Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1437–1443
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Fast RPLC-UV method on short sub-two microns particles packed column for the assay of tenoxicam in plasma samples

Iulia Sora [a], Toma Galaon [a], Stefan Udrescu [a], Jean Negru [b], Victor David [c], Andrei Medvedovici [a,c,*]

[a] *LaborMed Pharma S.A., Splaiul Independentei 319E, Bucharest 060044, Romania*
[b] *S.C. Agilrom Scientific S.R.L., Sos. Pipera Tunari 1H, Complex Class PO3, 077190 Voluntari, Romania*
[c] *University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, Sos. Panduri 90, Bucharest 050663, Romania*

Received 11 September 2006; received in revised form 29 October 2006; accepted 3 November 2006
Available online 4 December 2006

Abstract

An extraction-less sample preparation technique followed by a RPLC-UV method on sub-two microns particles packed short column were used for the assay of tenoxicam in plasma samples. Protein precipitation was made by means of trichloroacetic acid addition. Supernatant was injected to the chromatographic column without any further pH adjustment. The mobile phase consisted in a mixture of acetonitrile and aqueous 0.1% phosphoric acid, at 2 mL/min flow rate and gradient elution. The Zorbax SB-C18® column (50 mm length, 4.6 mm internal diameter and 1.8 μm particle size) was thermostated at 60 °C. The mobile phase gradient composition program allowed separation of tenoxicam and piroxicam (internal standard), column clean-up and re-equilibration within 4 min. UV detection was achieved at 368 ± 10 nm. The method is characterized by a low limit of quantitation of 25 ng/mL for tenoxicam, with a linearity interval up to 5500 ng/mL. The use of a low volume detection cell and detector high frequency data acquisition rate produced high precision and accuracy through a whole bioequivalence study of tenoxicam in two commercially available tablet formulations, after a single oral administration dose. Full method validation is presented. The high throughput characteristic of the proposed method allowed full validation and bioanalytical study completion within a 96 h period.

Keywords: Tenoxicam; Piroxicam; Plasma samples; Ultra-fast RPLC; Short sub-two microns particles column; Method validation; Extraction-less sample preparation method; Bioequivalence; High throughput

1. Introduction

Tenoxicam is a nonsteroidal anti-inflammatory drug (NSAIDs) from the oxicam group having also analgesic and antipyretic properties [1–5]. It is completely absorbed by the oral route and is about 99% bound to proteins in human plasma. Due to its accentuated hydrophilic character against other oxicams, tenoxicam is characterized by lower penetration into tissues, explaining its reduced incidence of adverse reactions [6]. Tenoxicam is mainly bound to the human serum albumin (HSA) simultaneously to both sites I and II [7]. Food intake on administration delays absorption without affecting bioavailability. Maximum plasma concentrations ranging from 2.3 to 3.0 mg/mL were reported after 1–5 h (the mean was 1.9 h) on fasted healthy volunteers after a single 20 mg oral dose [1,2]. A mean half-life elimination period of 67 h has been estimated [3]. The pharmacokinetics of tenoxicam is independent of patient age [8] or concurrent liver or renal diseases [9]. The effect of concurrent chronic, high dose aspirin therapy generates a decrease of about 24% of the mean half time elimination, and increases of about 49% and 98% of the volume of distribution and clearance, respectively [10]. There is no evidence for entero-hepatic recycling of tenoxicam in humans. Maximum concentrations of tenoxicam in synovial fluids are much lower compared to plasma ones and appeared significantly delayed [11]. Differences in tenoxicam concentration—time profiles after oral and intramuscular administrations are limited over the first 2 h period after dosing [12]. Bioavailability of pharmaceutical formulations based on polymorphs of tenoxicam was statistically insignificant [13]. Cytochrome P450(CYP)2C9, as product

* Corresponding author at: University of Bucharest, Faculty of Chemistry, Department of Analytical Chemistry, Sos. Panduri 90, Bucharest 050663, Romania.
E-mail address: avmedved@yahoo.com (A. Medvedovici).

0731-7085/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.11.008

of the polymorphic gene CYP2C9 provides major catabolic pathway for tenoxicam. An influence of these polymorphic expressions on the pharmacokinetics of tenoxicam has been evaluated [14].

