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Journal of Pharmaceutical and Biomedical Analysis
31 (2003) 589–596
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Chromatographic determination of high-molecular weight impurities in amoxicillin

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Received 6 June 2002; received in revised form 10 September 2002; accepted 10 October 2002

Abstract

The separation of high molecular weight impurities in amoxicillin has been examined by three methods of gel filtration chromatography (GFC) and reversed phase liquid chromatography (RPLC). Specificity of GFC was checked by applying the method for the determination of amoxicillin and its related impurities and linking of GFC and RPLC. The result shows that for the determination of polymers the gel filtration chromatography is a rather simple separation mode as compared to RPLC. Regarding the three gel filtration chromatographic methods, the separation on the Superdex peptide column is advantageous over other methods of determining amoxicillin polymers.
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Keywords: Amoxicillin high molecular weight impurities; Gel filtration chromatography; Reversed phase liquid chromatography

1. Introduction

Amoxicillin is a widely used semi-synthetic penicillin. The high molecular weight impurities (polymers) of amoxicillin are generated easily during production and storage of the material. It is of common knowledge that the polymers of β-lactam antibiotics are important with respect to the quality of the product. There are several reports on the isolation of amoxicillin polymers by gel chromatography on a Sephadex G-25 and by anion-exchange chromatography on a DEAE-Sephadex. However, these methods were used for the qualitative analysis of amoxicillin polymers [1,2] only. For quality control purposes, the separation of amoxicillin and its related substances containing dimer and trimer amoxicillin by a gradient elution on a C_{18} column and the identification of the polymer peaks by comparison of their relative retention times between amoxicillin and polymers were described in the BP 1998 edition [3]. Another separation was performed by HPLC-MS on a C_{18} column [4]. In addition, the use of high performance capillary electrophoresis (HPCE) has been reported for the separation and determination of ampicillin polymers [5]. However

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doi:10.1016/S0731-7085(02)00711-2

when the method was choiced as a routine quality control method, it is difficult to judge the polymer peaks if not using the reference substances. For the separation of high molecular weight impurities, a novel gel filtration chromatographic system based on the interaction between cephalosporins and Sephadex G-10 has been developed [6]. It is an easy way to find the high molecular weight impurities by gel filtration chromatography (GFC). The aim of the present study was to develop an optimized rutine quality control method for separation and analysis of amoxicillin high molecular weight impurities by means of different gel media for GFC and comparison of the separation of GFC and reversed phase liquid chromatography (RPLC).

2. Experimental

2.1. Materials

Amoxicillin sodium, amoxicillin trihydrate and amoxicillin standard were provided by the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP), People's Republic of China. Amoxicillin-dimer (ring-closed)-disodium, amoxicilloic acid, amoxicillin-dimer (open)-trisodium, amoxicillin-2(s)-piperazine-2,5-dione, amoxicillin-2(*R*)-piperazine-2,5-dione, amoxicillin-trimer-trisodium and *p*-hydroxyphenylglycine were provided by DSM Anti-infective Gist-brocades B.V. P.O. The acetonitrile used was HPLC grade, all other reagents were analytical grade and came from different commercial suppliers.

2.2. LC Equipment and columns

The equipment used consisted of three pumps, Waters 510, and two detectors, Waters 486. Chromatographic data analysis was performed by a Millennium 2010 chromatography manager.

The following columns were used: Superdex peptide HR 10/30, Sephadex™ G-10 XK16/46 (Pharmacia) and TSK G2000SW 7.5×30 mm, 10 m (TOSOH Japan). Zorbax Bonus-RP C_{18} 4.6×150 mm, 5 m, Diamonsil™ $C_{18,}$ 4.6×250 mm, 5 m, YWG C_{18}, 4.6×20 mm, 10 m packed inhouse.

Fig. 1 shows the column switching apparatus.

2.3. Sample preparations and mobile phase

Preparation of amoxicillin polymerized: dissolve a small amount of amoxicillin in water and store for 2 days. The sample concentrations used were: amoxicillin and amoxicillin polymerized 10 mg/ml, amoxicillin and its related substances 1 mg/ml, amoxicillin reference standard for assay 0.04 mg/ml.

