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Exploration of liquid and supercritical fluid chromatographic chiral separation and purification of Nutlin-3—A small molecule antagonist of MDM2

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Abstract

Inhibition of the MDM2–p53 interaction can stabilize the p53 protein and offer a novel strategy for cancer therapy. The imidazoline compound (Nutlin-3) is a promising small molecule antagonist of the MDM2–p53 interaction. This compound was synthesized as a racemic mixture, and one enantiomer is 100–200-fold more active than the other enantiomer. In this study, various enantiomeric separation approaches were explored to resolve the Nutlin-3 enantiomers using chiral supercritical fluid chromatography (SFC) as well as chiral liquid chromatography (LC) under normal phase mode, reversed phase mode and polar organic phase mode. The chiral SFC method based on Chiralcel OD column showed superior separation in terms of selectivity and efficiency. Optimization of the chiral separation method enabled high throughput preparative scale purification. Ultimately, 5 g of racemic mixture were purified on Prep-SFC in 75 min with the recovery rate above 92%.

Keywords: Anti-cancer drug; Chiral separation; Chiral purification; Liquid chromatography; Supercritical fluid chromatography

1. Introduction

The p53 protein is currently recognized as one of the dominant tumor suppressors, and its mutation to an inactive form is one of the most common genetic alterations observed in human cancer [1–3]. Under non-stressed conditions, cellular p53 is tightly controlled by the mouse double minute 2 (MDM2) gene which inhibits its transcriptional activity and promotes its degradation. Overexpression of MDM2 can impair the tumor suppressor function of p53 [4–6]. Thus, the p53 pathway has been a frequent target for the discovery of oncology therapeutics in the last several years. In addition to ongoing efforts to develop compounds that can reactivate mutant p53, inhibiting the interaction of MDM2 with p53 has been proposed as a target. An MDM2 antagonist could activate the p53 pathway and may offer a novel strategy for cancer therapy [7]. Vassilev et al. reported, for the first time a series of small molecule MDM2 inhibitors termed Nutlins, which possess the appropriate potency and drug-like properties [8]. These imidazoline compounds were synthesized as racemic mixtures. As shown with the most potent compound, Nutlin-3 (Fig. 1), only enantiomer (a) possessed potent binding activity with an IC_{50} of 0.09 μM. The enantiomer (b) was about 150-fold less active.

For early stage therapeutic programs of drug discovery, it has become of paramount importance to have a fast and flexible means for securing a desired single isomer in quantities of 100 mg to multi-grams for bio-assays, PK/PD, and toxicology studies. Quite a number of approaches have been used to isolate single enantiomers, including a limited scope of enantio-selective reactions, chiral salt resolution or separation by chiral chromatography. Both the enantio-selective reactions and the chiral salt resolution routes are potentially quite time consuming for method development and they often require expensive reagents. Currently, enantio-selective chromatography using high-performance liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) with chiral stationary phases (CSPs) has become the most widely utilized tool to obtain small quantities (up to low kilogram level) of pure enantiomers in pharmaceutical research. In this study, various chiral separation approaches, including normal phase chiral LC,

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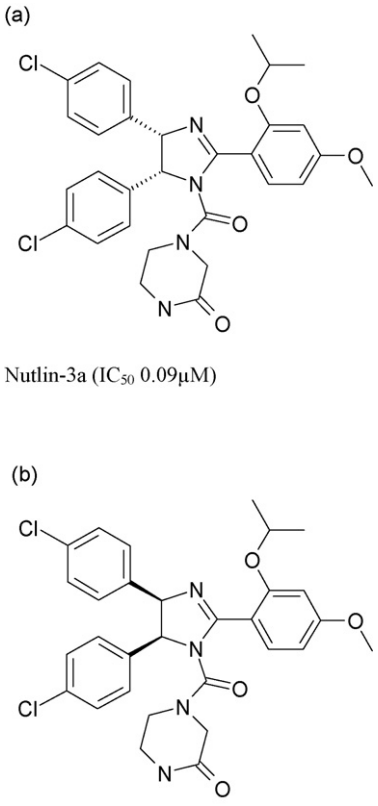

Fig. 1. Structures of Nutlin-3 enantiomers.

reversed phase chiral LC, polar organic mode chiral LC as well as chiral SFC, were explored to resolve the racemic mixture of Nutlin-3. The ultimate goal is to increase the throughput for the preparative chiral purification of this promising anticancer drug candidate, in order to obtain sufficient quantities of pure enantiomers for further pharmacological and toxicological evaluations.

2. Experimental

2.1. Materials and reagents

HPLC-grade methanol, acetonitrile, hexane, ethanol, isopropanol and water (J.T. Baker, Phillipsburg, NJ) were used. SFC-grade carbon dioxide (Air Products and Chemicals, Allentown, PA) was used as the primary mobile phase for SFC.

Nutlin-3 was synthesized in house. Its structure was characterized by ^{1}H NMR, ^{13}C NMR, and high-resolution MS.

2.2. Apparatus

HPLC was performed on an Agilent 1100 LC system (Wilmington, DE) equipped with an autosampler, binary pump, thermostatted column compartment and a diode array detector. SFC was performed on a Berger MiniGram SFC system analytical mode (Mettler-Toledo AutoChem, Newark, DE) with six-way column and solvent selection valves. The system consisted of an automatic liquid sampler (ALS) with a 5 μL loop used to make injections and a thermal control module (TCM) used to control the column temperature. Knauer variable wavelength UV detector (supplied by Mettler-Toledo) with a high-pressure flow cell was used for SFC-UV detection.

Table 1
The retention factor, selectivity and resolution data for the chiral separation of Nutlin-3 on normal phase LC

Entry	Column	Mobile phase (v/v)	k_1	α	Rs
1	OD	Hexane:ethanol 40:60	1.42	1.29	1.81
2		Hexane:ethanol 70:30	2.21	1.33	2.14
3		Hexane:2-propanol 40:60	1.76	1.55	1.95
4		Hexane: 2-propanol 60:40	2.01	1.56	2.09
5	AD	Hexane:ethanol 40:60	3.52	ns	ns
6		Hexane:2-propanol 40:60	3.06	ns	ns
7	OJ	Hexane:ethanol 70:30	2.04	1.21	1.17
8		Hexane:2-propanol 40:60	1.54	1.15	1.23
9	Chirobiotic T	Hexane:ethanol 20:80	3.87	1.47	2.01
10		Hexane:2-propanol 5:95[a]	5.17	1.40	1.12
11	Chirobiotic V	Hexane:ethanol 40:60	3.94	ns	ns
12		Hexane: 2-propanol 40:60	3.03	ns	ns

ns: no separation.
[a] Flow rate 0.9 mL/min.

