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Separation of maxi-K channel opening 3-substitued-4-arylquinolinone atropisomers by enantioselective supercritical fluid chromatography

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ABSTRACT

Many 3-substitued-4-arylquinolinones containing an *ortho* substituent on the aryl ring were known as a class of compounds with maxi-K opening activity. These quinolinones, which contained a stereogenic axis in their structures due to their bulky *ortho* substituents on the two aryl rings, exhibited atropisomerism. The rotationally hindered atropisomers could have differential biological and pharmacological activity, and it was highly desirable to separate them and test the individual atropisomers in biological assays. To explore the potential of supercritical fluid chromatography (SFC) to separate the atropisomers of this class of compounds, six 3-substitued-4-arylquinolinones with various hydrophilic and hydrophobic substituents in various positions were screened using three alcoholic modifiers (methanol, ethanol and 2-propanol) with four polysaccharide-based chiral stationary phases (Chiralpak AD-H and AS-H, Chiralcel OD-H and OJ-H). Our results showed that all six compounds studied were successfully resolved under multiple SFC conditions regardless of their structural differences and polarity. The majority of the separations were completed within 10 min. The Chiralpak AD-H column appeared to be superior to the other three chiral columns, and methanol and ethanol showed higher successful rate than 2-propanol in separating atropisomers of this class of compounds. These SFC methods were efficient and easily scalable for preparative separation. Thus, SFC was found to be the methodology of choice for resolving the atropisomers of this class of compounds.

1. Introduction

Within the potassium channel superfamily, the maxi-K channel subfamily is an extremely important therapeutic target. Compounds that can modulate the activity of maxi-K channels have the potential to be therapeutics for a number of diseases such as stroke, urinary incontinence, and irritable bowel syndrome [1–3]. During the screening of small molecules for maxi-K channel openers, several 3-substitued-4-arylquinolinones were discovered in our laboratories as a unique class of compounds with maxi-K opening activity [4]. Specifically, quinolinone **1** and its *N*-methyl derivative **2** (Fig. 1) were identified as potent maxi-K channel openers. In an effort to further identify novel maxi-K channel openers of this class, a series of analogs of **1** and **2** were also synthesized in our laboratories [5–7]. The structures of this class of compounds contain a stereogenic axis between the quinolinone ring and the aromatic ring. As a result, this class of compounds exhibited atropisomerism due to the hindered rotation around the aryl–aryl (Ar–Ar) single bond in the presence of bulky *ortho* substituents. When the energy barrier for rotation around the aryl–aryl single bond exceeds 16–20 kcal/mol, the two atropisomers could exist as non-inter-converting enantiomers at room temperature (20 °C) [8]. The energy barrier for rotation around the aryl–aryl single bond in **1** was estimated to be 31 kcal/mol, thus, the compound could exist as stable atropisomers at room temperature [9]. Since two stable, rotationally hindered atropisomers might display a difference in biological and pharmacological activity [9–13], it was highly desirable to separate them and test the individual atropisomers in biological assays. In addition, the individual atropisomers had to undergo stability studies before they could be advanced further in the drug discovery process. A fast and efficient chromatographic method was essential not only to scale-up the individual atropisomers, but also to monitor their stability and potential inter-conversion under

