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Analysis of *cis*-isomer-enriched dihydroquercetin sample by 1D and 2D NMR spectroscopy

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Abstract

Introduction. The structure of dihydroquercetin (DHQ) is characterized by two chiral centers at positions 2 and 3 of the benzopyran cycle, resulting in possible diastereomers: *trans*- and *cis*-isomers. Therefore, the development of methods for qualitative and quantitative control of DHQ diastereomers in analyzed samples is essential for patient safety management. Nuclear magnetic resonance (NMR) spectroscopy is one of the physicochemical methods that can be used for this purpose.

Aim. The study objective was to accumulate the analytical and structural characteristics of *cis*-DHQ by NMR spectroscopy of the spheroidal form of this flavonoid (DHQs).

Materials and Methods. 1D ^{1}H, ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-NOESY, and ^{1}H,^{13}C-HSQC NMR spectra were acquired at 298 K on an 800 MHz NMR spectrometer equipped with a TXI triple resonance probe. The number of scans was 32. The mixing time in the NOESY experiment was 400 ms. The ^{1}H and ^{13}C were analyzed using CcpNmr software. The dihedral angles were calculated by applying the Karplus equation.

Results and discussion. In *trans*-DHQ, the chemical shift values for H2 and H3 are 4.93 ppm and 4.52 ppm, respectively, and in *cis*-DHQ they are 5.31 ppm and 4.20 ppm, respectively. The spin-spin coupling constants between H2 and H3 of *trans*- and *cis*-DHQ are 12.00 Hz and 2.40 Hz, respectively. Thus, the dihedral angles for the *trans*- and *cis*-isomers are 154° and 64°, respectively. We found that DHQs contains 12.5 % of the *cis*-isomer.

Conclusion. Our experiments confirmed that NMR spectroscopy can discriminate between *trans*- and *cis*-DHQ based on the chemical shift values for the cross-peaks of H2 and H3. The second major finding was that this method can be considered as a more selective quantitative analysis than HPLC with UV detection without reference. One of the most important results of this study for drug development is the updated information on the structural parameters of DHQ diastereomers in the liquid phase.

Keywords: taxifolin, flavonoids, stereoisomerism, diastereomers, NMR spectroscopy, pharmaceutical analysis

Conflict of interest. The authors declare that they have no obvious and potential conflicts of interest related to the publication of this article.

Contribution of the authors. Roman P. Terekhov – formal analysis, project and data administration, funding acquisition, original draft preparation. Amir Taldaev – methodology, validation, investigation, review and editing. Eduard V. Bocharov – methodology, validation, review and editing. Denis I. Pankov – formal analysis; validation, review and editing. Anastasiya D. Savina – visualization, original draft preparation. Irina A. Selivanova – conceptualization, supervision, review and editing.

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Анализ *цис*-изомер-обогащенного образца дигидрокверцетина путем 1D- и 2D-ЯМР-спектроскопии

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Резюме

Введение. Структура дигидрокверцетина (ДКВ) характеризуется наличием двух центров хиральности в положениях 2 и 3 бензопиранонового цикла, что обуславливает возможность существования диастереомеров: *транс-* и *цис*-изомеров. Таким образом, разработка аналитических подходов к качественному и количественному контролю диастереомеров ДКВ в субстанции является необходимой для обеспечения безопасности пациентов. Спектроскопия ядерного магнитного резонанса (ЯМР) – один из физико-химических методов, который можно использовать для решения этой задачи.

Цель. Целью данного исследования было накопление аналитических и структурных характеристик *цис*-ДКВ при помощи спектроскопии ЯМР сфероидной формы данного флавоноида.

Материалы и методы. Спектры 1D-ЯМР ^{1}H, ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-NOESY и ^{1}H,^{13}C-HSQC были получены при 298 K на спектрометре, оборудованном датчиком тройного резонанса и работающем на частоте, составляющей 800 МГц на ^{1}H. Число сканирований для каждого спектра составило 32. Время смешения в ходе эксперимента NOESY – 400 мс. Спектры ^{1}H и ^{13}C были проанализированы и отнесены при помощи программы CcpNmr. Торсионные углы рассчитаны по уравнению Карплуса.

Результаты и обсуждение. В *транс*-ДКВ величина химического сдвига для H2 и H3 составила 4,93 м.д. и 4,52 м.д., а для *цис*-ДКВ – 5,31 м.д. и 4,20 м.д. соответственно. Константы спин-спинового взаимодействия между атомами водорода в положениях 2 и 3 *транс-* и *цис*-изомеров были 12,00 Гц и 2,40 Гц соответственно. Таким образом, торсионные углы между этими атомами водорода составили 154° в *транс*-ДКВ и 64° в *цис*-ДКВ. Установлено, что сфероидная форма ДКВ содержит 12,53 % *цис*-изомера.

