Analysis of different factors affecting a liquid chromatographic chiral separation of some imino-hesperetin compounds

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Abstract

An investigation was reported concerning the chiral analysis of some Imino-4-Hesperetin and the impact of different factors on their chiral high-performance liquid chromatographic separation. Using Schiff bases reactions, new Imino-4-Hesperetin derivatives had been elaborated by the reaction of Hesperetin with various diamines differ only in their carbon chain length which ranged between 4, 8 and 10 carbons; Yields were very acceptable and ranged between 77 and 89%. A chiral separation was then employed by a conventional HPLC with six different polysaccharide-based chiral stationary phases. The resolution, capacity and separation factors obtained were good ($2.11 \leq R_s \leq 4.5$); ($1.25 \leq k' \leq 8.71$) and ($1.16 \leq \alpha \leq 2.57$). Flow rate was about 0.3–0.5 mL/min. The factors affected the chiral resolution including the role of the carbon chain length which did have an impact on the resolution of these derivatives were discussed.

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Graphic abstract

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1 Introduction

Flavonoids are a group of polyphenolic compounds of low molecular weight, they are categorized into various subclasses including flavones, flavonols, flavanones, isoflavanones, anthocyanidins, and catechins. Among the classes of flavonoids, flavanones have been defined as citrus flavonoids due to their almost unique presence in citrus fruits. In addition, within the large family of flavonoids, flavanones

present a unique structural feature: a chiral center, which distinguishes them from all other classes of flavonoids [1].

Hesperetin (3',5,7-trihydroxy-4'-methoxyflavanone—Fig. 1) is one of aglycone flavonoids extracted from citrus fruits, with various pharmacological effects for the treatment of colds, stomachaches and coughs in traditional Chinese medicine [2, 3]. It could reduce neuronal cell death through antioxidant properties [4]. It can be also possess a potential prevention of Alzheimer's disease progression,

antidepressant activities, anti-inflammatory properties and the prevention of cancer and cardiovascular diseases [5].

About 20–25% of the optically active pharmaceuticals are sold and administered as pure enantiomers. US FDA, European Committee for Proprietary Medicinal Products and other drug controlling agencies have issued marketing guidelines for optically active pure drugs so, the demand for chiral separation techniques has increased greatly. Many pharmaceutical companies are manufacturing these pure forms of drugs by enantiomeric separations [6]. Enantiomers differ in their pharmacological and toxicological activities and only one of the enantiomers may be active whereas the other(s) can be inactive, toxic or ballast. For example, it has been reported that the (+)-threo-methylphenidate is 5–38 times more active than the (–)-threo-antipode, S-(–)-beta-adrenergic blockers are pharmacologically effective showing about 50–500-fold higher activities than their R-(+) antipode [6–8]. Therefore, the request for pure enantiomer of drugs is increasing rapidly because of the need for safe medications since racemic drugs cause side effects, toxicity or other problems in the human body. Some analytical techniques have been developed to monitor the chiral ratio of drugs and to increase throughput and reduce [9–19], liquid chromatography (LC) and capillary electrophoresis (CE) are the most used techniques because of their high efficiency, speed, preparative capability, wide range of applications, and reproducibility. Liquid chromatography, especially high-performance liquid chromatography (HPLC), has achieved a good reputation and considered as the backbone of chiral separation science, as it is being used in almost all industries. The development of the chiral stationary phases (CSPs) in HPLC has proved to be an effective modality in the resolution of racemic compounds; Using various chiral selectors in HPLC columns include polysaccharide, macrocyclic glycopeptide antibiotics, cyclodextrin, proteins, crown ethers and ligand exchanges [12, 20–22]. Several chiral columns have been used for the enantiomeric resolution of a wide variety of racemates among these, polysaccharide-based derivatives are currently the most useful and considered as the leaders in the chiral separation field thanks to their remarkable recognition capabilities and their wide range of applications [22–26]. The existence of a chiral selector is crucial for enantiomeric