As it can be observed, pharmacokinetics of tenoxicam has been extensively studied. Plasma concentrations of tenoxicam in humans are relatively high and subsequently a low limit of quantitation (LLOQ) around 20 ng/mL should be considered as satisfactory for analytical methods developed for bioequivalence purposes. However, "classic" approaches of assaying tenoxicam in plasma samples are based on liquid–liquid extraction procedures followed by RPLC separations. For this purpose, dichloromethane and ethyl acetate have been more often used as extraction media from acidified plasma samples [15–17]. Solid phase extraction (SPE) procedures have been also reported for plasma sample preparation. Thus, C18 Extrelut cartridges [18] as well as glycidylmethacrylate/divinylbenzene (GMA/DVB) and poly(divinylbenzene-*co*-*N*-vinylpyrrolidone) copolymers were successfully used [19]. LLOQ values around 25 ng/mL has been reached when using sample preparation procedures based on SPE. As a general consideration, detection limits (LOD) in the 5–20 ng/mL interval are achievable on using UV detection in the 355–371 nm range, while MS/MS (MRM) detection [17] allows LLOQ in the 0.5 ng/mL range.

Plasma levels of tenoxicam are not justifying tedious sample preparation procedures (such as liquid–liquid extraction or SPE) or special and expensive detection techniques (such as MS/MS). However, very few extractions-less sample preparation techniques have been proposed. Protein precipitation with 5% zinc sulfate/methanol [20] followed by RPLC method with spectrometric detection at 355 nm produced an LOD of 40 ng/mL. Protein precipitation by means of organic solvent addition [21] and UV detection at 365 nm lead to a surprisingly poor LOQ (200 ng/mL).

Recent literature data report pharmaceutical applications of ultra-fast liquid chromatographic separations. Speeding LC separations may be obtained in three different ways: (a) increasing flow rates and keeping column pressure drop in normal limits by using monolithic stationary phases; (b) using a high temperature regime applied to the chromatographic column; (c) using stationary phases having sub-two microns particle size packed in short column. Discussions on ultra-fast HPLC applications on sub-two microns packing particles have been already published [22–25]. When combined with simple protein precipitation sample preparation procedures, ultra-fast LC separations allow an unequalled throughput in bioanalytical applications (around 350 samples/day).

The present work refers to a bioequivalence study of tenoxicam in two commercially available pharmaceutical formulations (tablets) administrated orally, in a single dose. Piroxicam has been used as internal standard (IS). Sample preparation is based on a simple protein precipitation step by means of trichloroacetic acid addition. The supernatant was directly loaded to a Zorbax SB-C18® 1.8 μm particle size column, without any further pH adjustment. A fast gradient allows separation of target compounds in less than 1.2 min. A mobile phase gradient composition program having duration of 4 min includes separation, column clean-up, and re-equilibration periods, together. Aspects related to method validation are detailed. Validation of the analytical method was made in accordance to the recommendations of the guide "Bioanalytical Method Validation" [26]. This high throughput analytical solution leads to completion of both method validation and bioanalytical study within 96 h period.

2. Experimental

2.1. Instrumentation

Experiments were performed with an Agilent 1100 series LC/DAD (Agilent Technology, Waldbronn, Germany) system consisting of the following modules: degasser (G1379A), quaternary pump (G1311A), autosampler (G1313A), column thermostat (G1316A), and diode array detector SL series (G1315C). System control and data acquisition were made with the Agilent ChemStation Version A 10.02. The system was operationally qualified before and after the bioequivalence study.

As it can be observed, no major changes in the basic configuration of the Agilent 1100 series LC/DAD instrument were made. However, an 80 μL low internal volume mixer (5064-8273) replaces the normal version. The SL detector is characterized by an increased data acquisition rate (80 Hz). A semi-micro 5 μL flow cell (G1314-60011) replaces the basic one (13 μL). Tubing making connections between modules had 0.17 mm i.d. and minimized lengths.

2.2. Chromatographic method

A single Zorbax StableBond C18 column, 50 mm length, 4.6 mm internal diameter and 1.8 μm particle size, fitted with a Phenomenex C18 security guard cartridge (2 mm × 4 mm) was used during the validation stage and entire bioequivalence study. The column was thermostated at 60 °C. The column was validated before and after study completion, by computing the H–ū curves for piroxicam, using isocratic elution conditions (aqueous 0.1% phosphoric acid/acetonitrile = 65/35, v/v) and flow rates within 0.5–2.8 mL/min interval. The optimal flow rate corresponds to 2 mL/min. The reduced plate height (h) varies from 3.06 to 3.17 during study completion (about 1100 samples, including validation). Efficiency is reduced by a mean of 8% for an increase of 40% of the flow rate against its optimal value.

The bioanalytical method is based on gradient elution, using as mobile phase constituents aqueous 0.1% phosphoric acid and acetonitrile. According to a previous work, pK_a values for tenoxicam and piroxicam are 5.3 and 4.6, respectively [27]. The aqueous component of the mobile phase (containing 0.1% H_3PO_4) has a pH value around 2.5, forcing the analytes to elute as undissociated structures.