A total of 5.0 g of the Nutlin-3 was purified on chiral Prep-SFC. The purification was carried out on a Berger Multigram III SFC (Mettler-Toledo AutoChem, Newark, DE) equipped with two SD-1 Varian pumps, a Knauer K-2501 spectrophotometer. The sample was dissolved to a volume of 95 mL in methanol. Injection volume was 4.5 mL (230 mg per injection) and the sample was injected at intervals of 3 min onto a 25 cm × 50 mm × 5 μm preparative Chiralcel OD column

Table 2
The retention factor, selectivity, and resolution data for the chiral separation of Nutlin-3 on reversed phase LC

Entry	Column	Mobile phase (v/v)	k_1	α	Rs
1	OD-RH	Acetonitrile:H_2O 40:60	6.18	1.49	5.08
2		Methanol:H_2O 75:25	5.99	1.45	2.78
3		Ethanol:H_2O 75:25	3.49	1.40	2.49
4	AD-RH	Acetonitrile:H_2O 35:65	2.02	ns	ns
5		Methanol:H_2O 80:20	3.32	ns	ns
6		Ethanol:H_2O 85:15	2.44	ns	ns
7	OJ-RH	Acetonitrile:H_2O 35:65	2.31	1.11	1.08
8		Acetonitrile:H_2O 40:60	3.61	1.13	1.38
9		Methanol:H_2O 70:30	2.74	ns	ns
10		Ethanol:H_2O 60:40[a]	2.34	ns	ns
11	Chirobiotic T	Acetonitrile:H_2O 50:50	2.91	ns	ns
12		Methanol:H_2O 70:30	2.41	ns	ns
13		Ethanol:H_2O 70:30[a]	1.98	ns	ns
14	Chirobiotic V	Acetonitrile:H_2O 50:50	2.53	ns	ns
15		Methanol:H_2O 45:55[a]	3.65	1.06	0.77
16		Ethanol:H_2O 45:55[b]	1.59	ns	ns

ns: no separation.
[a] Flow rate 0.9 mL/min.
[b] Flow rate 0.8 mL/min.

Table 3
The retention factor, selectivity, and resolution data for the chiral separation of Nutlin-3 on polar organic phase LC

Entry	Column	Mobile phase (v/v)	k_1	α	Rs
1	OD-RH	Methanol	0.31	1.80	2.33
2		Ethanol	0.31	1.79	2.29
3		2-PrOH[a]	1.04	2.05	1.74
4		Acetonitrile	ns	ns	ns
5	AD-RH	Methanol	0.22	1.95	0.69
6		Ethanol	0.55	ns	ns
7		2-PrOH[a]	1.50	ns	ns
8		Acetonitrile	0.17	1.63	0.5
9	OJ-RH	Methanol	0.06	ns	ns
10		Ethanol	0.11	2.07	0.77
11		2-PrOH[a]	0.34	1.56	0.53
12		Acetonitrile	ns	ns	ns
13	Chirobiotic T	Methanol	0.41	1.28	1.53
14		Ethanol	3.66	1.24	1.43
15		2-PrOH[b]	5.05	ns	ns
16		Acetonitrile	2.45	ns	ns
17	Chirobiotic V	No separation	ns	ns	ns

ns: no separation.
[a] Flow rate 0.9 mL/min.
[b] Flow rate 0.8 mL/min.

Table 4
The retention factor, selectivity, and resolution data for the chiral separation of Nutlin-3 on SFC

Entry	Column	Mobile phase (v/v, vs. CO_2)	k_1	α	Rs
1	OD	30% MeOH	2.67	1.62	4.70
2		35% MeOH	1.97	1.59	4.12
3		35% EtOH	1.21	1.64	3.46
4		35% 2-PrOH	1.89	1.98	3.28
5		30% EtOH/ACN	2.65	2.76	4.41
6		35% EtOH/ACN	1.75	1.69	3.59
7	AD	40% MeOH	2.90	ns	ns
8		40% EtOH	3.51	ns	ns
9		40% 2-PrOH	3.64	ns	ns
10		40% EtOH/ACN	2.60	ns	ns
11	OJ	20% MeOH	1.11	2.35	2.95
12		20% EtOH	1.29	2.09	2.99
13		20% 2-PrOH	1.42	1.97	2.60
14		20% EtOH/ACN	1.03	2.12	3.17
15	Chirobiotic T	40% MeOH	1.31	1.36	1.71
16		40% EtOH	3.41	1.14	1.59
17		40% 2-PrOH	4.17	ns	ns
18		40% EtOH/ACN	1.29	ns	ns
19	Chirobiotic V	No separation	ns	ns	ns

ns: no separation.