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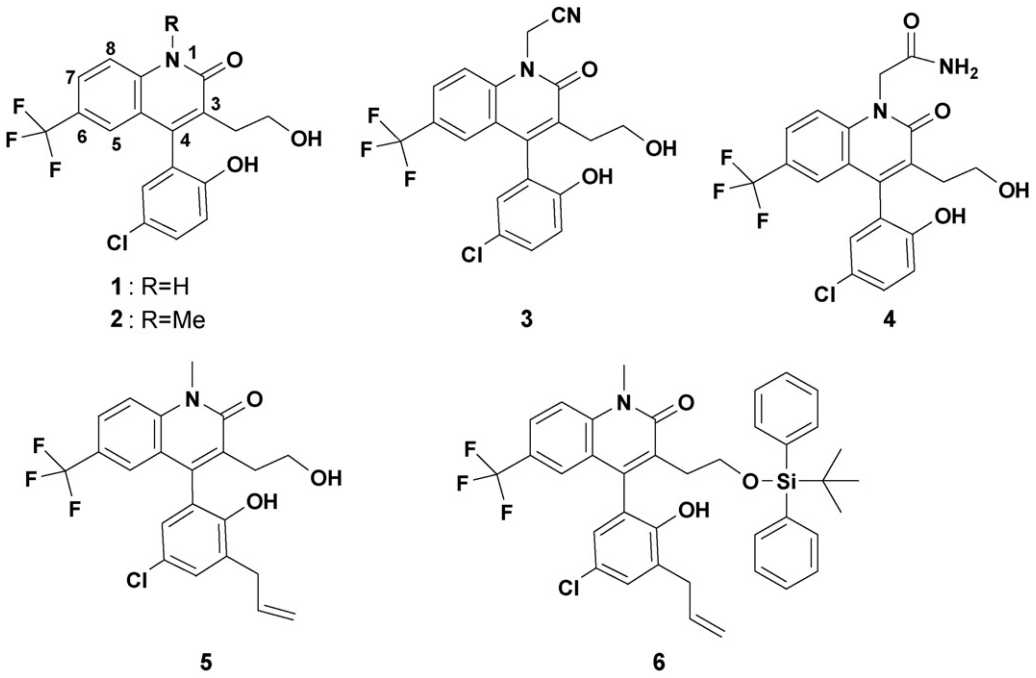

Fig. 1. Chemical structures of compounds **1**–**6**.

various environmental conditions, as well as in biological matrices.

The separation of atropisomers requires chiral resolution. HPLC using chiral stationary phases [14,15] appeared to be the most commonly used methodology for atropisomer resolution due to its capability in preparative purification, as well as its widespread presence in pharmaceutical research laboratories. Our method development for atropisomeric separation of **1** started also on HPLC by screening four polysaccharide-based CSPs (Chiralpak AD and AS, Chiralcel OD and OJ) using a mobile phase containing ethanol–hexane (1:9, v/v). Since this initial screening did not yield sufficient separation for **1**, the compound was subjected to extensive HPLC method development. Eventually, an HPLC method with a baseline separation was found for **1** (Fig. 2), and it provided sufficient quantities of individual atropisomers for the initial biological evaluation [9]. However, the HPLC method was time-consuming, requiring almost 60 min on an analytical scale, and 140 min for a preparative run. In addition, with scalability in the preparative mode being non-optimal due to the insufficient resolution, this method was inefficient for generating larger amount of individual atropisomers, and it also lacked robustness due to loss of resolution after multiple runs. In light of these observations, we sought to investigate alternative and more efficient methodology for the atropisomer separation of this class of compounds.

Supercritical fluid chromatography (SFC) has gained broad acceptance as an alternative separation technology in drug discovery, especially in the area of chiral separations [16–21]. We decided to explore its separation potential for the atropisomers of this class of quinolinone derivatives. SFC uses near-critical (supercritical and subcritical) fluid CO_2 and polar organic modifiers such as alcohols as mobile phases. Compared to liquid solvents, near-critical fluid CO_2 possesses the advantageous characteristics of higher diffusivity and lower viscosity, which result in more rapid re-equilibration, and higher speed and throughput. These characteristics also lead to a much lower column pressure drop, which allows higher flow rates and use of longer columns packed with smaller particle-size particles. Furthermore, since SFC uses CO_2 as major solvent with purified samples collected in small volume of organic solvents (alcohols in most cases), it significantly reduces time of sample work-up, as well as cost of solvent and solvent disposal, especially in the preparative application. During the last several years, there are quite a few of SFC application reports for chiral separation of compounds containing asymmetric centers as well as atropisomers [21–32]. In this study, compounds **1** and **2**, along with four additional derivatives belonging to the same class with various hydrophilic and hydrophobic substituents (Fig. 1) were screened with four polysaccharide-based SFC columns with 5-μm packing particles (Chiralpak AD-H and AS-H, Chiralcel OD-H and OJ-H) in combination with three alcoholic modifiers (methanol, ethanol and 2-propanol). The separation results of our SFC study on these compounds are reported in this article.