The composition of the mobile phases as well as the LC conditions are described in Table 1.

2.4. Separation of amoxicillin by GFC and RPLC

The test conditions are described in Table 1.

2.5. Specificity experiments of GFC regarding amoxicillin and its related impurities standard and linking of GFC and RPLC systems

GFC and RPLC test conditions are described in Table 1. The LC system for linking GFC and RPLC is shown in Fig. 1.

The amoxicillin-polymerized sample was first injected onto the GFC column. Each peak eluting from the gel column was collected on a trap column and subsequently separated on the RPLC system by applying corresponding position of the switching valve

2.6. Assay of amoxicillin high molecular weight impurities by GFC

The test conditions are described in Table 1. Amoxicillin standard was used for calculating the amount of impurities. In the case of the Superdex peptide and TSK G2000SW columns, the same mobile phase was used for the standard solution and the sample solution. For the Sephadex G-10 column, water was used as mobile phase (Table 1).

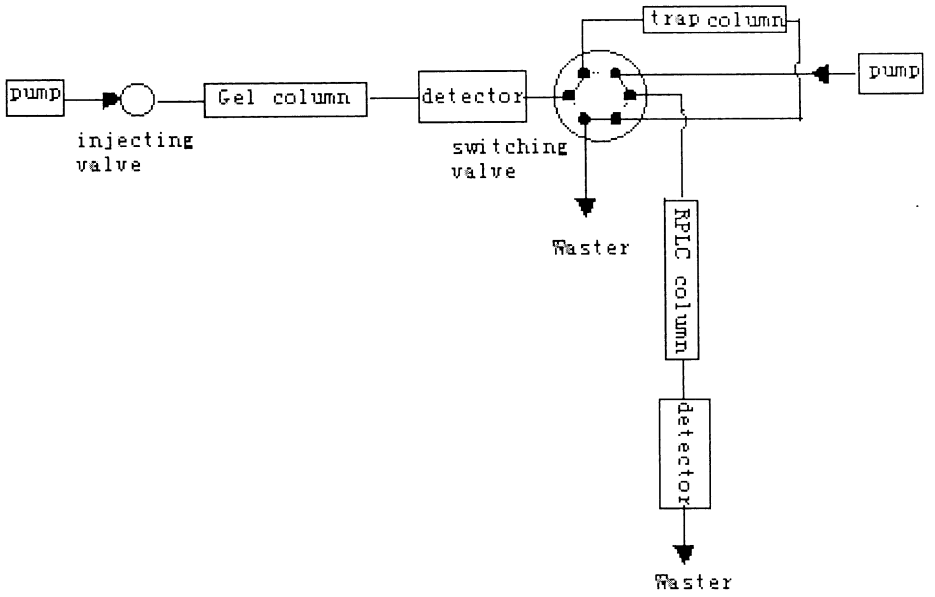

Fig. 1. The column switching apparatus: —, load;-- -, injection.

3. Results

3.1. Separation of amoxicillin polymers by gel filtration chromatography (GFC)

In this study, suitable mobile phases were evaluated for the separation and analysis of amoxicillin on a Superdex peptide, a TSK G2000SW and a Sephadex G-10 gel column (Table 1). The results show that many impurities of amoxicillin can be separated from each other on Superdex peptide and TSK G2000SW columns (Fig. 2a,b). As the molecular size exclusion limit of Sephadex G-10 media is 700 Da, the polymers (M_W: up to 766) in amoxicillin samples may be unable to pass through the gel and elute as one single peak (Fig. 2c). To the contrary, for the separation of the polymerized amoxicillin sample

Table 1
LC conditions

Method	Column	Mobile phase	Injection volume (l)	Flow rate (ml/min)	Detection UV (nm)
GFC	Superdex peptide	0.01 mol/l sodium phosphate buffer (pH 7.0)	50[a]	0.9	254
	TSK G2000SW	0.01 mol/l sodium phosphate buffer (pH 7.0)	50	0.9	254
	Sephadex G-10[a]	(A) 0.1 mol/L Sodium phosphate buffer (pH 7.0); (B) H_2O	200	0.9	254
RPLC[a]	C_{18}	(A) 25% 0.02 mol/l phosphate buffer (pH 5.0)–CH_3CN (99:1); (B) 25% 0.02 mol/l phosphate buffer (pH 5.0)–CH_3CN (80:20) [3]	20	1.0	254