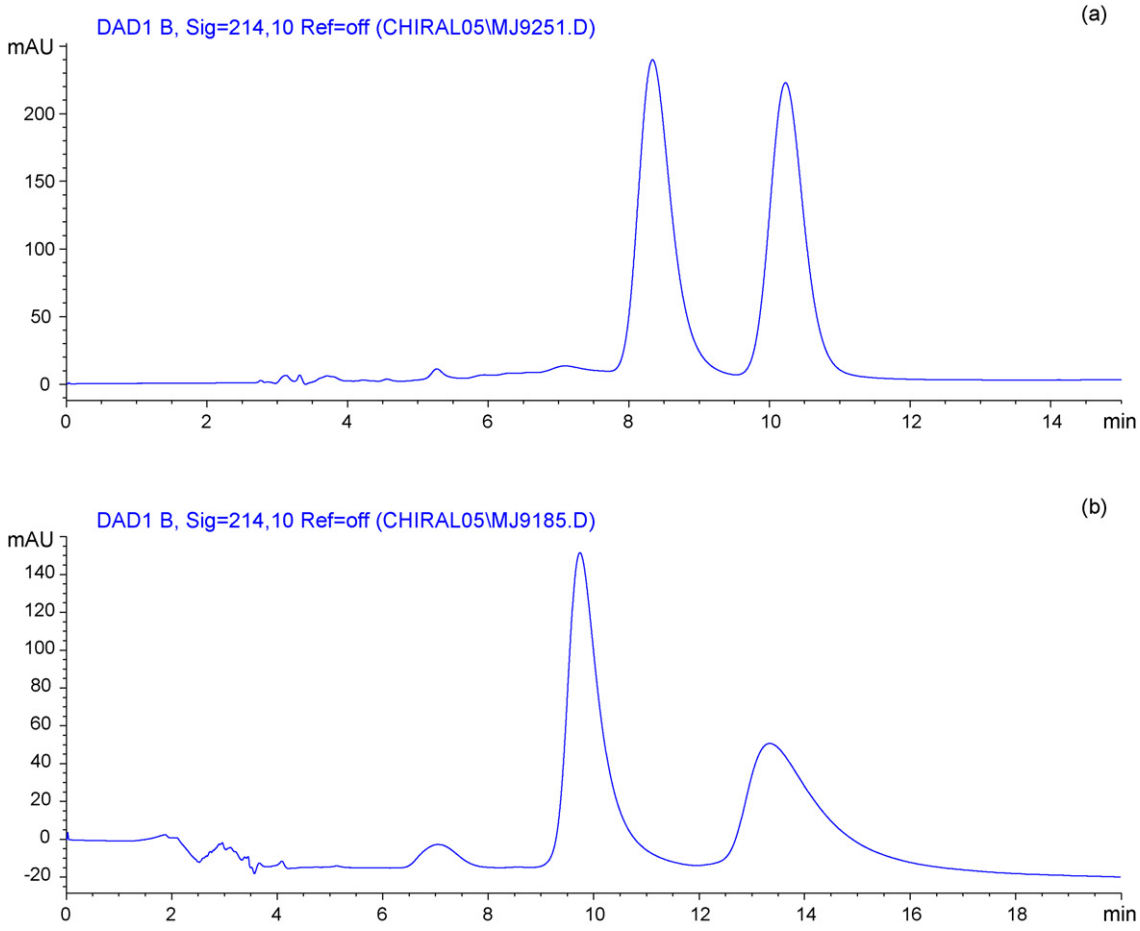

Fig. 2. Analytical separation of Nutlin-3 on normal phase chiral LC mode. (a) Chiralcel OD column with hexane:ethanol (70:30 v/v) as the mobile phase; (b) Chirobiotic T column with hexane:ethanol (20:80 v/v) as the mobile phase. Separation temperature 25 °C, flow rate 1.0 mL/min.

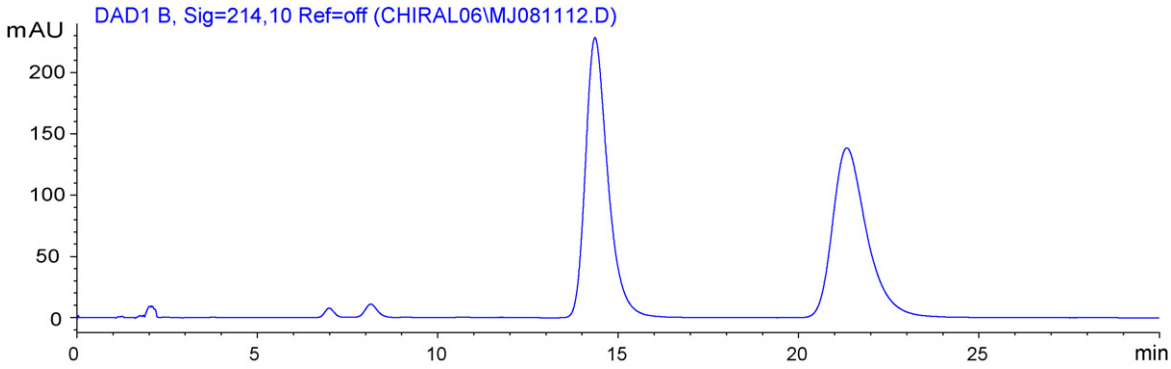

Fig. 3. Analytical separation of Nutlin-3 on reversed phase chiral LC mode. Chiralcel OD-RH column with acetonitrile:water (40:60 v/v) as the mobile phase. Separation temperature 25 °C, flow rate 1.0 mL/min.