2. Experimental

2.1. Reagents and compounds

Methanol, 2-propanol and hexane were all HPLC grade and purchased from J.T. Baker (Phillipsburg, NJ). Ethanol was 200 proof (99.98%) and purchased from Pharmco-AAPER

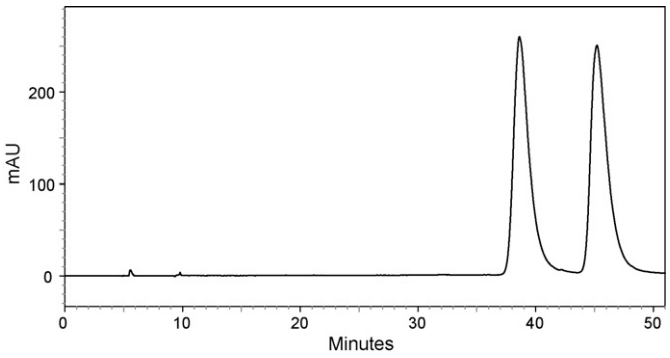

Fig. 2. HPLC chromatogram of **1** using Chiralpak AD column (4.6 × 250 mm, 10 μm), 2-propanol–hexane (5:95, v/v) at 0.6 ml/min, 25 °C, 230 nm detection.

(Brookfield, CT). The compounds (Fig. 1) studied are listed as follows: 4-(5-chloro-2-hydroxyphenyl)-3-(2-hydroxyethyl)-6-(trifluoromethyl)quinolin-2(1*H*)-one (**1**), 4-(5-chloro-2-hydroxyphenyl)-3-(2-hydroxyethyl)-1-methyl-6-(trifluoromethyl)quinolin-2(1*H*)-one (**2**), 2-(4-(5-chloro-2-hydroxyphenyl)-3-(2-hydroxyethyl)-2-oxo-6-(trifluoromethyl)quinolin-1(2*H*)-yl)acetonitrile (**3**), 2-(4-(5-chloro-2-hydroxyphenyl)-3-(2-hydroxyethyl)-2-oxo-6-(trifluoromethyl)quinolin-1(2*H*)-yl)acetamide (**4**), 4-(3-allyl-5-chloro-2-hydroxyphenyl)-3-(2-hydroxyethyl)-1-methyl-6-(trifluoromethyl)quinolin-2(1*H*)-one (**5**), and 4-(3-allyl-5-chloro-2-hydroxyphenyl)-3-(2-(*tert*-butyldiphenylsiloxyl)ethyl)-1-methyl-6-(trifluoromethyl)quinolin-2(1*H*)-one (**6**). These substances were synthesized and purified as previously described [5–7,9]. These samples met purity criteria (≥95% as determined by HPLC with UV detection) for in-house registration for discovery compound library. The structures of the compounds are shown in Fig. 1.

2.2. Instrumentation and method for chiral SFC screen

All analytical SFC screenings were performed on a Berger analytical SFC system (Mettler-Toledo Autochem, Newark, DE, USA) equipped with a dual pump fluid control module FCM 1200 with a six-position modifier switching valve, a thermal column module TCM-2000 with a six-port column switching valve, a Berger automatic liquid sampler ALS 3100 with a 5 μl sample loop, as well as an Agilent diode-array detector G1315A with a high-pressure flow cell (Agilent Technologies, Palo Alto, CA, USA.). Chromatographic data were acquired and processed with Berger SFC ProNTo software (Version 92.1). Liquid CO_2 was directly delivered from a dip-tube cylinder (SFC-grade CO_2, Airgas, CT, USA). All analyses were operated under isocratic conditions at a backpressure of 150 bar, a temperature of 35 °C, a flow rate of 2 ml/min, and UV detection at 210 and 230 nm. All compounds were analyzed on four polysaccharide-based chiral columns (Chiralpak AD-H and AS-H, Chiralcel OD-H and OJ-H, 4.6 mm × 250 mm, 5 μm) from Chiral Technologies (West Chester, PA, USA). Three organic modifiers (methanol, ethanol and 2-propanol) were employed in our SFC study. The equilibration time for each chromatographic condition was 10 min. The compounds were dissolved in ethanol at 1 mg/ml, and sample injection volume was 5 μl. For each organic modifier, an appropriate solvent strength (percentage) to elute the class of compounds within 20 min was first established by one or two experiments. All samples were subsequently screened by using these solvent systems with the four chiral columns described above. Thus, each compound was screened under at least 12 chromatographic conditions. The chromatographic data of the SFC screen using 10% methanol, 10% ethanol and 15% 2-propanol as the modifiers were recorded in Tables 1–3, respectively. The retention times of the first- and second-eluting atropisomers were represented as $t_{R,1}$ and $t_{R,2}$. The separation factor (α) was calculated from the equation: $\alpha = (t_{R,2} - t_0)/(t_{R,1} - t_0)$, where t_0 was the void time, which was estimated from time of the first peak disturbance of ethanol injection as 2.3 min. The resolution (R_s) was obtained from the equation: $R_s = 2\,(t_{R,2} - t_{R,1})/(W_1 + W_2)$, where W_1 and W_2 were the widths of the first- and second-eluting peaks at the baseline.

