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Research Article

Development of a chiral RP-HPLC method for identification of stereomers of newly synthesised xanthine-based hydrazide-hydrazone compound

Lily Peikova[1], Diana Tzankova[1], Alexandrina Dineva[1], Maya Georgieva[1], Alexander Zlatkov[1]

1 *Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Medical University-Sofia, 2 Dunav str. 1000, Sofia Bulgaria*

Corresponding author: *Lily Peikova* (lily_peikova@yahoo.com)

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Abstract

A reverse phase enantio-selective high performance liquid chromatographic method was developed for enantiomeric separation of 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)-propanehydrazide isomers. The enantiomers of 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)propanehydrazide were resolved on a ACE®Equivalence™ C18 (250 × 4.6 mm, 5 µm) column using a mobile phase system containing methanol, water, phosphate buffer pH 7.4 (50:46:4 v/v/v). The resolution between enantiomers was found to be more than 2.0. The sample solution and mobile phase were found to be stable for at least 48 h. The final optimised method was successfully applied to separate the (R)- and the (S)-enantiomers of 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)-propanehydrazide and was proven to be reproducible and accurate.

Keywords

xanthine-hydrazone, isomers, chiral RP-HPLC

Introduction

Theophylline, also known as 1,3-dimethylxanthine, is a proven bronchodilator drug used in therapy for respiratory diseases such as asthma or chronic pulmonary obstructive disease (COPD) (Black et al. 1990; Cazzola and Gabriella 2007; Majumdar and Sloan 2007a, b; Kawayama et al. 2008; Korzycka and Górska 2008).

Currently, the compounds with hydrazone structure have attracted the interest of many researchers for two reasons. Firstly, based on their facile synthesis, these compounds are important intermediary products in the development of new molecules with potential biological activity (Radunsky et al. 2015). Secondly, literature reports an important number of hydrazones which show interesting biological effects, such as antioxidant (Prathap et al. 2010), antitumour (Vicin et al. 2006), anti-inflammatory (Kumar and Chauhan 2014), anticonvulsive (Padmini et al. 2013), analgesic (Kheradmand et al. 2013), antimicrobial (Küçükgüzela et al. 1999; Singh and Raghav 2011) and antiviral (Vicin et al. 2009).

The idea of combining these two principles is interesting in order to obtain substances with diverse phar-

macological activity and improved toxicological profiles (Constantin et al. 2016).

Most often, the presence of a chiral carbon atom in the structure of the organic compounds is responsible for the appearance of at least two enantiomers. There is a well-known relationship between the pharmacological effect and the appearance of enantiomers in biologically active substances. Very often, an individual enantiomer of a chiral compound potentially exhibits different pharmacology, toxicology and metabolism activities in the living system (Nguyen et al. 2006) in comparison to the corresponding racemic mixture. Potentially only one of the two enantiomers may be responsible for the desired therapeutic response. Whereas, the other enantiomer may be inactive or may even have deleterious effect. Therefore, it is of most importance to develop analytical methods that can identify and determine the enantiomeric purity of a chiral active biologically active substance (Zhuang et al. 2016).

Use of chiral chromatography has proven to be immensely valuable for identification and quantification of chiral compounds. However, the development of the methods for the quantitative analysis of Chiral compounds and for the assessment of enantiomeric purity is extremely challenging, because the same physical and chemical properties of the two enantiomers make discriminating and separating them very difficult (Chimalakonda et al. 2012).

The aim of this study is to develop, validate and apply a chiral reverse phase liquid chromatography (RP-HPLC) method for identification of geometrical isomers of a newly synthesised theophylline-based hydrazide-hydrazone derivative.

Materials and methods

Chemicals and reagents

The necessary products for preparation of the mobile phase and solutions are of analytical grade, obtained from Sigma-Aldrich, Steinheim, Germany.