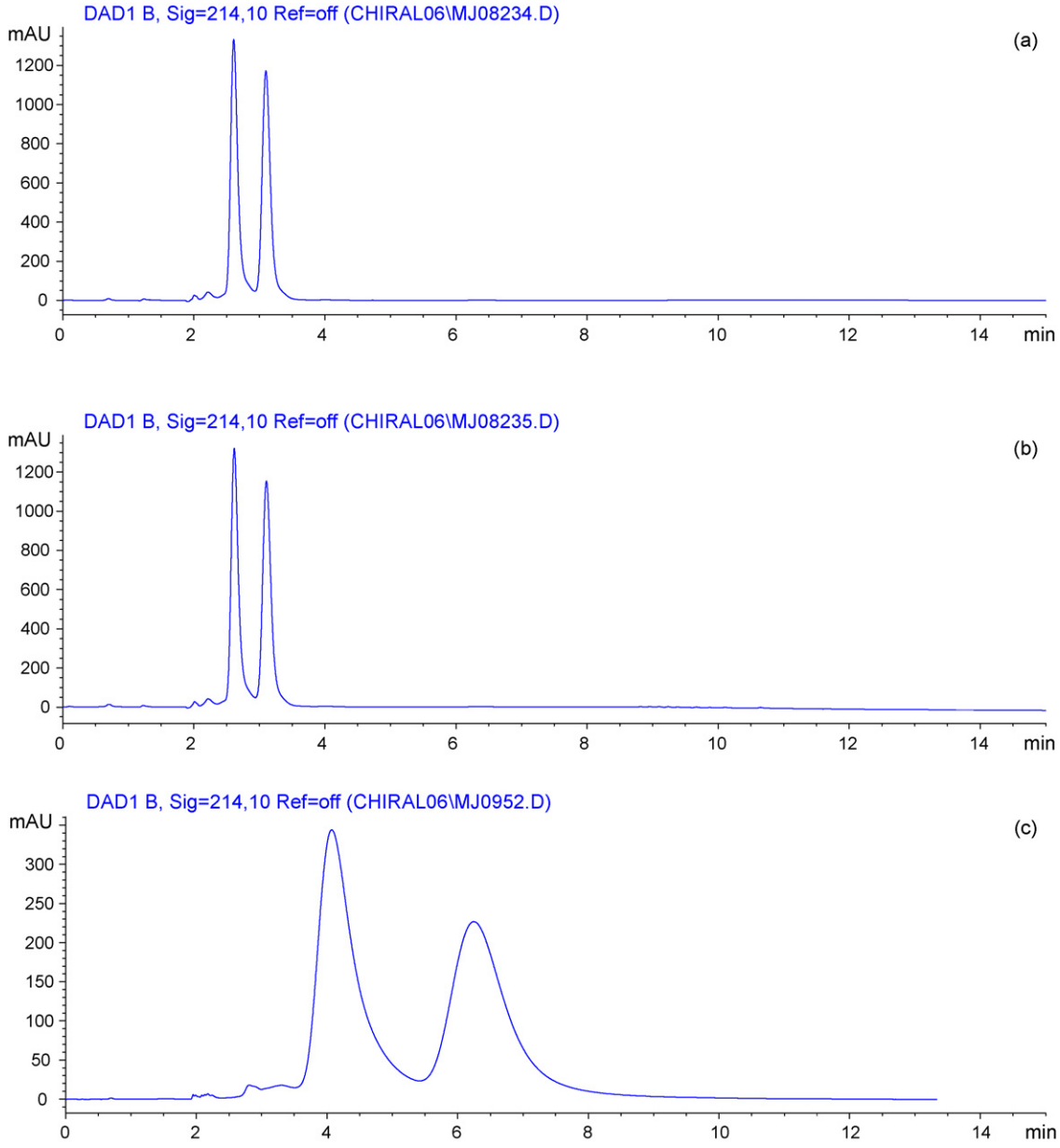

Fig. 4. Analytical separation of Nutlin-3 on chiral LC under polar organic phase mode with: (a) neat methanol; (b) neat ethanol; (c) neat isopropanol as the mobile phase. Separation temperature 25 °C, flow rate 1.0 mL/min, Chiralcel OD-RH column.

(Chiral Technologies, West Chester, PA). Sample peaks were collected using collection windows based on the predicted retention time.

Analytical Chiralcel OD, Chiralcel OD-RH, Chiralpak AD, Chiralpak AD-RH, Chiralcel OJ, and Chiralcel OJ-RH columns, were purchased from Chiral Technologies (West Chester, PA) with the dimensions of 25 cm × 4.6 mm × 5 μm. Chirobiotic T and V (25 cm × 4.6 mm × 10 μm) were purchased from Advanced Separation Technologies Inc. (Whippany, NJ).

2.3. Chromatographic conditions

Liquid chromatographic separations were performed at a temperature of 25 °C and a flow rate of 1.0 mL/min. Supercritical fluid chromatographic separations were performed at a temperature of 30 °C, a flow rate of 2.0 mL/min, and downstream mobile phase pressure of 100 bar. Detection in LC and SFC was attained by measurement of UV absorbance at 214 nm. The analyte was dissolved in methanol and the injection volume was 5 μL for both HPLC and SFC analysis. Duplicate injections were performed at each mobile phase concentration.

Preparative SFC separations were performed at a temperature of 30 °C, a mobile phase flow rate of 300.0 mL/min, and downstream mobile phase pressure of 100 bar.

3. Results and discussion

HPLC on CSPs has become the most widespread chiral separation technique used in the pharmaceutical industry in the past two decades [9]. The most commonly used CSPs include derivatized polysaccharide phases, macrocyclic-type phases, and pirkle-type pi-electron acceptor/pi-electron donor phases. Based on the mobile phase employed, the separation modes can be divided into normal phase mode, reversed phase mode and polar organic phase mode.