resolution as it reacts with the enantiomers in a specific way to be resolved. Chiral selectors issues chiral surfaces, which is indispensable for enantiomeric resolution. Enantiomers bind on this chiral surface to different extents. This binding is carried out by multiple types of fixings [27, 28]. Many factors affect the chiral separation's mechanisms and the chiral recognition between analytes and the CSPs; multiple researchers and scientists in literature gave some structural factors affecting chiral separation but a lot of other phenomenon can also affect the chiral separation and recognition mechanism, Interaction forces, mobile phase compositions and either the carbon chain length and some thermodynamic factors [29, 30]. The comprehension of chiral recognition mechanisms at molecular level is very importance in the chiral chromatographic domain. Moreover, search of literature divulge that some approaches have been done to find out the chiral recognition mechanism of different chiral stationary phases CSPs. Attempts have been made by NMR and computational methods to discuss the chiral recognition mechanism which have been developed for cyclodextrins Pirkle types and polysaccharides CSPs. Various rational models of interactions between CSP and enantiomers have been proposed. The interaction energies between CSPs and enantiomers have been calculated by quantum mechanical calculations and the chiral recognition mechanisms have been proposed based on these calculations and molecular simulation dynamics [31–33]. Although attempts have been made to predict the chiral recognition mechanism of these stationary phases at molecular level while the exact mechanism is still not known. NMR spectroscopy is the powerful and main technique for revealing the chiral recognition mechanism but the polysaccharide-based CSPs are soluble in the spectroscopic solvents. These solvents interact with the carbamate moieties, which is considered an essential adsorption sites for chiral recognition of the polysaccharide based CSPs and hence, the chiral recognition cannot be studied using these solvents. Amylose tris 3,5-dimethyl phenyl carbamate is a semi synthetic polymer which contain a polymeric chain of derivatized D-(+) glucose residues in alpha-1,4 linkage. These chains lie side by side in a helical fashion. The three-dimensional structures of cellulose and amylose-based CSPs were determined and compared using computational chemistry. Reports show that the possible structures were 3/2 helical chain conformation for cellulose tris phenyl carbamate and 4/1 helical chain conformation for amylose tris phenyl carbamate. The amylose CSP is more helical in nature and has well-defined cavities, making it considerably different from the corresponding cellulose analogue, which appear to be more linear and rigid in nature [26, 34–42].

Schiff bases represent an important class of ligands in coordination chemistry, by producing very stable complexes; Thus, they have been extensively investigated regarding their electronic properties, catalytic activity in

Fig. 1 Chemical structure of hesperetin

several chemical and photochemical reactions [43]. Schiff bases became a favored way for synthesis of new chemical compounds and have been used widely for pharmacological effects in different uses: antimicrobial, anticancer, antifungal, antiviral, antimalarial, anti-inflammatory, analgesic, antioxidant, antihypertensive and lipid-lowering activities [44]. Imines; a very important class of nitrogen entities due to their high reactivity. In different types of transformations, they have been used as nitrogen sources that can be further utilized in many fields. Hugo Schiff was the first who employed this kind of reactions since then lots of works have been started on Imine synthesis. Imines or iminium ions containing the reactive C=N moiety are also a key intermediate in multi-component reactions, asymmetric organo-catalysis, cross-dehydrogenative couplings and so on. Therefore, synthesis and applications of imines are essentially ever-appealing topics in chemistry. Imines, which are commonly referred to as Schiff bases or azomethines are important for many serious organic syntheses. Therefore, imine synthesis processes have lots of significance in organic and pharmaceutical chemistry. Imines are also known as biologically significant compounds because of their anti-inflammatory nature as well their applicability as anticancer agent, they also possess antibacterial and antifungal behavior [45, 46].

