parecoxib sodium, in healthy male volunteers (blood and urine samples).

A HPLC method with spectrofluorimetric detection was used to simultaneously quantify parecoxib and valdecoxib in canine plasma samples. Stability studies were performed for short-term romm temperature conditions (for 24 h), and long-term storage conditions (30 days at -20°C). Results indicated no stability-related problems would be expected during analyses for pharmacokinetic study [74].

A RP-HPLC method for determination of parecoxib in human plasma and pharmaceuticals was developed. Separation was carried out on CLC C18 column with mixture, acetonitrile : water (92:8, v/v) as a mobile phase, using UV detection at 200 nm [75].

Determination of the total radioactivity recovery in male and female mice following a single oral administration of [^{14}C] valdecoxib at 5 mg kg^{-1}, obtaining the metabolic profiles in selected mouse plasma RBC, urine, and fecal samples, the identification of the major metabolites of valdecoxib, estimation of plasma and RBC pharmacokinetic parameters for total radioactivity, and examination of gender difference in pharmacokinetics of valdecoxib and major metabolites were conduced. Pharmacokinetic parameters in plasma and RBC for total radioactivity, valdecoxib, and its metabolite were analyzed by non-compartmental modeling. The structures of metabolites were elucidated by electrospray LC-MS/MS using a combination of full and product ion scanning techniques [76].

3. Conclusions

The availability of an analytical method dedicated to the determination of coxibs is dependent on a variety of parameters including sensitivity, selectivity, cost effectiveness, availability of instrumentation, simplicity, rapidity, *etc*. but mainly purpose of the study. The clinically important differences between the drugs related to its purity, stability, bioavailability, and eventual pathways of metabolism, in terms of the possibility of their analysis were reported. Separation techniques, especially chromatography, were often used in the studies of drug metabolites and its pharmacokinetic studies. The presented papers covered both the determination of the active substance and its metabolites in various biological samples.

Abbreviations

^{1}H NMR, ^{13}C NMR: proton-1, carbon-13 nuclear magnetic resonance;
APCI: atmospheric pressure chemical ionization;
API: atmospheric pressure ionization;
BA: bioavailability;
CD: cyclodextrin;
COX: cyclooxygenase;
DMSO: dimethyl sulfoxide;
GC: gas chromatography;
HPLC: high-performance liquid chromatography;
HPTLC: high-performance thin-layer chromatography;
IR: infrared;
LC: liquid chromatography;
M: concentration [mol L^{-1}];
MRM: multiple reaction monitoring;
MS: mass spectrometry;
NSAIDs: non-steroidal anti-inflammatory drugs;
RP: reversed phase;
SIM: single ion monitoring;
TEA: triethylamine;
TLC: thin-layer chromatography;
UPLC: ultra-performance liquid chromatography;
UV: ultraviolet.

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