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ORIGINAL RESEARCH

Simultaneous quantitation of etoricoxib, salicylic acid, valdecoxib, ketoprofen, nimesulide and celecoxib in plasma by high-performance liquid chromatography with UV detection†

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ABSTRACT: A specific, accurate, precise and reproducible high performance liquid chromatography (HPLC) method was developed and validated for the simultaneous quantitation of etoricoxib, salicylic acid, valdecoxib, ketoprofen, nimesulide and celecoxib in human plasma. The method employed a simple liquid–liquid extraction of etoricoxib, salicylic acid, valdecoxib, ketoprofen, nimesulide and celecoxib and internal standard (IS, DRF-4367) from human plasma (500 μL) into acetonitirile. The organic layer was separated and evaporated under a gentle stream of nitrogen at 40°C. The residue was reconstituted in the mobile phase and injected onto a Kromasil KR 100-5C_{18} column (4.6 × 250 mm, 5 μm). The chromatographic separation was achieved by gradient elution consisting of 0.05 M formic acid (pH 3)–acetonitrile–methanol–water at a flow rate of 1.0 mL/min. The eluate was monitored using an ultraviolet (UV) detector set at 235 nm. The ratio of peak area of each analyte to IS was used for quantification of plasma samples. Nominal retention times of etoricoxib, salicylic acid, valdecoxib, ketoprofen, nimesulide, IS and celecoxib were 15.63, 17.20, 21.66, 24.95, 26.27, 30.24 and 32.22 min, respectively. The standard curve for etoricoxib, salicylic acid, valdecoxib, ketoprofen and celecoxib was linear ($r^2 > 0.999$) in the concentration range 0.1–50 μg/mL and for nimesulide ($r^2 > 0.999$) in the concentration range 0.5–50 μg/mL. Absolute recovery was >83% from human plasma for all the analytes and IS. The lower limit of quantification (LLOQ) of nimesulide was 0.5 μg/mL and for etoricoxib, salicylic acid, valdecoxib, ketoprofen and celecoxib the LLOQ was 0.1 μg/mL. The inter- and intra-day precisions in the measurement of QC samples, 0.1, 0.3, 15.0 and 40.0 μg/mL (for all analytes except nimesulide), were in the range 2.29–9.37% relative standard deviation (RSD) and 0.69–10.28% RSD, respectively. For nimesulide the inter- and intra-day precisions in the measurement of quality control (QC) samples, 0.5, 1.5, 15.0 and 40.0 μg/mL, were in the range 3.21–7.37% RSD and 0.97–7.06% RSD, respectively. Accuracy in the measurement of QC samples for all analytes was in the range 91.03–106.38% of the nominal values. All analytes including IS were stable in the battery of stability studies, viz. bench top, autosampler and freeze–thaw cycles. Stability of all analytes was established for 21 days at –20°C. The application of the assay in an oral pharmacokinetic study in rats co-administered with celecoxib and valdecoxib is described.

KEYWORDS: non-steroidal anti-inflammatory drugs; coxibs; co-administration; pharmacokinetics; HPLC-UV detection

INTRODUCTION

Rheumatoid arthritis is an autoimmune disease that causes inflammation in the lining of the joints, which results in pain, stiffness, swelling, joint damage and loss of function in the joints. Osteoarthritis is the result of wear and tear of the material that cushions joints, usually in weight-bearing joints. Osteoarthritis is often accompanied by some inflammation but not to the degree seen in rheumatoid arthritis. Presently millions of people live with the pain of arthritis, which may be mild or debilitating. There are many medications available to relieve arthritis pain and slow the progression of the disease.

Non-steroidal anti-inflammatory drugs (NSAIDs) are agents that reduce inflammation by inhibiting the COX enzymes, which are mediators of inflammation. However, in spite of their beneficial effects, NSAIDs have a tendency to interfere with the body's ability to protect the stomach lining as well as protect platelet function. Therefore, manifestations of toxicity may be unacceptable in many patients. This paved the way for the discovery and development of newer agents called

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Abbreviations used: CC, calibration curve; CCX, celecoxib; DMSO, dimethyl sulfoxide; EDTA, ethylene diamine tetra acetic acid disodium salt; ETX, etoricoxib; KPF, ketoprofen; NMS, nimesulide; NSAID, non-steroidal anti-inflammatory drugs; SCA, salicylic acid; VDX, valdecoxib.