To reach our objectives new *n*-alkyl-imino-4-hesperetin compounds are synthesized from the condensation of hesperetin and several diamines vary among themselves in the length of their carbon chain, using Schiff bases reaction to form the wanted imines; Then achiral separation was set up by HPLC on several polysaccharide stationary phases under polar and normal isocratic mode.

2 Materials and methods

2.1 Reagents

Synthesis of proposed compounds required the hydrolysis hesperidin to obtain hesperetin. Hesperedin was purchased from Alfa Aesar (Karlsruhe, Germany), primary diamines used in the reactions: A (1,4-diaminobutane); B (1, 8-diaminooctane); C (1,10-diaminodecane) had been purchased from Sigma-Aldrich (St Louis, MO, USA). All other solvents and reagents including HPLC solvents were obtained from Sigma-Aldrich (St Louis, MO, USA) and Riedel-deHaën (Sleeze, Germany).

2.2 UV-VIS

UV spectra were obtained in several solvents with UNICAM UV300 spectrophotometer assisted to desktop computer.

n = 4 Product 1 Yield% = 89%	n = 8 Product 2 Yield% = 81%	n = 10 Product 3 Yield% = 77%
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Fig. 2 Structures and yields of imino-hesperetin derivatives

2.3 IR

IR-Spectra were recorded with an AGILENT Cary 630 FTIR spectrophotometer with a diamond ATR accessory for solid and liquid samples, requiring no sample preparation; wavenumbers are given in cm^{-1} .

2.4 Melting point

Melting points were determined by Büchi® melting point apparatus Model B-545 with capillary tubes, temperature range up to 400 °C.

2.5 Synthesis of hesperetin derivatives

One mmol of hesperetin (0.302 g) and 2 mmol of each suitable primary diamine were refluxed in methanol (7 mL) for 15 h. The mixture was cooled to room temperature, 10 mL of diethyl ether was then added to the mixture, a solid precipitate was formed, filtered and recrystallized from ethanol and water to give the desired products (Fig. 2). The completion was monitored by TLC.

2.6 HPLC

The HPLC employed was a SHIMADZU Scientific Instruments' system LC-20A (Shimadzu, Kyoto, Japan) with an injector of 20 μL Rheodyne 1907 sample loop, a pump LC-20A, a vacuum degasser DGU-20A5, and a Shimadzu SPD-20 with variable wavelength ultraviolet (UV) detector. Chromatographic data were acquired, stored, and analyzed by the LC Lab solution software (Shimadzu, Tokyo, Japan). The injection volume was 20 μL and the UV wave length was changed according to λ_{max} of each compound.

Fig. 3 Structures of the polysaccharide-based chiral stationary phases used in this study

Chromatographic separations were carried out under isocratic mode at different flow rates at ambient temperature.

2.7 HPLC operating conditions

Flow rate ranged from 0.3 to 0.5 mL/min; several mobile-phase systems were used in this study. The mobile phases were prepared in a volume/volume relation, and before delivering into the system, it was filtered through a Millipore membrane filter (0.5 µm; Millipore, Bedford, Massachusetts, USA) and degassed daily before use.

2.8 Stationary and mobile phases

In this work seven analytical stationary phases were employed: an Exsil C-18 ODS stationary phase (250 × 4.6 mm ID, particle size 5 µm) as achiral stationary

phase, six polysaccharide based CSPs were also used, namely cellulose derivatives: Chiralpak®IB [cellulose tris (3,5-dimethylphenylcarbamate) immobilized on 5 µm silica-gel], Chiralcel®OD [cellulose tris (3,5-dimethylphenylcarbamate) coated on 10 µm silica-gel], Chiralcel®OD-H [cellulose tris (3,5-dimethylphenylcarbamate) coated on 5 µm silica-gel] Chiralcel®OZ-3 [cellulose tris (3-chloro-4-methylphenylcarbamate) coated on 3 µm silica-gel] Chiralcel®OJ [cellulose tris (4-methylbenzoate) coated on 10 µm silica-gel] and amylose derivatives: Chiralpak®IA [amylose tris (3,5-dimethylphenylcarbamate) immobilized on 5 µm silica-gel] and Chiralpak®AD [amylose tris (3,5-dimethylphenylcarbamate) coated on 10 µm silica-gel] purchased from Chiral Technologies Europe (Illkirch, France) (Fig. 2). HPLC-grade solvents used were: isopropanol (ISP) and hexane (HEX) (Fig. 3).

