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Gradient elution of organic acids on a β-cyclodextrin column in the polar organic mode and its application to drug discovery

Peter J. Simms[a,*], Cynthia T. Jeffries[b], Xumin Zhao[c], Yujin Huang[c], Thomas Arrhenius[c]

[a] *Drug Development Department, Valeant Pharmaceuticals Int., 3300 Hyland Avenue, Costa Mesa, CA 92626, USA*
[b] *Department of Chemistry, Chembridge Research Laboratories, Rancho Bernardo, CA 92128, USA*
[c] *Department of Chemistry, Chugai Pharma USA, LLC San Diego, CA 92121, USA*

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Abstract

A high performance liquid chromatographic method was developed that separated organic acids using the polar organic mode. The separation was obtained using a β-cyclodextrin stationary phase with a mobile phase that was composed of acetonitrile/methanol/triethylamine (TEA)/acetic acid. The compounds were eluted under gradient conditions and the elution order depended on the number, type and position of the hydrogen bonding functional groups present in the molecule. Adjusting the acid to base ratio resulted in the biggest change in selectivity. In addition, increasing the methanol concentration decreased the retention times of the analytes, which had little effect on the selectivity. Using a certain set of conditions one could separate a large number of organic acids, which allowed these acids to be detected by UV and mass spectrometry. These conditions were used to evaluate the purity of potential pharmaceutical drug candidates that showed activity towards a kinase target vascular endothieal growth factor (Vegf). Each compound contained a carboxylic acid group that was critical to the activity. The method was able to give purity estimates of these samples, which were difficult to determine by other HPLC methods.

Keywords: Gradient elution; Cyclodextrins; Organic acids; Drug discovery

1. Introduction

Development of pharmaceutical drug candidates depends on how accurate the data obtained on the compound is during the discovery process. HPLC plays an important role in determining the purity and identity of the compounds of interest. Most HPLC analyses use reversed-phase chromatography coupled to different detectors [1] or LC/MS [2–7]. Methods that are developed using C18 stationary phases have produced rugged and dependable separations. These separations can also be very rapid and allow for high throughput analysis. Separation of pharmaceutical compounds can be also performed using normal-phase chromatography. However these conditions are not always compatible with LC/MS, which is often used to identify the candidates. Solvents like hexane are not miscible with many HPLC solvents. Halogenated solvents like chloroform and methylene chloride can attack and corrode the stainless steel in an HPLC system, which can lead to signal suppression or clogging of the LC/MS system. This makes it difficult to develop a normal-phase method that can be used to evaluate the purity of a large number of pharmaceutical candidates.

Due to their unique structure cyclodextrins have been used for a variety of chromatographic separations. Effective separations have been developed using cyclodextrins as mobile phase additives [8–15]. Cyclodextrins were attached to HPLC stationary phases in 1983 using spacers that contained no nitrogen groups [16–18]. Removal of the nitrogen from the spacer allowed for a more stable support, which gave better resolution of difficult to resolve isomers. These stationary phases have been shown to separate compounds in three different modes, reversed-phase [16,19–24], normal-phase [25] and the polar organic mode [26–29].

* Corresponding author. Fax: +1 714 641 7201.
E-mail address: pjsimms@valeant.com (P.J. Simms).

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The separation mechanism depended on the type of mobile phase that was used. Reversed-phase separations used mobile phases that consisted of an organic modifier and some aqueous buffer. Normal-phase separations typically used hexane/isopropanol mobile phases. The polar organic mode used mixtures of acetonitrile/methanol/TEA/acetic acid mobile phases to obtain the desired separation [28].

The polar organic mode used non-aqueous solvents; it contained anhydrous polar solvents such as TEA and acetic acid [29]. The solvents used were miscible, which resulted in the development of a set of gradient conditions for the separation. The presence of methanol provided a protic solvent to help with ionization of the samples when performing LC/MS analysis. Therefore, one could examine a large number of compounds using gradient conditions, which allowed for a better assessment of the sample purity.

The polar organic mode was developed primarily for the separation of enantiomers that contained a hydrogen bonding functional group on the chiral center and another functional group in the molecule. β-blockers have been successfully separated under these conditions using various cyclodextrin stationary phases [28]. Most separations developed using the polar organic mode were compound specific and there was no set of conditions that could be applied to a large number of compounds. Organic acids have been analyzed by HPLC using reversed-phase [30–32], ion exchange [33–36] and normal-phase chromatography [35,37,38]. However, there was little information available using the polar organic mode to separate organic acids.

