Enantioseparation of 3-phenylacetylamino-2,6-piperidinedione and related chiral compounds

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Abstract

This paper reports HPLC methodology for the first successful enantiomeric separations of 3-phenylacetylamino-2,6-piperidinedione (PAP), a naturally occurring peptide derivative used for inhibiting the growth of cancer tissues. The chiral separation of four related hydrolysates is also described. A commercially available tris-4-methylbenzoate cellulose (Chiralcel OJ) column was used as the chiral stationary phase, operated in the normal-phase mode. The results demonstrated that hydrolyzed products of PAP, each of which has a carboxylic acid functionality present in its structure, eluted in a reasonable time and are enantiomerically resolved only when a trace amount of organic acid is present in the mobile phase. Different alcohols (ethanol and isopropanol) and acid additives (trifluoroacetic acid, trichloroacetic acid and acetic acid) were evaluated. In general, for the separation of the acidic enantiomers, ethanol is superior to isopropanol and stronger acids enhance the resolution more effectively. However, chiral separation of PAP could only be accomplished with isopropanol in the mobile phase and no acidic additive was needed.

Keywords: Enantiomer separation; Mobile phase composition; Phenylacetylaminopiperidinedione; Glutamines; Glutamic acids

1. Introduction

3-Phenylacetylamino-2,6-piperidinedione (PAP) is a naturally occurring peptide derivative. It was originally extracted as a mixture from human urine in an attempt to find the effective constituent that can inhibit the growth of cancer tissues. It was later isolated from its mixture, structurally studied and reported to be the effective inhibitory component on the progression of neoplastic diseases [1–7]. Due to its antineoplastic property, 3-phenylacetylamino-2,6-piperidinedione is also called Antineoplaston A10 although controversy over its effectiveness persists

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[8,9]. The imide group in the piperidine ring causes the ring to be susceptible to hydrolysis, forming N-phenylacetyl-DL-glutamine (PAG) and N-phenylacetyl-DL-isoglutamine (PAiG) [2,4]. In our laboratory, N-phenylacetyl-DL-glutamic acid (PAGA) along with phenylacetic acid and glutamic acid were also generated when hydrolysis was further carried out [10]. The antineoplastic effect of PAP hydrolysates has been studied [2–4] and patients have been administered soluble sodium salts of PAG and PAiG [3].

There is a stereogenic center on the third position of the piperidine ring. Hendry et al [5,6] studied the possible stereogenic interaction between PAP and base pairs in DNA and suggested that the L-isomer,

proposed to be the naturally occurring isomer of PAP derived from L-glutamine, was stereochemically complementary to DNA. Thus, separation of PAP enantiomers is important not only to ensure that only L-enantiomer is administered to patients but also in the study of possible racemization of PAP after its administration. In this paper, we report the first chiral separation of PAP and its hydrolysates, PAG, PAiG and PAGA by HPLC. Due to its structural similarity to the PAP hydrolysates, N-phthalyl-DL-glutamine (PHTH), a hydrolysis product of thalidomide, was also included. The study focuses on the manipulation of mobile phase composition to achieve optimum resolution for enantioseparation. All chiral compounds were enantiomerically separated using a cellulose-based chiral stationary phase (CSP) (Chiralcel OJ) in the normal-phase mode, while preliminary result indicated that another cellulose-based CSP, Chiralcel OD, was inferior to Chiralcel OJ. This method has provided an effective way to isolate pure enantiomers of PAP and its related compounds.

2. Experimental

2.1. Chemicals

3-Phenylacetylamino-2,6-piperidinedione, triethylamine (TEA), trifluoroacetic acid (TFA) (protein sequencing grade), trichloroacetic acid (TCA) (ACS reagent grade) and acetic acid (HOAc) (99+% grade) were purchased from Sigma (St. Louis, MO, USA). Anhydrous ethanol (200 proof of U.S.P.) was from Quantum (Tuscola, IL, USA). Phosphoric acid (85% w/w, ACS reagent) was from Aldrich (Milwaukee, WI, USA). Acetonitrile, methanol, isopropanol and hexane were of OmniSolv grade and obtained from EM Science (Gibbstown, NJ, USA). Potassium hydroxide (85%) and ammonium acetate (analytical reagent) were obtained from Taylor (St. Louis, MO, USA).

