

A Validated HPLC Method for the Determination of Tiludronate Disodium on a Novel Brominated Stationary Phase

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Abstract

An analytical method for analysis of Tiludronate; [4-chlorothiophenyl) methylene] bisphosphonate known as Tildren[®] is developed without pre or post derivatization on a newly introduced halogenated stationary phase namely pentabromobenzyl column (PBr column) for the first time.

The optimum HPLC conditions used are: A mobile phase composed of acetonitrile: Water: triethylamine: Acetic acid (50: 50: 0.05: 0.05; v: v: v: v), at a flow rate 0.5 mL/min, at temperature 35°C. Two wavelengths were selected from the UV-spectrum namely 197 and 267 nm.

The method is linear in the range of 0.06-0.6 mg/mL with square of the regression coefficient (r²) 0.9998 at 197 nm. The limit of quantification (LOQ) is 0.040 mg/mL and the limit of detection (LOD) is 0.013 mg/mL. The method is precise with %RSD 0.41-1.36 (intra-day) and %RSD 0.38-1.91 (inter-day). The method is found to be robust at temperature 35°C ± 3°C, wavelength 197 nm ± 1 nm, pH of the aqueous phase ± 0.03 and %ACN 50% ± 1%.

At 267 nm, the method is linear in the range of 0.08-0.8 mg/mL with r² 0.9997, LOQ is 0.050 mg/mL and LOD is 0.016 mg/mL. The %RSD for intra-day precision ranges from 0.96-1.74, while for inter-day precision ranges from 1.09-2.32. The method is proved robust in temperature 35°C ± 3°C, wavelength 297 nm ± 3 nm, pH of the aqueous phase ± 0.03 and %ACN 50% ± 1%.

The method is accurate with % recovery ranged from 98.82-99.42% with %RSD ranging from 0.210-1.023.

This method is suitable for the assay of tiludronate in both bulk and in its veterinary pharmaceutical formulations.

Keywords: Tiludronate disodium; Halogenated stationary phases; Pentabromobenzyl group bonded (PBr) column; Veterinary pharmaceutical analysis; Non-nitrogen containing bisphosphonates

Abbreviations:

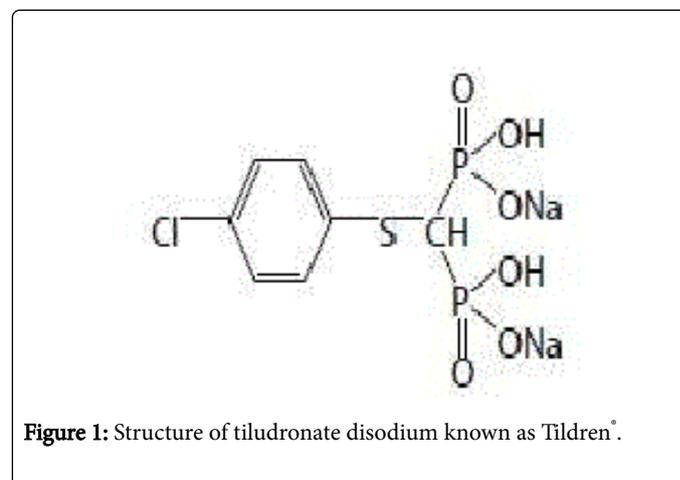
Ac: Acetic Acid; CAN: Acetonitrile; FDA: Food and Drug Administration; LOD: Limit of Detection; LOQ: Limit of Quantification; MeOH: Methanol; %RSD: % Relative Standard Deviation; PBr column: Pentabromobenzyl column; TEA: Triethylamine

Introduction

Bisphosphonates are a class of drugs that act as inhibitors of bone resorption in humans [1]. The study of their action began in 1960 and they can be classified into Nitrogen-containing e.g., Neridronate, Alendronate, Pamidronate, Olpadronate, Risedronate, Zoledronate, Ibandronate or non-Nitrogen containing bisphosphonates e.g., Clodronate, Etidronate, Tiludronate [2].

Tiludronate; [4-chlorothiophenyl) methylene] bisphosphonate known as Tildren[®] belongs to the non-Nitrogen-containing

bisphosphonates (Figure 1) which is introduced in 2011 as veterinary medications for the treatment of horses [3]. The drug was approved by the Food and Drug Administration (FDA) in 2014 introduced in the United States markets [4].