3 Results and discussion

3.1 Synthesis of hesperetin derivatives

Schiff bases are typically formed by the condensation of primary diamines with the carbonyl group of hesperetin. The synthesis of imino-hesperetin was carried out by refluxing hesperetin with the appropriate primary diamines in methanol. The results showed that the yields depend on the primary diamine and its carbon chain length, yields ranged between 77 and 89%.

Reactions were confirmed by IR we can easily notice the disappearance of (C=O) and the formation of (C=N).

3.2 Spectroscopic data

4-((4-aminobutyl)imino)-2-(3-hydroxy-4-methoxyphenyl) chromane-5,7-diol (Product 1) yellow, yield 89%, MP: 220–221 °C, UVmax (MeOH, nm): 283 (band I); 335 (band II), IR (neat, cm⁻¹): (O–H), 3276 (C–H arom), 3089 (C=N), 1633 (NH₂), 1603 (C=C arom), 1491 (C–N), 1147.

4-((8-aminooctyl)imino)-2-(3-hydroxy-4-methoxyphenyl) chromane-5,7-diol (Product 2) dark yellow, yield 81%, MP: 225–226 °C, UVmax (MeOH, nm): 287 (band I); 340 (band II), IR (neat, cm⁻¹): (O–H), 3319 (C–H arom), 3011 (C=N), 1678 (NH₂), 1601 (C=C arom), 1520 (C–N), 1133.

4-((10-aminodecyl)imino)-2-(3-hydroxy-4-methoxyphenyl) chromane-5,7-diol (Product 3) dark yellow, yield 77%, MP: 227–228 °C, UVmax (MeOH, nm): 287 (band I); 341 (band II), IR (neat, cm⁻¹): (O–H), 3121 (C–H arom), 3053 (C=N), 1642 (NH₂), 1549 (C=C arom), 1427 (C–N), 1183.

3.3 Chiral separation

We tried to optimize the HPLC conditions by changing the chromatographic parameters to have the best possible results to better understand the chiral recognition mechanism. During this investigation, two possible chiral separation modes were used namely, normal and polar organic phase under isocratic elution technique. The commonly used mobile phases for this type of CSPs are pure

Table 1 Chromatographic parameters of the chiral separation of the imino-hesperetin

Chiral stationary phases	Products	Mobile phase	Flow rate (mL/min)	T ₁ (min)	T ₂ (min)	k ₁	k ₂	α	Rs
Chiralpak®IB	1	100% ISP	0.5	07.14	12.47	2.07	5.34	2.57	3.29
	2	85% ISP 15% HEX	0.4	11.06	12.76	4.52	5.38	1.19	3.02
	3	100% ISP	0.5	07.10	07.95	2.54	2.98	1.17	2.83
Chiralcel®OD-H	1	100% ISP	0.5	07.11	07.97	2.59	3.01	1.16	3.05
	2	70% ISP 30% HEX	0.5	10.90	12.46	4.50	5.23	1.16	2.76
	3	70% ISP 30% HEX	0.5	11.42	12.53	4.47	5.27	1.18	2.57
Chiralcel®IA	1	70% ISP 30% HEX	0.5	13.85	19.42	5.93	8.71	1.47	2.11
	2	–	0.3	–	–	–	–	–	–
	3	–	–	–	–	–	–	–	–
Chiralcel®OJ	1	85% ISP 15% HEX	0.3	07.21	12.80	1.25	2.60	2.07	4.50
	2	100% ISP	0.5	08.16	9.03	1.86	2.01	1.08	0.93
	3	–	–	–	–	–	–	–	–
Chiralpak®OD	1	100% ISP	0.5	07.15	13.30	2.58	5.65	2.19	3.96
	2	100% ISP	0.5	09.24	11.12	3.61	4.56	1.26	2.54
	3	–	–	–	–	–	–	–	–
Chiralpak®AD	1	–	–	–	–	–	–	–	–
	2	–	–	–	–	–	–	–	–
	3	–	–	–	–	–	–	–	–

