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Short communication

An improved HPLC method overcoming Beer's law deviations arising from supramolecular interactions in tolfenamic acid and cyclodextrins complexes

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Abstract

Inclusion complexes of tolfenamic acid (TA), a non-steroidal anti-inflammatory drug, with methyl-βcyclodextrin and hydroxypropyl-β cyclodextrin were prepared and characterised. Spectrophotometric, chromatographic (RP-HPLC) and ^{1}H NMR studies of the complexes were conducted. It was observed that cyclodextrins influence TA's molar absorptivity leading to Beer's law deviation. Consequently, the accuracy problem arose, urged for the application of specific chromatographic conditions for the determination of TA in the presence of CDs. A new HPLC method was developed and validated. TA was analysed on a C18 column 5 μm (150 × 4.6 mm), using a column thermostat regulated at 30°C. The mobile phase consisted of methanol-phosphate buffer solution (pH 3.2; 0.07 M) (90:10 v/v) and the flow rate was set at 2.0 ml min^{-1}. The detector was operated at 286 nm. TA was successfully determined, overcoming the problems arising from the presence of cyclodextrins.

Keywords: Tolfenamic acid; Methyl and hydroxypropyl-βcyclodextrin complexes; Reversed-phase chromatography; Method validation

1. Introduction

Tolfenamic acid (TA) is a non-steroidal anti-inflammatory drug (NSAID), which along with the other derivatives of anthranilic acid (mefenamic acid, meclofenamic acid, flufenamic acid), are widely used as analgesic, anti-inflammatory and antipyretic drugs. These agents inhibit the biosynthesis of prostaglandins (PG), as a consequence of interfering within the arachidonic acid cascade, as well as the PG receptors in certain

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tissues [1,2]. It is obvious that TA, like the rest of the NSAIDs, may provoke chronic gastrointestinal (g.i.) irritation or local damage of the g.i. mucosa. When administered p.o., TA is absorbed by the g.i. tract, though the rate of absorption is relatively slow [3]. It presents rather extensive binding (99.7%) to the plasma proteins and undergoes enterohepatic circulation [1]. TA is practically insoluble in water resulting in serious problems of bioavailability and making the preparation of new formulations difficult.

The complexation of TA with macromolecules, such as the cyclodextrins, may resolve some of these problems and may also reduce the undesirable effect on the g.i. mucosa. Cyclodextrins (CDs) are naturally occuring cyclic oligosaccharides, known for their effect on stability, solubility and bioavailability of various drugs, as well as for the reduction of drugs' side-effects [4–6]. The α-1,4-linked D-glucopyranose units form a CD ring, which is shaped like a truncated cone. Entire molecules or parts of them can penetrate in the semipolar cavity forming an inclusion complex. Complexes of different stoichiometries can be formed, depending on the polarity and the size of the guest molecule and on the dimensions of the CD's cavity.

Although the studies of CDs complexes are very extensive, there is no reference on analytical methods that can be applied in order to assay the active ingredient in the presence of CDs. The quantitative determination of the compounds of a pharmaceutical (or other) preparation is the most important task of the quality control analyst.

In this work, complexes of TA with hydroxypropyl-βCyclodextrin (HPβCD) and methyl-βCyclodextrin (MeβCD) were prepared and characterized. During the spectrophotometric determination of TA in the presence of cyclodextrins, Beer's law deviation of the measurements was observed. In order to counterbalance the accuracy problems arising from this phenomenon, we developed and evaluated an HPLC method, suitable for the determination of TA in the presence of cyclodextrins. Appropriate corrections were made in order to improve the UV method.

2. Experimental

2.1. Materials and reagents

TA, as well as the reagents and the solvents (HPLC grade) were kindly offered by ELPEN S.A. Pharmaceutical (Attica, Greece). HPβCD and MeβCD were purchased from Sigma–Aldrich. The water used, was deionized and filtered by a Milli-Q Plus water purifying system (Millipore). Mobile phases were vacuum filtered and degassed by Milli-Q Plus system (Millipore) through a 0.45 μm pore PTFE membrane filter (Waters). The column used for the analysis of TA was purchased from MZ (Mainz, Germany) and the alternative columns used for the control of the ruggedness of the method were purchased from Merck (Darmstadt, Germany).