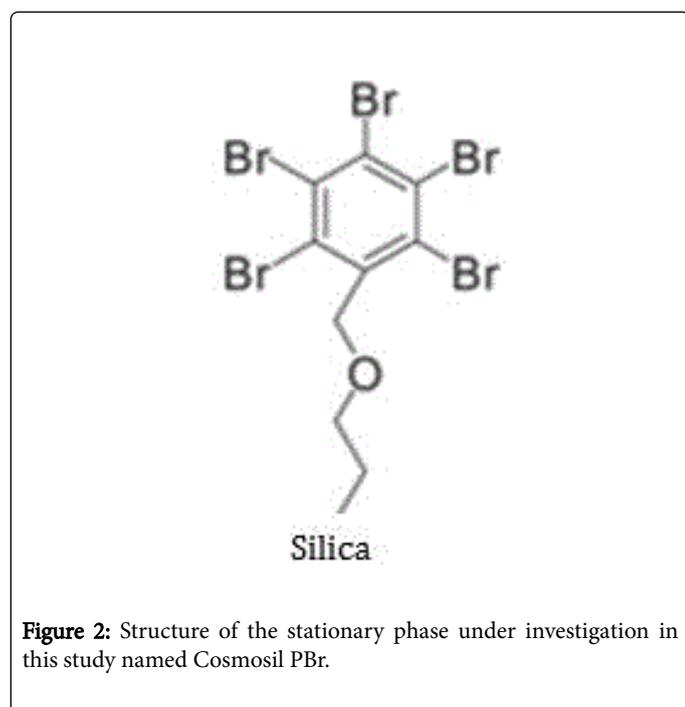


It is used in the treatment of the clinical symptoms of navicular syndrome in horses. Navicular syndrome is caused by increases in mechanical stress on the navicular bone and leads to increase in osteoclastic activity, followed by disruption in normal bone remodeling those results in the process of bone lysis overcoming bone formation.

Tildren[®] is administrated intravenously; it penetrates the osteoclasts by endocytosis resulting in decreasing the osteoclastic activity and ultimately decreasing bone resorption [5]. Lately tiludronate; due to its particular anti-inflammatory and analgesic properties, has also been used to treat other equine osteo-degenerative pathologies [6].

To date, few methods have been reported in literature for the analysis of Tildren[®] such as liquid chromatographic analysis using mass spectrometry by Wong et al. [3] and Popot et al. [1] and HPLC-UV for tiludronate by Fel et al. [7].

Pentabromobenzyl-bonded silica stationary phase known as Cosmosil PBr is a newly introduced halogenated stationary phase. The structure of this new phase is shown in Figure 2.



The aim of this work is to develop a validated method for the direct analysis of Tildren[®], without pre- or post-derivatization on a newly halogenated stationary phase namely pentabromobenzyl column (Cosmosil PBr column). To the best of our knowledge, this is the first report describes the analysis of this drug on Cosmosil PBr column.

Experimental Procedure

Equipment

The analysis was performed on Thermo Fisher UHPLC Dionex Ultimate 3000. It consists of pump (ISO-3100SD), autosampler (WPS 3000 SL), column thermostat (TCC-3000 SD) and Diode Array Detector (DAD-3000 RS). The data acquisition is collected using Chromeleon 6.8 software.

The column used is Cosmosil PBr, pentabromobenzyl group bonded phase, (150 × 4.6 mm, 5 μm), purchased from Nacalai Tesque (Kyoto, Japan).

Materials and reagents

Methanol (MeOH) (HPLC grade), acetonitrile (ACN) (HPLC grade), triethylamine (TEA) and glacial Acetic acid (AC) were purchased from Sigma-Aldrich, Germany.

Tiludronate disodium and its veterinary formulation Tildren[®] and were obtained from Ceva Santé Animale (Libourne, France).

Methods

For the standard stock solution (0.1 mg/mL) was prepared by dissolving 10 mg in 100 mL deionized water.

Each Tildren[®] vial contains 500 mg tiludronic acid as tiludronate disodium and 250 mg mannitol USP as excipient. For its assay, 10 mg was dissolved into 100 mL deionized water. The solutions were kept at 5°C and covered with foils for weeks. The injection volume in all runs was 10 μL.