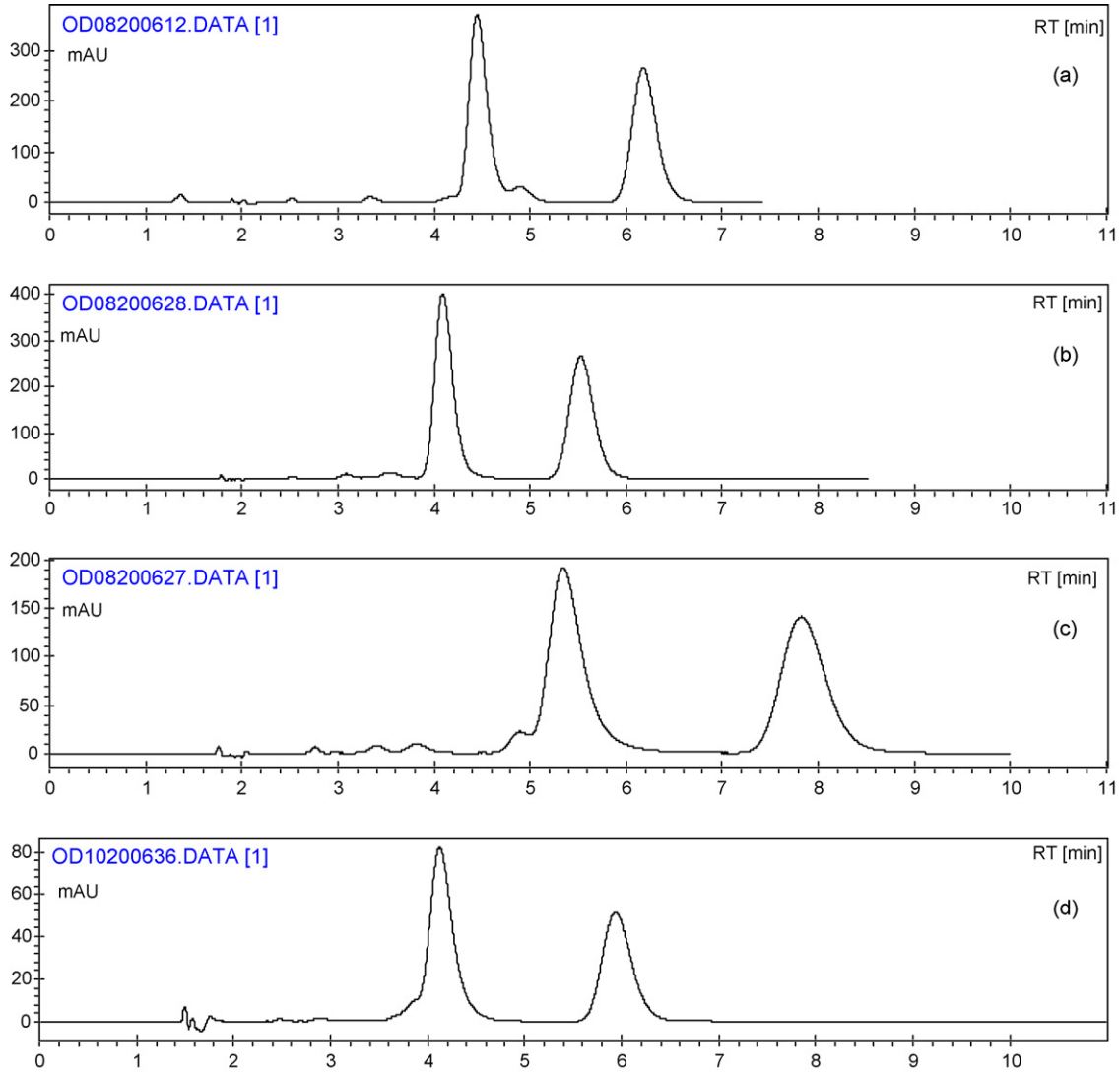

Fig. 5. Analytical separation of Nutlin-3 on chiral SFC on Chiralcel OD column with 35% (v/v) of: (a) methanol; (b) ethanol; (c) isopropanol; (d) ethanol:acetonitrile (1:1) as the CO_2 modifier. Downstream mobile phase pressure of 100 bar, 30 °C, flow rate 2.0 mL/min.

3.1. Chiral separation of Nutlin-3 on HPLC

Normal phase separations are most widely used in chiral HPLC. The common normal phase mobile phases are the mixtures of alcohol and alkanes. Although the chiral separation mechanism is not fully clear, the formation of hydrogen bonds between the analytes and CSPs is believed to be one of the major interactions that contribute to chiral separation. Increasing the proportion of alcohol modifier enhances the polarity of the mobile phase and as a result, increases the solubility of the analyte in the mobile phase, reduces the formation of hydrogen bonding, and shortens the retention time. Based on the available CSPs in this lab, the enantioseparation of Nutlin-3 was investigated first on three derivatized polysaccharide phases Chiralcel OD, OJ, Chiralpak AD, and two macrocyclic-type phases Chirobiotic T and V analytical columns under normal phase separation condition. Hexane/ethanol, and hexane/isopropanol were tried as the mobile phases, respectively. As listed in Table 1, among the polysaccharide phases, only the OD column, which is a particulate silica-based column coated with (3,5-dimethylphenyl carbamate) derivatized cellulose, gave baseline enantiomeric separation with resolution above 1.5. Another cellulose-based column, Chiralcel OJ only showed partial separation. In the mobile phase, when the same percentage ethanol was replaced with isopropanol, a slightly longer retention time of the analyte was observed, but there was no significant gain in selectivity. Interestingly, as the mobile phase polarity increased, e.g. from 30 to 60% ethanol, or from 40 to 60% isopropanol, the retention time was reduced, but the enatioselectivity was less affected by the change of the alcohol percentage. This may suggest that hydrogen bonding may not be the only interaction and at least one other type of interaction that is independent of solvent polarity is responsible for enantiomeric selectivity [10]. It has been reported that derivatized cellulose stationary phases exhibit inclusion-type retention that lead to enantioselectivity, and this could be the other dominant contribution here towards chiral discrimination [11]. Under the attempted conditions, the best separation was obtained on the OD column using

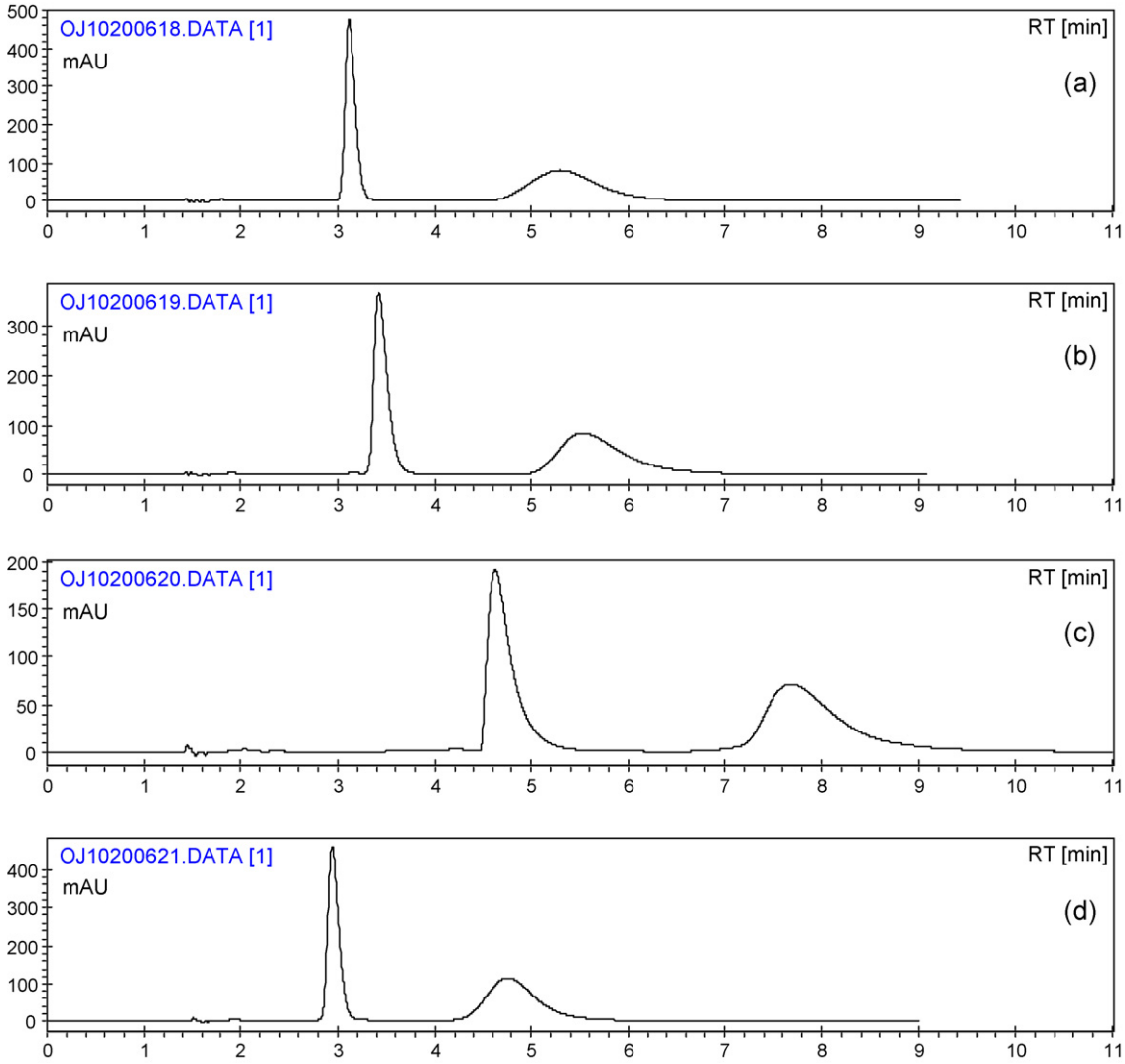

Fig. 6. Analytical separation of Nutlin-3 on chiral SFC using a Chiralcel OJ column with 35% (v/v) of: (a) methanol; (b) ethanol; (c) isopropanol; (d) ethanol:acetonitrile (1:1) as the CO_2 modifier. Downstream mobile phase pressure of 100 bar, 30 °C, flow rate 2.0 mL/min.