In this study, we examined the utility of separating organic acids using the polar organic mode. We then applied this method to drug discovery samples that were difficult to analyze using reversed-phase HPLC. These samples were very hydrophobic and had extremely low solubility in aqueous solvents. When these samples were analyzed using reversed-phase HPLC it was difficult to assess the sample purity due to the complex chromatograms that were generated during the analysis. We now report on a HPLC method that used the polar organic mode to separate various organic acids. The assay used a gradient that was composed of acetonitrile/methanol/triethylamine/acetic acid to elute the acidic compounds. In addition to HPLC-UV detection this assay worked well with LC/MS-ESI detection.

All the compounds produced very good negative ion spectra. The separation was effected by the acid to base ratio and the concentration of methanol.

2. Experimental

2.1. Reagents

Glacial acetic acid, anhydrous triethylamine (TEA), ibuprofen, nalidixic acid, myristic acid, naproxen, flurbiprofen, hydrocortisone-2,1-hemisuccinate and palmitic acid were purchased from Sigma Chemical Co., St. Louis, MO, USA. 1,4-Naphthalene dicarboxylic acid, 2,3-dihydroxybenzoic acid, *N*-(1-phenylethyl)succinamic acid, 2-methoxycinnamic acid, 2-carboxycinnamic acid, 1-naphthalene sulfonic acid, 2,4,6-trihydroxybenzoic acid, 3,4-dihydroxycinnamic acid, 4-carboxyphenoxy acetic acid, 2-naphthylacetic acid, citronellic acid, 2,5-dihydroxybenzoic acid, 3,4-dimethylbenzoic acid, 1-naphthalene acetic acid, 2,5-naphthalene disulfonic acid tetrahydrate, 2,3,4-trihydroxybenzoic acid, 2-naphthalene sulfonic acid, cinnamic acid, 4-methoxycinnamic acid, 2-hydroxycinnamic acid, 3-phenoxycinnamic acid, 4-acetoxybenzoic acid, phenoxybutyric acid, 2,3-naphthalene dicarboxylic acid, 3,5-dimethoxycinnamic acid, 4-acetylbenzoic acid, furoic acid, *p*-anisic acid, *N*-[1-(1-naphthyl)ethyl]succinamic acid, nicotinic acid, hydroxyisobutyric acid and 2-pyrrolidone-5-carboxylic acid were purchased from Aldrich Chemical Co., Milwaukee, WI, USA. The 3,4-dimethyl benzoic acid was purchased from Lancaster laboratories, Windham, NH, USA. Acetonitrile and high purity water were purchased from Fisher Scientific Co., Pittsburgh, PA, USA.

2.2. Equipment

HPLC samples were run on a Dionex Summit HPLC system that was equipped with a UVD170S variable wavelength detector. The data was collected using Chromeleon vs. 6.2 software. In addition, samples were analyzed using a Waters Alliance 2690 HPLC system that was equipped with a 996 photodiode array detector, and the data was collected using Millennium32 software. LC/MS analysis was performed on a Finnigan LCQ mass spectrometer using an electrospray ionization (ESI) probe. The system was equipped with a Waters Alliance 2690 HPLC system for sample injection and mobile phase control. The UV data was collected from a Waters 2487 variable wavelength detector that was equipped with a micro flow cell. The Cyclobond I 2000 columns 100 mm × 4.6 mm and 150 mm × 2.0 mm were purchased from Advanced Separations Technology (ASTEC) Whippany, NJ, USA. The C18 reversed-phase column was purchased from Phenomenex Inc., Torrance, CA, USA.

2.3. Preparation of mobile phase

The 100:0.3:0.2 acetonitrile/TEA/acetic acid was prepared by taking 1000 ml of acetonitrile and adding 3 ml of TEA and 2 ml of glacial acetic acid. The 85:15:0.8:1.0 acetonitrile/methanol/TEA/acetic acid was prepared by adding 150 ml of methanol to 850 ml of acetonitrile. Afterwards, 8 ml of TEA and 10 ml of acetic acid were added to give the final proportions. Acetonitrile (0.1% formic acid) was prepared by adding 1 ml of formic acid to 1000 ml of acetonitrile. The water (0.1% formic acid) was prepared by adding 1–1000 ml of water. All solvents were filtered through a 0.2 μm nylon filter prior to analysis.