The optimum condition was achieved using mobile phase consisting of acetonitrile: Water: triethylamine (TEA): Acetic acid (AC) (50: 50: 0.05: 0.05; v: v: v: v), at a flow rate 0.5 mL/min, at temperature 35°C. The mobile phase was filtrated using 0.45 μm membrane filter, and then allowed to remain in ultrasonic bath for 30 min. The UV detector was adjusted at wavelength 197 nm and 267 nm.

The method validation was performed according ICH guidelines [8]. The parameters studied were linearity, Limit of Detection (LOD), Limit of Quantification (LOQ), precision (Inter- and Intra-day), accuracy and robustness. It is worth mentioning that the validation steps were performed at two wavelengths 197 nm and 267 nm.

For linearity, 12 calibrations standards were prepared by serial dilution of tiludronate, covering a range of 0.06 mg/mL to 0.8 mg/mL. Each of these 12 concentrations was injected 3 times. Then, a calibration curve was constructed by plotting the peak area against the corresponding concentrations (mg/mL) to study the linearity. Results were evaluated by calculating square of the regression coefficient (r²).

The Limit of Detection (LOD) is the lowest amount that can be detected. It was calculated from the calibration curve using this equation:

$$\text{LOD}=3.3 (\sigma/S)$$

LOQ is the lowest amount of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. It was calculated from the calibration curve using this equation:

$$\text{LOQ}=10 (\sigma/S)$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve [8].

Precision is describing the degree of repeatability of an analytical method. In this work, the precision of the methods was evaluated in terms of inter- and intra-day precision. Both were expressed by calculating % relative standard deviation (%RSD) [9]. This was performed by analyzing 3 different concentrations (0.08, 0.1, 0.2 mg/mL) three times on the same day (n=9): Intra-day precision (repeatability), for 3 different days: Inter-day precision. Each of these 3

concentrations has been prepared three times and each was injected three times.

Accuracy is the closeness of the accepted value and the found value. Tildren® (0.1 mg/mL) was spiked with known amount of standards 0.10 mg/mL, 0.20 mg/mL and 0.30 mg/mL, so that the final concentrations are 0.2 mg/mL, 0.3 mg/mL and 0.4 mg/mL, respectively. Each sample was injected three times. The results were expressed as percentage recovery (% recovery) and %RSD [10]. For robustness, of the method, it was evaluated by the capacity of the method to remain uninfluenced by small changes in the experimental parameters [8].

Results and Discussion

Method development

Initially, the condition applied was water: ACN (50: 50; v: v) at flow rate 0.7 mL/min and temperature 25°C, the tiludronate peak eluted early at 1.643 min as shown in Figure 3A. To increase the retention time, an increase in the percent aqueous phase was investigated, but it was observed that it led to peak split as shown in Figure 3B; this could be attributed to the high polarity of tiludronate. There is a competition between the aqueous phase mobile and tiludronate on the stationary phase. This split appeared even when the mobile phase composition was water: ACN (60:40; v: v).

Methanol was investigated as an alternative solvent based on the fact that it falls in a different part of the solvent selectivity triangle than CAN, and hence could create a dramatic change in analysis [11]. The mobile phase condition was water: MeOH (50: 50; v: v) at flow rate 0.7 mL/min and temperature 25°C. The peak eluted slightly later in comparison to ACN (at tR 2.393 min) however, the peak was broad as shown in Figure 3C. Accordingly, ACN was selected to be the organic solvent in this work.

Different conditions were then investigated to achieve optimum specifications for this HPLC method. As lowering the flow rate will increase tR, but broaden the peak, we decreased flow rate to 0.5 mL/min accompanied by increase of the column temperature up to 35°C, leading to enhanced peak shape.

Therefore, the condition: ACN: water (50: 50; v: v) at flow rate 0.5 mL/min and temperature 35°C was investigated. The tiludronate peak appeared at 2.157 min (Figure 3D). Varying the conditions to: ACN: water: AC (50: 50: 0.05; v: v: v; pH4.05) at flow rate 0.5 mL/min and temperature 35°C lead to a slight increase in tR (2.770 min) (Figure 3E).