hexane–ethanol (70:30) (Fig. 2a). As for the two macrocyclic columns, Chirobiotic T achieved baseline separation of the two enantiomers (Fig. 2b). However, the peaks were relatively broad with the peak width around 2 and 3.5 min each. In addition, the total run time was up to 18 min. The broad peak and long analysis time are not suitable for purification purposes, since a wide collection window and a long injection cycle time will be required, which excludes this method for purification.

Three types of polysaccharide-based reversed phase columns (OD-RH, AD-RH and OJ-RH) and two macrocyclic columns (Chirobiotic T and V) were then evaluated under reversed phase mode, using acetonitrile/water, methanol/water and ethanol/water as the mobile phases. There was no separation on AD-RH and Chirobiotic T at all. While on OJ-RH and Chirobiotic V there was only partial separation with the mobile phase of acetonitrile/water and methanol/water (see Table 2). OD-RH column showed baseline separation in all three mobile phase systems. Compared to the alcohol/water mobile phase system, acetonitrile/water gave better separations in general. Acetonitrile has lower hydrogen bond donating ability and accepting ability than those of methanol and ethanol, but its polarity is close to that of methanol. As a result, the interaction of hydrogen bonding between analyte and CSPs may be stronger in an acetonitrile/water environment than in an alcohol/water environment. The best result came from the acetonitrile/water mobile phase on the OD-RH column (Fig. 3). The retention time difference in the two peaks was up to 5 min, which allows for higher sample loading in the purification process. However, the long elution time (around 24 min) will increase the total run time of the purification. In addition, since water is part of the mobile phase, from the point of longer solvent evaporation time, reversed phase LC is not the first choice for chiral purifications unless no other separation mode works competitively.

The use of neat alcohol or acetonitrile as the mobile phase is referred as polar organic phase mode in chiral separations. Although the separation mechanism is not clear, using polar organic mobile phases is attractive for preparative chiral purification due to enhanced solubility and simplicity of solvent removal [12]. In this study, all three polysaccharide-based reversed phase columns (OD-RH, AD-RH and OJ-RH) afforded differing degrees of separation of Nutlin-3 (Table 3). Over all, alcohols (neat methanol, ethanol and isopropanol) provided higher selec-

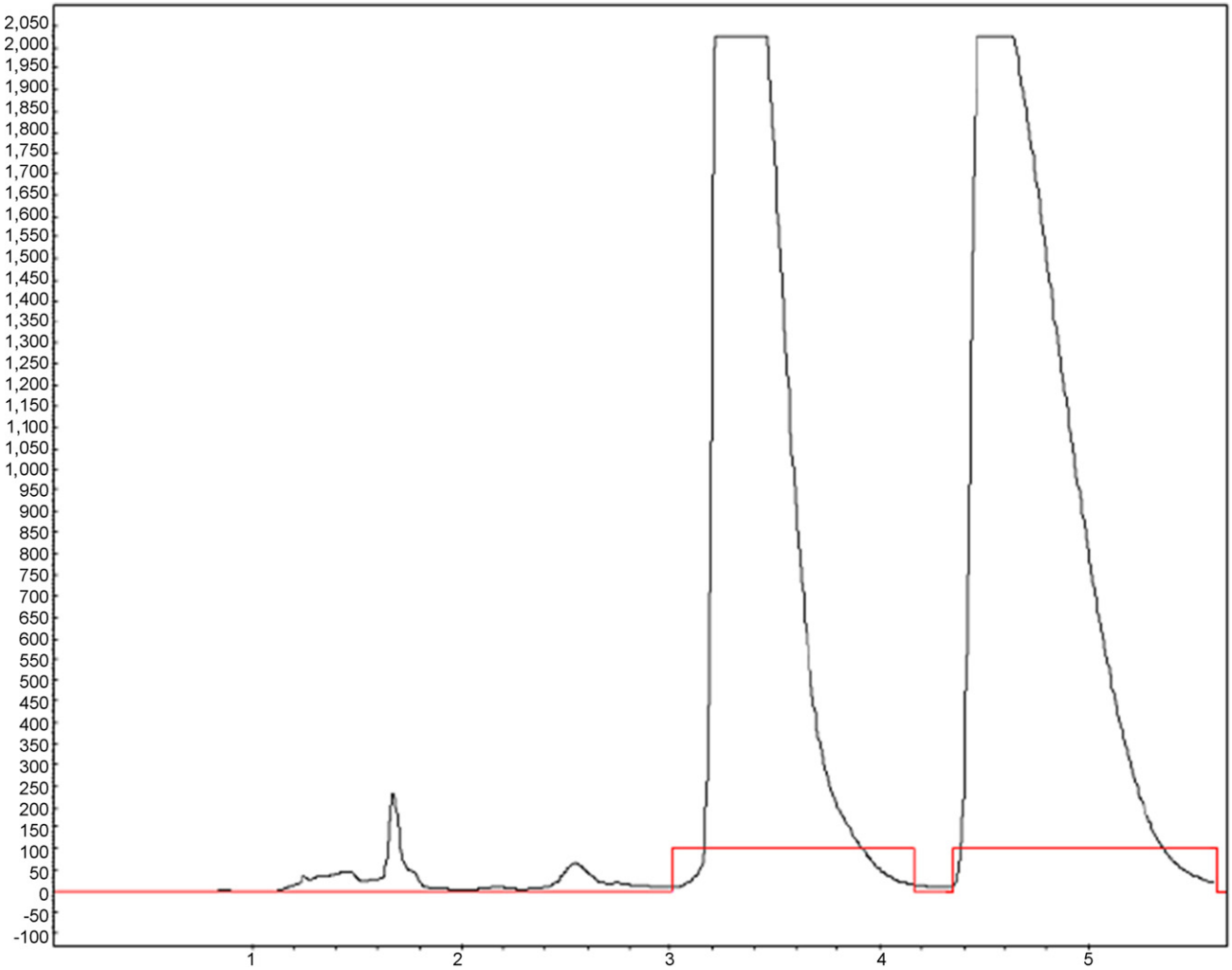

Fig. 7. Single injection on Prep-SFC with 5 cm i.d. Chiralcel OD prep column. Two hundred and thirty milligrams per injection, downstream mobile phase pressure of 100 bar, 30 °C, flow rate 300.0 mL/min, 35% (v/v) methanol as the CO_2 modifier (the collection windows were indicated by the offset baseline).