2.2. Preparation of solutions

Aqueous solutions of 1.0 M KOH and 10% TEA, used in hydrolysis of PAP, were prepared by dissolving sufficient amounts of KOH or TEA in Milli-Q water. Ammonium acetate buffer solution (0.1 M,

pH=5.0), used as a mobile phase component in HPLC achiral separations of PAP hydrolysates, was obtained by dissolving solid $NH_4C_2H_3O_2$ directly in Milli-Q water and adjusting the pH with acetic acid. The buffer was filtered through 0.45 μm cellulose acetate membrane. An acetonitrile solution of PAP and methanol solutions of all acidic chiral compounds were prepared at a concentration of about 1 mg/ml.

2.3. Preparation and purification of PHTH, PAG, PAiG and PAGA

N-Phthalyl-D-glutamine and N-phthalyl-L-glutamine were prepared as previously reported [11], and mixed in equal proportions to provide N-phthalyl-DL-glutamine for experiment use.

The hydrolysis of PAP, the syntheses of PAP hydrolysates and their characterizations have been discussed by Revelle et al. [10]. In the current work, an effort was focused on obtaining sufficient N-phenylacetyl-DL-glutamine (PAG), N-phenylacetyl-DL-isoglutamine (PAiG) and N-phenylacetyl-DL-glutamic acid (PAGA) in a relatively pure form to prepare HPLC sample solutions for chiral separations. About 45 mg of PAP was placed in a small amber vial. To this vial was added 2 ml of 10% TEA aqueous solution. The vial was then loosely covered and placed on a 60 °C hot plate for 2.5 h. A small portion of the solution was diluted with methanol and then analyzed by reversed-phase HPLC. PAG, PAiG and PAGA were identified by peak spiking with a corresponding pure sample synthesized by Revelle [10]. It was found that PAG and PAiG accounted for about 94% of the total products and the ratio of PAG to PAiG was about 4:1. The semipreparative HPLC method described below was conducted to separate PAG and PAiG. Fractions of PAG and PAiG, collected after each HPLC semipreparative separation, were combined, and the mobile phase removed at reduced pressure. The semi-solid product was then collected in a small vial and further vacuum-dried at room temperature for about 18 h. The dried product was dissolved in methanol in a small vial which was then wrapped with aluminum foil and stored in a refrigerator for future use. An HPLC analysis, conducted for purity

determination of final products, gave an assay of 97.7% for PAG and 94.8% for PAiG. To obtain PAGA, stronger basic solution was used. About 60 mg of PAP was dissolved in 2 ml of 1.0 M KOH at room temperature. The hydrolysis was allowed for 2 h. Due to the strong basicity of KOH, PAiG was further hydrolyzed into PAGA. The major hydrolysates therefore were PAGA and PAG. The HPLC analysis showed that the ratio of PAG to PAGA was about 3:1. In a similar manner to that described earlier, PAG and PAGA were separated, fraction-collected, purified and stored. The purities were found to be 99.9% for PAGA and 84.8% for PAG.

2.4. HPLC apparatus and methods for achiral semipreparative separations and analysis of the hydrolysates

An Hewlett-Packard HPLC 1050 series system equipped with a multiple wavelength UV detector, an autosampler and an HP ChemStation were used for monitoring the ratio of hydrolysates when different hydrolysis procedures were applied and the purity measurements of the final products. A C_{18} column, Ultracarb 3 ODS (20) (150×4.6 mm I.D., 3 μm), manufactured by Phenomenex (Torrance, CA, USA) was used. The mobile phase was composed of 90% ammonium acetate buffer (0.1 M, pH=5.0) and 10% acetonitrile. The analyses were carried out at a flow-rate of 0.5 ml/min. Under these reversed-phase HPLC conditions, the hydrolysates were eluted in the order of PAGA, PAG and PAiG.