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COX-2-specific inhibitors (coxibs), viz. celecoxib, rofecoxib, etoricoxib and valdecoxib, which are like NSAIDs in that they inhibit the inflammatory conditions while they preserve the integrity of the stomach lining or platelet control. The new class of agents has efficacy comparable to NSAIDs, but with a much improved safety profile such that their use in both the treatment of acute and chronic pain, with or without inflammatory conditions, has been widely accepted.

Most experts on osteoarthritis consider acetaminophen as the initial medication of choice for controlling pain. When acetaminophen is not able to control pain, NSAIDs are typically used as the next line of defense to control pain and inflammation associated with osteoarthritis. Coxibs are effective in reducing inflammation and relieving pain associated with arthritis, and appear to have a lower risk of gastrointestinal side effects, when compared with traditional NSAIDs. Coxibs are appropriate second-line agents when a patient has specific risk factors that preclude NSAID use. The choice of appropriate NSAID or coxib should be based on patient risk factors, adverse effects and cost.

Today, there is an abundant availability of NSAIDs and coxibs that patients may be initially treated with, and perhaps switches to other NSAIDs and/or coxibs may occur during the therapy. Therefore, development of an assay that has a generic application for quantitative determinations of a number of NSAIDs and coxibs has significant utility. We believe simultaneous determination of coxibs and NSAIDs offers the following advantages: (a) ease and convenience of clinical routine monitoring; (b) applicability to routine pharmacokinetic investigations, including drug–drug interaction studies; (c) simultaneous analysis of pharmaceutical dosage forms and/or fixed dose combination products. Several methods for the simultaneous determination of NSAIDs or coxibs in plasma or serum have been published, such as HPLC with UV (Nageswara Rao *et al.*, 2004; Sen *et al.*, 2003; Mikami *et al.*, 2000; Martin *et al.*, 1999; Hirai *et al.*, 1997; Owen *et al.*, 1987), HPLC with electrochemical detector (Kazemifard and Moore, 1990) or capillary electrophoresis (Heitmeier and Blaschke, 1999) or GC-MS (Maurer *et al.*, 2001; El Haj *et al.*, 1999; Kim and Yoon, 1996) or LC-MS/MS (Werner *et al.*, 2004; De Kanel *et al.*, 1998). Our aim was to develop a generic method that allowed for the determination of NSAID or coxib without the need for the development of a separate and distinct method for each analyte with different structural features. To the best of our knowledge, there is no single bioanalytical HPLC method reported in the literature describing the validation and quantitation of NSAIDs and coxibs simultaneously in biological fluids. In order to have a universal acceptance and ensure ease of applicability, we have used a UV detector with no special assembly for the detection of NSAIDs and coxibs.

The validated generic HPLC-UV assay can be used for the simultaneous determination of etoricoxib, salicylic acid, valdecoxib, ketoprofen, nimesulide and celecoxib in human plasma. The method offers the advantage of simplicity with adequate sensitivity, selectivity, precision and accuracy for the simultaneous determination of NSAIDs and coxibs.

EXPERIMENTAL

Chemicals and reagents. Etoricoxib (ETX), valdecoxib (VDX), celecoxib (CCX) and DRF-4367 (IS) (Fig. 1) were synthesized by the Medicinal Chemistry Group, Dr Reddy's Laboratories Ltd (DRL), Hyderabad and were characterized using chromatographic and spectral techniques by Central Instrumentation Laboratory, DRL, Hyderabad. Nimesulide (NMS) and ketoprofen (KPF) (Fig. 1) were procured from Bulk Actives unit of DRL, Hyderabad. Purity was found to be more than 99% for all the compounds. Salicylic acid (SCA) was purchased from Loba Chemicals, Mumbai, India. Acetonitrile, methanol (HPLC grade), potassium dihydrogen orthophosphate, ethylene diamine tetra acetic acid disodium salt (EDTA) and phosphoric acid (analytical reagent grade) were purchased from Qualigens, Glaxo (India), Mumbai, India. Dimethyl sulfoxide (DMSO) was obtained from Sigma Chemical Co. (St Louis, MO, USA). All aqueous solutions including the buffer for the HPLC mobile phase were prepared with Milli Q-grade (Millipore, Milford, MA, USA) water. The control human plasma was obtained from Cauvery Diagnostics and Blood Bank, Secunderabad, India.