Заключение. Результаты исследования подтверждают возможность использования спектроскопии ЯМР для идентификации *транс-* и *цис*-ДКВ по величинам химических сдвигов кросс-пиков H2 и H3. Другим важным результатом стало обнаружение большей селективности данного количественного метода анализа в сравнении с УФ-ВЭЖХ в отсутствие стандартного образца на *цис*-изомер. Одним из значимых результатов данного исследования для разработки лекарственных средств стало уточнение информации о структуре диастереомеров ДКВ в жидкой фазе.

Ключевые слова: таксифолин, флавоноиды, стереохимия, диастереомеры, ЯМР-спектроскопия, фармацевтический анализ

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Вклад авторов. Р. П. Терехов – формальный анализ, администрирование данных и проекта, получение финансирования, создание и редактирование черновика рукописи. А. Х. Тальдаев – методология, верификация данных, проведение исследования, редактирование рукописи. Э. В. Бочаров – методология, верификация данных, редактирование рукописи. Д. И. Панков – верификация данных, формальный анализ, редактирование рукописи. А. Д. Савина – визуализация, создание и редактирование черновика рукописи. И. А. Селиванова – концептуализация, руководство исследованием, редактирование рукописи.

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INTRODUCTION

Dihydroquercetin (DHQ), also known as taxifolin, is a chiral compound that present in numerous natural objects. The systematic name of this flavonoid is 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydro-4*H*-1-benzopyran-4-one. Due to its wide range of biological effects, this natural polyphenol is a promising object for the design of new phytopharmaceutical drugs [1].

DHQ is characterized by high antioxidant activity [2] and low bioavailability [3]. There are several ways to increase the bioavailuability of this flavonoid, including design of new pharmaceutics [4–5], synthesis of chemical derivatives [2], and phase modification [6–7]. It has been shown, that pseudopolymorphs of DHQ are characterized by different biological effects.

The spheroidal form of DHQ (DHQs) was obtained by spray drying [8]. In a recent study, we found that this substance can be considered as *cis*-isomer-enriched form of DHQ [9]. The structure of this flavonoid is characterized by two chiral centers in positions 2 and 3 of benzopyran cycle, which results in possible diastereomers of DHQ (Figure 1): *trans-* and *cis*-forms.

It is known that diastereomers demonstrate unequal biological activity and toxicity. There are separate studies on the stereospecific pharmacokinetics of DHQ and the asymmetric distribution of stereoisomers in the body [10]. Therefore, the development of analytical methods for qualitative and quantitative control of DHQ diastereomers in a substance is essential for patient safety management.

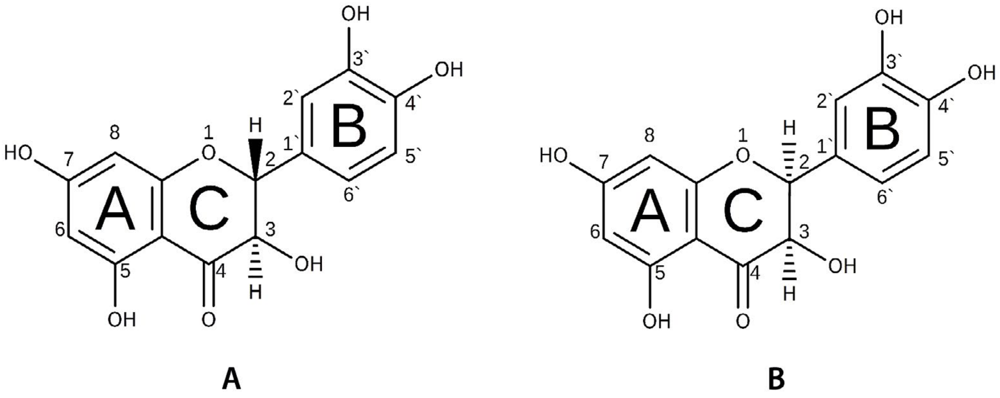

Figure 1. **Molecular structures of DHQ diastereomers: trans-DHQ (A) and cis-DHQ (B)**

Nuclear magnetic resonance (NMR) spectroscopy is one of the physicochemical methods, that can be utilized for this purpose. NMR ^{1}H and ^{13}C spectroscopies are useful approaches for determining the configuration of organic compounds in solution. Enantiomers have identical NMR parameters, which makes them indistinguishable. However, it can be used to identify diastereomers.