For semipreparative separation of hydrolysates, the instrumentation consisted of a Waters 600 multisolvent delivery system, an HP 1040A diode-array detector, a Rheodyne sampling valve for manual sample solution delivery and an HP 79994A HPLC ChemStation as a controller. In the separation of PAGA and PAG, generated by hydrolysis of PAP with 1.0 M KOH solution, a semipreparative C_{18} column, Techsil 5 (250×8.0 mm I.D., 5 μm), purchased from Phenomenex was employed. The mobile phase composition was ammonium acetate buffer (0.1 M, pH=5.0)–methanol (80:20). About 180 μl (2×90 μl) of sample solution was injected at a flow-rate of 0.5 ml/min. The flow-rate was then increased immediately after the injection to 2.0 ml/

min to complete the separation. In the separation of PAG and PAiG, the major hydrolysates generated by hydrolysis of PAP with 10% TEA solution, Techsil 5 column could not separate PAG from PAiG. Therefore, the same C_{18} column and mobile phase composition used in the analysis of hydrolysates described above were employed. The flow-rate was 0.75 ml/min and 75 μl of the sample solution was injected.

2.5. HPLC apparatus and methods for chiral separations

Enantioseparations were performed on an Hewlett-Packard (HP) HPLC system consisting of an HP 1050 series pumping system, an HP 1040A diode-array detector, and an HP 79994A HPLC ChemStation as a controller. Waters 712 Wisp autosampler was connected to the system to deliver sample solutions, usually 5–10 μl. A Chiralcel OJ column (250×4.6 mm I.D.), packed with 10 μm porous silica gel coated with tris(4-methylbenzoate) derivatized cellulose, was purchased from Chiral Technologies, (Exton, PA, USA). A Brownlee Newguard silica cartridge (15 mm×3.2 mm I.D., 7 μm) held in a Brownlee Newguard holder (from P.J. Cobert Associates, St. Louis, MO, USA) was used as a guard column. The mobile phase consisted of hexane and an alcohol as indicated individually in the figures and tables. When a trace amount of an acidic additive was used in the mobile phase, the additive was first added at certain concentrations to a pure alcohol reservoir. The mobile phase was then pumped from pure hexane and alcohol reservoirs in a proportionate manner to achieve appropriate compositions. To prevent the deterioration of the column, a hexane–isopropanol (90:10) mixture was always used as the storage solvent. This was extremely important if the mobile phase containing additive was used. When mobile phase containing acidic additive was used, the column was flushed with a large quantity (about 600 ml or 10 h) of hexane–isopropanol (90:10) before it was used for the mobile phase which did not contain an acidic additive or before it was stored. The flow-rate was kept at 1.0 ml/min.

All HPLC experiments were carried out at room temperature with detection at 220 nm.

3. Results and discussion

3-Phenylacetylamino-2,6-piperidinedione has a similar structure to that of thalidomide (3-phthalimido-2,6-piperidinedione). Both compounds contain a piperidine ring which has carbonyl groups on its 2 and 6 positions. The third position of the piperidine ring is substituted by either a phenylacetylamino group (PAP) or phthalimido group (thalidomide), resulting in a stereogenic center. The structure of PAP along with those of acidic chiral compounds studied in this work are shown in Fig. 1. N-Phthalyl-DL-glutamine, a compound which possesses some of the structural characteristics of thalidomide and N-phenylacetyl-DL-glutamine, was included.

Aboul-Enein and Islam reported a baseline separation of thalidomide enantiomers using a mobile phase of hexane–ethanol (50:50) on a Chiralcel OJ chiral stationary phase (CSP) [12]. On the same type of CSP, enantioseparation of PAP was accomplished ($R_s = 1.60$) with a mobile phase of hexane–iso-
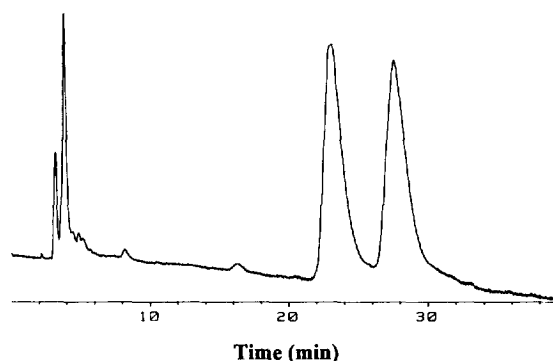

Fig. 2. Enantioseparation of 3-phenylacetylamino-2,6-piperidinedione on Chiralcel OJ CSP. Mobile phase= hexane–isopropanol (70:30); flow-rate= 1.0 ml/min; room temperature.