Chromatography. The HPLC system was a Dinoex (Dionex GmbH, Waldbronn, Germany) LC system equipped with degasser (G1322A), isopump (P680A LPG), auto-injector with sample cooler (ASI-100T) and photodiode array detector (UVD340U). The data were acquired and processed with Chromeleon® software (version 6.60 SP1a). A Kromasil KR 100-5C_{18} column (4.6 × 250 mm, 5 μm) was used for the analysis. The ternary mobile phase system consisting of reservoir-A (0.05 M formic acid, pH-3.0), reservoir-B (Milli Q water–acetonitrile, 5:95) and reservoir-C (Milli Q water–methanol, 10:90) were run as per the gradient program (Table 1) with a flow rate of 1.0 mL/min through the column to elute the analytes. The eluate was monitored by the UV detector set at 235 nm.

Standard solutions. Standards and QC stock solutions of ETX, SCA, VDX, KPF, NMS, CCX and IS were prepared in DMSO (10 mg/mL). The primary stock solutions of the analytes and IS were stored at –20°C. Appropriate dilutions were made in DMSO to produce working stock solutions of 1000, 400, 200, 100, 40, 20, 10, 4 and 2 μg/mL for ETX, SCA, VDX, KPF and CCX, 1000, 400, 200, 100, 40, 20, 10 μg/mL for NMS, and these stocks were used to prepare calibration curve (CC). Another set of working stock solutions was made in DMSO (from primary stock) at 800, 300, 6 and 2 μg/mL for ETX, SCA, VDX, KPF and CCX, and at 800, 300, 30 and 10 μg/mL for NMS for preparation of QC samples. Stock solutions were stored at approximately 5°C. Individual QC