2.2. Instrumentation

Absorption spectra were recorded with a Hitachi UV spectrophotometer, model U2000. The measurements were carried out at room temperature. ^{1}H-NMR spectra were recorded on a Bruker DRX 400 MHz spectrometer in 20 μl NaOD 40% and 9.80 ml 99.9% D_2O (Sigma). The HPLC chromatograms were obtained with a Waters 600E pump + controller, a mobile phase degassing system using Helium sparging, Waters 996 photodiode array detector and the Millenium 2010 as software. The same system was also used for the analyses of TA's solutions.

2.3. Chromatographic conditions

TA was analyzed on a reversed-phase Lichrospher C18 column (150 × 4.6 mm, 5 μm), using a column thermostat regulated at 30°C. The mobile phase consisted of methanol–phosphate buffer solution (pH 3.2;0.07 M) (90:10 v/v) and the flow rate was set at 2.0 ml min^{-1}. The detector was operated at 286 nm and a 25 μl loop was used for sample injection. The injections were carried out by Waters 717 plus autosampler.

Previous methods reported for the determination of TA [1,7–11], were not appropriate for the analysis of TA in the presence of cyclodextrins. In

solutions of TA's CD complexes, the main goal was to detect TA in its unionized and totally decomplexed form. For this purpose the optimal conditions were chosen; 90% of the mobile phase consisted of Methanol, which is known to be an agent competitive for the complexation, and 10% of a phosphate buffer solution with a pH value 3.2, in order to obtain an extensive percentage of TA unionized ($pK_a = 4.3$). A characteristic chromatogram obtained, is shown in Fig. 1. Also, phase solubility studies (manuscript in preparation) proved that the complexation process is less intense in acidic pH, leading us to the choice of the specific pH value. Finally, the use of temperature, does not only improve the appearance of the peak, but also facilitates the decomplexing process.

3. Results

3.1. Characterization of the complexes

Proton NMR spectrometry is commonly used for the investigation of the structures of CDs complexes. The chemical shifts variations of the spectra of both TA and CDs, indicate the formation of the complexes. CDs' H3 and anomeric protons undergo upfield shift, allowing the assumption that the guest molecule penetrates into the CDs' cavity and that there is some kind of interaction between H1 and the guest. The chemical shift changes of TA spectrum suggest that the 1:2 complex is formed.

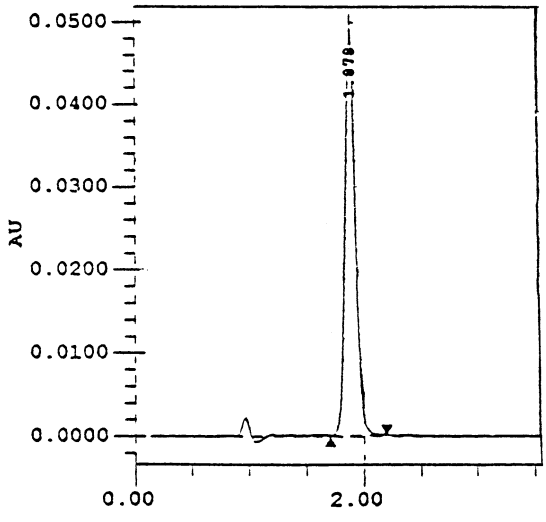

Fig. 1. Characteristic chromatogram obtained with the HPLC method, used for the determination of TA in the presence of cyclodextrins.

The stoichiometry of the complexes is determined with the continuous variation technique (Job's method). This procedure is extensively employed using NMR data. The stoichiometry of the complexes can be determined considering the protons of both TA and CD. Consequently, by the Job plots obtained, the 1:2 stoichiometry was verified. In Fig. 2, an indicative Job plot obtained by analyzing a series of solutions of TA and MeβCD at different ratios, is depicted. The 1:2 stoichiometry is confirmed since $r = 0.63$.