propanol (70:30) (Fig. 2). The use of hexane–ethanol mobile phase, however, resulted in a comparable retention time with no indication of enantiomeric separation. The different enantioselectivity caused by different alcohols in the mobile phase indicated, as discussed by Wainer et al. [13,14], that the structure of the alcohol in the mobile phase can alter the steric

DL-3-phenylacetylamino-2,6-piperidinedione
(PAP)

N-phenylacetyl-DL-glutamine
(PAG)

N-phenylacetyl-DL-isoglutamine
(PAiG)

N-phenylacetyl-DL-glutamic acid
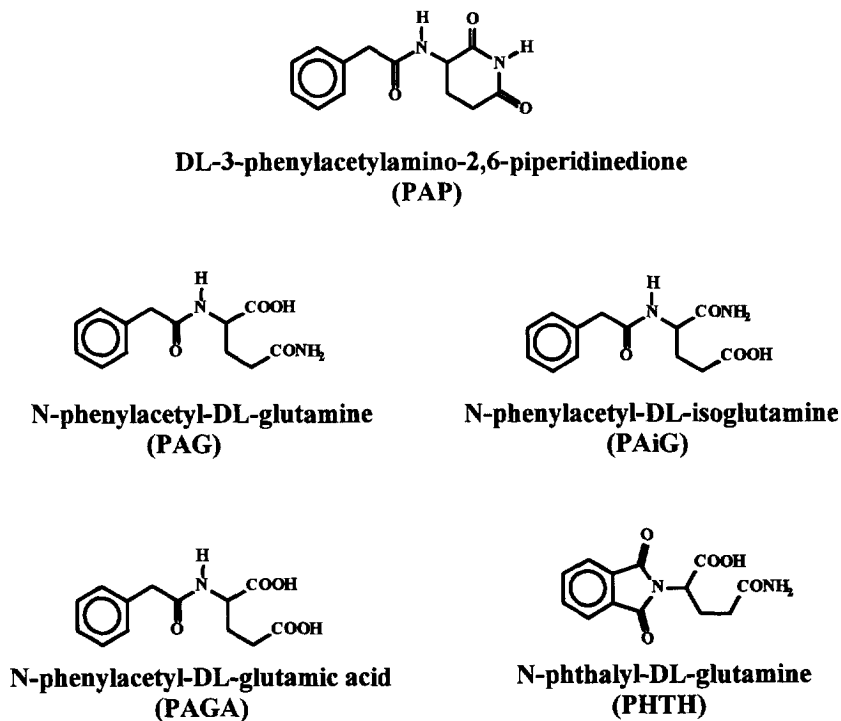(PAGA)

N-phthalyl-DL-glutamine
(PHTH)

Fig. 1. Structures of PAP and related chiral acids studied.

nature of the chiral cavities on a CSP. In this case, the use of isopropanol may allow the asymmetric portion of the enantiomeric solutes to fit into the chiral cavity and interact stereogenically with other effective parts of the CSP to achieve chiral separation. In another experiment, Chiralcel OD (3,5-dimethylphenylcarbamate cellulose) column, was also utilized and found to be inferior to Chiralcel OJ for the separation of thalidomide and PAP enantiomers. The elution order of PAP was not determined due to the unavailability of pure enantiomers.

All hydrolysates, PAG, PAiG, PAGA, and PHTH, contain a carboxylic acid functionality. In a previous investigation [15], it was demonstrated that the addition of organic acid into the mobile phase was essential for the separation of acidic and basic enantiomers on Chiralcel OJ CSP. The similar observation was obtained on other cellulose-based CSPs [16]. In the current study, the addition of organic acid to the mobile phase reduces the retention and improves the chiral resolution of all acidic chiral compounds. Without a proper acidic additive in the mobile phase, all acidic chiral compounds investigated were retained so strongly by the stationary phase that they were not eluted in a reasonable time (>150 min). However, if a trace amount of a strong acid, e.g., trifluoroacetic acid, is added in the mobile phase, the chiral separation is achieved in a much shorter retention time.