Table 1
Gradient conditions for HPLC-UV analysis using the polar organic conditions

Time (min)	Flow rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0	1	100	0
1	1	100	0
5	1	0	100
13	1	0	100
13.1	4	100	0
16	4	100	0

The samples were analyzed using a Cyclobond I 2000 column 100 mm × 4.6 mm, with UV detection at various wavelengths. The injection volume was 10 μl at 25 °C. Mobile phase A was 100:0.3:0.2 acetonitrile/TEA/acetic acid and mobile phase B was 85:15:0.8:1.0 acetonitrile/methanol/TEA/acetic acid.

Table 2
Reversed-phase chromatography gradient conditions

Time (min)	Flow rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0	3.5	90	10
0.5	3.5	60	40
1.5	3.5	60	40
4.5	3.5	10	90
5.5	3.5	10	90
5.6	3.5	90	10
8.0	3.5	90	10

Samples were analyzed on a Phenomenex C18 Hypersil column 50 mm × 4.6 mm with UV detection at various wavelengths. Mobile phase A was acetonitrile (0.1% formic acid) and mobile phase B was water (0.1% formic acid).

2.4. HPLC conditions

HPLC analysis was performed as follows. All the HPLC-UV analysis was performed using a Cyclobond I 2000 column 100 mm × 4.6 mm at 28 °C with the gradient conditions listed in Table 1. The samples were detected by UV at various wavelengths. The injection volume was varied. The reversed-phase HPLC conditions are listed in Table 2.

2.5. LC/MS analysis

LC/MS was performed using a Finnigan LCQ equipped with an electrospray ionization probe (ESI) in the negative ionization mode. The HPLC column used was a Cyclobond I 2000 column 150 mm × 2.0 mm column using the mobile phase conditions listed in Table 3. The compounds were detected using UV detection in addition to mass spectrometry. The compounds were detected at various wavelengths.

3. Results and discussion

The polar organic mode was selected to examine drug discovery compounds that showed activity towards a kinase target vascular endothelial growth factor, VegF. Prior to using this assay on the drug discovery samples, we first examined what affect these conditions had on the separation of standard compounds. The first study used hydrocortisone and some of its derivatives (Fig. 1). Using isocratic conditions we examined what affect changing the acid–base ratio had on these compounds. The hydrocortisones were separated using mixtures of acetonitrile/TEA/acetic acid. The neutral compounds, hydrocortisone and hydrocortisone 21-cypionate eluted in approximately 4 min when the acid–base ratio was 1:1. The acidic compound, hydrocortisone 21-hemisuccinate, eluted within 12 min using the same conditions. Hydrocortisone and hydrocortisone 21-cypionate eluted at approximately the same retention time when the acid–base ratio was changed to 1.3:1. However, the hydrocortisone 21-hemisuccinate eluted in less than 10 min. The data for this study was tabulated in Table 4.

Table 3
HPLC conditions used to perform LC/MS analysis

Time (min)	Flow rate (ml/min)	Mobile phase A (%)	Mobile phase B (%)
0	0.5	100	0
1	0.5	100	0
7	0.5	0	100
12	0.5	0	100
12.1	0.5	100	0
18	0.5	100	0

The samples were analyzed using a Cyclobond I 2000 column 150 mm × 2.0 mm, with UV detection at various wavelengths. The injection volume was 10 μl at 25 °C. Mobile phase A was 100:0.3:0.2 acetonitrile/TEA/acetic acid and mobile phase B was 85:15:0.8:1.0 acetonitrile/methanol/TEA/acetic acid.