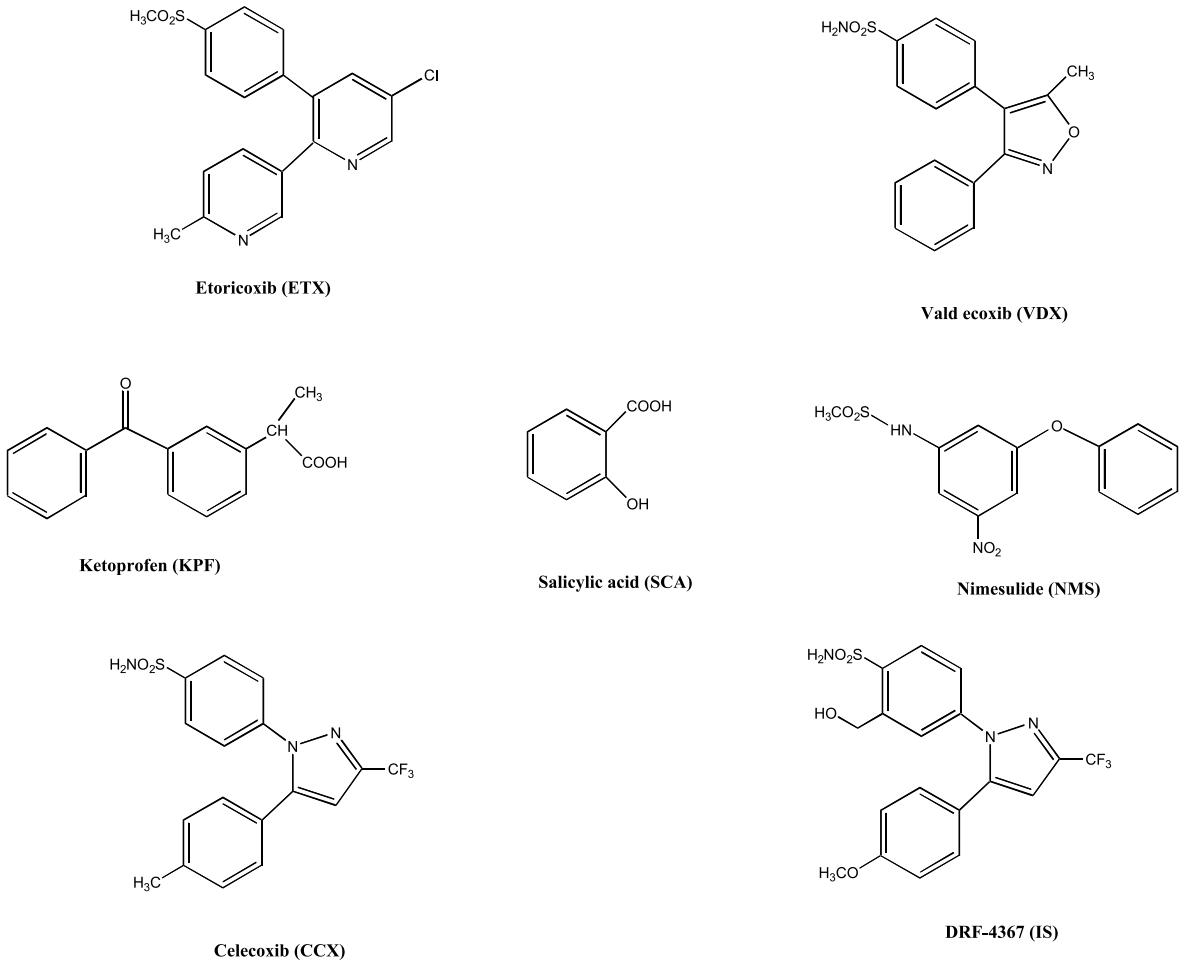

Figure 1. Structural representation of etoricoxib (ETX), salicylic acid (SCA), valdecoxib (VDX), ketoprofen (KPF), nimesulide (NMS), celecoxib (CCX) and DRF-4367 (IS).

Table 1. Gradient-time program for the HPLC method with a ternary mobile phase system

Time (min)	Flow rate (mL/min)	0.05 M formic acid (pH 3.0) (%)	Milli Q water–acetonitrile, 5:95, v/v (%)	Milli Q water–methanol, 10:90, v/v (%)
0	1.0	100	—	—
2	1.0	90	—	10
9	1.0	50	20	30
25	1.0	30	50	20
35	1.0	10	85	5
36	1.0	100	—	—
45	1.0	100	—	—

and CC two-in-one or composite working stock solutions of all analytes (ETX, SCA, VDX, KPF, NMS and CCX) were made before spiking into QC and CC samples accordingly. A working IS solution (2.0 µg/mL) was prepared in DMSO. Calibration samples were prepared by spiking 500 µL of control human plasma with the appropriate amount of the analytes and IS on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control human plasma in bulk at appropriate concentrations (at 0.1, 0.3, 15.0 and 40.0 µg/mL for ETX, SCA, VDX, KPF and CCX; at 0.3, 1.5, 15.0 and 40.0 µg/mL for NMS) and 500 µL volumes were aliquoted into different tubes and, depending on the nature of experiment, samples were stored at –20°C until analysis.

Sample preparation. To 500 µL of plasma sample, solution of DRF-4367 (IS) equivalent to 1 µg was added and mixed for 15 s on a cyclomixer (Remi Instruments, Mumbai, India). After

the addition of 3 mL acetonitirile, the mixture was vortexed for 2 min, followed by centrifugation for 5 min at 3200 rpm on a tabletop centrifuge (Remi Instruments, Mumbai, India). The organic layer (2.8 mL) was separated and evaporated to dryness at 40°C using a gentle stream of nitrogen (Zymark® Turbovap®, Kopkinton, MA, USA). The residue was reconstituted in 200 µL of the Milli Q–water:methanol (50:50) and 100 µL were injected onto the HPLC column.

Calibration curves. Calibration curves were acquired by plotting the peak area ratio of each analyte–IS against the nominal concentration of calibration standards. The concentrations used were 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 µg/mL for ETX, SCA, VDX, KPF and CCX; for NMS 0.5, 1.0, 2.0, 5.0, 10.0, 20.0 and 50.0 µg/mL concentrations were used. The results were fitted to linear regression analysis using $1/X$ as weighting factor. The calibration curve had to have a correlation coefficient (r^2) of 0.99 or better. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value, except at LLOQ, which was set at ±20%.

Precision and accuracy. The intra-assay precision and accuracy were estimated by analysing six replicates containing ETX, SCA, VDX, KPF and CCX at four different QC levels, i.e. 0.1, 0.3, 15.0 and 40.0 µg/mL, and for NMS 0.5, 1.5, 15.0 and 40.0 µg/mL. The inter-assay precision was determined by analyzing the four levels of QC samples on three different runs. The criteria for acceptability of the data included accuracy within ±15% deviation (DEV) from the nominal values and a precision of within ±15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of CV (United States Pharmacopoeia, 1995; Shah *et al.*, 1992).