Instrumentation and chromatographic conditions

The chromatographic separation was performed on a modular HPLC system UltiMate Dionex 3000 SD, Chromeleon 7.2 SR3 Systems, Thermo Fisher Scientific Inc. The separation was achieved isocratically with chiral ACE®Equivalence™ C18 (5 µm), 250 mm x 4.6 mm, 5 µm column eluted with a mixture of methanol, water and 0.5 M potassium dihydrogen phosphate buffer pH 7.4, 50:46:4 v/v/v, as the mobile phase at flow rate of 1.0 ml/min. The mobile phase was filtered through a 0.45 µm membrane filter and degassed. Detection was carried out by absorbance at 272 nm. The analysis was carried out at column temperature of 25°C and injection volume of 20 µl.

Mobile phase composition

CH_3OH:H_2O:phosphate buffer pH 7.4 = 50:46:4 (v/v/v).

Preparation of the phosphate buffer with pH = 7.4

The used buffer was prepared according to the methodology described in European Pharmacopoea 7.0 (2016).

Preparation of the test solution

A 2 mg sample of the tested compound was weighed and dissolved in 10 ml of methanol. A 2.5 ml portion of this solution was diluted to 25.0 ml with methanol and 20 µl of the obtained solution was injected into the apparatus.

Results and discussion

In the current research, 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)-propanehydrazide was selected as a model compound whose chemical structure revealed the possibility of formation of optical isomers, based on the presence of an asymmetric carbon atom, as seen on Figure 1.

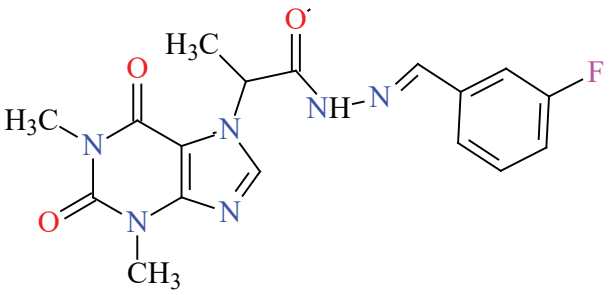

Figure 1. Chemical structure of the evaluated 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)propanehydrazide.

For identification of the possible isomers, a chiral RP-HPLC method was developed and validated.

Development of the chiral RP-HPLC analytical procedure

The main purpose of this work was to develop a fast Reverse Phase HPLC method to separate and quantify the two enantiomers in 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)propanehydrazide. During the method development, several chiral HPLC columns were selected on the basis of the chemical structure and location of the chiral centre of the analyte and also on the characteristics of the chiral phase of the analytical columns. The best chiral selectivity and resolution were obtained on an ACE®Equivalence™ C18

column. Therefore, the ACE®Equivalence™ C18 column was selected for further optimisation.

For optimisation of the mobile phases, the effect of a variety of organic modifiers such as methanol, ethanol, glacial acetic acid and phosphate buffers with different pH were investigated. The finally selected mobile phase was methanol : water : phosphate buffer pH 7.4 (50:46:4, v/v/v). Table 1 summarises the final chromatographic conditions. The final method was then used for analysis of both 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)propanehydrazide isomers in the racemic mixture.

Table 1. Final chromatographic conditions of the method.

Chiral HPLC column	ACE®Equivalence™ C18 (250 x 4.6 mm), 5µm
Mobile phase	CH_3OH:H_2O:phosphate buffer pH 7.4 = 50:46:4 (v/v/v)
Temperature	25 °C
Flow rate	1.0 ml/min
Wavelength	272 nm
Injection volume	20 µl
2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)propanehydrazide	0.2 mg/ml

As indicated in Figure 2, the separation of the two enantiomers was obtained using isocratic elution by use of CH_3OH:H_2O:phosphate buffer pH 7.4 = 50:46:4 (v/v/v) as mobile phase and an ACE®Equivalence™ C18 column as the chiral stationary phase.

Validation of the developed chiral RP-HPLC analytical procedure

The method was validated according to ICH Q2 (R1) guidelines (ICH 2005). The system's suitability, precision, linearity, accuracy and selectivity were evaluated during method validation.

