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Sensitive high performance liquid chromatographic assay for nitazoxanide metabolite in plasma

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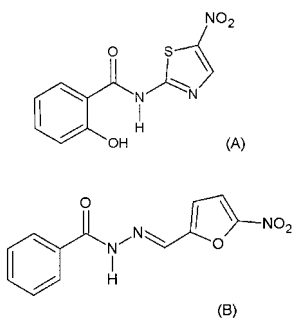
A sensible and specific HPLC analytical method for the determination of tizoxanide (TZO), the active metabolite of nitazoxanide (NTZ) in rat plasma was developed and validated. Samples of 200 μL were efficiently deproteinized with acetonitrile. Assay was performed using a C_{18} CC with a ternary gradient elution of 50 mmol · L^{-1} KH_2PO_4 : acetonitrile : methanol and UV/Vis detection at 416 nm. The analytical method was linear in a range of 10–1280 ng · mL^{-1}, precise (RSD % > 2.2), accurate (RE % < 7.8) and with high recovery (% > 95%). Stability studies showed that TZO was stable in plasma for short and long-time period (45 days) and proved to be suitable for pharmacokinetic studies of NTZ in rats. The method was also evaluated using human plasma samples and no statistical differences were found in the response-curve between rat and human samples.

1. Introduction

Nitazoxanide (NTZ) is a broad-spectrum antihelmintic agent, which is effective against a wide variety of parasites and bacteria infecting animals or humans (Fox et al. 2005). It has been available for several years in developing countries where tapeworm and liver fluke infestations are common (Rossignol et al. 2006). In USA, it has been approved for the treatment of *Cryptosporidium parvum* in patients with AIDS and for the treatment of *Giardia lamblia* (Fox et al. 2005). The antiprotozoal activity of NTZ is believed to be due to interference with the pyruvate ferredoxin oxidoreductase (PFOR) enzyme-dependent electron transfer reaction, which is essential to anaerobic energy metabolism (Sisson et al. 2002). It has been shown that once orally administered, NTZ is rapidly metabolized to tizoxanide (TZO) which also shows parasiticidal activity.



(A) Tizoxanide (B) Nifuroxazide

Recently, *in vitro* studies have shown that the cestocidal effect of albendazole sulphoxide in combination with tizoxanide is additive (Palomares-Alonso et al. 2007), therefore preclinical pharmacokinetic studies are required to confirm the results. Few methodologies have been developed for the quantification of TZO in plasma. A couple of procedures using HPLC UV/Vis for pharmacokinetic studies in humans (using a volume of 500 μL of sample) have been described elsewhere (Stockis et al. 1996; Agarwal et al. 2007). Other methods using LC/MS/MS and TLC with densitometry for detection reported a lower limit of quantification (LOQ) around 20 and 400 ng · mL^{-1} respectively (Stockis et al. 2002; Namur et al. 2007). On the other hand, there are no reports of the pharmacokinetic parameters of TZO in rats in the published literature so far.

In this paper we describe a sensitive HPLC assay for TZO determination using an analytical wavelength of 416 nm with nifuroxazide (NFZ) as internal standard (IS). A reproducible protein precipitation methodology for rat and human plasma with a volume of 200 μL of sample was probe; LOQ was 10 ng · mL^{-1} and stability studies that have not been reported before were performed. The method was validated according to the procedures and acceptance criteria based on FDA guidelines (FDA guidelines 2001). After validation, samples from a pharmacokinetic study in rats were analyzed using this method.

2. Investigations, results and discussion

TZO and IS are compounds with almost equal polarities, instead of an isocratic mobile phase method a ternary gra-

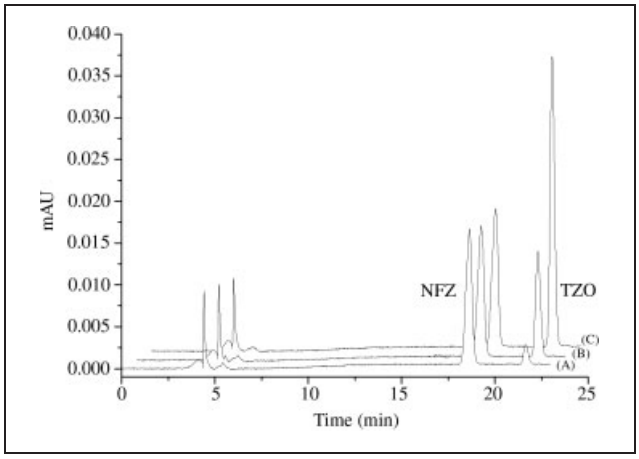


Fig. 1: Chromotograms quality control points 70 (A), 400 (B) and 1000 (C) ng · mL^{-1}