The initial composition of 30% organic solvent is changed to 100% acetonitrile after 1.5 min. The final composition is kept constant for 0.5 min (column clean-up) and stepwise brought to the initial value in 0.01 min. Column equilibration takes 2 min. The flow rate was set to the optimal value of 2 mL/min. Column was thermostated at 60 °C. The choice of a gradient elution mode may be considered excessive, as the separation between

the target analyte and the IS is not critical. However, the following main goals have been reached by applying such a solution: (a) to maintain an appropriate retention for the first eluting peak (tenoxicam), resulting in reduced interfering effect brought by the co-isolated matrix; isocratic separation trials lead to an exponential first order decrease function relating capacity factor characterizing the first eluting peak and the % content of acetonitrile in the mobile phase ($k' = 12.8 \times 10^{-(\%\ \mathrm{ACN}/10.51)} - 0.036$); (b) column washing after each injection, allowing removal of endogenous compounds from the matrix (detectable or not at the analytical wavelength), leading to preservation of the column properties over the whole study; column washing up to 100% acetonitrile in the mobile phase involves no risks as long as no inorganic buffer has been used in its composition; (c) the use of an increased injection volume of 50 μL, resulting in a subsequent increase in terms of sensitivity; the column producer recommend injection volumes typically of 5 μL or less, usually lower than 2 μL; however, in gradient elution mode, injection volume requirements are less critical, in case of an acceptable retention of the first eluting peak; this particular feature allows elimination of a concentration step during sample preparation (for instance, evaporation to dryness of 500 μL of the supernatant resulting after protein precipitation with acetonitrile takes about 20 min, at 60 °C under nitrogen flow).

2.3. DAD parameters

The analytical wavelength was set at 368 nm and the bandwidth to 20 nm. A reference wavelength of 560 ± 10 nm was used. The detector slit was increased to 8 for a better sensitivity. The response time was fixed at 0.1 s.

2.4. Materials

All solvents were HPLC grade from Merck (Darmstadt, Germany). Water for chromatography (minimum resistivity 18.2 MΩ and maximum TOC 30 ppb) was produced within the laboratory by means of a TKA Lab HP 6UV/UF instrument and used during experiments. Tenoxicam and IS (piroxicam) as standard reference substances were purchased from European Pharmacopoeia, Council of Europe, Strasbourg, France (tenoxicam, batch 1a, code. no. EPT0040800, and internal standard, batch 2, code no. EPP2130000).

2.5. Methodology and pharmacokinetic application

The developed method was applied to an open-label, analytically blinded, single dose, randomized, two-periods, two-sequences, fast state, and crossover bioequivalence study. Twenty-four healthy volunteers (male/female ratio = 18/6) with an mean age of 21.5 years and a mean weight of 71.5 kg, received one dose of 20 mg tenoxicam of the tested product (T) and one of the reference product (R), in the sequence determined by randomization, with a 21 days wash-out period between consecutive administrations. The protocol of the study was formally accepted by the evaluation department of the Romanian National Drug Agency and received the approval of the Institutional Ethics Committee. Venous blood samples were collected pre-dose (0 h) and at the following post-dose intervals of time: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 24, 72 and 144 h. Medical examinations were performed in the screening and at the beginning of each study period (in-house day), in every single blood sampling days and at the end of each study period.

The pharmacokinetic parameters considered for evaluation of the bioequivalence between tested and reference products were—C_{max}: observed maximum plasma concentration of tenoxicam; T_{max}: sampling time of the maximum plasma concentration; t_{half}: terminal elimination half life time; AUC_{last}: area under plasma concentration/time plot until the last quantifiable value; AUC_{total}: area under plasma concentration/time plot extrapolated to infinity. Pharmacokinetic parameters were determined by means of the Kinetica™ software (Version 4.4.1.) from Thermo Electron Corporation, U.S.A. The analysis of variance was performed on the pharmacokinetic parameters. Then, the 90% confidence intervals of the pharmacokinetic parameters characterizing the tested/reference products were determined.

3. Results and discussions

3.1. Choice of internal standard

The importance of a right choice of the internal standard is obvious. Because the hydrophobic character of the target compounds controls their separation and may influence the sample preparation step (by means of their solubilities in water), it seems practical to find out among the oxicam class, a homologous of tenoxicam having similar log K_{ow} (log of the partition coefficient between 1-octanol and water, as an indicator of the molecular hydrophobic character). Computed log K_{ow} values for tenoxicam and piroxicam are 2.4 and 2.58, respectively. Both compounds exhibit strong adsorption bands in the 300–400 nm interval, resulting in a sensitive and relatively selective detection.