Table 1 lists the retention and enantioresolution for the four chiral acids obtained using the mobile phases with different concentrations of trifluoroacetic acid. It shows that the retention was reduced significantly with the initial addition of TFA (0.01%), and progressively increased with an increase in TFA concentration to 0.04%, then dropped slightly at 0.05% TFA. The enantioresolutions for all acidic compounds shows a similar trend to that of retention, i.e., reaching a maximum at about 0.03% TFA concentration. It was also noted that enantioresolution (R_s) was changed more dramatically than enantioselectivity (α) when the concentration of TFA was increased. A typical example is the separation of PAiG in which the α value is constant, while the R_s value shows a gain of about 40% when the TFA concentration is increased from 0.01% to 0.04%. This indicates that the appropriate amount of TFA is necessary to ensure the optimal mass transfer process of the solute between the stationary phase and the mobile phase. Thus, two adjacent enantiomer peaks could be sharpened and resolution could be improved.

The effects of other mobile phase composition changes, including the substitution of TCA or HOAc for TFA and the substitution of ethanol for isopropanol, on the retention and separation of all four hydrolysate enantiomers are shown in Table 2. It is known that acidity decreases in the order of TFA $(pK_a=0.3$ [17]), TCA $(pK_a=0.70$ [18]) and HOAc $(pK_a=4.74$ [19]). When compared to TFA, the mobile phase containing TCA results in higher retention, comparable selectivity and lower resolution (PAGA is an exception in the last two points). The poor resolution was caused by lower column

Table 1
The influence of TFA concentrations on retentions and enantioseparations for acidic compounds

| Acidic compound | Concentration of TFA in mobile phase (%)[a] | | | | | | | | | | | | | | |
| | 0.01 | | | 0.02 | | | 0.03 | | | 0.04 | | | 0.05 | | |
	k_1'[b]	α[c]	R_s[d]	k_1'[b]	α[c]	R_s[d]	k_1'[b]	α[c]	R_s[d]	k_1'[b]	α[c]	R_s[d]	k_1'[b]	α[c]	R_s[d]
PAG	3.30	1.35	1.54	4.46	1.38	2.12	5.29	1.42	2.35	5.70	1.40	2.31	5.50	1.38	2.23
PAiG	2.93	1.12	0.75	4.74	1.12	0.99	5.92	1.12	1.06	6.05	1.12	1.04	5.83	1.12	1.00
PHTH	7.50	1.07	0.68	10.34	1.12	0.76	10.70	1.13	1.08	11.96	1.13	1.04	11.41	1.11	0.87
PAGA	2.70	1.09	0.40	3.55	1.12	0.67	4.02	1.14	1.04	4.32	1.13	0.96	4.32	1.13	0.79

[a] The mobile phase composition is hexane–(ethanol+TFA) (90:10). The result obtained using the mobile phase containing 0.00% TFA is not listed here. Under that mobile phase condition, all chiral acidic compounds were retained more than 150 min.
[b] k_1' is the capacity factor for the first eluted enantiomer.
[c] α is the ratio of the capacity factor of the second eluted enantiomer to that of the first eluted enantiomer. In each case, the D-enantiomer eluted first.
[d] R_s values are calculated according to the USP method. When less than 0.70, values were estimated.

Table 2
The influence of mobile phase compositions on retentions and enantioseparations for acidic compounds

Acidic compound	Mobile phase compositions[a]											
	EtOH–TFA (9.97:0.03)			EtOH–TCA (9.97:0.03)			EtOH–HOAc (9.95:0.05)			IPA–TFA (9.97:0.03)		
	$k_1'^{b}$	α^{c}	R_s^{d}	$k_1'^{b}$	α^{c}	R_s^{d}	$k_1'^{b}$	α^{c}	R_s^{d}	$k_1'^{b}$	α^{c}	R_s^{d}
PAG	5.29	1.42	2.35	5.89	1.42	2.11	e			7.33	1.75	2 15
PAiG	5.92	1.12	1.06	6.37	1.11	0.79	14.20	1.05	0.71	6.21	1.07	0 72
PHTH	10.70	1.13	1.08	12.14	1.13	0.88	e			17.87	1.05	0 66
PAGA	4.02	1.14	1.04	4.74	1.18	1.31	e			5.22	1.30	1 57

[a] Mobile phase composition contains 90% hexane, 10% of (alcohol+organic acid modifier) as listed in this table. EtOH stands for ethanol, and IPA for isopropanol.
[b,c,d] The same as in Table 1.
[e] Under this mobile phase composition, the acidic enantiomers were retained more than 150 min.