NMR spectroscopy is approved for quality control of active pharmaceutical ingredients (APIs) by numerous regulatory guides, including the pharmacopeias of Russia, the Eurasian Economic Union, the European Union, and the United States of America. It is used in pharmaceutical practice for identification [11], qualitative control [12], and determination of impurities [13] and residual organic solvents. There are two ways of compound identification by NMR spectroscopy: by spectral data interpretation and via comparison of the obtained NMR spectrum with a reference. The European Pharmacopoeia noted, that solid-state NMR spectroscopy provides information about the molecules conformations. Also, 2D NMR spectroscopy is widely used in structural chemistry to describe the conformation of small molecules [14–15], biopolymers [16–17], and their interactions [18].

The academic literature has revealed a gap in NMR spectral data of *cis*-DHQ. To the best of our knowledge, the 2D NMR spectra of this compound were not previously published. Therefore, the complex analysis of DHQs by NMR spectroscopy looks promising for future pharmaceutical analysis of this substance.

The purpose of current research was to accumulate the analytical and structural characteristics of *cis*-DHQ by NMR spectroscopy of DHQs.

MATERIALS AND METHODS

Materials

The initial substance of DHQ (99.1 %) was provided by Ametis JSC (Russia) hereinafter referred to as raw DHQ (DHQr). The synthesis of DHQs was described previously [6, 8]. The deuterated methanol (99.8 %, Cambridge Isotope Laboratories, USA) was used as solvent.

NMR Spectroscopy

DHQ samples in amount of 15 mg were dissolved in 450 µL of methanol. The solutions were placed in a 5 mm NMR tube. NMR spectra were acquired at 298 K on an 800-MHz Bruker Avance III NMR spectrometer (Germany) equipped with a TXI triple resonance probe. The pulse programs for 1D ^{1}H, ^{1}H,^{1}H-COSY, ^{1}H,^{1}H-NOESY, and ^{1}H,^{13}C-HSQC were zg, cosydfetgp.2, noesyetgp, and hsqcetgpsp, respectively. In all cases, the number of scans was 32. The mixing time in NOESY experiment was 400 ms. The ^{1}H and ^{13}C spectra were analyzed with CcpNmr software [19].

The dihedral angles (φ) were calculated using Karplus equation:

$$J = A + B \cdot \cos(\varphi) + C \cdot \cos(2\varphi),$$

where J is spin-spin coupling constant (SSCC) and A, B, and C are empirically derived parameters, which in our case equal to 4, (–1), and 9.5, respectively.

The interatomic distances (r_{ij}) were calculated by formula:

$$r_{ij} = \sqrt[6]{\frac{I_{std}}{I_{ij}} \cdot r_{std}^6},$$

where I_{ij} is the volume of cross-peak in NOESY spectrum of analyzed atoms, I_{std} and r_{std} are volume of cross-peak in NOESY spectrum and interatomic distance of reference atoms. In current experiment H5′ and H6′ were used as reference, and these interatomic distance was equal to 2.47 Å.

RESULTS

The complex analysis, included 1D and 2D NMR spectroscopy, was performed for the initial DHQr and *cis*-isomer enriched DHQs. As DHQr is characterized by higher diastereomeric homogeneity, it can be considered as a reference of *trans*-isomer.

Figure 2 presents the results of 1D NMR ^{1}H spectroscopy. Regardless of configuration, the spectrum profile of DHQ in MeOH-d_4 with water includes five signals associated with the following hydrogen atoms: H8 ($\delta = 5.90$ ppm; 1H; d), H6 ($\delta = 5.94$ ppm; 1H; d), H5′ and H6′ ($\delta = 6.86$ ppm; 6H; m), H2′ ($\delta = 6.99$ ppm; 1H; d), and OH5 ($\delta = 11.75$ ppm; 1H; s). Additionally, *trans*-DHQ can be identified by signal of hydrogens in chiral centers: H3 ($\delta = 4.52$ ppm; 1H; d) and H2 ($\delta = 4.93$ ppm; 1H; d). In contrast, in *cis*-DHQ, these signals have different characteristics: H3 ($\delta = 4.20$ ppm; 1H; d) and H2 ($\delta = 5.31$ ppm; 1H; d). The SSCC between hydrogen atoms in positions 2 and 3 for *trans*- and *cis*-DHQ were 12.00 Hz and 2.40 Hz, respectively. Further calculations with Karplus equation showed that observed SSCC of hydrogen signals of *trans*-DHQ can be a result of a dihedral angle of 154° between these atoms, while in *cis*-DHQ this structural parameter should be 64° according to our data. Based on the peak volumes in spectrum of DHQs, it is found that the analyzed substance contains 12.5 % of *cis*-isomer.