3.2. Sample preparation procedure

A 0.25 mL plasma aliquot was mixed with 25 μL of the IS stock solution (20 μg/mL, a mixture 1/3 (v/v) of acetonitrile and water being used as solvent). After vigorous vortex mixing, 25 μL of a 70% trichloroacetic acid solution (w/v) are added as a protein precipitation agent. After vortex-mixing, the sample is centrifuged at 7500 × *g*. The supernatant is transferred to a vial without any further pH adjustment, for injection in the chromatographic column.

The potential problem relating to the sample preparation procedure arises from the fact that both analytes exhibit hydrophobic characteristics and have to be maintained soluble in an aqueous strongly acidic media. The precipitated proteins may further absorb the target compounds, resulting in their poor recovery.

In order to evaluate all these combined effects, recovery studies were carried out. Tenoxicam was spiked to blank plasma samples at 75, 750 and 3000 ng/mL levels while IS was added at 2000 ng/mL level. Five replicates were processed according to the sample preparation procedure for each concentration level of

Table 1
Recoveries for tenoxicam and IS, after applying the sample preparation procedure to spiked plasma samples

Compound	Concentration (ng/mL)	Recovery (%) (against water spiked samples)	R.S.D.% ($n=5$)	Recovery (%) (against samples spiked in bulk protein precipitated blank plasma)	R.S.D.% ($n=5$)
Tenoxicam	75	30.8	1.3	91.2	2.3
Tenoxicam	750	30.2	1.9	95.5	1.8
Tenoxicam	3000	30.3	0.1	96.3	1.7
Mean values for tenoxicam		30.4	2.9	94.3	2.9
Piroxicam	2000	27.1	1.9[a]	94.6	1.8[a]

Calculations were made by comparison to water spiked samples and samples spiked to bulk protein precipitated blank plasma.
[a] Relative standard deviation – R.S.D.% – was calculated for a set of 15 replicates.

tenoxicam. Same spiking operation was achieved for both compounds in HPLC grade water and in bulk protein precipitated blank plasma. These samples were injected as such. Results are presented in Table 1.

It clearly results that about two thirds from both analytes are adsorbed on the precipitated proteins. Adsorption does not depend upon the concentration of the target compound. We can conclude that recoveries from processed plasma samples calculated against water spiked ones are around 30% for both analyte and IS and the adsorption process on precipitated proteins is reproducible. As the method was found sensitive enough for the declared purpose, relatively poor recoveries should be accepted, when considering the reproducibility of the sample preparation procedure. An additional element sustaining process reproducibility was brought by the variation of the peak areas of the IS during study completion: for 600 processed samples, the calculated relative standard deviation (R.S.D.%) was found 6.6%.

Protein precipitation by means of organic solvent addition appears to be a more realistic alternative for plasma sample preparation. Recoveries of tenoxicam and piroxicam from plasma samples processed by addition of acetonitrile (volumetric ratio between plasma and organic solvent is 1/2) are about 88% compared to spiked water samples. However, this results in a three-folds dilution of the plasma sample. Achieving injection of volumes higher than 20 μL without solvent focusing effects imposes an additional dilution step with water (volumetric ratio 1/1), in order to ensure agreement between the sample solvent and the mobile phase composition at the beginning of the separation process. The overall process will lead to a reduced sensitivity. According to our experience, acidic protein precipitation leads to "cleaner" samples characterized by a lower matrix carryover, producing advantages in terms of selectivity.

3.3. Selectivity of the chromatographic method

The chromatographic method separates target compounds with an increased apparent resolution ($R_S = 18.2$). Spectrometric detection at 368 nm adds its own inherent selectivity against the plasma endogenous components still remaining in samples after preparation. During the method validation the selectivity has been proved for six blank plasma samples. Additionally, in all pre-dose collected plasma samples from volunteers participating to the study, no endogenous interference was observed. In Fig. 1 two overlaid chromatograms are given in order to prove the selectivity of the chromatographic method.

Residual peak areas in blank samples over the whole study ranged from 3.3% to 18.6% from the peak area of tenoxicam corresponding to LLOQ (25 ng/mL).