2.3. Instrumentation and method for preparative SFC separation

The chiral preparative separation was carried out on a Berger MultiGram II (Mettler-Toledo Autochem, Newark, DE, USA). The preparative SFC system consisted of two Varian SD-1 pumps (Walnut Creenk, CA, USA) with one modified to pump CO_2, a phase separation control module SCM 2500, an electronic control module ECM-2500, a chiller Julabo FT 401 (Labortechnik GmbH, Seelback, Germany), and a variable wavelength UV detector Knauer 2501 (Knauer, Berlin, Germany) with high-pressure flow cell. The system was further equipped with an automatic injection system equipped with a 2 ml sample loop and a fraction-collection cabinet. Liquid CO_2 was delivered by a Berger GDS-2000 system. The preparative separation of compound **1** was carried out on a Chiralcel OJ-H

Table 1
Retention times ($t_{R,1}$, $t_{R,2}$), separation factor (α) and resolution (R_s) of compounds **1**–**6** on Chiralpak AD-H and AS-H, Chiralcel OD-H and OJ-H, using 10% methanol in carbon dioxide at 2 ml/min, 35 °C, 230 nm detection, 150 bar backpressure

Compound	Column	$t_{R,1}$ (min)	$t_{R,2}$ (min)	α	R_s
1	AD-H	6.20	7.37	1.31	2.93
	OD-H	11.50	11.77	1.03	0.60
	OJ-H	5.15	–	1.00	0.00
	AS-H	10.56	11.84	1.16	1.60
2	AD-H	5.39	7.04	1.55	3.67
	OD-H	9.65	10.96	1.18	2.18
	OJ-H	8.88	12.92	1.62	6.21
	AS-H	5.15	–	1.00	0.00
3	AD-H	5.42	7.22	1.63	5.00
	OD-H	9.18	10.50	1.19	3.30
	OJ-H	4.16	5.42	1.72	3.15
	AS-H	5.02	–	1.00	0.00
4	AD-H	8.02	9.79	1.31	3.54
	OD-H	15.82	17.80	1.15	3.19
	OJ-H	4.20	4.50	1.17	0.75
	AS-H	9.08	–	1.00	0.00
5	AD-H	6.51	8.62	1.51	3.52
	OD-H	12.82	17.76	1.47	6.59
	OJ-H	5.01	6.10	1.42	2.42
	AS-H	5.58	6.04	1.06	1.15
6	AD-H	5.50	6.69	1.39	2.67
	OD-H	13.21	15.72	1.23	5.56
	OJ-H	5.51	6.19	1.22	1.51
	AS-H	6.22	–	1.00	0.00

Table 2
Retention times ($t_{R,1}$, $t_{R,2}$), separation factor (α) and resolution (R_s) of compounds **1**–**6** on Chiralpak AD-H and AS-H, Chiralcel OD-H and OJ-H, using 10% ethanol in carbon dioxide at 2 ml/min, 35 °C, 230 nm detection, 150 bar backpressure