The system's suitability requirements (blank baseline: no interfering peaks above 0.1% level at the retention time of two 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)propanehydrazide enantiomers, 0.2% standard solution having signal-to-noise ratio (S/N) ≥ 10 for each enantiomer; standard solution preparation agreement (≤ 2.0%), resolution factor (≥ 1.2), injector agreement (difference between two injections ≤ 2.0%)) were met prior to performing the method validation experiments.

Specificity

No significant interfering peaks (peak area > 0.1%) was observed at the retention time of the two enantiomers in blank solution. In addition, no evidence of co-elution was noted, using peak purity analysis for the two enantiomers.

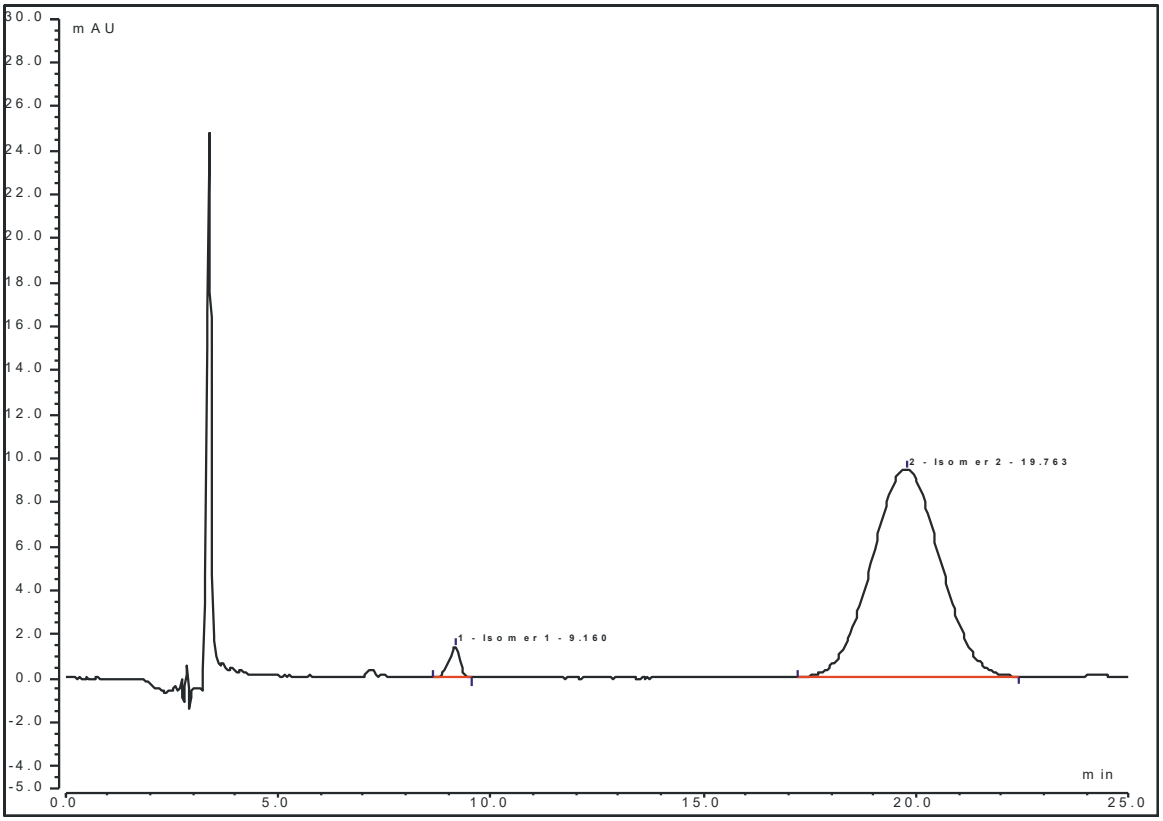

Figure 2. A typical chromatogram of 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)propanehydrazide racemate analysed using the final chromatographic conditions.