The data of 2D HSQC NMR spectroscopy (Figure 3) generally confirmed the hydrogens assignments to signals in 1D NMR 1H spectra. Five hydrogens form cross-peaks with aromatic carbon atoms: from H8 to C8 ($\delta = 94.72$ ppm), from H6 to C6 ($\delta = 95.81$ ppm), from H5′ and H6′ to C5′ and C6′ ($\delta = 114.52$ ppm), and from H2′ to C2′ ($\delta = 119.61$ ppm). Also, there are two cross-peaks with sp^3-hybridized carbon atoms, bounded with oxygen atoms: from H3 to C3 ($\delta = 72.18$ ppm) and from H2 to C2 ($\delta = 83.60$ ppm). What stands out in this figure is the presence of two additional cross-peaks in DHQs spectrum, that are not observed in DHQr. They characterized by the δ-values of carbon atoms, that are similar to C3 and C2, however, the δ-values of hydrogen atoms are equal to the parameters of *cis*-configuration of DHQ.

The profile of 2D COSY NMR spectra exhibited three spin systems in the DHQ molecule (Figure 4), that are associated with A-, B-, and C-rings. The cross-peak at δ 4.56/4.95 ppm is associated with H2 and H3 of *trans*-DHQ. H6 showed in COSY spectrum a cross-peak to H8 (δ 5.94/5.98 ppm). The last, B-ring can be described by the cross-peak at δ 6.85/6.90 ppm (H5′ and H6′/H2′). The additional cross-peak in COSY spectrum of DHQs at δ 4.23/5.34 ppm can be assigned to H2 and H3 of *cis*-DHQ.

Comparing with COSY spectra, the 2D NOESY NMR spectra were enriched with several new cross-peaks (Figure 5). In the spectrum of DHQr, the H2 of the *trans*-diastereomer showed cross-peaks with OH5 (δ 4.93/11.75), H2′ (δ 4.93/6.87 ppm), H6′ (δ 4.93/6.98 ppm), and H8 (δ 4.93/5.90 ppm). Furthermore, H3 of the same configuration forms H2′ (δ 4.53/6.87 ppm), H6′ (δ 4.53/6.98 ppm), and OH5 (δ 4.53/11.75 ppm). The hydrogens of C-ring in *cis*-isomer also give cross-peaks with the same atoms.

Based on the 2D NOESY NMR spectra profile, the interatomic distances in the analyzed compounds were calculated and compered with X-ray diffraction data (XRD), published in literature [20–22], and molecule parameters are estimated using Dreiding models (Table 1). Due to the method's limitation the distance between H3 and H8 could not identified for both diastereomers. For the molecule of *trans*-DHQ, the correlation between structural parameters, obtained by NOESY NMR spectroscopy and XRD, was moderate ($r^2 = 0.5388$). Dreiding models allow to build two conformers for *trans*-isomer: The first structure is characterized by the axial positions