[a] For examining the specificity by column switching, an injection volume of 200 l was used. Mobile phase A was used for testing the samples and mobile phase B for testing the reference standard on the Sephadex G-10 column. The following gradient elution profile was applied for RPLC: linear gradient elution, 0–25 min, ratio A:B/0:100; 25–40 min, isocratic elution with a ratio of A:B/0:100; 40–55 min, re-equilibration with A:B of 82:8. The gradient elution was started immediately after the elution of the amoxicillin peak.

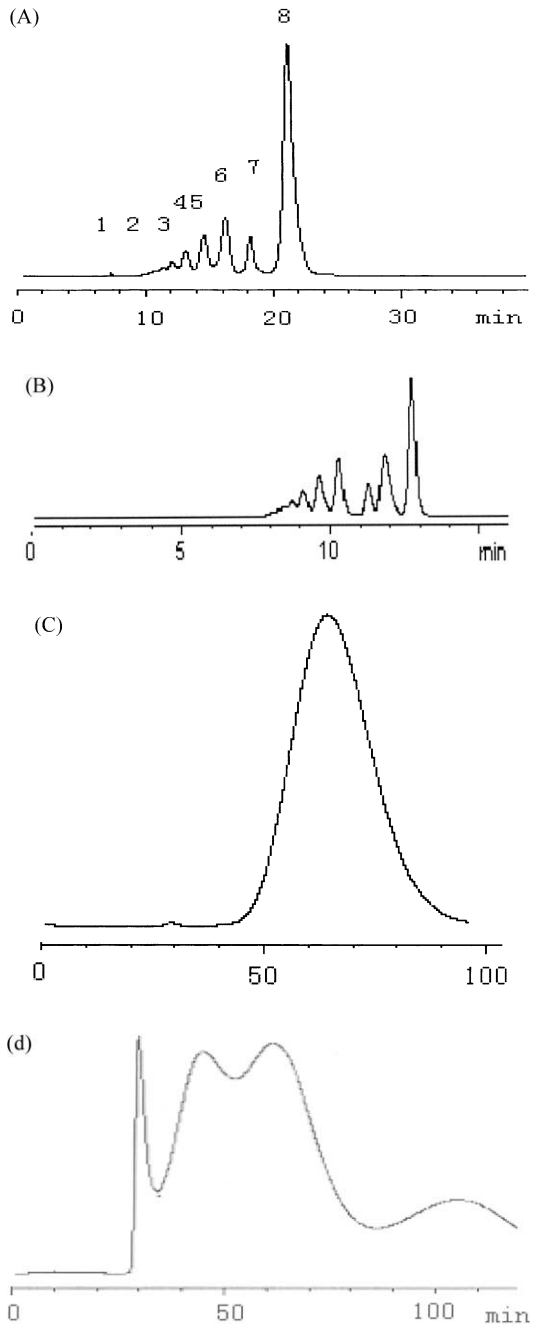

Fig. 2. (a) Chromatogram of the polymerized amoxicillin, column: Superdex peptide. (b) Chromatogram of the polymerized amoxicillin, column: TSK G2000SW. (c) Chromatogram of amoxicillin, column: Sephadex™ G-10. (d) Chromatogram of the polymerized amoxicillin, column: Sephadex™ G-10.

on the Sephadex G-10 column, a poor separation was observed. Additional peaks of impurities were eluted between the amoxicillin peak and the single peak with a small peak width due to the many degradation products in the polymerized amoxicillin sample. (Fig. 2d).