Ion-pair chromatography is a very common technique in analyzing polar organic compounds on RP-LC [2], and alteration in the mobile phase has been accomplished by adding Ac and TEA (0.05:0.05; v: v). Because of this, the following condition were applied using a mobile phase consisting of ACN: water: AC: TEA (50: 50: 0.05: 0.05; v: v: v: v; pH 5.09) at flow rate 0.5 mL/min and temperature 35°C.

The tiludronate peak then eluted at 2.290 min (Figure 4A). It was observed that when the pH increased, the retention time (tR) slightly decreased. This can be attributed to the fact that at higher pH tiludronate becomes more ionized, and hence would elute earlier. The UV spectrum of tiludronate under the above-mentioned conditions was monitored. The optimal wavelengths were found to be 197 nm and 267 nm (Figure 4C).

Because our investigation found the condition ACN: water: AC: TEA (50: 50: 0.05: 0.05; v: v: v: v) at flow rate 0.5 mL/min and temperature 35°C showed an optimum retention time and better peak shape in comparison to other conditions assessed, it was selected to be the optimum condition and the validation was accomplished under these specifications on both wavelengths (197 nm and 267 nm).

The identified optimum condition for HPLC of Tildren® on Cosmosil PBr column was investigated at two wavelengths: 197 nm (Figure 4A) and 267 nm (Figure 4B). At 197 nm, it showed the highest sensitivity toward the analyte and at 267 lower sensitivity but the baseline was more stable, since it was above the cutoff of the mobile phase as shown in Figures 4A and 4B respectively.

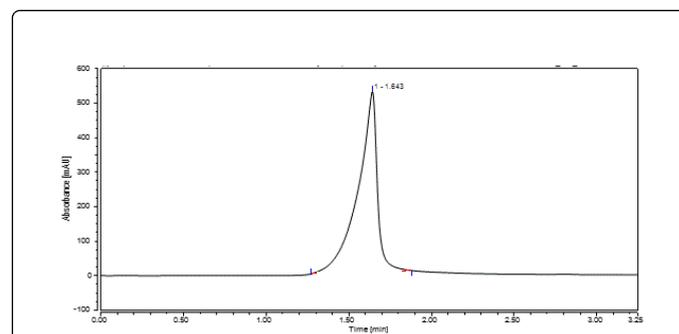


Figure 3A: Chromatograms of tiludronate disodium on PBr column showing different conditions: (A): Mobile phase ACN: Water (50: 50; v: v); flow rate 0.7 mL/min, T=25°C.

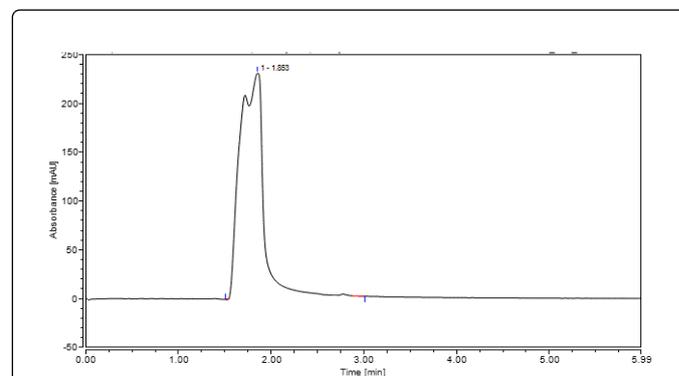


Figure 3B: Mobile phase ACN: Water (60: 40; v: v); flow rate 0.7 mL/min, T=25°C.

Interaction between the analyte and the stationary phase can be explained from the following interactive forces: π - π interactions between the pentabromobenzyl group on the stationary phase and the para-chlorophenyl group on the analyte. A competition between the two phosphonate groups and water content in the mobile phase on the stationary phase due to the formation of hydrogen bonding. This could explain the peak splitting associated with the higher percentage of the aqueous phase. London Dispersion force, a weak intermolecular force that results from dipoles temporarily induced from random unsymmetrical electron positions in two adjacent atoms, also known as "instantaneous dipole-induced dipole force".