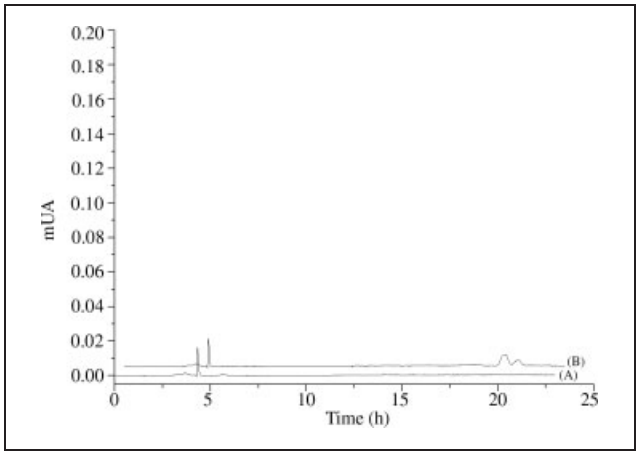


Fig. 2: Chromatograms of blank human plasma (A) and blank rat plasma samples

dient elution was employed to obtain better resolution and peak shape (Fig. 1).

The method was validated by evaluating selectivity, linearity, accuracy, precision, recovery, quantification limit and short and long term stability. The LOQ was 10 ng · mL^{-1} and all calibration curves were linear in a range of 10–1280 ng · mL^{-1} ($y = 0015x + 0.0003$) with an average determination coefficient of 0.9995 + 3.6 E-04. Extraction efficiency mean value was $101 \pm 2.8\%$, $101 \pm 0.6\%$ and $104 \pm 2.3\%$ for low, medium and high QC samples respectively, while the recovery for the IS was $95 + 0.4\%$.

In order to determine precision and accuracy of the method, three replicates of each QC sample were evaluated. Results are shown in Table 1. It can be seen that the coefficient of variation of the intra-day precision did not exceed 2.2% and for the inter-day precision (3 days) the maximum value was 8.1%. In the case of accuracy, the values were within −0.4 and 7.8% at each concentration level.

TZO stability in plasma has not been reported so far. In this study short and long stability tests under FDA requirements were performed. Table 2 summarizes the results. It can be observed that processed samples can be stored for 72 h at 15 °C in the autosampler prior to analysis. On the other hand, results of processed and freeze and thaw samples did not show a significant loss of the compound after analysis; therefore, samples can be analyzed after 24 h after being processed and stored at 4 °C and after two freeze and thaw cycles at −70 °C. Stability test performed at room temperature showed that after 24 h at 25 °C neither low nor high concentration of QC samples presented a significant degradation, and therefore no stability-related problems were found during routine sample analysis. Long-term stability results showed that samples were stable at least for 45 days at −70 °C.

Figure 3 shows the pharmacokinetic profile of TZO in rats after oral administration of NTZ (7.5 mg/kg). No plasma levels were found after 6 h. Results of pharmacokinetic parameters were: maximum plasma concentration of TZO (C_{max}) 258 ng · mL^{-1}, time of maximum plasma concentration (t_{max}) 0.25 h, area under the plasma concentration versus time curve from 0 to the last measured concentration (AUC) 366.89 ng · mL/h, area under the plasma concentration versus time from 0 to infinity ($AUC_{0\ to\ \infty}$), 450.1361 ng · mL/h, half life ($t_{1/2}$) 1.64 h and mean residence time (MRT) 2.31 h.

To determine if this analytical procedure could be used for human plasma samples, selectivity (Fig. 2) and linearity were studied. No interferences in peak retention times of

Table 1: Intra- and inter-day precision and accuracy results for assay (n = 3)

Added, C (ng · mL^{-1})	Found, C (ng · mL^{-1})	Inter-day R.S.D.(%)	Intra-day R.S.D.(%)	RE (%)
70	71.6	2.2	4.5	−0.4
400	392.8	0.9	8.1	7.8
1000	987.25	0.8	7.3	5.9

Table 2: Short-term and long-term results for assay stability (n = 3)