3.2. Effect of the complexation process on the spectroscopic data of TA

The influence of CDs on the spectral behaviour of several molecules is extensively reported in the literature [12–16]. These perturbations are similar, to those observed with the TA complexes studied in this work.

Inclusion complexes of TA with βCD, MeβCD and HPβCD were prepared in aqueous solutions. After 48 h of continuous stirring at room temperature the equilibrium is reached. Remarkable is the fact that the complexation with MeβCD is achieved faster than with HPβCD. The presence of two aromatic rings in TA's molecule contributes to its hydrophobic properties, facilitating the complexation with MeβCD [15].

Upon addition of increasing amounts of CDs the λ_{max} of TA is red shifted. In Fig. 3, the UV absorption spectra of TA's aqueous solutions containing various concentrations of HPβCD is depicted. The shift of λ_{max} is ascribed to the change of the environment of TA, caused by the complex formation and is more apparent with the HPβCD complexes, because of the hydrogen bonds that can be formed between TA's carboxyl-group and CDs' secondary hydroxyls and the hydroxypropyl-groups of HPβCD. TA's carboxyl-group, which is probably located outside the CD's cavity,

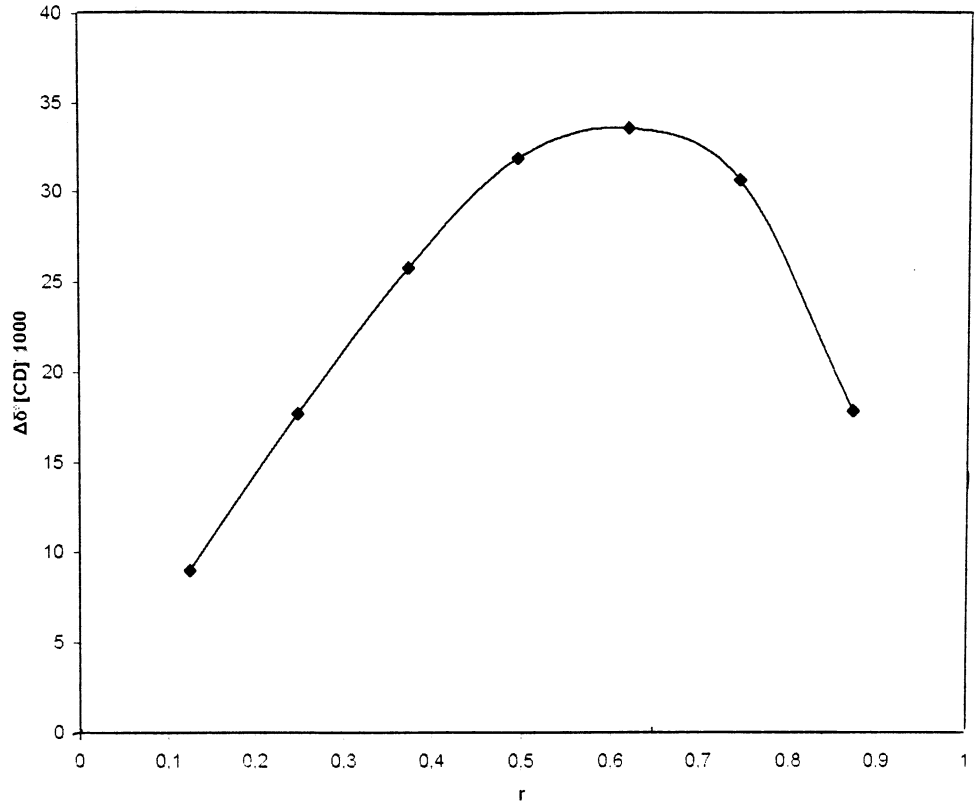

Fig. 2. Job plot obtained using chemical shift variations from selected protons of MeβCD.

remains in a more polar environment because of the presence of the secondary hydroxyls of CDs and water molecules. The bathochromic shift of the K-band (286 nm) of the chromophore is, possibly, a result of this polarity.