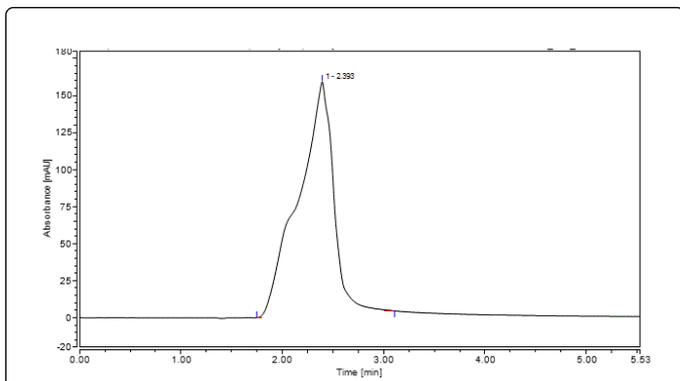


Figure 3C: Mobile phase MeOH: Water (50: 50; v: v); flow rate 0.7 mL/min, T=25°C.

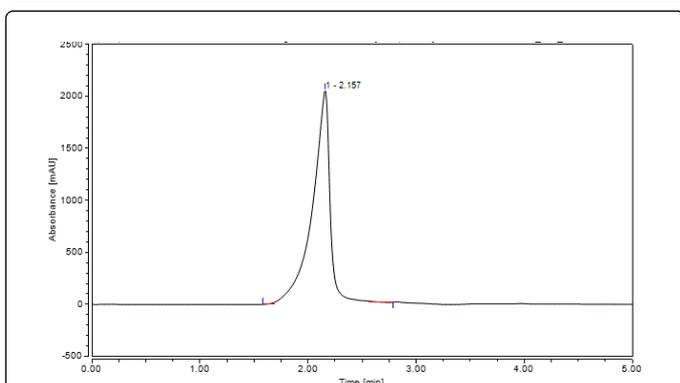


Figure 3D: Mobile phase ACN: Water (50: 50; v: v); flow rate 0.5 mL/min, T=35°C.

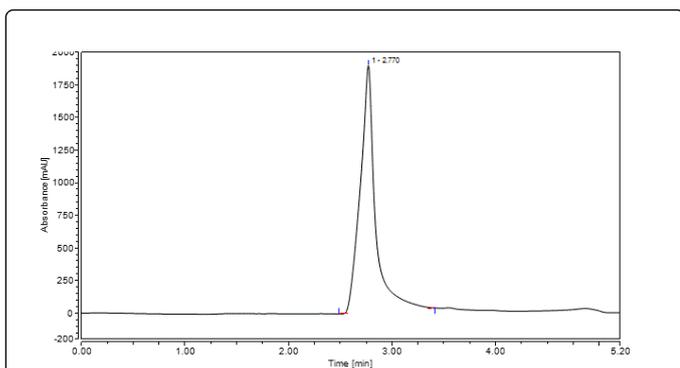


Figure 3E: Mobile phase ACN: Water: AC (50: 50: 0.05; v: v: v); flow rate 0.7 mL/min, T=25°C.

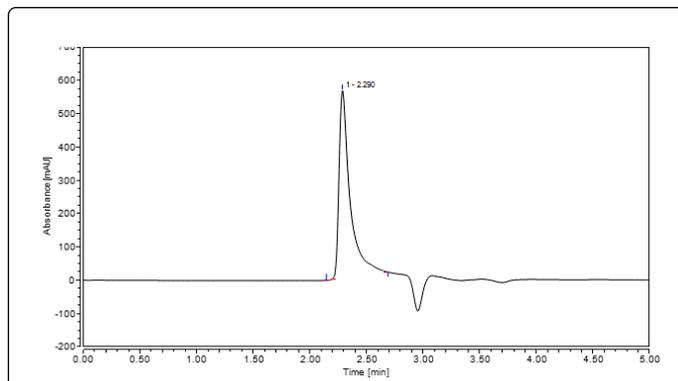


Figure 4A: Chromatograms of Tiludronate disodium on PBr column at optimum condition: Mobile phase ACN: Water: TEA: AC (50: 50: 0.05: 0.05; v: v: v: v); flow rate 0.5 mL/min, T=35°C, UV=197 nm, C: Mobile phase MeOH: Water (50: 50; v: v); flow rate 0.7 mL/min, T=25°C.