tivity than acetonitrile. This observation is opposite to what we found in the reversed phase separation mode, which indicates the separation mechanism in the polar organic mode may be different than that of the reversed phase mode. The shape and/or the size of the chiral cavity on the CSPs may be modified as the mobile phase is changed from an organic–aqueous mixture to a neat organic phase. This result cannot be explained by simply using the strength of hydrogen bond donating/accepting ability of the mobile phase. On the OD-RH column, baseline separation with an elution time of 4 min was obtained by using neat methanol or ethanol (Fig. 4). However, for the purpose of purification, such a baseline separation is still not sufficient as the two eluted peaks are too close to increase the loading-ability in the purification process, although the run time is short.

3.2. Chiral separation of Nutlin-3 on SFC

SFC is generally considered as a normal phase separation technology. A polar organic solvent such as methanol, ethanol or isopropanol, is used as the CO_2 modifier to increase the polarity of the mobile phase. A condensed modifier layer (usually an alcohol) is adsorbed on the stationary phase [13]. As a result, the mobile phase is almost always less polar than the modified stationary phase. In the past several years, the rapidly emerging technique of SFC with CSPs has become the major chiral separation tool in the drug discovery arena [9]. The low viscosity and high diffusivity of the SFC mobile phase allow for separations to be achieved with higher flow rates, higher column efficiencies, improved resolution and faster column re-equilibration. Packed column SFC typically provide a three to fivefold reduction in analysis time over conventional HPLC [9].

Three polysaccharide-based columns (OD, AD and OJ) and two macrocyclic columns (Chirobiotic T and V) were tested using SFC. There was no separation with the Chirobiotic V and Chiralpak AD columns with various modifiers. While on the Chirobiotic T column, separations were achieved only when methanol or ethanol were used as modifiers. Baseline separations were obtained on OD and OJ columns, with all of the modifiers tested. When three types of solvents: methanol, ethanol or the mixture of ethanol and acetonitrile (v/v, 1:1) were used as modifiers, the separations were accomplished within 7 min with the selectivities all above 1.5 (Table 4). Based on the retention time of the analytes, methanol, ethanol and the ethanol/acetonitrile mixture showed similar solvent strength. Isopropanol has a weaker solvent strength and resulted in longer retention of the analyte. On the OD column, the second-eluted peak showed a slightly broader peak width than the first eluted peak, as an isocratic elution was used (Fig. 5). The selectivity is generally

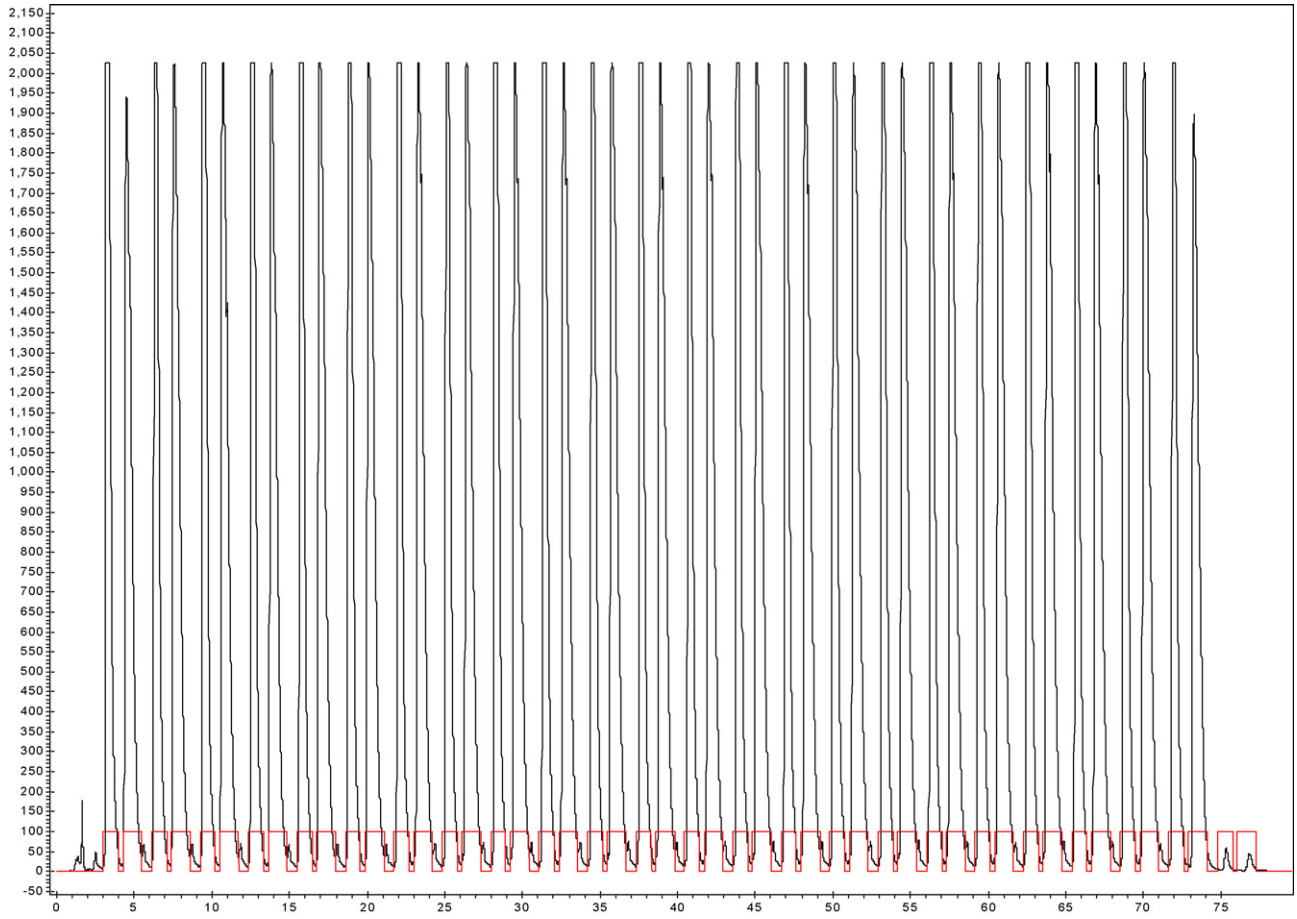

Fig. 8. Twenty-three-stacked injection on Prep-SFC with 5 cm i.d. Chiralcel OD prep column. Two hundred and thirty milligram/injection, 3 min per injection, downstream mobile phase pressure of 100 bar, 30 °C, flow rate 300.0 mL/min, 35% (v/v) methanol as the CO_2 modifier (the collection windows were indicated by the offset baseline).