Limit of quantification and limit of detection

The limit of quantification (LOQ) and limit of detection (LOD) were calculated from the standard deviations and slopes of the responses, using the signal-to-noise ratio. The LOQ for 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene) propanehydrazide was found to be 0.02 µg/ml, while the LOD was 0.002 µg/ml.

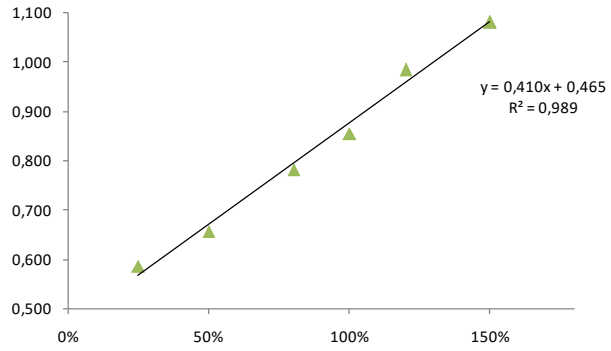

Figure 3. Linearity of the developed chiral RP-HPLC method.

Linearity/accuracy

In this study, "Enantiomer 2" is treated as the main structure and "Enantiomer 1" is treated as impurity. The linearity of both enantiomers was evaluated. Since "Enantiomer 2" was treated as the main structure, its linearity was evaluated from 25% to 150% of the nominal concentration. Linear regression was used to process the calibration data. The correlation coefficients of linearity were 0.989 for 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene)-propanehydrazide, which indicated good correlation between the peak areas and the range of concentrations studied (Figure 3).

A linear least square analysis of the data gave a correlation coefficient (R^2) of 0.989. The *y*-intercept obtained was ≤ 0.5% of the 100% level, indicating that there is no significant bias for quantification for "Enantiomer 2". The method is considered accurate for "Enantiomer 2" as per ICH guidelines.

"Enantiomer 1" is treated as an impurity and the linearity and recovery were studied together by preparing a series of "Enantiomer 1" solutions. A linear least square analysis of the data gave a correlation coefficient (R^2) of 0.988. The *y*-intercept obtained was ≤ 3.0% of the 100% level indicating that there is no significant bias for quan-