Fig. 4 Simple schematic representation of the reaction

alcohols, and mixtures of alcohols with hexane. Several types of mobile phase compositions were investigated by changing the nature and percentage of the mixture components. To simplify the presentation, only the chromatographic results with the optimal mobile phase composition and/or conditions that gave the best resolution on different (CSPs.) are summarized in Table 1.

3.4 Mechanism of reaction

Mechanism of the transformation of Aldehydes or Ketones to Imines via Schiff bases reaction relies on a set of steps,

nucleophilic attack of a primary amine on carbonyl carbon will take place that affords hydroxyl compound which on dehydration gives Schiff bases. So, the Schiff bases formation is really a sequence of two types of reactions: addition followed by elimination. The formation of Schiff bases in this method is largely depends on the rate of removal of water from the reaction mixture. Originally, the classical synthetic route for preparation of Schiff bases was reported by Schiff which involves the condensation of primary amines with carbonyl compounds under azeotropic distillation with the simultaneous removal of water [2, 29, 47–49] (Fig. 4).

Fig. 5 HPLC chromatograms of the enantiomeric separation of compounds 1, 2 and 3 on CSP Chiralcel®OJ, Chiralpak®IB and Chiralpak®IB; Mobile phases were 85%ISP–15%HEX, 100% ISP and 85%ISP–15%HEX, flow rates were 0.3 mL/Min, 0.5 mL/Min and 0.5 mL/Min respectively. Temperature 25 °C, UV detector set at 283 nm

3.5 HPLC analysis

The preliminary separation with a conventional C18-bonded achiral stationary phase gave us conclusive evidence of the purity of the compounds. The chiral separation of these imino-hesperetin derivatives has been attempted by HPLC using various polysaccharide CSPs. These compounds have one chiral center so only two enantiomers are expected to be resolved.

The majority of the compounds were base-line separated with $R_s > 1.5$ (2.11–4.50) on cellulose based CSPs; while poor resolution or no resolution could be achieved on amylose derivatized CSPs.

For compound 1; It was well separated on all CSPs except Chiralpak®AD the best separation was obtained using Chiralcel®OJ with a mixture of (85% isopropanol–15% hexane) under 0.3 mL/min flow rate; while the shortest retention time was observed on Chiralpak®IB with 100% isopropanol.

Compound 2 could not be separated on the amylose CSPs namely, Chiralcel®IA and Chiralpak®AD; but is well separated on almost all cellulose based CSPs.

Compound 3 was separated on 50% of the CSPs where Chiralpak®IB and Chiralcel®OD-H were the most powerful phases for all the chiral separation. Although these phases possess the same chiral selector is coated onto silica in case of Chiralcel®OD-H, while it is immobilized onto silica in case of Chiralpak®IB. However, the retention times and separation factors of these enantiomers on both CSPs. Under the same conditions were different, even with the similarity in structure of the chiral selector in both CSPs. The immobilization of the cellulose tris-(3, 5-dimethylphenylcarbamate) on silica did affect the chiral recognition ability possibly due to the change of the polysaccharide configuration during the immobilization process (Fig. 5).