Compound	Column	$t_{R,1}$ (min)	$t_{R,2}$ (min)	α	R_s
1	AD-H	8.38	12.12	1.62	6.23
	OD-H	8.43	12.58	1.66	7.55
	OJ-H	5.01	5.99	1.36	3.31
	AS-H	8.39	9.21	1.14	1.37
2	AD-H	6.68	8.99	1.54	4.62
	OD-H	6.83	9.38	1.57	5.10
	OJ-H	4.78	5.80	1.41	2.91
	AS-H	5.80	–	1.00	0.00
3	AD-H	6.58	8.82	1.54	6.40
	OD-H	6.78	9.20	1.55	6.91
	OJ-H	4.80	5.82	1.43	3.64
	AS-H	5.61	–	1.00	0.00
4	AD-H	10.55	10.82	1.03	0.30
	OD-H	10.21	10.78	1.07	0.63
	OJ-H	4.60	5.05	1.20	0.82
	AS-H	11.58	12.90	1.14	2.20
5	AD-H	7.19	9.86	1.56	5.93
	OD-H	7.10	9.92	1.60	6.26
	OJ-H	5.90	6.58	1.19	1.51
	AS-H	6.66	7.21	1.13	1.10
6	AD-H	6.50	6.69	1.39	2.67
	OD-H	13.21	15.72	1.23	5.56
	OJ-H	5.51	6.19	1.22	1.51
	AS-H	6.22	–	1.00	0.00

Table 3
Retention times ($t_{R,1}$, $t_{R,2}$), separation factor (α) and resolution (R_s) of compounds **1**–**6** on Chiralpak AD-H and AS-H, Chiralcel OD-H and OJ-H, using 15% 2-propanol in carbon dioxide at 2 ml/min, 35 °C, 230 nm detection, 150 bar backpressure

Compound	Column	$t_{R,1}$ (min)	$t_{R,2}$ (min)	α	R_s
1	AD-H	5.28	5.44	1.06	0.53
	OD-H	7.20	–	1.00	0.00
	OJ-H	3.45	4.00	1.52	1.38
	AS-H	5.68	6.01	1.10	0.70
2	AD-H	5.48	8.69	2.04	6.42
	OD-H	6.71	7.60	1.21	1.98
	OJ-H	3.60	4.03	1.36	1.43
	AS-H	4.40	4.48	1.04	0.20
3	AD-H	5.51	8.68	2.02	7.93
	OD-H	6.77	7.69	1.21	2.63
	OJ-H	3.59	4.01	1.35	1.56
	AS-H	4.44	–	1.00	0.00
4	AD-H	5.51	5.90	1.13	1.30
	OD-H	10.40	11.55	1.14	2.30
	OJ-H	3.58	–	1.00	0.00
	AS-H	6.08	6.65	1.15	1.32
5	AD-H	5.38	7.48	1.69	4.24
	OD-H	9.16	–	1.00	0.00
	OJ-H	3.93	–	1.00	0.00
	AS-H	5.09	5.47	1.14	0.84
6	AD-H	3.70	5.22	2.09	5.13
	OD-H	7.47	8.11	1.13	1.60
	OJ-H	4.08	4.28	1.16	0.40
	AS-H	4.33	–	1.00	0.00

SFC prep-column (30 mm × 250 mm, 5 μm), using 10% ethanol as the modifier, at a flow rate of 70 ml/ml. The sample (420 mg) was dissolved in 28 ml ethanol.

3. Results and discussion

3.1. SFC separation of *1* and *2*

Both compound **1** and its *N*-methylated derivative **2** were well resolved under multiple SFC conditions. Using methanol as modifier, baseline separation was achieved for **1** on AD-H and OJ-H columns, while there was little and no separation on OD-H and AS-H columns. However, using ethanol as the modifier, separation was improved on almost all four chiral columns, especially on OD-H column, on which resolution increased from 0.6 to 7.6. On the other hand, when the modifier was changed from ethanol to 2-propanol, all columns, except OJ-H column, lost their resolution efficiency, and yielded little or no separation. These results indicated that the separation of **1** could be greatly influenced by the properties of the modifiers, such as their polarity as well as their hydrogen-donating/accepting ability. Ethanol with its values of these properties between methanol and 2-propanol appeared to be the most efficient modifier for **1**. For **2**, although ethanol appeared to be slightly better modifier in terms of the resolution, no significant difference was observed in the separation profiles with all three modifiers, all of which yielded baseline separations on AD-H, OD-H and OJ-H columns, but no separation on AS-H column. The most efficient SFC separation for both **1** and **2** was obtained with ethanol on an OJ-H column, under which both compounds were excellently resolved in less than 7 min (Fig. 3).