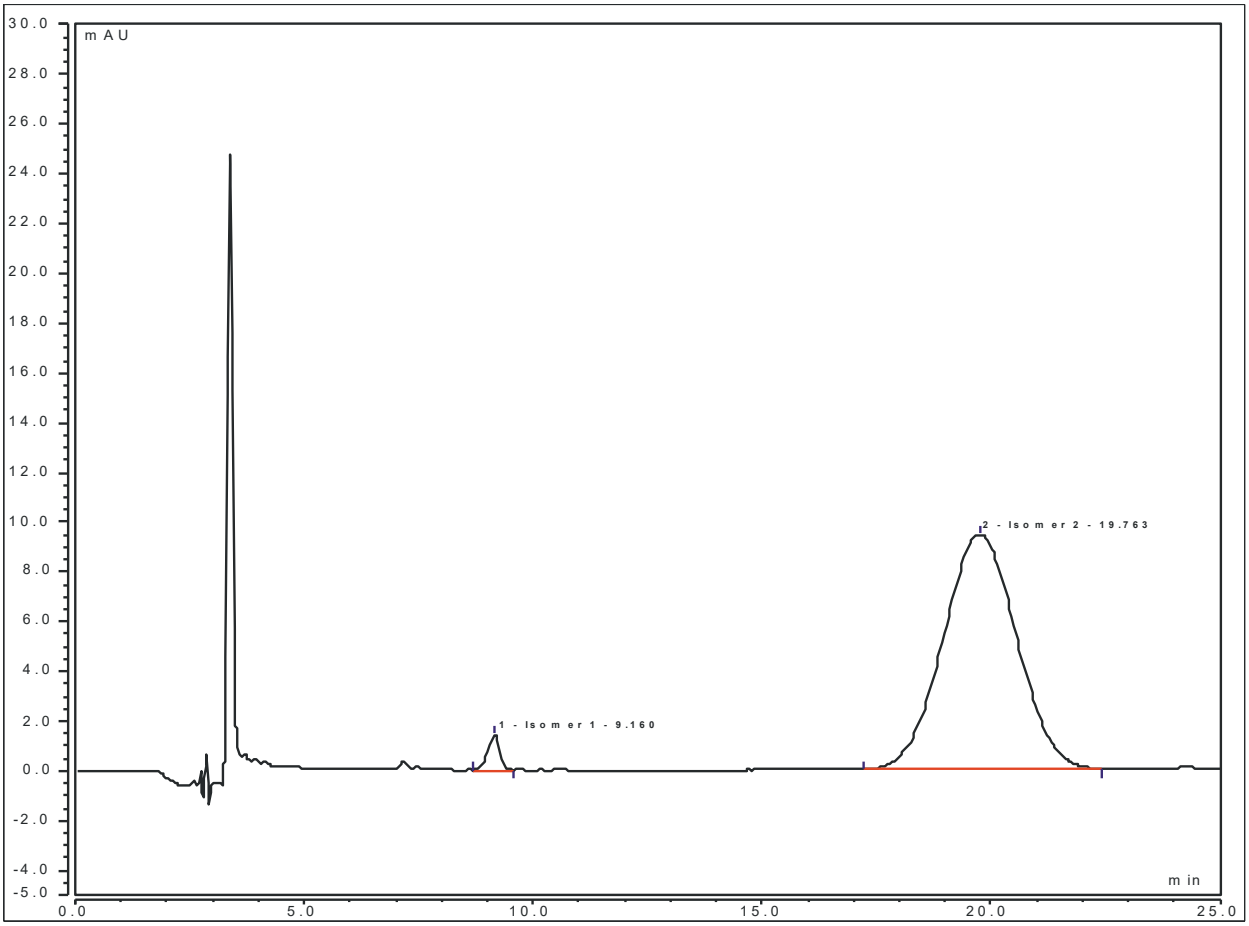

Figure 4. Representative chromatogram of the analyzed 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluoro-benzylidene)propanehydrazide.

tification for "Enantiomer 1". The method is proven to be linear within the investigated range.

Precision

The method's precision was evaluated by analysing six solutions of Enantiomer 2 at 100% level. The enantiomer purity value was calculated for each preparation. The average enantiomer purity was 99.3% and RSD = 0.645% (n = 6), thus meeting the criteria of RSD ≤ 2%. These results demonstrate that the method is precise for determination of the enantiomer purity of the 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluoro-benzylidene)propanehydrazide samples.

Application of the developed and validated chiral RP-HPLC method for separation and identification of enantiomers

The developed chiral RP-HPLC method was applied for identification and separation of enantiomers in newly synthesised 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluoro-benzylidene)propanehydrazide. The result is presented in Figure 4.

The chromatogram shows that the developed method is applied for successful separation and identification of the possible enantiomers of theophylline- and xanthine-based hydrazide-hydrazones.

Conclusion

A new chiral RP-HPLC method was successfully developed and validated for 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluorobenzylidene) propanehydrazide. The method was determined to be linear, accurate, precise, robust and sensitive. The method can be used to verify that 2-(1,3-dimethyl-2,6-dioxo-2,3-dihydro-1H-purine-7(6H)-yl)-N'-(3-fluoro-benzylidene) propanehydrazide is a racemic mixture, as well as to determine the enantiomeric purity of single enantiomer samples of xanthine derivatives, containing a hydrazine moiety in their structure. The method is also considered QC friendly as it is robust, uses isocratic mobile phase with both enantiomers eluting in less than 20 minutes and employs commonly used solvents as the mobile phase.

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