Experimental condition	Added, C (ng · mL^{-1})	TZO	
		Found, C (ng · mL^{-1}) + S.D.	RE (%)
Short-term stability			
Autosampler samples, 72 h at 15 °C	70	73.6 ± 2.7	−5.1
	1000	955.4 ± 2.1	4.4
2 Freeze-thaw cycles	70	74.4 ± 5.8	−6.4
	1000	945.6 ± 0.5	5.4
Processed sample, 24 h at 4 °C	70	78.4 ± 1.6	12.0
	1000	1137.3 ± 3.1	−13.7
Room sample, 24 h at 28 °C	70	64.5 ± 9.9	7.7
	1000	976.0 ± 9.8	12.0
Long-term stability			
Plasma sample, 45 days storage at −70 °C	70	64.3 ± 7.8	8.0
	1000	984.0 ± 3.0	3.0

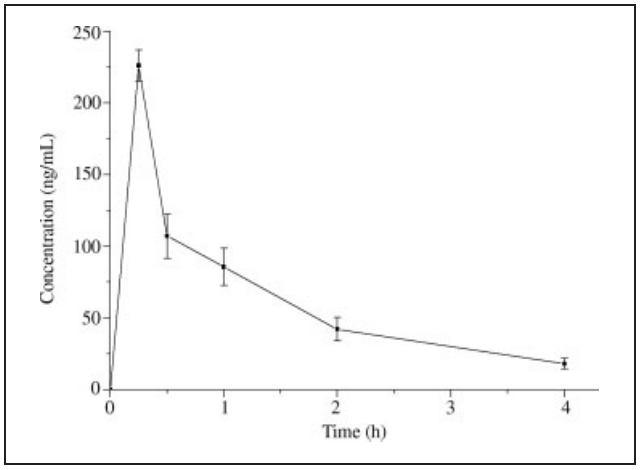


Fig. 3: TZO plasma concentration Vs time obtained after a dose of 7.5 mg · kg oral administration of NTZ in rats

TZO and IS (21.7, 18.6 min) were found from blank plasma samples. From curves analyzed in human plasma a linear relationship ($r = 0.9997$) in a range of 10–1280 ng · mL^{-1} ($y = 0.0014x - 0.013$) was found. No statistical differences were found in slope or intercept between rat and human plasma samples, indicating that human plasma samples could also be analyzed using this methodology. The method is sensitive and simple and can be used for pharmacokinetic or bioavailability studies.

3. Experimental

3.1. Chemicals

TZO was synthesized by Dr. Rafael Castillo-Bocanegra at the Facultad de Química, UNAM. Chemical identity was confirmed by NMR, MS analysis and m.p. determination. NFZ was purchased from Sigma-Aldrich Co., U.S.A. Me and Ac were HPLC grade (Sigma-Aldrich Co., USA). Potassium dihydrogenphosphate (KH_2PO_4) (Sigma-Aldrich Co., USA) and dimethylsulphoxide (DMSO) (Merck, 99%) were of analytical reagent-grade. Water was obtained in-house from a Milli-Q Water System (Millipore Corporation, USA). Rat plasma was obtained from healthy Sprague-Dawley rats from the Instituto Nacional de Neurología y Neurocirugía (México, D.F.). Human plasma was obtained from healthy volunteers.

3.2. Chromatographic method

The chromatographic system consisted of a Waters Alliance 2690 HPLC pump (Waters, Milford, MA, USA), coupled to a photodiode array detector 2996 (Waters, Milford, MA, USA) and a Waters Alliance 2690 autosampler equipped with a 100 µL loop. Separation was performed using an X-Terra C_{18} CC (150 mm × 4.6 mm, 5 mm; Waters Milford, MA, USA) protected with a security guard column (Phenomenex C_{18} ODS). Autosampler and CC oven were held at 15 °C and 25 °C respectively. The initial mobile phase was composed of a mixture 90 : 5 : 5 (v/v) of phosphate buffer (KH_2PO_4 50 mmol · L^{-1}, pH = 5.7):methanol : acetonitrile (PAM) pumped at 0.4 mL · min^{-1}. The gradient was achieved varying linearly the mobile phase composition over 23 min up to final mixture (v/v) 10 : 45 : 45 of PAM.

3.3. Preparation of standard and quality control samples

From a stock solution of 1 mg · mL^{-1} in methanol a dilution was made for the IS working solution (6000 ng · mL^{-1}). Stock solution of TZO (1 mg · mL^{-1}) was prepared by dissolving a weighed amount of 10 mg TZO in a DMSO/acetonitrile solution (30 : 70, v/v). After use, stock solutions of TZO and NFZ were stored at 4 °C. Standard solutions in methanol at concentrations of 10 and 100 mg · mL^{-1} were prepared from serially dilutions of TZO stock solution. Calibration curves were prepared by spiking 100 µL of the appropriate standard solution in 900 µL of drug-free rat plasma in order to obtain the desired concentrations (10, 20, 40, 80, 160, 320, 640 and 1280 ng · mL^{-1}). The same procedure was made for quality control samples (QC) at low, medium and high concentrations (70, 400 and 1000 ng · mL^{-1}). The spiked samples (calibration points and QC) were then processed by the protein precipitation procedure.