Stability experiments. The stability of ETX, SCA, VDX, KPF, NMS, CCX and IS 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. The peak-areas of each analyte and IS obtained at initial cycle were used as the reference to determine the relative stability at subsequent points. Stability of each analyte in the biomatrix during 6 h (bench-top) was determined at ambient temperature (25 ± 3°C) at four concentrations in quadruplicate. Freezer stability of ETX, SCA, VDX, KPF, NMS and CCX in human plasma was assessed by analyzing the QC samples stored at –20°C for 21 days. The stability of ETX, SCA, VDX, KPF, NMS and CCX in human plasma following repeated freeze–thaw cycles was assessed using QC samples spiked with ETX, SCA, VDX, KPF, NMS and CCX. The samples were stored at –20°C between freeze–thaw cycles. The samples were thawed by allowing them to stand at room temperature for approximately 2 h. After drawing out the required volume, the samples were then returned to the freezer. The stability of ETX, SCA, VDX, KPF, NMS and CCX was assessed after three freeze–thaw cycles. The samples were processed using the same procedure as described in the Sample Preparation section. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e. ±15% DEV) and precision (i.e. 15% RSD), except for LLOQ, where it should not exceed ±20% of CV.

Extraction recovery. The recovery of all analytes and IS, through the liquid–liquid extraction procedure, was determined by comparing the responses of the analytes extracted from replicate QC samples (n = 4) with the response of analytes from non-extracted standard solutions at equivalent concentrations. Recoveries of ETX, SCA, VDX, KPF and CCX at 0.1, 0.3, 15.0 and 40.0 µg/mL and for NMS at 0.5, 1.5, 15.0 and 40.0 µg/mL were determined. The recovery of the IS was determined at a single concentration of 1.0 µg/mL.

Application of the method. Male Wistar rats, 12–14 weeks of age and weighing between 190 and 200 g, were fasted overnight (~14 h) before the dosing day and they had free access to water throughout the experimental period. VDX and CCX were administered orally at a dose of 100 mg/kg/compound, as a suspension in 0.25% sodium carboxy methyl cellulose. Animals were provided with standard diet 3 h post-dosing. The rats were anesthetized in ether and blood samples (~1 mL, sparse sampling protocol) were collected from the retro-orbital plexus into microfuge tubes (containing 30 µL saturated EDTA) at 0.5, 1, 2, 3, 5, 8 and 24 h post-dosing. Plasma was harvested by centrifuging the blood in a microcentrifuge (Biofuge, Hereaus, Germany) at 12,800 rpm for 5 min and stored at –20°C until analysis. Plasma (500 µL) samples were spiked with IS and processed as described above.

Pharmacokinetic analysis. Pharmacokinetic parameters were calculated by employing a non-compartmental analysis (Gibaldi and Perrier, 1982). The peak plasma concentration (C_{max}) and the corresponding time (T_{max}) were directly obtained from the raw data. The area under the plasma concentration vs time curve up to the last quantifiable time point, $AUC_{(0-t)}$, was obtained by the linear and log–linear trapezoidal summation. The $AUC_{(0-t)}$ extrapolated to infinity (i.e. $AUC_{(0-\infty)}$) by adding the quotient of C_{last}/K_{el}, where C_{last} represents the last measurable time concentration and K_{el} represents the apparent terminal rate constant. K_{el} was calculated by the linear regression of the log-transformed concentrations of the drug in the terminal phase. The half-life ($t_{1/2}$) of the terminal elimination phase was obtained using the relationship $t_{1/2} = 0.693/K_{el}$.

RESULTS AND DISCUSSION

Optimization of the experimental conditions

Preliminary experiments were carried out to optimize the experimental parameters affecting both the chromatographic separation of the target compounds in the LC-column selected and their detection by UV. The UV spectra of the analytes were independently determined (Table 2). Each compound exhibited different maximum UV absorbance and, in order to detect NSAIDs and coxibs simultaneously with good sensitivity, 235 nm was selected as UV_{max} for all the compounds. The feasibility of different mixtures of solvents, such as acetonitirile and methanol, using different buffers such as phosphate and ammonium acetate, along with different flow-rates (in the range

Table 2. Maximum UV absorption of test compounds

Compound	λ_{max} (nm)
ETX	284
SCA	238
VDX	240
KPF	256
NMS	300
CCX	255
IS	247

0.5–1.0 mL/min) was tested for complete chromatographic resolution of all six analytes and IS.