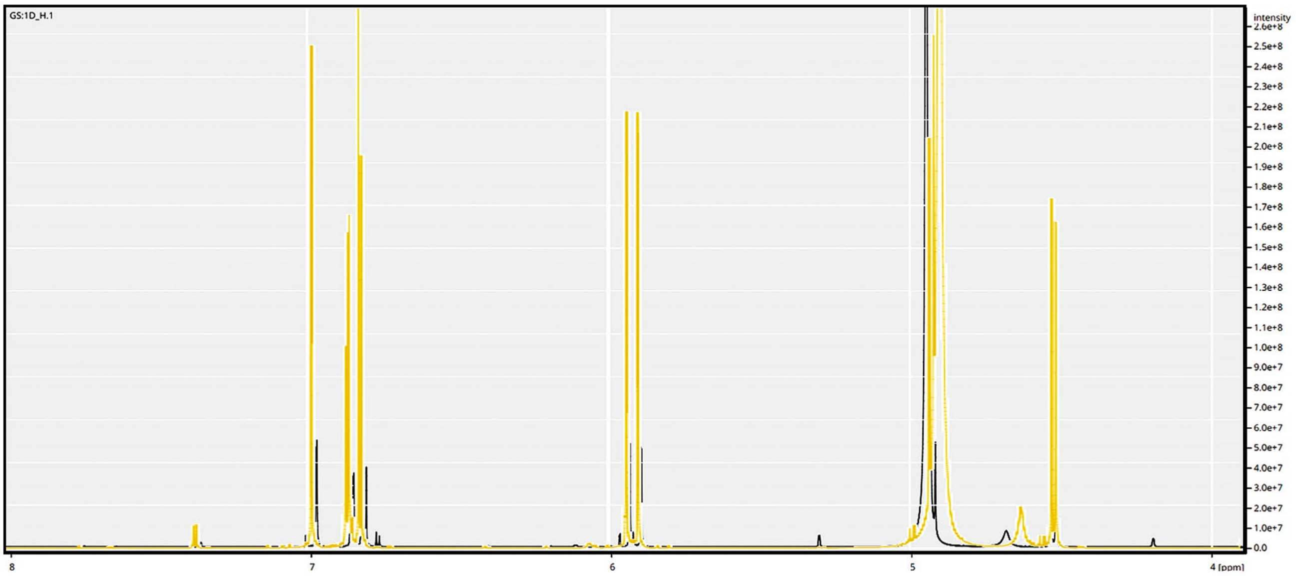

Figure 2. **1D NMR ^{1}H spectra of DHQs (gray) and DHQr (yellow)**

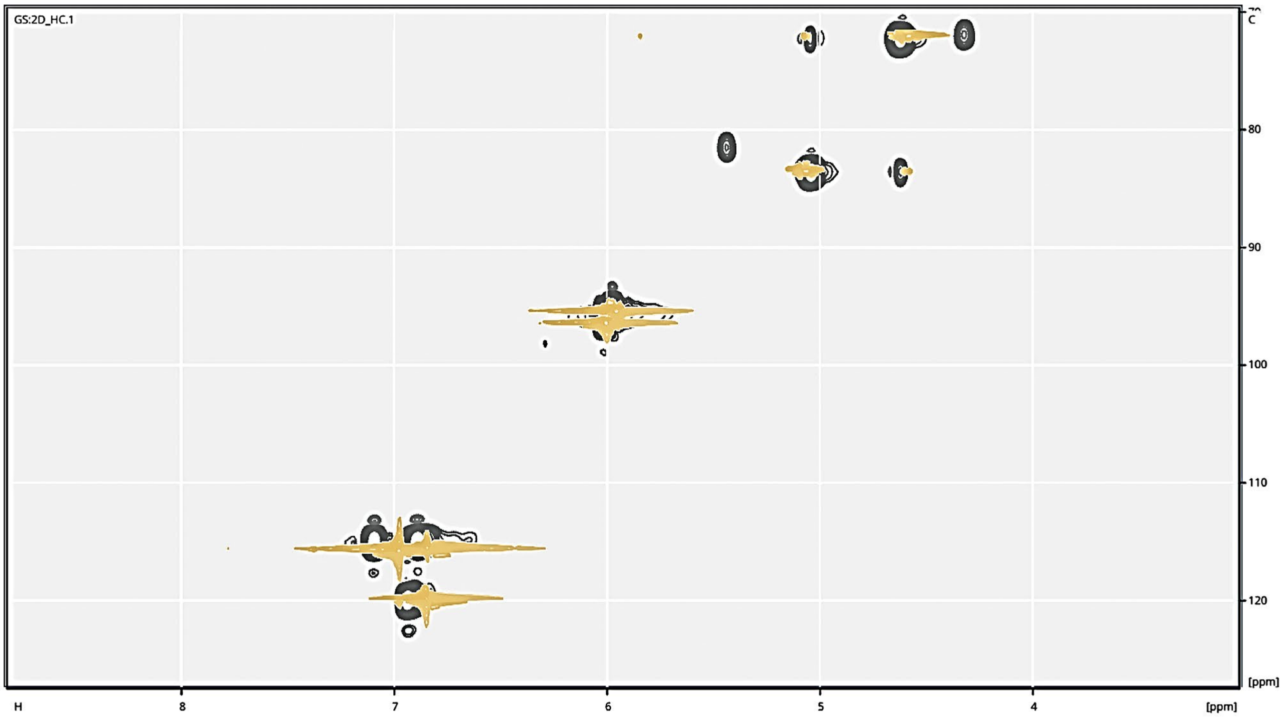

Figure 3. **2D HSQC NMR spectra of DHQs (gray) and DHQr (yellow)**

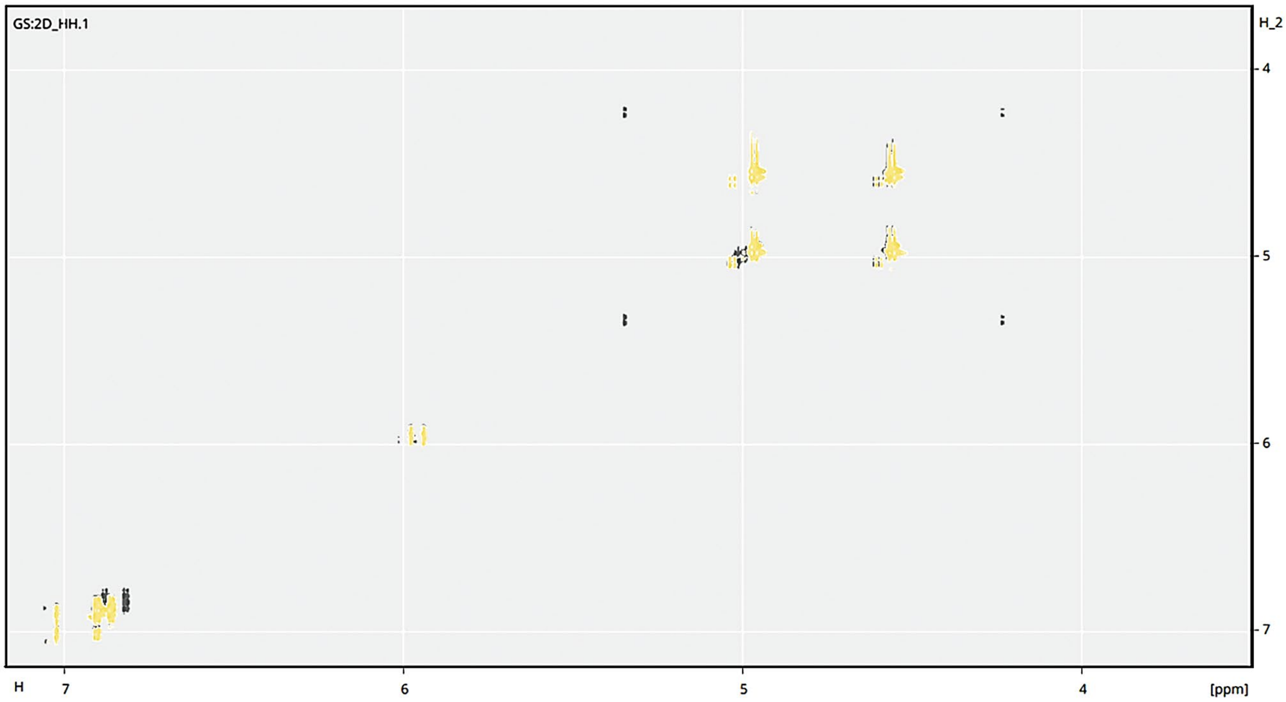

Figure 4. **COSY 1H spectra of DHQs (gray) and DHQr (yellow)**

of both H2 and H3, and the second one has these atoms in equatorial positions. The correlation coefficients between NMR data and interatomic distances obtained using Dreiding models for H2*a*H3*a* and H2*e*H3*e* conformers were 0.8260 and 0.6977, respectively. Also, the structure of *cis*-DHQ gives an opportunity to generate two conformers via Dreiding models. The correlation between NMR data and characteristic of H2*e*H2*a*-conformer was insignificantly higher ($r^2 = 0.8016$) comparing with H2*a*H2*e*-conformer ($r^2 = 0.7723$).