3.4. Protein precipitation procedure

A volume of 200 µL of spiked plasma sample was transferred into 1 mL plastic tubes and spiked with 100 µL of IS solution (6000 ng · mL^{-1}). After the addition of 200 µL of acetonitrile, samples were shaken in a vortex for 2 min, and centrifuged for 20 min at 15000 rpm. Supernatant was separated to a clean plastic tube, an aliquot of 200 µL was transferred into the autosampler and 100 µL were injected into the chromatographic system.

3.5. HPLC method validation

3.5.1. Selectivity

In order to determine the selectivity, blank plasma samples from three different rat sources, and hemolyzed plasma samples were analyzed.

3.5.2. Sensitivity

Accuracy and precision of the LOQ were validated with the extraction of five spiked samples of 10 ng · mL^{-1} of TZO.

3.5.3. Linearity in rat plasma

For linearity three calibration curves at concentrations of 10, 40, 80, 160, 320, 640 and 1280 ng · mL^{-1} were subjected to preparation procedure and injected to the HPLC system. Calibration curves were plotted using the relationship between the area of TZO/IS versus concentration and analyzed by unweighted least squares linear regression.

3.5.4. Recovery

The % recovery of TZO and IS after protein precipitation procedure from spiked plasma samples was determined by analyzing five extracted samples of concentrations 70, 400 and 1000 ng · mL^{-1}. The peak area ratio of TZO/IS of these samples was compared with standard solutions in 40 : 20 : 40 v/v water : methanol : acetonitrile at the same concentration as plasma samples.

3.5.5. Precision and accuracy

Accuracy and inter-day precision were calculated on a single day using three replicates of the QC samples. For the intra-day precision three replicates of the low, medium and high quality control points were evaluated over three consecutive days. Accuracy was evaluated as follows: [mean theoretical concentration − mean calculated concentration/mean theoretical concentration] × 100 (% of relative error, %RE), while the precision was given by the inter-day and intra-day in % of relative standard deviation (%RSD).

3.5.6. Stability

The stability of the active metabolite TZO was evaluated using different temperature and time conditions. Short-term stabilities (autosampler, freeze-thaw, processed sample and room temperature) as well as long-term stability (45 days) were carried out during the validation of the analytical method. For all of studies, three replicates of the QC samples at low and high concentrations were processed and analyzed according requirements. Stability test results were compared with fresh QC samples. Autosampler stability was evaluated keeping the processed samples in the autosampler during 72 h at 15 °C. In case of stability of the processed sample, QC were extracted and stored at 24 h at 4 °C. For testing freeze and thaw analyte stability aliquots of QC were stored at −70 °C for 24 h and thawed unassisted at room temperature. This procedure was replicated twice. Finally, for the room temperature stability test, aliquots remained 24 h at room temperature before analysis. Long-term stability was evaluated by storing QC during 45 days at −70 °C.

3.5.7. Pharmacokinetic study in rats

The study protocol complied with the "Guide to the Care and Use of Experimental Animal Care" and was approved by the Animal Ethics Committee of the Universidad Nacional Autónoma de México (UNAM). Six male Sprague-Dawley rats weighing 290 + 10 g received a single oral dose of NTZ in suspension at 7.5 mg · kg. Blood samples of 0.4 mL were drawn via a catheter implanted into the vein tail and collected into heparinized tubes before administration and at 0.25, 0.5, 1.0, 2.0, 4.0, 6.0 h. After sample collection, 0.4 mL of physiologic saline solution was administered for compensation of loss blood. Samples were centrifuged at 3500 rpm for 15 min; plasma was separated and stored frozen at −70 °C until analysis using the validated HPLC method. Pharmacokinetic parameters were determined by a non compartmental model using WinNonLin 4.0 program (Pharsight, Mountain View, CA, USA), and C_{max}, t_{max}, AUC, $AUC_{0\ to\ \infty}$, $t_{1/2}$ and the MRT, were calculated.

3.5.8. Linearity and selectivity in human plasma

Two calibration curves were prepared from a pool of three different sources of human plasma, and analyzed using the same procedure for rat plasma. Slope, intercept and correlation coefficient were determined.

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