efficiency and broad peak width. Significantly better resolution for PAGA was obtained using TCA instead of TFA ($R_s = 1.31$ versus $R_s = 1.04$). This indicates that in addition to acidity, the chlorine substituent on acetic acid may play a role in enantioseparation. With the exception of PAiG, the chiral acids were not eluted with mobile phases containing acetic acid as a modifier. PAiG may be the weakest acid among the four chiral acids because the carboxylic acid group is farther from the amide bond. When the amide group is attached directly to the carbon atom alpha to the carboxyl group, as in the case of PAG, PAGA, and PHTH, it attracts the electron cloud of the carboxylic acid functionality and facilitates deprotonation of the acid. The acidity of PAiG may be sufficiently weak that the presence of HOAc could allow the PAiG molecules to remain unionized and to be eluted from the column.

Earlier in this paper, it was demonstrated that a mobile phase composed of hexane and isopropanol can separate PAP, but not a mobile phase of hexane and ethanol. Therefore, isopropanol was used in place of ethanol to evaluate the effect of this alcohol on the chiral separation of all four acidic chiral compounds. It was found that except for PAGA, this substitution produced deterioration of enantioresolutions (e.g., $R_s = 0.72$ for hexane–isopropanol versus $R_s = 1.06$ for hexane–ethanol in the case of PAiG). However, PAGA showed a different behavior from the other chiral acids. This exception along with those described earlier might be due to the fact that PAGA possesses two carboxylic acid functionalities. Better enantioselectivity was achieved for PAG ($\alpha = 1.75$ for hexane–isopropanol versus $\alpha = 1.42$ for

hexane–ethanol) although enantioresolution declined ($R_s = 2.15$ versus $R_s = 2.35$). This indicates that the use of isopropanol caused better selectivity but poor column efficiency. Fig. 3 illustrates the separations achieved for the enantiomers of all four chiral acidic
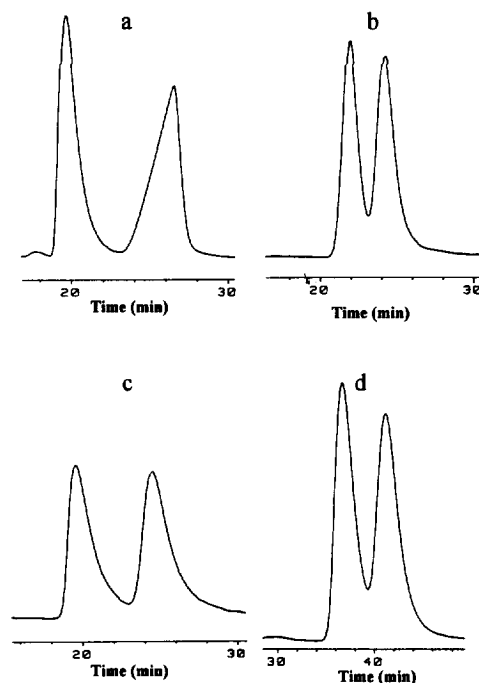

Fig. 3. Separations of PAP related acidic enantiomers. D-isomer eluted before L-isomer in all separations. (a) PAG, mobile phase: hexane–ethanol–TFA (90:9.97:0.03); (b) PAiG, mobile phase: hexane–ethanol–TFA (90:9.97:0.03); (c) PAGA, mobile phase: hexane–isopropanol–TFA (90:9.97:0.03); (d) PHTH, mobile phase: hexane–ethanol–TFA (90:9.97:0.03).

compounds using optimum mobile phase conditions. By spiking with the pure L-isomer, it was determined that the D-enantiomer was eluted before the L-enantiomer in the enantioseparation of all four hydrolysates.

In conclusion, Chiralcel OJ CSP when used in conjunction with a hexane–alcohol–organic acid mobile phase offers a feasible means for the enantio-separation of PAP, and its hydrolysis products. Although 3-phenylacetylamino-2,6-piperidinedione, like thalidomide, is a substituted 2,6-piperidinedione, the mobile phase used for its chiral discrimination was composed of hexane–isopropanol (70:30), different from that for thalidomide which was hexane–ethanol (50:50). However, in the enantioseparations of the hydrolysates, the results demonstrated that except for PAGA, ethanol was a better mobile phase modifier than isopropanol.

Acknowledgments

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