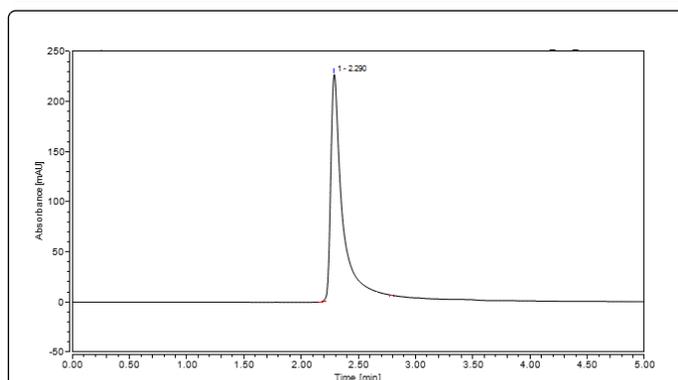


Figure 4A: Chromatograms of Tiludronate disodium on PBr column at optimum condition: Mobile phase ACN: Water: TEA: AC (50: 50: 0.05: 0.05; v: v: v: v); flow rate 0.5 mL/min, T=35°C, UV=197 nm.

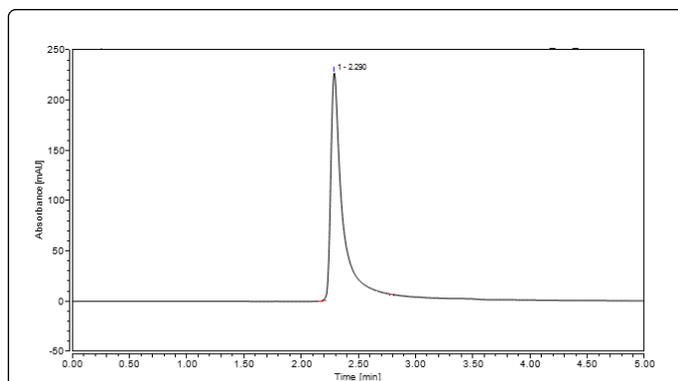


Figure 4B: Chromatograms of Tiludronate disodium on PBr column at optimum condition: Mobile phase ACN: Water: TEA: AC (50: 50: 0.05: 0.05; v: v: v: v); flow rate 0.5 mL/min, T=35°C, UV=267 nm.

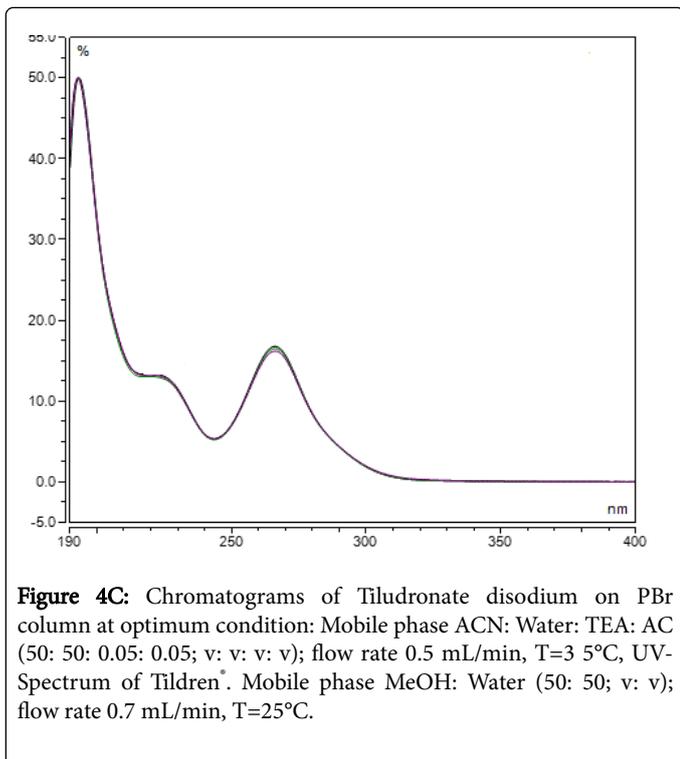


Figure 4C: Chromatograms of Tiludronate disodium on PBr column at optimum condition: Mobile phase ACN: Water: TEA: AC (50: 50: 0.05: 0.05; v: v: v: v); flow rate 0.5 mL/min, T=3 5°C, UV-Spectrum of Tildren[®]. Mobile phase MeOH: Water (50: 50; v: v); flow rate 0.7 mL/min, T=25°C.