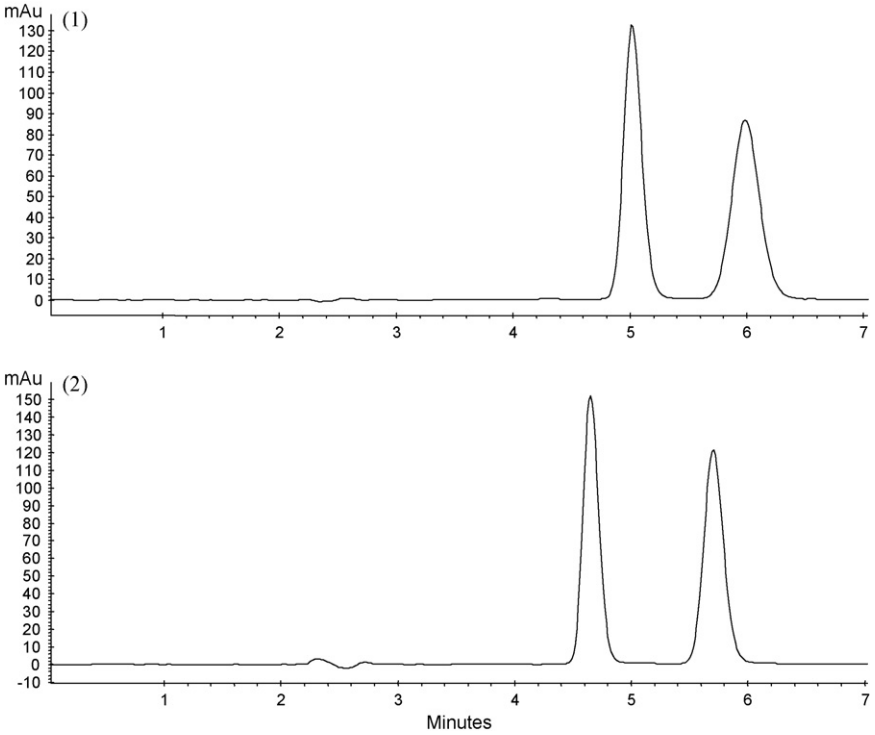

Fig. 3. Chromatograms of **1** and **2** using Chiralcel OJ-H column (4.6 mm × 250 mm, 5 μm), 10% ethanol in carbon dioxide at 2 ml/min, 35 °C, 230 nm detection, 150 bar backpressure.

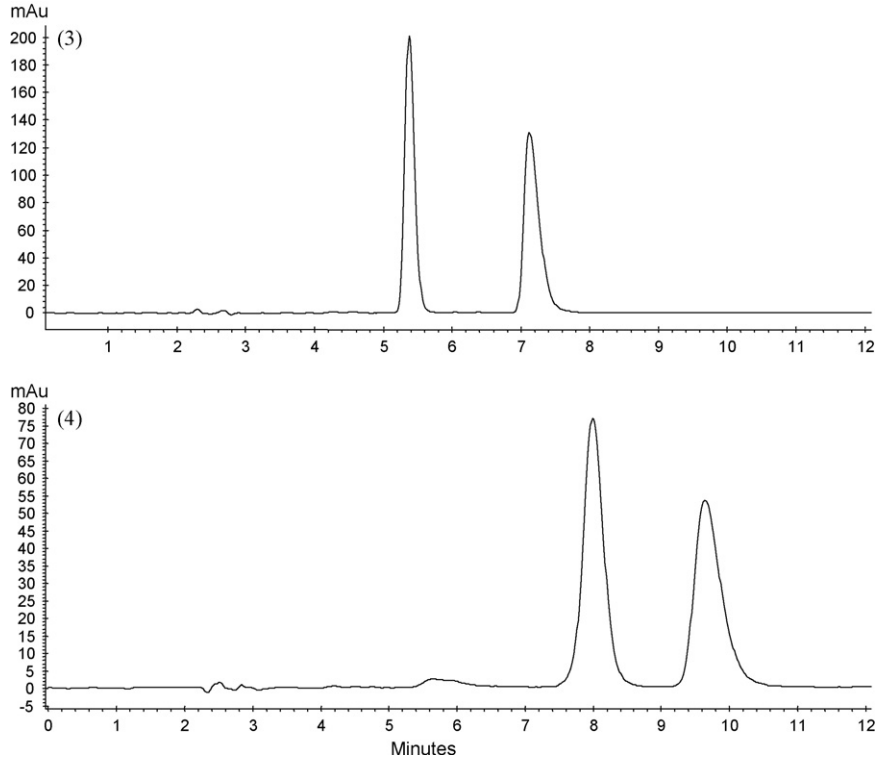

Fig. 4. Chromatograms of **3** and **4** using Chiralpak AD-H column (4.6 mm × 250 mm, 5 μm), 10% methanol in carbon dioxide at 2 ml/min, 35 °C, 230 nm detection, 150 bar backpressure.