Specificity and chromatography

In the chosen completely optimized chromatographic conditions, specificity was indicated by the absence of any endogenous interference at retention times of peaks of interest as evaluated by chromatograms of blank rat plasma and plasma spiked with ETX, SCA, VDX, KPF, NMS, CCX and IS. When single analytes were injected at the highest concentration in the chromatographic system, at the retention times of all analytes no interference was observed (data not shown). All analytes, viz. ETX, SCA, VDX, KPF, NMS and CCX, were well separated with retention times of 15.63, 17.20, 21.66, 24.95, 26.27 and 32.22 min, respectively. Figure 2 shows a typical overlaid chromatogram for the control human plasma (free of analyte and IS) and human plasma spiked with ETX, SCA, VDX, KPF, NMS and CCX at their respective LLOQs.

Calibration curve

Peak area ratios of each analyte spiked in human plasma to the IS were measured and acted as a surrogate for quantitation. A representative calibration graph of peak-area ratio (each analyte to IS) vs each analyte concentration in the range 0.1–50 μg/mL for all analytes except for NMS, where it was in the range 0.5–50 μg/mL, was found to be linear. The average regression ($n = 3$) was 0.999 for all the analytes. The standard curve had a reliable reproducibility for each analyte across the calibration range. The lowest concentration with the RSD <20% was taken as the LLOQ (Shah *et al.*, 1992) and was found to be 0.1 μg/mL for ETX, SCA, VDX, KPF and CCX and 0.5 μg/mL for NMS.

Accuracy and precision

As displayed in Table 3, the assay had adequate accuracy and precision for intra- and inter-day simultaneous determination of six analytes from plasma samples.

Stability

Autosampler and bench-top stability. Over a period of 24 h injection time in the auto-sampler at 5°C and on the bench-top for an 8 h period, the predicted concentrations for ETX, SCA, VDX, KPF and CCX at 0.1, 0.3, 8.0 and 15.0 μg/mL and NMS at 0.5, 1.5, 8.0 and 15.0 μg/mL samples deviated within the nominal concentrations. The results were found to be within the assay variability limits (Table 4).

Freeze–thaw stability. Table 4 shows the results of the analyses of the QC samples following three

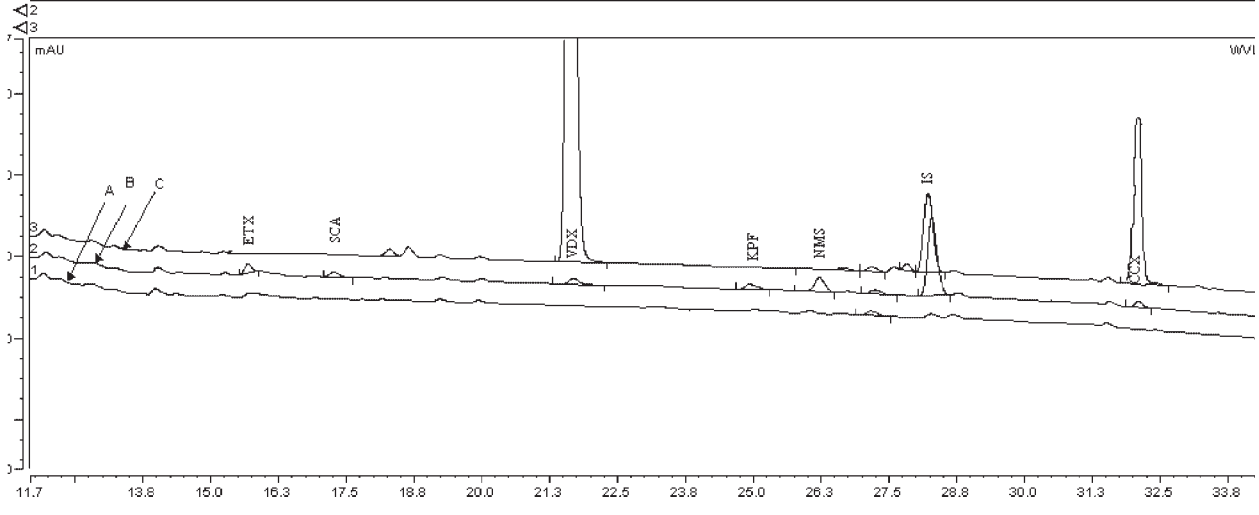

Figure 2. HPLC chromatograms of a 100 μL injection of (a) human blank plasma, (b) human blank plasma spiked with ETX, SCA, VDX, KPF, NMS and CCX at LLOQ (0.1 μg/mL) and 1 μg/mL of internal standard, and (c) a 3.0 h *in vivo* plasma sample obtained from a rat dosed with VDX and CCX at 100 mg/kg p.o.