Method validation

At 197 nm, the calibration curve plotted for tiludronate was linear in the concentration range of 0.06 mg/mL to 0.6 mg/mL. The regression equation for the calibration curve is $y=622.13 \times -0.9841$. The square correlation coefficient (r^2) was found to be 0.9998, which indicates good linearity of the method in this range. The limit of quantification (LOQ) and Limit of Detection (LOD) was found to be 0.040 mg/mL and 0.013 mg/mL respectively for standard drug solutions.

Intra-day precision was investigated by preparing three concentrations: 0.08, 0.1 and 0.2 mg/mL; three times each; then each was injected three times and the %RSD was 1.34, 1.36 and 0.41, respectively.

The three previously mentioned concentrations were injected over 3 consecutive days to assess the inter-day precision and the %RSD was 1.91, 1.48 and 0.38, respectively, as shown in Table 1.

The robustness of the method was assessed by RSD values by carrying out small changes in the following parameters: Temperature, wavelengths and %ACN and the results were as follow:

Temperature $35^\circ\text{C} \pm 3^\circ\text{C}$ with %RSD 0.47%.

Wavelength $197 \text{ nm} \pm 1 \text{ nm}$ with %RSD 2.56%.

% Acetonitrile $50\% \pm 1\%$ with %RSD 0.87 %.

pH of the aqueous phase ± 0.03 with %RSD 1.51%.

While at 267 nm, the calibration curve plotted for tiludronate was linear in the concentration range of 0.08 mg/mL to 0.8 mg/mL. The regression equation for the calibration curve was $y=353.15 \times -3.9178$, the correlation coefficient (r^2) was 0.9997, the LOQ was 0.051 mg/mL and the LOD was 0.016 mg/mL as shown in Table 1.

Parameters	Wavelength 197 nm		Wavelength 267 nm	
	%RSD (Intra-day precision)	%RSD (Inter-day precision)	%RSD (Intra-day precision)	%RSD (Inter-day precision)
Regression Equation, r^2	$y=622.13 \times -0.9841$ 0.9998		$y=353.15 \times -3.9178$ 0.9997	
LOQ (mg/mL)	0.04		0.051	
LOD (mg/mL)	0.013		0.016	
Precision				
0.08 mg/mL	1.34	1.91	1.69	2.32
0.1 mg/mL	1.36	1.48	1.74	2.11
0.2 mg/mL	0.41	0.38	0.96	1.09

Table 1: Validation parameters of Tildren[®] using different wavelengths (197 nm and 267 nm).

For intra-day precision, the %RSD of the three concentrations: 0.08, 0.1 and 0.2 mg/mL were 1.69, 1.74 and 0.96 respectively; while for inter-day precision, the %RSD was 2.32, 2.11 and 1.09.

For the robustness, the %RSD for the following parameters was as follow:

Temperature $35^\circ\text{C} \pm 3^\circ\text{C}$ with %RSD 0.48%.

Wavelength $267 \text{ nm} \pm 3 \text{ nm}$ with %RSD 2.13%.

% Acetonitrile $50\% \pm 1\%$ with %RSD 1.39 %.

pH of the aqueous phase ± 0.03 with %RSD 1.93%.

Similar results were obtained for accuracy using detection for both wavelengths. The % recovery, as shown in Table 2, ranged from 98.82% to 99.42%, and the %RSD ranged from 0.21 to 1.02.

Theoretical concentration (mg/mL)	Actual concentration*(mg/mL)	%RSD	%Recovery*	%RSD
0.2	0.198	0.982	99.42	1.023
0.3	0.297	0.28	99.25	0.211
0.4	0.395	0.67	98.82	0.53
*Average of 3 determinations				

Table 2: Accuracy of Tildren[®].

Application on preparation

A 10 mg Tildren[®] of was dissolved into 100 mL deionized water. 10 μL was injected under the identified optimum condition on PBr column and t_R was 2.307 min, as presented in Figures 5A and 5B.