Especially interesting is the influence of the CDs' concentration on the molar absorptivity (ε) of TA (Fig. 4). TA exhibits a continuous increase in absorption at stoichiometries 1:1 and 1:2. Further increase of the CDs' concentration results in a continuous decreasing of the drug's absorption. These pronounced changes of molar absorptivity (ε) result in Beer's law deviation of the measurements and lead to inaccurate measurements with UV spectrophotometry. It is possible, also, that the presence of both complexed and free forms of TA in the solution, contributes to this phenomenon. By applying the relationship $A_x = A_{obs}(\varepsilon_x/\varepsilon_{obs})$ separately for each stoichiometric ratio, the correct values of A and ε are calculated.

Preliminary developed HPLC methods were also affected by the spectroscopic changes of TA in the presence of CDs, urging for the use of specific chromatographic conditions. Therefore, in order to make possible the quantitative determination of TA in the presence of CDs, an HPLC

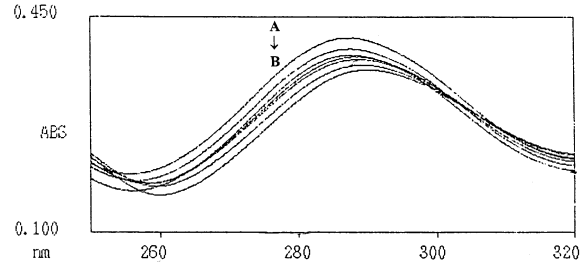

Fig. 3. UV absorption spectrum of TA (3.93×10^{-5} M) at various HPβCD concentrations. Read from A to B: 7.88×10^{-5}, 3.93×10^{-5}, 1.96×10^{-5}, 2.36×10^{-4}, 3.94×10^{-4}, 7.88×10^{-4}, 1.57×10^{-3} M.

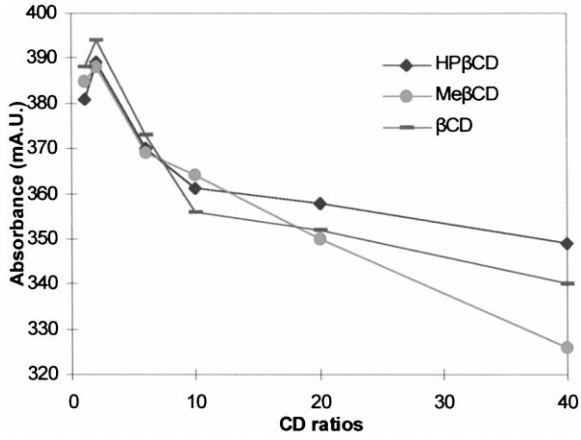

Fig. 4. Effect of various CDs' concentration on TA's UV absorbance.

method was developed and validated, with which TA is detected at its unionized and fully decomplexed form.

3.3. Validation of the chromatographic method

The definition of the parameters for the validation of a method vary between different authors, hence the validation of the specific method was carried out according to the criteria described in USP XXIII [17]. System suitability tests [18] were also performed in order to validate the chromatographic system.

The suitability of the chromatographic system was tested by injecting ten times at 3 different days a test solution of TA. The relative standard deviation (RSD) of the peak response was $\leq$ 1.0%, the tailing Factor (T) was around 1.0 and the theoretical plate number (N_p) was not less than 2000.

The linearity of the developed method was determined by analyzing a series of standard aqueous solutions of TA at six different concentrations in the range of 50–150% of the expected working assay. The correlation coefficient and the regression line were found to be excellent. The repeatability of the method was assessed by analysing a series of six aqueous solutions of TA at 2 different days. The analyses were carried out by the same operator, in the same laboratory, using the same equipment. RSD was calculated to be less than 1.0%, even though the acceptable limits for the calculated concentrations are 7.3–11.0% [19]. Intermediate precision was defined within the same laboratory and the RSD of the peak response was less than 1.0%.