The second study used the gradient conditions to study the elution of some cinnamic acid derivatives. The cinnamic acid derivatives were separated using the optimized mobile phase conditions (Fig. 2). In order to elute all the compounds the methanol concentration was increased from 0 to 15% over an 8-min period. The acid–base ratio was also changed during the gradient in order to ensure all the acidic compounds eluted off the column. This separation followed the same elution pattern observed with the hydrocortisones; the more neutral and positively charged compounds eluted earlier than the acidic compounds. Further examination of the selectivity showed that the position of the carboxylic acid group also affected the retention of the analyte. A chromatogram of 1,4-naphthalene dicarboxylic acid and 2,3-naphthalene

Table 4
Effect of changing the acid–base ratio on the separation of hydrocortisones

Compound	Retention time (min)	
	Condition A	Condition B
Hydrocortisone 21-cypionate	3.41	3.36
Hydrocortisone	4.55	4.50
Hydrocortisone 21-hemisuccinate	11.53	9.87

Condition A was 100:0.8:0.8 acetonitrile/TEA/acetic acid and condition B was 100:0.8:1.0 acetonitrile/TEA/acetic acid. The separation was performed on a Cyclobond I 2000 column 100 mm × 4.6 mm using a flow rate of 1 ml/min and the compounds were detected using UV at 254 nm. The column was kept at constant temperature of 28 °C and the sample volume was 10 μl.

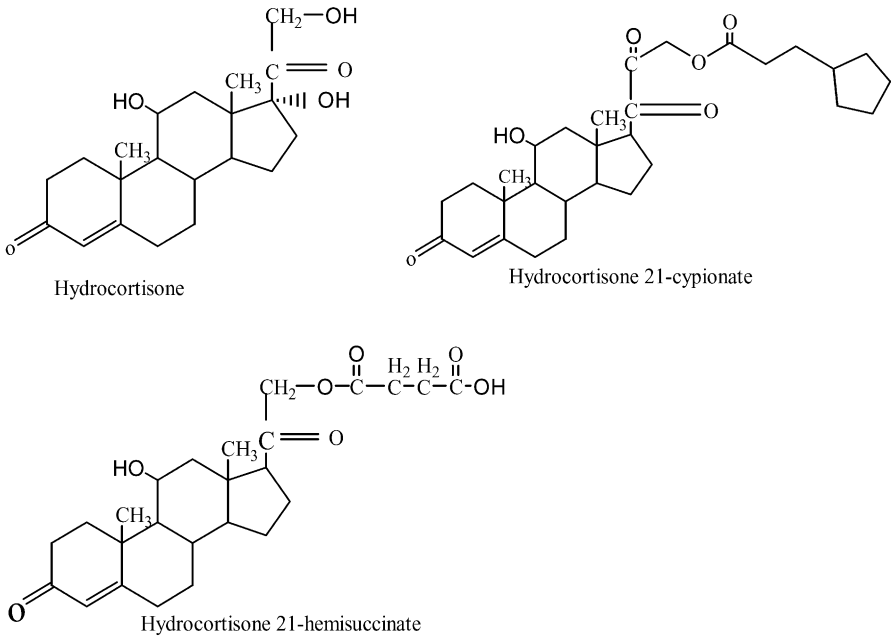

Fig. 1. Structures of hydrocortisone and its derivatives.

dicarboxylic acid showed very different retention times for the two compounds. The 2,3-naphthalene dicarboxylic acid eluted in approximately 4.5 min. While the 1,4-naphthalene dicarboxylic acid eluted in 12.8 min. This demonstrated that the position of the functional group was important to the selectivity of the method. The retention times and asymmetry for a diverse set of organic acids was tabulated in Table 5. The data showed that the method had a good selectivity for fatty acids, sulfonic acids and other small organic acids.

Our goal was to develop an alternate way to separate the potential drug candidates. Previous efforts to analyze these hydrophobic compounds using reversed-phase HPLC were unsuccessful. Under these conditions the chromatogram shown in Fig. 3 was very complex and it appeared to contain multiple peaks. LC/MS analysis was performed on this sample using the reversed-phase method. In this case, each peak showed the mass of interest, which was 496 amu. This indicated that there was a secondary interaction occurring during the separation. The compounds had very low solubility in aqueous solvents (>1 μg/ml). This could have caused the sample to precipitate during the high aqueous portions of the gradient, which lead to the complex chromatogram. Efforts to improve the chromatography by changing the gradient conditions were unsuccessful.