According to the bioequivalence study protocol, no co-medication has been considered to avoid eventual potential adverse effects generated by the administration of the studied drug. None of the healthy volunteers involved in the study reports intake of concurrent medication during the testing periods. However, some usual active substances have been considered for method selectivity evaluation. Plasma spiked samples at 1 μg/mL level, containing the following active products (acetylcysteine, acetylsalicylic acid, ascorbic acid, bromhexine, caffeine, chlorpheniramine, codeine, omeprazole, paracetamol and ranitidine) have been prepared and analyzed according to the method. The apparent capacity factors calculated for the tested active products are null for acetylcysteine, ascorbic acid, codeine and ranitidine, 0.13 for paracetamol, 0.23 for caffeine, 0.34 for omeprazole, 0.35 for chlorpheniramine, 1.24 for aspirin and 2.49 for bromhexine with respect to 1.91 for tenoxicam and 4.03 for IS. At the analytical wavelength, none of the previously mentioned compounds is detectable.

3.4. Robustness of the chromatographic method

Several operational parameters of the chromatographic method have been varied for assessment of their impact on the final results.

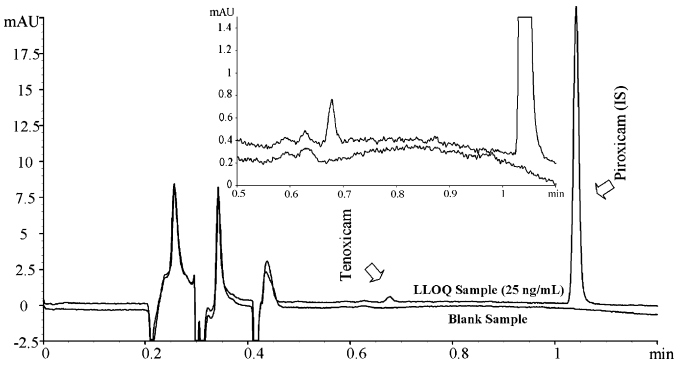

Fig. 1. Chromatogram of a plasma sample spiked with 25 ng/mL tenoxicam and 2000 ng/mL internal standard, with a zoomed window for the analyte of interest, overlaid to the blank plasma (gray line).

It was proved that pump solvent mixing accuracy within ±0.7% demonstrated during OQ induces variations of the retention times of the target compounds within their normal variation intervals.

The fast gradient formation (46.7% min^{-1}) could be considered somewhat critical for the method. Absolute retention time of the IS (eluting at the end of the gradient program) was monitored over the whole study. The relative standard deviation of the absolute retention time values characterizing the IS peak during study completion ($n = 600$) was 0.42%, which proves the accurate and reproducible formation of fast gradients by the qualified high pressure pump.

The influence of temperature on the chromatographic separation was evaluated in the 50–70 °C interval. Variation of retention parameters fits within their normal variation intervals. No noticeable effects were observed on apparent efficiency and chromatographic resolution.

The concentration of the phosphoric acid in the aqueous constituent of the mobile phase has been also considered. Reducing concentration by 50% or increasing it by 100% has no significant impact on retention, apparent efficiency, and chromatographic resolution.

3.5. Calibration and quantitation limit

A calibration study was first made for the IS, to acknowledge that the planned concentration level spiked to plasma samples (2000 ng/mL) fits within the linearity domain. The studied concentration interval ranged from 1000 to 4000 ng/mL of IS spiked to plasma samples. The linear regression ($y = a + bC$, y: peak area of the IS; C: concentration of IS spiked to plasma samples in ng/mL; a: intercept = -1.5 ± 1.6; b: slope = 0.0087 ± 0.0008) was characterized by a correlation coefficient of 0.9957.

Calibration was realized over a wide range of tenoxicam concentrations in spiked plasma samples (25–5500 ng/mL) and a fixed concentration of IS (2000 ng/mL). Calibration function ($Y = A + BC$; Y: tenoxicam/IS peak area ratio; C: tenoxicam concentration in plasma samples, ng/mL) was characterized by the following parameters: $B = 8.2 \times 10^{-4} \pm 6.8 \times 10^{-6}$; $A = -16 \times 10^{-4} \pm 86 \times 10^{-4}$; $r = 0.99993$.

Over the linearity procedure carried out for tenoxicam (nine concentration levels, six replicates per concentration), the R.S.D.% characterizing IS peak areas was 4.7%.

Evaluation of the quantification limit (LOQ) has been achieved as following: $LOQ = [2t(s_A + s_B C_{av})]/(B + 2ts_B)$, where s_A, s_B are the standard deviations calculated for A and B, C_{av} the mean concentration value from the set used for the linear regression and t is the Student coefficient considered for $n - 2$ ($n = 9$) degrees of freedom and a confidence level of 99% ($t = 1.415$).

From the experimental dataset, the computed LOQ is 48.3 ng/mL.