The same procedure was followed for the aqueous solutions of TA's complexes with MeβCD and HPβCD. The calculated RSDs for both repeatability and intermediate precision studies were $\leq 1.3\%$.

The accuracy of the method was examined with recovery experiments, by applying the analytical method to samples to which known amounts of the analyte have been added. The mean recovery was 99.73%. Recovery experiments were also carried out, by adding known amounts of the analyte to mixtures of sample with the matrix (CDs). The average recovery of TA from aqueous solutions containing the complex TA/MeβCD was 99.13% and from aqueous solutions containing the complex TA/HPβCD was 98.60%. The method is considered suitable for the determination of TA in solutions containing CDs. In order to verify the results obtained from the recovery experiments, two series of aqueous solutions containing the same concentrations of TA and various concentrations of HPβCD and MeβCD, were assayed, with a spectrophotometer and a high performance liquid chromatograph. The results obtained spectrophotometrically, differ within the same series of solutions, indicating that the complexation affects the spectroscopic behaviour of TA. By applying the HPLC method, TA is successfully determined even in the presence of different concentrations of CDs. The accuracy problems are eliminated.

Finally, the ruggedness of the analytical method was studied, by examining the influence of a variety of different operational and environmental conditions on the analysis results. Flow rate ($\pm$ 0.4 ml min^{-1}), column temperature ($\pm$ 5°C), pH value ($\pm$ 0.4 pH units), detector's wavelength (220 nm) and mobile-phase composition were changed within a realistic range. The nominal conditions of the analysis were applied by a different operator, with a different piece of equipment, with a different column (5 μm Lichrosorb C18 125 × 4.0

Table 1
Study of the ruggedness of the HPLC method

Various conditions	Precision (%)	RSD (%) ($n = 3$)	Retention time (min)
Different column			
125 × 4.0 mm, 5 μm	99.10	0.34	1.4
Two different lots of the column of the same manufacturer			
1st	99.14	0.03	1.7
2nd	98.95	0.03	1.7
Column of different age	98.84	1.36	1.9
Flow rate			
−0.4 ml min^{-1}	99.61	0.92	2.5
+0.4 ml min^{-1}	99.45	0.26	1.6
Temperature			
+5°C	102.32	0.05	1.9
−5°C	101.29	1.94	1.9
pH			
+0.4 pH units	100.78	0.62	2.0
−0.4 pH units	103.0	1.87	2.1
Different ratios of mobile phase			
CH_3OH:phosphate buffer pH 3.2 (80:20)	98.93	0.02	3.6
CH_3OH:phosphate buffer pH 3.2:CH_3CN (80: 10: 10)	98.91	0.02	1.8
Detection wavelength (220 nm)	100.60	0.03	1.8
Different HPLC system			
Alliance 2690 separation module/waters	103.9	0.33	1.8
Different operator	98.78	0.40	2.0

mm), with two different lots of the column of the same manufacturer and finally with a column of different age. The degree of reproducibility was expressed as a function of the assay parameters and compared to the assay under the nominal chromatographic conditions (Table 1).

The affect of the parameters was within the specified tolerance range, hence within the method's ruggedness range.

4. Conclusions

The presence of CDs in the solution influence the spectroscopic data of TA, making difficult the quantitative determination of TA with the usual analytical methods. When working with CDs' complexes, the probability of getting inaccurate results because of the spectrophotometric changes that CDs may bring to the guest-molecule, must be considered. It is very important to keep in mind that the guest must always be detected completely decomplexed. This can be achieved when working under specific chromatographic conditions.

First of all, the completion of the complexation must be verified by NMR or solubility methods, thus the spectroscopic changes can be observed. Then, in order to obtain the guest-molecule decomplexed, the pH of the mobile-phase must favor the decomplexation. The choice of the specific pH depends on the determination of K_{st} of the complex at different pH values, performed by various methods [20,21]. The competitive action of organic solvents on the complexation, when used in large proportions, can also be advantageous for the preparation of the mobile-phase. Finally, the use of a column thermostat regulated

at a chosen temperature can facilitate the decomplexing process.

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