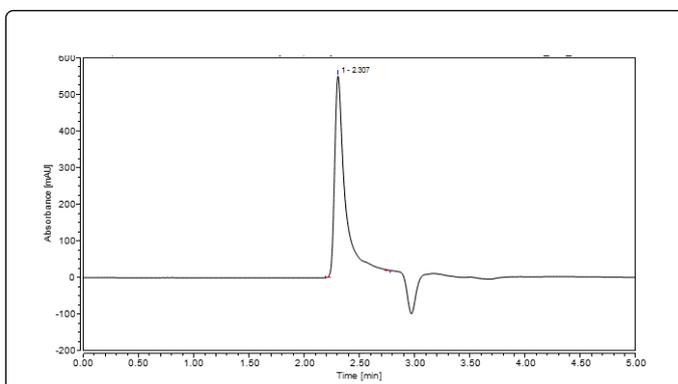


Figure 5A: Chromatograms of Tildren[®] on PBr column at optimum condition: Mobile phase ACN: Water: TEA: AC (50: 50: 0.05: 0.05; v: v: v: v); flow rate 0.5 mL/min, T=35°C, UV=197 nm.

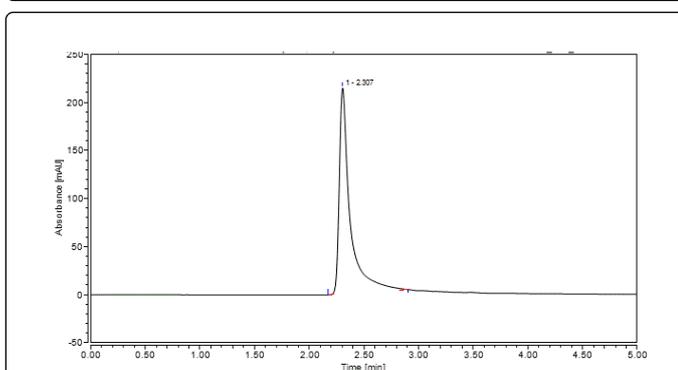


Figure 5B: Chromatograms of Tildren[®] on PBr column at optimum condition: Mobile phase ACN: Water: TEA: AC (50: 50: 0.05: 0.05; v: v: v: v); flow rate 0.5 mL/min, T=35°C, UV=267 nm.

Conclusion

A method development for tiludronate disodium on PBr column without any prior- or post-derivatization on PBr column was described for the first time.

The optimum chromatographic condition was acetonitrile: Water: TEA: AC (50: 20: 0.05: 0.05; v: v: v: v), at a flow rate 0.5 mL/min, at temperature 35°C. From the UV-spectrum, two optimum wavelengths were selected: 197 and 267 nm. The interactive forces which are involved in the separation included π - π interactions and hydrogen bonding.

The proposed method was validated according to ICH guidelines in terms of linearity, LOD, LOQ, precision (Inter- and Intra-day), accuracy and robustness.

At 197 nm, the method was linear in the range of 0.06-0.6 mg/mL with r_2 0.9998. The LOQ was 0.040 mg/mL and the LOD was 0.013

mg/mL. The %RSD for intra-day precision ranged from 0.41-1.36, while for inter-day precision ranged from 0.38-1.91. The method was found to be robust at temperatures 35°C \pm 3°C, wavelength 197 nm \pm 1 nm, pH of the aqueous phase \pm 0.03 and % CAN of 50% \pm 1%.

At 267 nm, the method was linear in the range of 0.08-0.8 mg/mL with r_2 0.9997. The LOQ was 0.050 mg/mL and the LOD was 0.016 mg/mL. The %RSD for intra-day precision ranged from 0.96-1.74, while for inter-day precision ranged from 1.09-2.32. The method was found to be robust at temperatures 35°C \pm 3°C, wavelength 297 nm \pm 3 nm, pH of the aqueous phase \pm 0.03 and % CAN of 50% \pm 1%.

Accuracy of the % recovery ranged from 98.82%- 99.42% with %RSD ranging from 0.210-1.023.

The proposed method was applied on Tildren[®] formulation and was found to be in good correlation with the standard. Accordingly, the proposed method was applicable for the analysis of tiludronate in bulk and preparation in less than 3 min.

Conflicts of Interest

The authors declare no conflict of interest.

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