During study completion, a calibration was performed for each two volunteers. Bulk blank plasma samples were spiked at 50, 100, 250, 500, 1000, 2500, 3000 and 4000 ng/mL with tenoxicam and 2000 ng/mL with IS. Twelve aliquots at each concentration level from bulk spiked plasma samples were placed in separate vials and frozen at −40 °C. One set of calibration plasma samples was thaw at the same time as samples from two volunteers, prepared in the same manner and analyzed within the same chromatographic sequence. The normal variation interval of the slopes resulting by computation of the linear regressions was $[7.29 \pm 0.17] \times 10^{-3}$, while the same interval for intercepts was [0.0021 ± 0.0062] ($n = 20$).

3.6. Precision

Precision was checked on spiked plasma samples at three concentration levels of tenoxicam (70, 750, and 2000 ng/mL). Table 2 enlists experimental results obtained during the evaluation of precision, considering as parameters the absolute peak area of tenoxicam, the peak area ratio between tenoxicam and IS, and the corresponding calculated concentration (applying the regression equation obtained under the linearity study). Results for IS peak area values are also given.

During the study completion, for each analytical sequence, quality control (QC) samples were considered at three concentration levels (70, 750 and 2000 ng/mL, respectively). Intra-sequence precision was evaluated in terms of concentration for 20 QC sets (calculation was made by using the linear regression equation obtained for the calibration corresponding to the sequence). The following results were obtained: for 70 ng/mL

Table 2
Intra- and inter-day precision for spiked plasma samples

Spiked concentration (ng/mL)	Parameter	Intra-day ($n = 10$)		Inter-day ($n = 6$)	
		Mean ± 2 S.D.	R.S.D.%	Mean ± 2 S.D.	R.S.D.%
75	Peak Area	1.04 ± 0.034	1.63	0.93 ± 0.05	2.54
	Analyte/IS peak area ratio	0.061 ± 0.003	2.24	0.061 ± 0.003	2.69
	Experimental concentration (ng/mL)	75.8 ± 2.8	1.87	75.9 ± 4	2.61
750	Peak area	10.44 ± 0.42	2.03	10.86 ± 1.04	4.81
	Analyte/IS peak area ratio	0.601 ± 0.009	0.74	0.63 ± 0.01	1.17
	Experimental concentration (ng/mL)	733 ± 5.5	0.75	770 ± 18	1.18
2000	Peak area	28.6 ± 1.1	1.93	28.1 ± 2.6	4.7
	Analyte/IS peak area ratio	1.63 ± 0.03	0.9	1.68 ± 0.05	1.4
	Experimental concentration (ng/mL)	1991 ± 18	0.9	2050 ± 62	1.5
(IS) 2000	Peak area	17.4 ± 0.9	2.47	16.4 ± 2.2	6.76

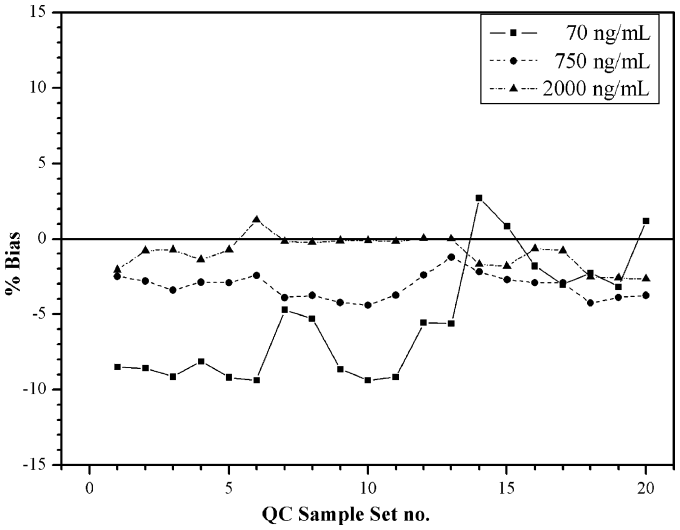

Fig. 2. % bias obtained for QC samples over the study, as an accuracy indicating tool.

level, the mean calculated concentration was 67.3 ± 6.4 ng/mL, with an R.S.D.% of 4.7%; for 750 ng/mL level, the mean calculated concentration was 726 ± 17 ng/mL, with an R.S.D.% of 1.2%; for 2000 ng/mL level, the mean calculated concentration was 1982 ± 50 ng/mL, with an R.S.D.% of 1.3%.