*3.2. SFC separation of compounds containing polar substituents, **3** and **4***

Similar to compound **2**, both **3** and **4** were *N*-substituted analogs of **1**, except with more polar groups. Compound **3** with a cyanomethyl substituent, was separated very well under all but three screened SFC conditions. There was no significant difference between methanol, ethanol and 2-propanol, and all three modifiers gave good separations on AD-H, OD-H and OJ-H columns, but no separation on AS-H column. Compound **4**, substituted with a

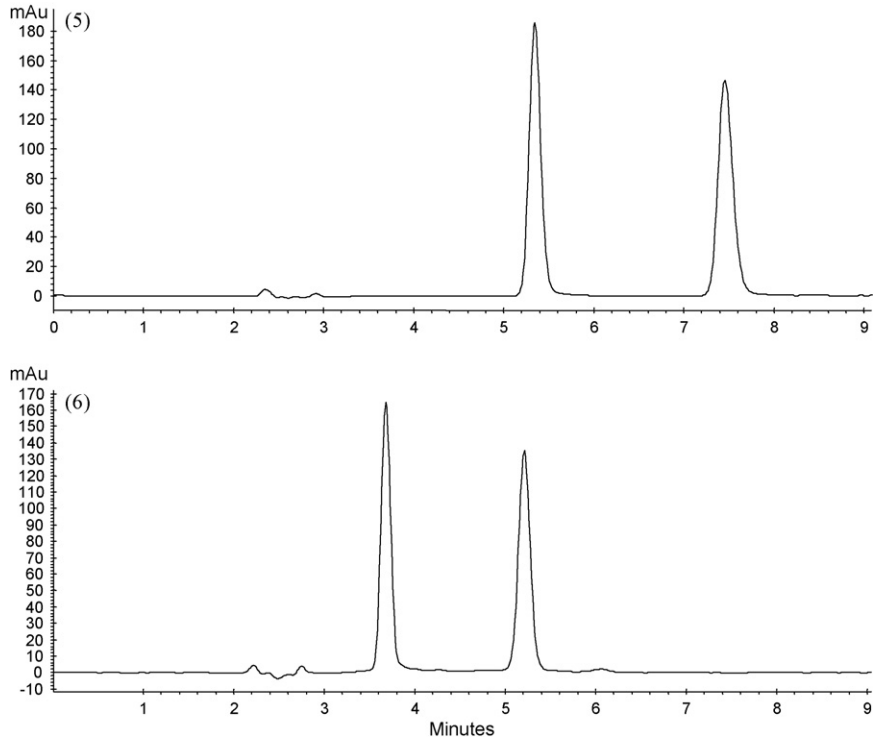

Fig. 5. Chromatograms of **5** and **6** using Chiralpak AD-H column (4.6 mm × 250 mm, 5 μm), 15% 2-propanol in carbon dioxide at 2 ml/min, 35 °C, 230 nm detection, 150 bar backpressure.

polar amide moiety, was resolved to the baseline under four SFC conditions. The right combination of the modifier and type of the column appeared to be the key to the separation of **4**. Methanol gave good separations on the AD-H and OD-H columns, and no separation on the AS-H column. On the contrary, when the modifier was switched to ethanol, poor separations were obtained on the AD-H and OD-H columns, but a baseline separation was observed on AS-H column. Among all the methods that yielded baseline separations, the combination of methanol and an AD-H column appeared to be the most suitable and efficient condition for separating these more hydrophilic analogs, under which the atropisomers of both **3** and **4** were well separated within 11 min with a resolution of 3.5 and higher (Fig. 4).