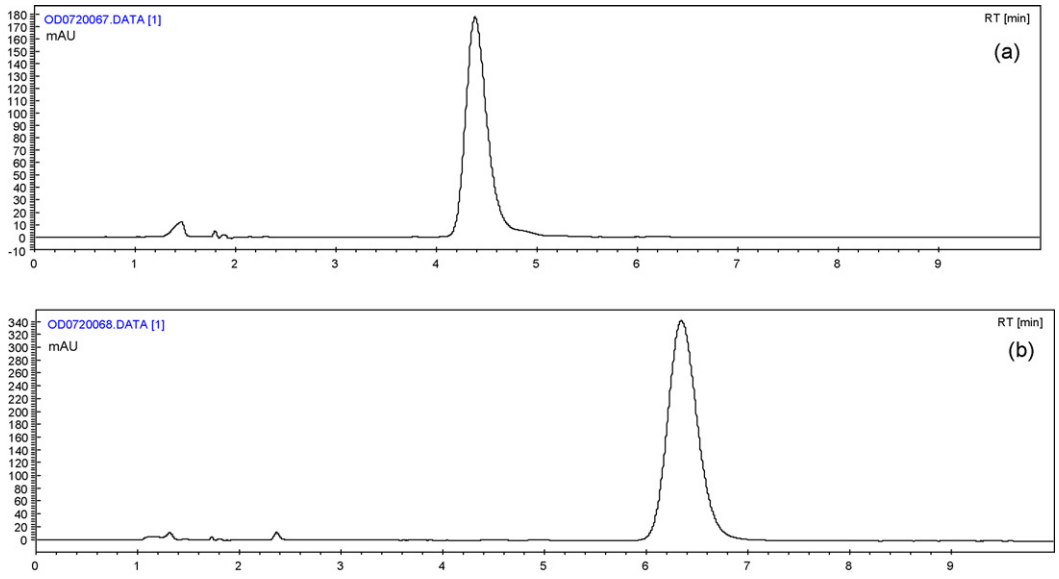

Fig. 9. Re-analysis of Nutlin-3 after chiral SFC purification (a) Nutlin-3a; (b) Nutlin-3b. Chiralcel OD column with 35% (v/v) of methanol as the CO_2 modifier. Downstream mobile phase pressure of 100 bar, 30 °C, flow rate 2.0 mL/min.

higher on the OJ column than on the OD column. However, lower resolution on the OJ columns was observed as the second-eluted peak was much broader than the first peak (Fig. 6). The theoretical plates (N) of the second peak were dramatically reduced to around 1/10th, compared to that of the first eluting peak. The broad peak also requires a wider collection window and consequently a longer solvent evaporation time.

After balancing speed (the retention time of the late-eluting peak), selectivity (the gap between the two peaks) and efficiency (the peak width), the SFC method using the OD column with 35% methanol as the CO_2 modifier, was considered as the best compromise in purifying Nutlin-3.

3.3. Chiral purification of Nutlin-3 on SFC

SFC is becoming quite popular for chiral purifications, not only because of its speed (high flow rate, efficiency and ease of solvent removal), but also because of lower expense in terms of organic solvent usage and disposal [9]. After the analytical method development as discussed above, the optimized analytical SFC method was transferred to prep-SFC on a 5 cm i.d. Prep OD column (25 cm length) at a mobile phase flow rate of 300.0 mL/min. The loading study showed a satisfactory separation which allows up to 230 mg per injection (10 mL loop). The total run time was 6 min for a single injection (Fig. 7). The stacked injection mode was used in the purification process. The injection cycle was determined based on the time range from the starting point of the first peak to the ending point of the second peak, and in the present study, a 3 min injection cycle was used for chromatographic purification. In order to increase the recovery, collection was set up based on the predicted retention time window instead of a threshold. The collection window length was set to 1.2 min for the first peak and 1.5 min for the second peak. As shown in Fig. 8, a total of 23-stacked injections were performed where each peak eluted exactly at the predicted time yielding high purity for each collected fraction. Five grams of Nutlin-3 were purified in 75 min with a recovery of 92%. Both isomers were obtained with an enantiomeric purity above 99.5% in the re-analysis (Fig. 9). By using the powerful capabilities of prep-SFC, the overall productivity rate was 2 g/h or 48 g/day for optically pure Nutlin-3. The organic solvent (methanol) usage was ca. 3.15 L per collected gram of each enantiomer. If CO_2 had been substituted with hexane the total solvent consumption would have been be 9 L. Consequently ~65% more time would have been needed for solvent removal and greater expense would be required for the usage and disposal of the organic solvent.

4. Conclusions

In the early stage of drug discovery, the tool of chiral separation is far more efficient than using asymmetric synthesis or related resolution procedures to obtain a small quantity of single enantiomeric drug candidate for the purposes of bioassay and toxicity evaluation. In this study, while searching for an optimized method for the purification of the Nutlin-3 racemic mixture, several different chiral separation methods were explored. Under the normal phase, reversed phase, as well as polar organic phase liquid chromatographic separations, baseline resolutions were achieved by using Chiralcel OD column. However, long elution time or broad peak width were also observed, thus preventing those separation conditions from becoming an ideal high throughput purification method. Supercritical fluid chromatography shows its intrinsic advantages in preparative purification, in terms of higher flow rates, quicker column equilibration, simpler solvent removal and less expense for solvent usage and disposal. A method was successfully developed on a prep-SFC using a Chiralcel OD column and 35% methanol as the CO_2 modifier. The daily throughput could be as high as 48 g of each enantiomer at one-third the time and expense compared to the HPLC method.

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