We developed a set of conditions to separate the potential drug candidates. When the same sample was analyzed using the polar organic conditions the chromatogram showed two sharp peaks. Amore accurate assessment of the purity could be made for this sample (Fig. 3). This sample was further examined by LC/MS, using the polar organic conditions,

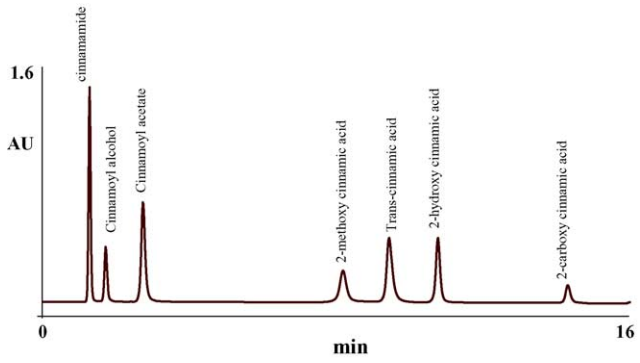

Fig. 2. Chromatogram of cinnamic acid derivatives using a Cyclobond I 2000 column 100 mm × 4.6 mm. The mobile phase conditions used are listed in Table 1. The compounds were detected at 295 nm. The injection volume was 5 μl.

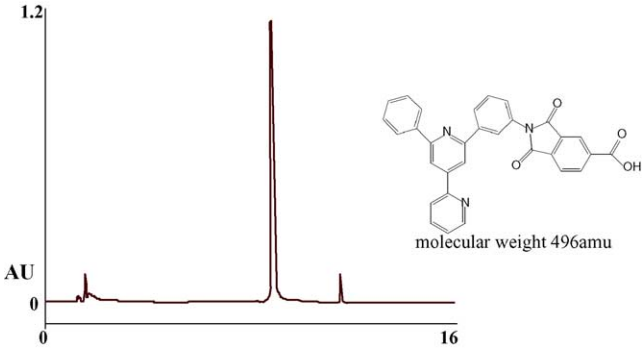

Fig. 3. Chromatograms of a drug discovery sample 30881 generated using a Cyclobond I 2000 column. The gradient conditions are listed in Table 3. The samples analyzed on the Cyclobond column were analyzed using the conditions in Table 1. The compounds were detected using UV detection at 280 nm. The injection volume was 5 μl.