Table 3. Intra- and inter-day accuracy, precision and recoveries of analytes spiked in human plasma

Compound	Nominal (μg/mL)	Found concentration (μg/mL)	Accuracy (%)	RSD (%) Intra-day (n = 6)	RSD (%) Inter-day (n = 6)	Recovery (%)
ETX	0.10	0.09	91.03	2.83–10.15	7.32	89
	0.30	0.27	91.29	2.97–4.20	5.45	87
	15.0	15.96	106.38	1.05–2.89	2.72	112
	40.0	39.76	99.40	1.68–3.23	2.57	109
SCA	0.10	0.10	100.31	6.42–10.28	9.37	111
	0.30	0.31	103.16	2.26–5.19	4.95	104
	15.0	15.92	106.15	1.13–2.36	5.06	127
	40.0	39.84	99.59	1.74–3.45	4.48	135
VDX	0.10	0.10	101.00	3.66–8.67	6.51	111
	0.30	0.28	94.18	3.45–4.98	3.98	102
	15.0	15.30	102.03	0.69–1.83	2.29	109
	40.0	38.36	95.90	1.00–3.16	2.57	106
KPF	0.10	0.10	103.09	3.95–7.49	7.68	100
	0.30	0.29	96.11	1.36–9.51	8.72	100
	15.0	15.64	104.24	1.29–2.52	6.44	108
	40.0	39.41	98.53	2.13–3.21	4.66	106
NMS	0.50	0.47	94.61	3.37–7.06	7.37	90
	1.50	1.46	97.34	1.15–2.93	6.30	112
	15.0	15.15	101.00	0.97–2.30	4.67	116
	40.0	38.69	96.73	1.70–3.13	3.21	113
CCX	0.10	0.10	99.62	5.08–7.43	7.30	82
	0.30	0.28	92.12	2.04–9.86	6.26	100
	15.0	15.26	101.74	1.00–1.85	2.85	108
	40.0	38.25	95.62	0.91–3.50	2.98	104
IS	1.00	—	—	—	—	100

Table 4. Stability data of each analyte quality controls in human plasma

Compound	QC (spiked) concentration (μg/mL)	0 h (for all)	3 F/T	6 h (B.T)	24 h (in-injector)	21 day (–20°C)
ETX		0.09	0.09	0.1	0.090	0.09
SCA		0.10	0.09	0.11	0.082	0.10
VDX	0.1	0.10	0.10	0.10	0.086	0.10
KPF		0.10	0.10	0.10	0.083	0.11
CCS		0.10	0.10	0.10	0.11	0.10
NMS	0.5	0.45	0.46	0.43	0.48	0.51
ETX		0.27	0.31	0.33	0.26	0.28
SCA		0.31	0.29	0.33	0.29	0.32
VDX	0.3	0.28	0.28	0.33	0.28	0.28
KPF		0.29	0.31	0.28	0.26	0.27
CCS	0.3	0.28	0.28	0.26	0.29	0.27
NMS	1.5	1.46	1.37	1.32	1.53	1.56
ETX		15.96	16.03	15.21	15.53	16.26
SCA		15.92	16.62	15.21	15.28	16.85
VDX	15.0	15.30	16.62	15.21	15.04	15.54
KPF		15.64	15.93	14.51	14.81	15.49
NMS		15.15	15.17	14.25	14.26	15.57
CCS		15.26	14.56	14.49	14.99	16.01
ETX		39.76	42.03	39.84	39.73	39.54
SCA	40.0	39.84	42.03	39.84	39.05	41.56
VDX		39.36	42.03	39.84	38.63	37.90
KPF		39.41	40.43	38.68	38.93	40.89
NMS		38.69	39.98	38.78	38.28	37.55
CCS		38.25	37.93	38.38	38.74	39.33