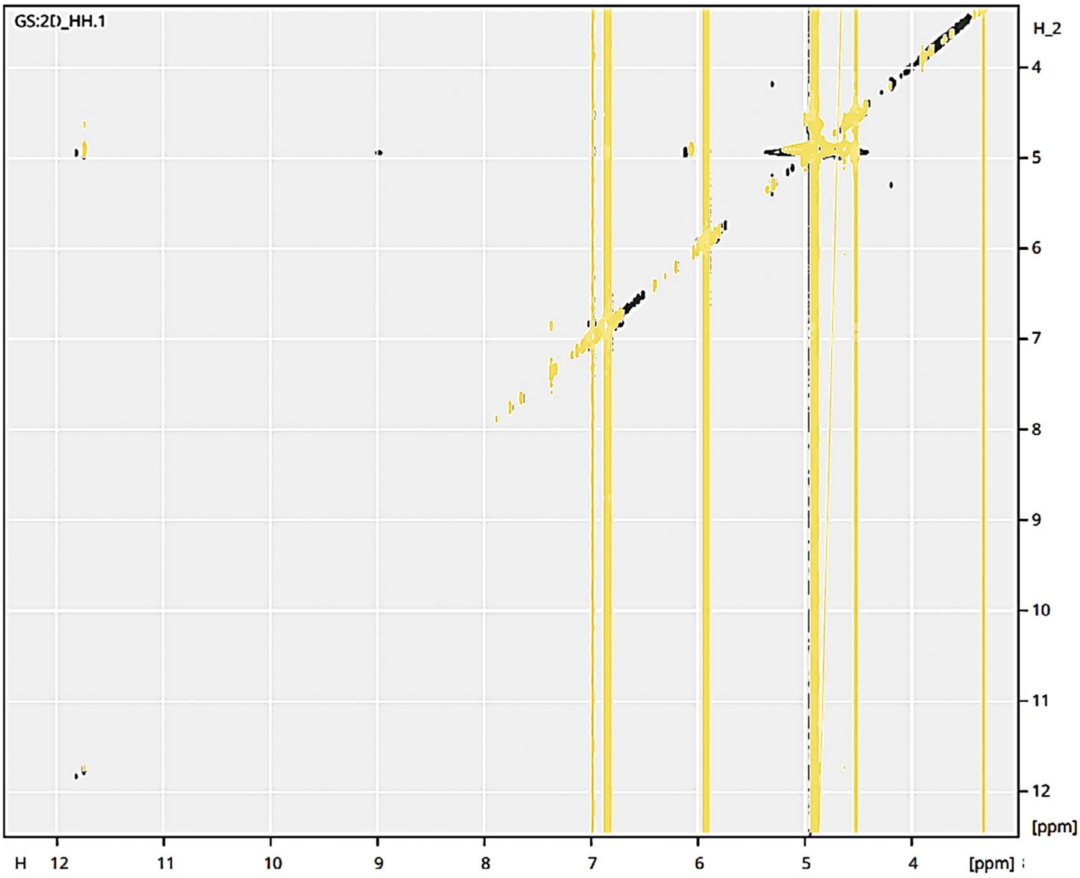

Figure 5. NOESY spectra of DHQs (gray) and DHQr (yellow)

Table 1. Interatomic distances in molecules of conformers of DHQ diastereomers

DHQ molecule		Source of data	Interatomic distance, Å								
			H2					H3			
Configuration	Conformation		H3	OH5	H8	H2′	H6′	OH5	H8	H2′	H6′
trans-	–	NMR	3.14	4.42	3.52	2.75	2.50	3.98	–	2.74	2.86
	H2*a*H3*a*	XRD	2.84	4.76	4.11	3.65	2.64	4.36	4.71	2.31	4.35
		DM	3.07	4.54	3.98	3.35	2.71	4.34	5.14	3.51	3.47
	H2*e*H3*e*		2.47	5.70	4.54	3.59	2.39	4.34	5.78	3.19	3.90
cis-	–	NMR	2.40	–	3.41	3.14	2.88	3.58	–	3.43	3.15
	H2*a*H3*e*	DM	2.47	4.62	3.98	3.47	2.27	4.14	5.74	3.78	3.59
	H2*e*H3*a*		2.47	5.70	4.46	3.43	2.43	4.34	5.26	3.59	3.63

Note. *a* – axial position of hydrogen; *e* – equatorial position of hydrogen; XRD – X-ray diffraction (mean, calculated on the basis of literature data [20–22]); DM – Dreiding models.

Together, these results provide important insights into structures of DHQ diastereomers, which will be discussed in the next section.

DISCUSSIOIN

This study set out with the aim of assessing the opportunities of NMR spectroscopy in qualitative and quantitative control of DHQ diastereomers in API.

A number of NMR data was found in literature. In general, our spectra's profiles reproduced the results of previous researches, and signal were assigned correctly [23–25]. The absence of the majority signals associated with hydrogen atoms in hydroxyl functional groups can be explained by hydrogen-deuterium exchange between solvent and flavonoid.

The majority of authors focused on the *trans*-DHQ; however, information about the spectral properties of *cis*-isomer is also available [26–29]. Most articles reported data obtained for deuterated methanol solutions of DHQ, the same way as for our research. According to this literature, the chemical shift values for H2 and H3 in methanol solutions of *trans*-DHQ varied from 4.89 ppm to 5.00 ppm and from 4.49 ppm to 4.60 ppm, respectively [26, 28–32]. In contrast, hydrogen atoms in positions 2 and 3 of *cis*-diastereomer are characterized by δ 5.30 ppm and δ 4.20 ppm, respectively [29]. The results of current study are in line with the literature data: In *trans*-isomer the chemical shift values for H2 and H3 were 4.93 ppm and 4.52 ppm, respectively, and in *cis*-DHQ they were 5.31 ppm and 4.20 ppm, respectively. So, we confirmed, that 1D NMR spectroscopy can be used in pharmaceutical analysis to identify the diastereomers of DHQ in API.