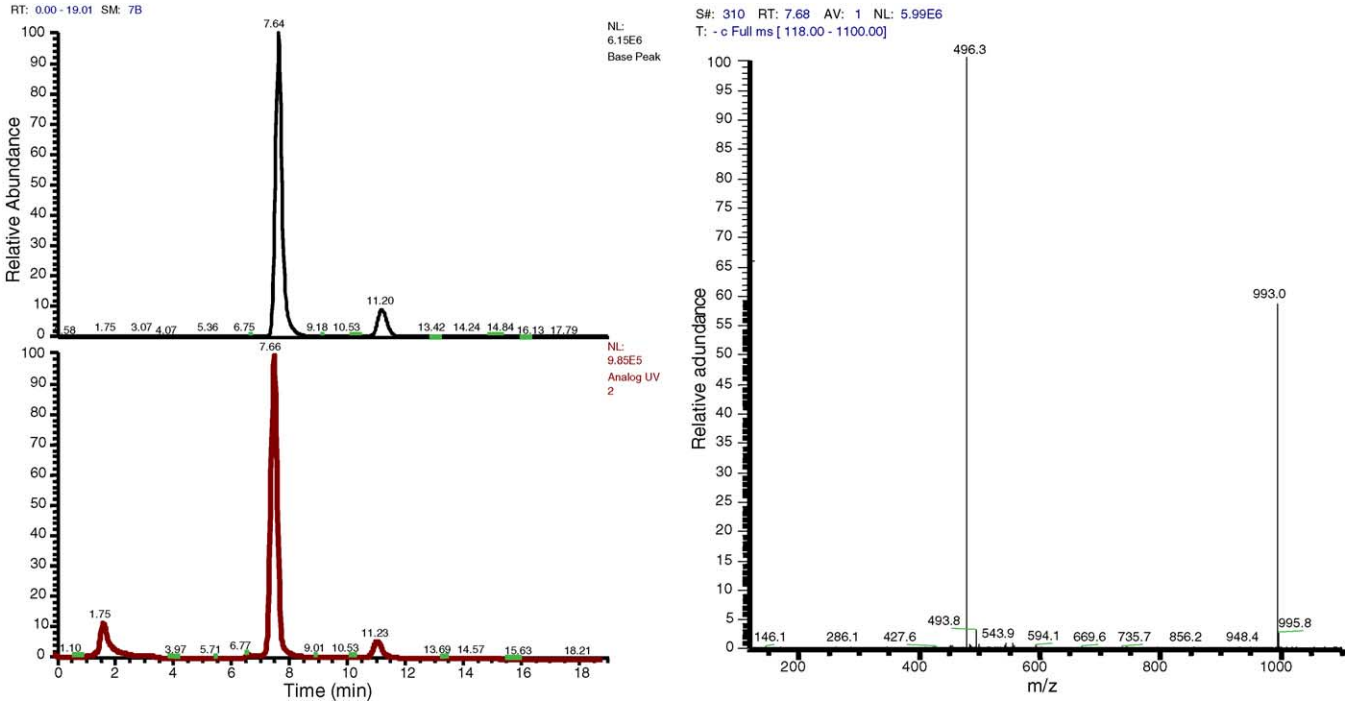

Fig. 4. TIC of sample 30881 using a Cyclobond I 2000 column 150 mm × 2.0 mm with the mobile phase conditions listed in Table 2. The sample was detected using electrospray ionization in the negative mode. In addition, UV data was also collected at 280 nm on this sample during LC/MS analysis. The injection volume was 2.00 μl.

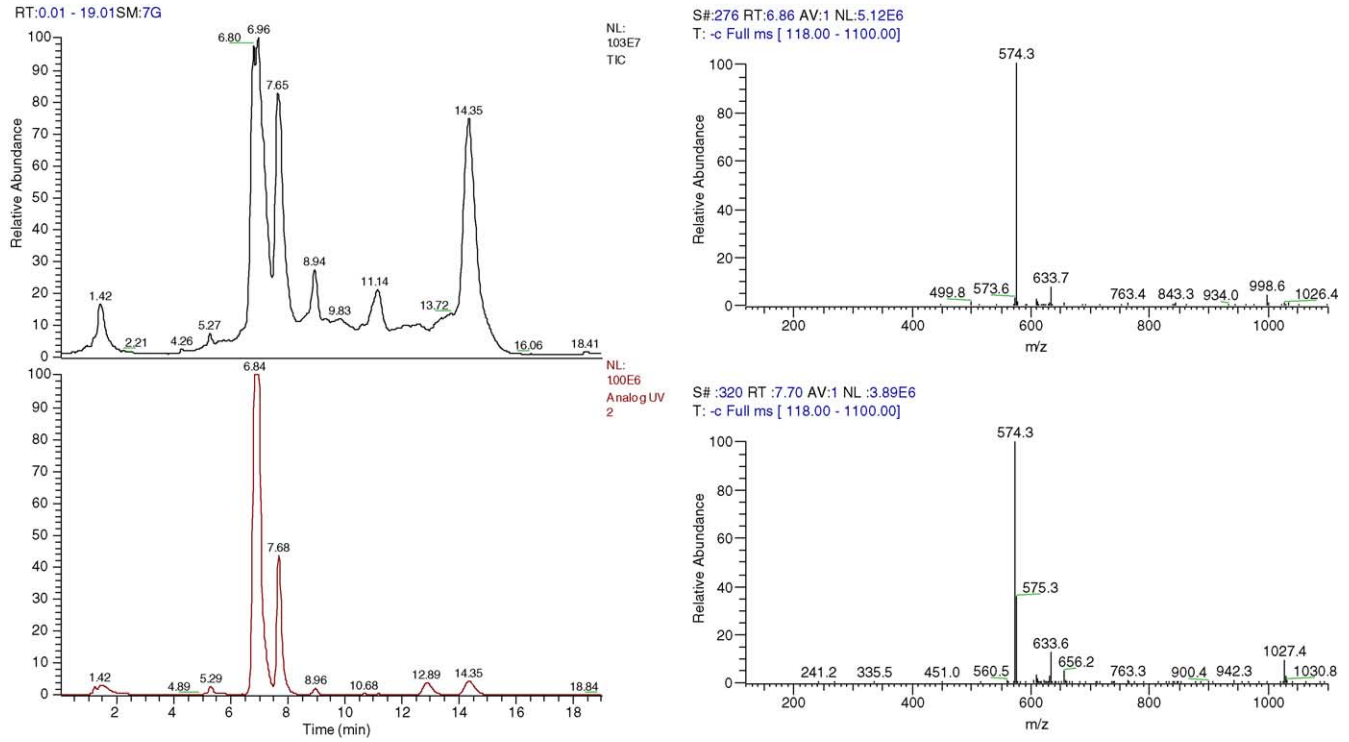

Fig. 5. TIC of sample 30858 using a Cyclobond I 2000 column 150 mm × 2.0 mm with the mobile phase conditions listed in Table 2. The sample was detected using electrospray ionization in the negative mode. In addition, UV data was also collected at 280 nm on this sample during LC/MS analysis. The injection volume was 2.00 μl.