3.7. Accuracy

The accuracy of the method may be evaluated from the QC samples analyzed over the study (20 sets). Intra-sequence accuracy, estimated as the bias (calculated as percentage) of the QC samples against the theoretical concentration values, acts as an accuracy indicator. Fig. 2 indicates the variation of the % bias, at the three concentrations chosen for the QC sets (low level: 70 ng/mL; medium level: 750 ng/mL and high level: 2000 ng/mL), over the whole study. All results are within the accepted interval.

3.8. Stability of analytes and samples

Stability studies for tenoxicam were made on spiked plasma samples having concentrations of 100, 1000, and 3000 ng/mL. The stability of the IS stock solution in acetonitrile (20 μg/mL) was also checked over a 10 days period, at 48 h sampling interval. Before each analysis, the IS stock solution was spiked to a blank plasma sample at 2000 ng/mL level; sample was processed according to the procedure and injected to the chromatographic column.

Freeze and thaw stability was studied for five consecutive cycles, from −40 °C to ambient (thaw process was unassisted).

Long term stability was studied over 60 days, at 15 days sampling interval (five samplings) and a temperature of −40 °C.

Short term stability was made over 24 h interval. Frozen spiked plasma samples were thawed unassisted at room temperature and analyzed after 2, 4, 8, 12, and 24 h, respectively.

Post-preparative stability was evaluated by analyzing processed spiked plasma samples after preparation and at 4, 8, 12, 24 and 48 h after preparation, on storage bench top, at room temperature.

Results obtained during stability evaluation study are given in Table 3.

3.9. Bioequivalence study

The main pharmacokinetic parameters obtained on study completion are given in Table 4.

It is worthwhile to note that the determined pharmacokinetic parameters are in perfect agreement with literature data [2,3,5].

Table 3
Results obtained during the stability study

Procedure	Concentration of tenoxicam						IS	
	100 ng/mL		1000 ng/mL		3000 ng/mL		Peak area	
	Mean	R.S.D.%	Mean	R.S.D.%	Mean	R.S.D.%	Mean	R.S.D.%
Freeze and thaw ($n=5$)	95.1	3.02	1066	0.3	3123	0.6	–	–
Long term ($n=5$)	93.1	1.1	1061	0.5	3129	0.2	–	–
Short term ($n=6$)	95.5	0.9	1068	0.7	3134	0.2	–	–
Post-preparative ($n=6$)	95.9	2.8	1059	0.4	3126	0.3	–	–
IS stock solution	–	–	–	–	–	–	16.599	2.62

Table 4
Statistics of pharmacokinetic parameter

Drug	Statistic	C_{max} (ng/mL)	T_{max} (h)	T_{half} (h)	AUC_{last} (ng/mL h)	AUC_{tot} (ng/mL h)
Tested (T)	Mean	3123.8	3.2	78.4	202362.5	294553.5
	R.S.D.%	23.0	15.9	52.5	24.8	46.1
Reference (R)	Mean	3302.2	1.70	79.7	204726.8	300885.8
	R.S.D.%	19.0	57.6	61.8	25.3	49.1
90% confidence interval for the ratio of the means T/R		87.6–100.7	–	–	96.2–101.6	92.7–104.6

4. Conclusions

A high throughput method for determination of tenoxicam in plasma samples is proposed. Both stages of the method (sample preparation procedure and chromatographic separation) are simple and fast. Sample preparation procedure is based upon protein precipitation with concentrated trichloroacetic acid. Tenoxicam and IS are adsorbed on the precipitated proteins, but the process is highly reproducible and the resulting sensitivity largely satisfy requirements. No pH adjustment prior to injection is necessary. Chromatographic separation is achieved on a 1.8 μm particle size sterically protected octadecyl modified high purity silicagel, especially created to enhance on the chemical stability in acidic media. The optimal flow rate is 2 mL/min, column being operated at 60 °C in order to control the pressure drop in the system. A normal high pressure pump has been used (maximum allowed pressure 400 bar). A dynamic mixer with reduced internal volume has been used in order to maintain increased efficiency. Tubing connections between modules had minimized internal volumes, too, for exactly the same purposes. Analytical separation, column clean-up, and re-equilibration are achieved within 4 min. Analytes are separated within 1.2 min with high apparent resolution, by means of a fast gradient. Properties of the column remain practically unchanged over the analysis of more than 1000 plasma samples (validation + study completion). Detection at 368 nm produced an enhanced selectivity, minimizing interference effects. Detector was equipped with a 5 μL reduced volume flow cell and exhibits high frequency data collection characteristics (80 Hz).

Method validation demonstrates an LOQ of 48 ng/mL and a linearity interval up to 5500 ng/mL. Very good precision and accuracy were obtained. Precision and accuracy were also supported by data obtained on calibration and QC sample evaluation.