The quantitative analysis of DHQ diastereomers was performed by method of internal normalization of 1D NMR ^{1}H spectrum of DHQs. It shows that molecular ratio between *trans*- and *cis*-isomers in the analyzed sample was 7 : 1. This finding is contrary to our previous study [9], which reported the results of HPLC analysis of the same substance. The quantity of *cis*-DHQ was lower (9.5 %), and *trans*/*cis*-isomers ratio was 8 : 1. Several studies noted that validation parameters values for the quantitative analysis performed via NMR spectroscopy and HPLC are comparable [33–36]. Taking into account the fact that diastereomers may be characterized by different values of molar extinction coefficients at the same wavelengths [37–39], it can be considered that without a reference sample of *cis*-DHQ, NMR spectroscopy is a more specific and preferable method for quantitative analysis of DHQ diastereomers. Based on the results of current study, we suppose that ratio of molar extinction coefficients at 288 nm of *trans*- and *cis*-isomers of DHQ should be 1 : 0.875.

Our results are relevant not only for applied science but also for basic science. The 2D NMR spectra (COSY, HSQC) of *trans*-DHQ were available before our research [40]. However, according to our data, it is the first case when traces of *cis*-DHQ are observed by these methods. As 2D COSY NMR spectroscopy is characterized by high sensitivity, it can make sense for impurities control in pharmaceutical analysis.

We found that SSCC between H2 and H3 of *trans*- and *cis*-DHQ are 12.00 Hz and 2.40 Hz, respectively. So, the dihedral angles for *trans*- and *cis*-isomers are 154° and 64°. Our findings match those observed in earlier studies. In the literature, the SSCC of these signals *trans*- and *cis*-DHQ are 11.4 Hz and 2.9 Hz, respectively [26, 28–32], which corresponds to dihedral angles of 151° and 61°. However, the structural parameters in this investigation are far below those obtained by XRD. According to crystallographic data, the dihedral angle between hydrogens in *trans*-configuration varies from 165.0° to 178.6° [20–22]. Furthermore, the interatomic distances in the molecule of *trans*-isomer, obtained by NMR spectroscopy and XRD, are not identical. This contradictory observation might be explained by the fact that crystallographic data were collected exploring the solid samples, while NMR spectroscopy analyzed solutions. Firstly, the conformation of molecules in the lattice cell is the result of complex intramolecular interactions, which may be absent in solutions. Secondly, the molecules of the solvent affect the geometry of the dissolved compound. Thirdly, the fluctuations of interatomic distances due to the atomic resonance should also be considered. Anyway, in view of liquid phase inside of living organisms, the structures of DHQ diastereomers obtained by NMR spectroscopy may be more relevant for drug design using molecular modeling [41]. Nevertheless, the limitation of these data applicability is associated with the difference in physicochemical properties between deuterated methanol and bioliquids.

Based on the interatomic distances, the conformations of DHQ diastereomers can be identified. Our findings support the idea that *trans*-DHQ is set up in a form characterized by axial position of both H2 and H3. This accords with previous crystallographic data [20–22] and explained by thermodynamic efficiency of the equatorial position for bulky substituents. Contrary to expectations, this study did not find a significant evidence for equatorial position of 3,4-dihydroxyphenyl in *cis*-DHQ. The values of correlation coefficients between NMR data and parameters of Dreiding models do not highlights this point. The absence of cross-peak between H2 and OH5 in 2D NOESY NMR spectrum of DHQs supporting the H2*e*H3*a* conformation of *cis*-isomer, as spin-spin coupling is not registered in NOESY method if interatomic distance is more than 5 Å. However, this observation can be explained by low concentration of *cis*-DHQ in analyzed sample.

In general, the complex NMR analysis we performed has several important outcomes for basic and applied research and highlights the importance of further research in this area.

CONCLUSION

This study set out to assess the opportunities of NMR spectroscopy in qualitative and quantitative analysis of DHQ diastereomers using the model of DHQs. Our experiments confirmed that this method can discriminate between *trans*- and *cis*-diastereomers based on the chemical shift values for the cross-peaks of H2 and H3 (δ 4.56/4.95 ppm and δ 4.23/5.34 ppm for *trans*- and *cis*-isomer, respectively). The second major outcome was that NMR spectroscopy can be considered as more selective quantitative analysis than HPLC with UV-detection and without reference. We found that DHQs contains 12.5 % of *cis*-isomer. One of the more significant findings for drug development to emerge from this study is the updated information about the structural parameters of DHQ diastereomers in the liquid phase. The empirical findings in this research project provide a new understanding of *cis*-isomer-enriched DHQ sample. The present study lays the groundwork for future stereoisomer control in promising chiral APIs based on the DHQ. What is now needed is the design of a reference sample for *cis*-DHQ. This would be a fruitful area for further work.

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