Table 5
List of organic acids analyzed using the polar organic mode

Compound	Retention time (min)	Asymmetry
1,4-Naphthalene dicarboxylic acid	11.97	2.35
2,3-Dihydroxybenzoic acid	2.75	3.89
N-(1-Phenylethyl)succinamic acid	6.12	1.65
Ibuprofen	5.80	1.85
2-Methoxycinnamic acid	5.02	1.14
2-Carboxycinnamic acid	11.72	0.70
1-Naphthalene sulfonic acid	1.72	0.95
Nalidixic acid	1.46	2.74
2,4,6-Trihydroxybenzoic acid	2.16	0.85
3,4-Dihydroxycinnamic acid	7.53	1.63
4-Carboxyphenoxy acetic acid	6.97	1.19
2-Naphthylacetic acid	5.82	1.19
Citronellic acid[a]	4.92	1.33
2,5-Dihydroxybenzoic acid	4.32	1.29
3,4-Dimethylbenzoic acid	4.94	1.55
1-Naphthalene acetic acid	5.33	1.14
1,5-Naphthalene disulfonic acid tetrahydrate	3.10	0.80
2,3,4-Trihydroxybenzoic acid	4.87	1.42
Palmitic acid[a]	5.35	1.55
2-Naphthalene sulfonic acid	2.32	0.69
Cinnamic acid	5.79	1.13
4-Methoxycinnamic acid	5.51	1.25
2-Hydroxycinnamic acid	7.10	1.27
3-Phenoxypropionic acid	5.29	1.33
4-Acetoxybenzoic acid	4.63	1.47
2-Phenoxybutyric acid	4.74	1.19
3,4-Dimethylbenzoic acid	4.96	1.56
Myristic acid[a]	3.81	1.29
2,3-Naphthalene dicarboxylic acid	3.88	3.42
3,5-Dimethoxycinnamic acid	4.89	1.22
Naproxen	4.93	1.27
4-Acetylbenzoic acid	4.70	1.20
Furoic acid	5.24	1.06
p-Anisic acid	4.96	1.62
Hydrocortisone 21-hemisuccinate	8.39	2.24
N[1-(1-Naphthyl)ethyl]succinamic acid	6.08	1.25
Flurbiprofen	5.00	1.81
Nicotinic acid	6.37	2.76
Hydroxyisobutyric acid[a]	4.18	2.20

[a] Parameters determined by LC/MS using a 150 mm × 2.0 mm Cyclobond I 2000 column with the conditions listed in Table 2. Parameters for all the other acids were determined using a Cyclobond I 2000 column (100 mm × 4.6 mm) using the conditions in Table 1.

to confirm its identity (Fig. 4). The total ion chromatogram (TIC) trace showed the two major peaks that were observed using HPLC-UV. The resulting spectra for this sample gave a $[M - 1]$ of 496 amu for the most intense peak, which indicated that the desired compound was present as the major component. The peak at 993 amu was determined to be the dimer of this compound. The desired compound had a relative purity of approximately 90% by LC/MS. Further examination of these conditions showed that this method was able to resolve *cis* and *trans* isomers as indicated by similar mass spectra for the two major components in Fig. 5.

To examine the reproducibility of the gradient a system suitability test was performed. We made six replicate injections of 1-naphthalene acetic acid and 2-naphthalene acetic acid and calculated the values for retention time, resolution, peak area and asymmetry. The average values and the standard deviations were listed in Table 6. The percent R.S.D. for the retention times was less that 2%, which indicated that the gradient conditions used were reproducible and the column was being equilibrated back to the initial conditions. The consistent and reproducible values for retention time, asymmetry, and resolution showed that the column was stable under these conditions. Fig. 6 compared chromatograms obtained using a new column and a column that had approximately 1500 injections on it. The data showed that there is very little loss in retention or resolution over this time period, indicating that the column had very long lifetime under these conditions.