3.3. SFC separation of compounds with hydrophobic substituents, *5* and *6*

Compounds **5** and **6**, are more hydrophobic than their parent molecules **1** and **2**. Compound **5**, is an olefinic analog of **2**, and **6** is a silyl intermediate of **5**, in which the primary hydroxyl group was protected as *tert*-butyldiphenylsilyl ether. Baseline separations were achieved for **5** under 7 out 12 SFC conditions. There were no significant differences in the separation profiles between methanol and ethanol, which gave **5** reasonably good separations across almost all columns. Using 2-propanol as modifier led to an efficient separation with excellent resolution on AD-H column, but little or no separation on other columns. Compound **6** was well resolved under eight SFC conditions. The type of the modifier seemed to have little effect on the separation profiles of **6**, since all three modifiers gave similar separation profiles cross four columns. The right column appeared to be the key for the separation success of **6**. While AD-H and OD-H gave excellent separations using all modifiers, AS-H was found to be the least efficient with little or no separation. Among all the methods, the SFC condition using 2-propanol on an AD-H column was found to be the most efficient for separating this subgroup of compounds. Using this method, all **5** and **6** were well resolved within 8 min (Fig. 5).

3.4. SFC preparative purification of (−)-*1*

The most efficient SFC method developed for **1** (Fig. 3) was directly transferred to a Berger MultiGram II preparative SFC system. Although the flow rate at 2.0 ml/min with an analytical column (4.6 mm × 250 mm, 5 μm) could be proportionally scaled up to ca. 85 ml/min for a preparative column (30 mm × 250 mm, 5 μm), instrument limitation allowed a rate of 70 ml/min, which was used in the preparative separation of (±)-**1**. The sample load, 30 mg (2 ml) per injection, was established directly on the preparative column with trial injections. Instead of maximizing the load, advantage of the power of the stacked injections was taken for the preparative separation. Sample was injected into the column at an interval of 11 min. A total of 14 injections was needed to complete the separation, which yielded 205 mg of (−)-**1** (peak 1) and 202 mg of (+)-**1** (peak 2). The recovery rate of the sample was around 97%. The absolute stereochemistry of (−)-**1** and (+)-**1** was established by the X-ray crystallographic analysis [9]. The purified atropisomers, (−)-**1** and (+)-**1**, showed enantiomeric excess (ee) values of >99.9% and >98.9%, respectively. Atropisomer (−)-**1** demonstrated significantly higher activity than (+)-**1** as maxi-K channel openers [9].

3.5. Stability studies of (−)-*1*

Atropisomer (−)-**1** (Fig. 3, $R_{t,1}$ = 5.01 min), the more potent maxi-K channel opener, was subjected to the stability studies at 37 °C in human serum and at 80 °C in *n*-butanol. Stability study with (−)-**1** using pooled human serum indicated that the atropisomer was stable and did not racemize during 30 h, which was the duration of the experiment. Thermal stability was also investigated applying the same analytical conditions. Atropisomer (−)-**1** at 80 °C in *n*-butanol underwent a 19% conversion to (+)-**1** over 72 h. The experimental details of the studies were described in Ref. [9].

4. Conclusion

In conclusion, our results showed that the atropisomers of all six 3-substitued-4-arylquinolinones studied can be resolved under four or more SFC conditions by using supercritical fluid chromatography, irrespective of their structural variations and polarity, and the majority of the separations were completed within 10 min. The AD-H column appeared to be superior to the other three chiral columns, while methanol and ethanol showed higher successful rate than 2-propanol for atropisomer separation of this class of compounds. However, it was proven worthwhile to explore systematically all four columns and three modifiers for faster and more efficient methods, especially when preparative scale-up was required. Our SFC screen yielded three efficient generic SFC methods for the parent molecules (**1**, **2**), the more hydrophilic analogs (**3**, **4**), and the more lipophilic derivatives (**5**, **6**), respectively. These SFC methods could potentially be utilized to separate additional analogs with similar structural properties within the class. The SFC method developed for the parent molecules was successfully used for the preparative purification of the individual atropisomers, (−)-**1** and (+)-**1**. Furthermore, the fast analytical capability of the SFC method facilitated the successful evaluation of the stability of (−)-**1** at 37 °C in human serum and at 80 °C in *n*-butanol [9]. Finally, this study took into consideration the possibility that two stable atropisomers could have differential biological and pharmacological activities. Should such differential activities of stable atropisomers be encountered, there could be a distinct possibility of advancing a chiral drug into pharmaceutical development. From this perspective, SFC served as a valuable methodology for the investigation of this class of compounds, and it should be further explored for applicability to the other classes of compounds.

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