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

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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

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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