The method was successfully applied to an open-label, single dose, randomized, two-periods, two-sequences, fast state, and crossover bioequivalence study of two commercially available tenoxicam tablets formulations. Pharmacokinetic parameters being obtained are in very good agreement with data from literature. The bioequivalence of the two products was demonstrated.

The major advantage of the proposed method relies to its simplicity and speed. Practically, a whole analytical study, including method development and validation can be realized within 96 h.

References

[1] K.T. Olkkola, A.V. Brunetto, M.J. Mattila, Clin. Pharmacokinet. 26 (1994) 107–120.
[2] C. Crevoisier, P. Heinzmann, I. Forgo, U.C. Dubach, Eur. J. Drug Metab. Pharmacokinet. 14 (1989) 23–27.
[3] T.W. Guentert, T. Stabler, L. Banken, R. Defoin, M. Schmitt, Arzneimittelforschung 44 (1994) 1051–1054.
[4] A. Corvetta, R. Della Bitta, M.M. Luchetti, G. Pomponio, V. Ciufoletti, Clin. Exp. Rheumatol. 9 (1991) 143–148.
[5] O.G. Nilsen, Clin. Pharmacokinet. 26 (1994) 16–43.
[6] M. Fenner, Scand. J. Rheumatol. Suppl. 65 (1987) 97–101.
[7] F. Bree, P. Nouven, S. Urien, P. Riant, E. Albengres, M. Fener, J.P. Tillement, Fundam. Clin. Pharmacol. 3 (1989) 267–279.
[8] O.G. Nilsen, R.A. Walstad, M. Eckert, P. Heinzmann, A. Buckert, T. Am, I. Loge, J. Unnvik, E. Thue, Eur. J. Clin. Pharmacol. 35 (1988) 563–566.
[9] O.G. Nilsen, K. Aasarod, T.E. Wideroe, T.W. Guentert, Pharmacol. Toxicol. 89 (2001) 265–272.
[10] R.O. Day, P.D. Paull, S. Lam, B.R. Swanson, K.M. Williams, D.N. Wade, Br. J. Clin. Pharmacol. 26 (1988) 455–462.
[11] R.O. Day, K.M. Williams, S. Graham, M. Handel, Arthritis Rheum. 34 (1991) 751–760.
[12] T. Stebler, T.W. Guentert, Biopharm. Drug Dispos. 14 (1993) 483–490.
[13] M.S. Salem, H.N. Alkaysi, A.M. Gharaibeh, N.M. Gharaibeh, A.A. Badwan, J. Clin. Pharm. Ther. 19 (1994) 203–207.
[14] R. Vianna-Jorge, J.A. Perini, E. Rondinelli, G. Suarez-Kurtz, Clin. Pharmacol. Ther. 77 (2005) 587–588.
[15] M.I. Munera-Jaramillo, S. Botero-Garces, J. Chromatogr. 616 (1993) 349–352.
[16] P. Heizmann, J. Korner, K. Zinapold, J. Chromatogr. 374 (1986) 95–102.
[17] H.Y. Ji, H.W. Lee, Y.H. Kim, D.W. Jeong, H.S. Lee, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 826 (2005) 214–219.
[18] D. Dell, R. Joly, W. Meister, W. Arnold, C. Partos, B. Guldimann, J. Chromatogr. 317 (1984) 483–492.
[19] M. Sultan, G. Stecher, W.M. Stoggl, R. Bakry, P. Zaborski, C.W. Huck, N.M. El Kousy, G.K. Bonn, Curr. Med. Chem. 12 (2005) 573–588.
[20] J.L. Mason, G.J. Hobbs, J. Chromatogr. B: Biomed. Appl. 665 (1995) 410–415.
[21] J.I. Troconiz, L.G. Lopez-Bustamante, D. Fos, Arzneimittelforshung 43 (1993) 679–681.
[22] Y. Xiang, D.R. Maynes, M.L. Lee, J. Chromatogr. A 991 (2003) 189–196.
[23] M. Martin, G. Guiochon, J. Chromatogr. A 1090 (2005) 16–38.
[24] R.E. Majors, LC–GC Europe 6 (2006) 1–9.
[25] J.J. Kirkland, J. Chromatogr. Sci. 38 (2000) 535–544.
[26] Guidance for Industry. Bioanalytical Method Validation. U.S. Department of Health and Human Sercices, FDA, CDER, CVM, 2001. http://www.fda.gov/cder/guidance/index.htm.
[27] V. David, F. Albu, A. Medvedovici, J. Liq. Chromatogr. Liq. Technol. 27 (2004) 965–984.