Table 6
System suitability results

	Retention time (min)	Resolution	Area	Asymmetry
1-Naphthalene acetic acid				
Average	6.13	1.69	525865.3	1.17
s	0.02	0.03	4912.81	0.01
%R.S.D.	0.29	1.73	0.93	1.18
2-Naphthalene acetic acid				
Average	6.60	NA[a]	509143.5	1.34
s	0.03	NA[a]	2770.41	0.02
%R.S.D.	0.52	NA[a]	0.54	1.30

[a] Resolution was calculated and reported using the first peak in the chromatogram.

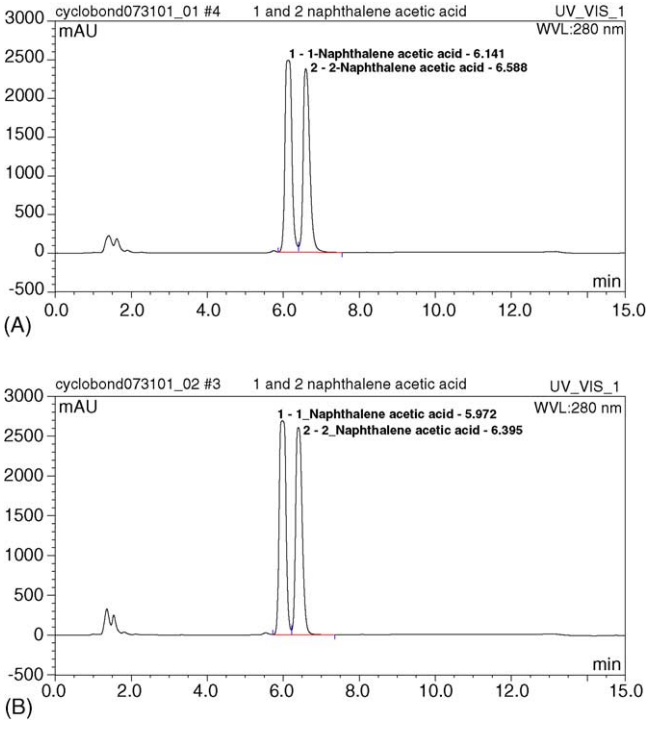

Fig. 6. Chromatogram of (A) new and (B) old Cyclobond I 2000 column using the mobile phase conditions listed in Table 2. The sample was detected with UV at 280 nm. The injection volume was 10 μl.

4. Conclusion

Better purity estimates were obtained for this class of drug discovery compounds using the polar organic mode. These hydrophobic compounds gave better peak shape and less complex chromatograms in the polar organic mode compared to the reversed-phase method. LC/MS analysis of these samples showed a single mass ion, which indicated that the peak being examined contained one component. Retention and selectivity depended on the position and type of hydrogen bonding functional groups that were attached to the molecule. Both straight chain and aromatic acids could be separated under these conditions. The method was able to separate both mono- and di-substituted sulfonic acids. Increasing the concentration of methanol could be used to elute highly retained compounds. In addition, acidic compounds were eluted faster by making adjustments in the acid to base ratio. The mobile phase was compatible with LC/MS and the acidic compounds gave clear molecular ion peaks using electrospray ionization in the negative mode. The volatile solvents allowed for faster sample desolvation, which helped sample ionization in the mass spectrum. The method had good selectivity; it separated *cis* and *trans* isomers from each other. The low column back pressure allowed for the column to be equilibrated back to the initial conditions at a high flow rate, which helped decrease the total analysis time. The reproducibility demonstrated in the system suitability study showed that the equilibration time was sufficient. Also, the column, when properly cared for showed excellent stability with little loss of resolution after 1500 injections.

The method is limited at low UV wavelengths. Compounds that absorb at these wavelengths, typically below 230 nm, could be difficult to detect due to baseline drift caused by the increased concentration of methanol over the gradient during the analysis. Using other detectors such as mass spectroscopy or evaporative light scattering would allow for better detection of these low absorbing compounds. Water soluble compounds could be difficult to analyze due to the polarity difference between water and the acetonitrile in the mobile phase.

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