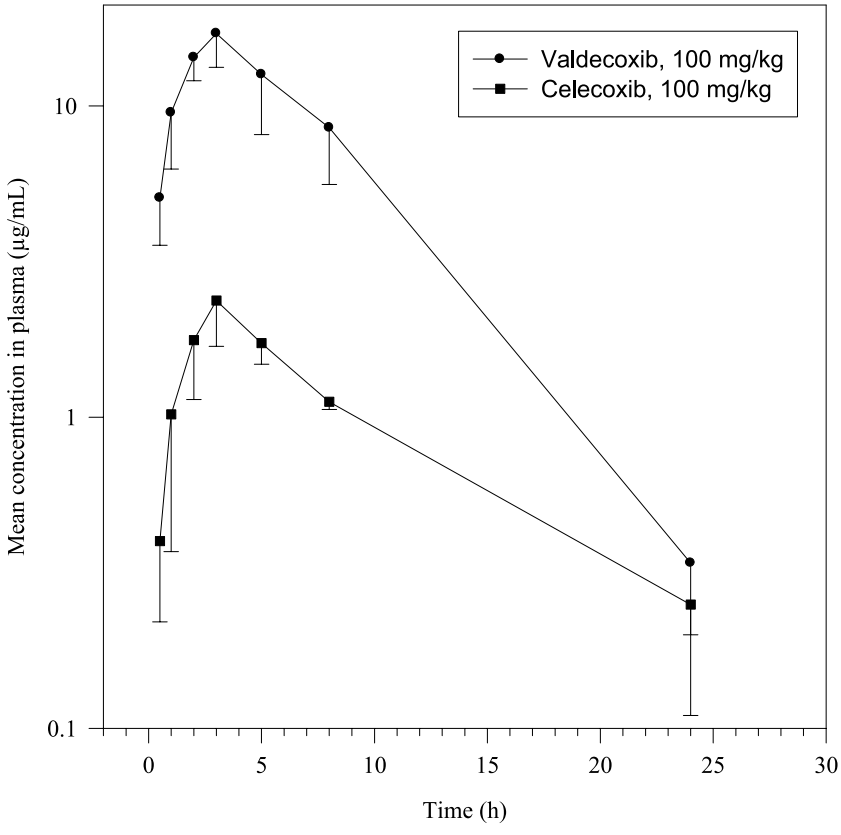

Figure 3. Plasma concentration vs time profiles of VDX and CCX after single dose oral administration of 100 mg/kg in male Wistar rats. The data points are means and standard deviation bars of four observations.

freeze–thaw cycles. All analytes were shown to be stable in the frozen plasma at –20°C for at least three freeze–thaw cycles.

Freezer stability. ETX, SCA, VDX, KPF, NMS and CCX were found to be stable when stored at –20°C for at least 21 days. Both accuracy and precision of QC samples in this evaluation were within the assay variability of ±15% (Table 4).

Extraction recovery

The results of the comparison of neat standards vs plasma-extracted standards were estimated at LLOQ, low, medium and high QC concentration for all the analytes. The absolute recoveries (>83%) are shown in Table 3. The absolute recovery of internal standard at 1.0 µg/mL was 100%.

Application of the method

Although the complete validation was done in human plasma, we selected rodents for *in vivo* study to show the applicability of the newly developed bioanalytical method. The rat plasma samples generated following concurrent administration of VDX and CCX were analyzed by the newly developed validated method along with QC samples. All QC samples prepared in rodent plasma met the criteria as per the guidelines (data not shown). After a single oral administration of 100 mg/kg each of VDX and CCX to male Wistar rats, the plasma concentrations of VDX and CCX were determined by the described method. The mean plasma concentration vs time profiles for VDX and CCX are depicted in Fig. 3. Inspection of Fig. 3 revealed that the newly developed analytical method had the required sensitivity to characterize the absorption, distribution and elimination phases of both VDX and CCX following oral dosing. The pharmacokinetic parameters were calculated using a non-compartmental analysis. The maximum plasma concentration in plasma (C_{max}) was achieved at 2.80 ± 0.45 and 3.25 ± 1.26 h for VDX and CCX, respectively. The half-life ($t_{1/2}$) was found to be 3.10 ± 0.66 and 5.84 ± 1.76 h for VDX and CCX, respectively. The $AUC_{(0-\infty)}$ for VDX and CCX was found to be 100.67 ± 8.9 and 28.17 ± 3.36 µg h/mL, respectively.

CONCLUSION

A HPLC-UV method utilizing optimized gradient elution with single-wave length has been developed for simultaneous analysis of NSAIDs and coxibs in human plasma. The validated method is specific, accurate, precise and reproducible. We have used the method successfully to determine the pharmacokinetic profile of two coxibs in rats and demonstrated that this assay is an effective and inexpensive analytical tool. In addition, the proposed method may be also useful for the quantitation of rofecoxib as is and with slight modification may be extended for the measurement of paracetamol. Thus, our method has the potential to be used for the routine analysis and pharmacokinetic investigations of various NSAIDs and coxibs in various biological fluids.

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