3.6 The study of separation

Several combinations were employed, different mobile-phases compositions and various CSPs listed previously were all pulled together to have the best chiral separation possible. Baseline separations with good resolutions were observed for the majority of products. The best results were obtained using Chiralcel®OJ which gave a very high resolution values with $R_s = 4.50$ for compound 1 as the maximum value, and $R_s = 0.93$ for compound 3 as the minimum value under flow rate 0.3 mL/min using 85% isopropanol–15% hexane and 0.5 mL/min using 100% isopropanol respectively. The poorest separations were observed on amylose CSPs namely Chiralpak®AD and Chiralpak®IA which gave unsatisfactory results for the compounds under study. These differences in chiral recognition

mechanism between the amylose and cellulose derivatives are due to the different configurations of the glucose residues (and linkages) and higher-order structures of CSPs of cellulose and amylose CSPs. It seems clear in this case that the amylose derivatives, which possesses a more helical configuration, did not effectively contribute to the separation of these imino-hesperetin enantiomers compared to the cellulose derivatives, which has a more rigid and linear configuration resulting in its ability to resolve all the enantiomers. Cellulose and amylose-based CSPs are different in nature that changes the capability of separation of the CSPs by influencing the formation of transient diastereomeric complexes between the analytes and CSPs. The discrimination power of these polysaccharide-based CSPs stems from complex interactions with the solutes. The chiral grooves sited in the chiral selectors provide a stereoselective environment to the enantiomers; thus the enantiomers fit in these chiral grooves to different extents as glove and hand arrangement. These polysaccharide CSPs has many of chiral active sites and thus have a relative high probability of interaction with the solutes, leading to the separation of the stereoisomers. In summary, a combination of hydrophobic interactions, Van der Waals forces and attractive forces (e.g., hydrogen bonding, dipole–dipole interactions and charge transfer (π – π) formation) are believed to explain the molecule recognition process [50–53]. It is of interest to mention that elution under normal phase mode is more efficient than the polar phase mode in achieving the separation of the imino-hesperetin compounds. It seems that the hydrogen bonding and π – π interactions of the aromatic moieties of the CSPs with the aromatic rings of the analytes are preferred in the normal phase mode separation system [30, 54–56].

3.7 Effect of carbon chain length

The chiral separation mechanism on polysaccharide CSPs was affected also by the length of the carbon chain of the compounds; The addition of ($-\text{CH}_2-$) groups seems to change the chiral recognition and the resolution capacity between the solutes and the CSPs; That can be explained by the augmentation of hydrophobicity of compounds when ($-\text{CH}_2-$) groups increases, where the hydrophobic interactions plays a major role in the product's resolution from one another; By looking at the R_s values, one notice that shorter the carbon chain length is (low hydrophobicity) higher is the resolution. The carbon chain length variation affects also the polarity of the products, which it decreases by adding ($-\text{CH}_2-$) groups; these differences in polarity affects the chiral separation mechanism, therefore, resulted in achieving high resolution and separation values of products 1 and 2 compared to product 3. The carbon chain length has an effect on the volume of

molecules, which creates a steric effect which makes the chiral separation of large molecule difficult compared to small ones. This explains the graduation in ease of the chiral separation of the compounds; one can notice that smaller molecules are easily resolved and chiral separated than the larger ones [57, 58]. It is well known that the variation of carbon chain length affects enthalpy and entropy compensation of molecules; which affects in turn the intermolecular forces in binding, so Van der Waals forces and hydrogen bonds in first place then the other interaction forces between CSPs and solutes. It was concluded that smaller is the carbon chain length, fewer are the interaction forces which cause a shorter is the retention time. This explains why small molecules elutes faster than larger molecules [59, 60].

4 Conclusion

Three new imino-hesperetin derivatives were synthesized from hesperetin and several diamines. Chiral separation of these derivatives were carried out using HPLC under polar and normal phase modes with various cellulose and amylose derivatized polysaccharide-based CSPs. The cellulose derivatives showed efficient and more performant capabilities in resolving the racemic imino-hesperetin compounds. The chiral recognition mechanism was affected by several factors where the carbon chain length has great impact on the resolution of these derivatives.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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