The analytical procedure for testing amoxicillin using the Superdex peptide column was validated. For calculating the linearity, the total number of analyses was 16. A calibration curve in the range from 0.6 to 10 g was tested: a regression equation $y = 422520x - 32780$ with $y =$ peak area, $x =$ amount injected in g, was calculated, with a correlation coefficient $r = 0.9999$ and a relative standard deviation (RSD) = 0.16%; the RSD of the slope was 0.85% and the RSD of intercept was about 53% ($n = 4$). For quantification, the detection limit was 30 ng with a signal-to-noise ratio of 3 ($n = 5$) and the quantitation limit was 100 ng with a signal-noise ratio of 10 ($n = 5$). The repeatability was tested by analyzing the solution corresponding to 2.7 and 0.6 g, the RSD for the peak area of amoxicillin was 1.04 and 2.19%, respectively ($n = 4$). The RSD for the intermediate precision of the assay of amoxicillin was 0.31% ($n = 5$). Although ruggedness/robustness of the method did not evaluate in our laboratory, the Superdex peptide GFC system had been introduced to several pharmacetical quality control laboratories in China and had been successfully carried out in those labotatories.

A 0.04 mg/ml amoxicillin reference standard solution was injected serially to the GFC system to check the stability of amoxicillin solution. After four operations, an impurity peak could be finding clearly before the amoxicillin peak in the chromatogram. The result indicates that amoxicillin solution is unstable and each sample solution should be injected immediately after preparation during the actual analysis.

3.2. Separation of amoxicillin polymers by RPLC

The method for the separation of amoxicillin polymers by RPLC was described in the BP 1998. In this study, the separation of amoxicillin and its related substances was examined with different columns and mobile phases prepared on different

Table 2
Retention times of amoxicillin and its related substances on RPLC column

Compound name	Zorbax Bonus-RP C_{18}		Diamonsil™ C_{18}	
	Rt_R	RSD (%) $n=3$	Rt_R	RSD (%) $n=3$
Amoxicillin standard	1	0	1	0
Amoxicillin-dimer (closed)	5.1	2.8	3.4	4.2
Amoxicillin-dimer(open)	4.5	5.4	3.1	4.6
Amoxicilloic acid	0.7	9.4	0.9	8.3
Amoxicillin-2(s)-piperazine-2,5-dione	4.1	3.4	3.0	2.4
Amoxicillin-2(*R*)-piperazine-2,5-dione	3.8	3.7	2.9	3.7
p-Hydroxyphenylglycine	0.35	2.3	0.28	2.5
Amoxicillin-trimer	7.1	5.9	3.9	5.5

Rt_R = relative retention time of the related substances as compared to that of amoxicillin

days (Table 2). The results show the relative retention times of impurities as compared to the retention time of amoxicillin. The tests were performed on different columns and on different days in order to establish an accurate procedure for the identification of the impurities.

3.3. Specificity experiments of GFC

3.3.1. Specificity experiments with amoxicillin and its related impurities standard

Firstly, the specificity experiments of GFC were examined with amoxicillin and its related impurity reference standards. The results are shown in Table 3. On the Superdex peptide and TSK G2000SW columns the retention time of amoxicilloic acid (MW383) is close to that of the ring-closed dimer of amoxicillin (MW730) eluting before amoxiclllin. The ring-open dimer (MW748) and trimer (MW1114) amoxicillin have the same position, too. In addition, the retention times of amoxicillin-2(s)-piperazine-2, 5-dione (MW365) and amoxicillin-2(*R*)-piperazine-2,5-dione were shorter than the retention time of amoxicillin on the TSK G2000SW column. However, on the Sephadex G-10 column, the ring-closed dimer cannot be excluded due to adsorption and cannot be separated from amoxicillin due to extensive peak broadening. The analysis of the single peak with a small peak width on the Sephadex G-10 column shows that this peak does not contain the ring-closed dimer.

From the above results it can be derived that the chromatographic behavior of amoxicillin and its related impurities is affected by size exclusion as well as by adsorption, because they do not follow an elution order according to the corresponding molecular size.

Table 3
Retention time of amoxicillin and its related substances

Compound name	Retention time (min)			
	Sephadex G-10	Superdex	TSK G2000PW	C_{18} (Diamonsil™)
Amoxicillin standard	58.2	20.9	12.7	10.2
Amoxicillin-dimer (closed)	62.1	18.0	11.2	35.5
Amoxicillin-dimer (open)	33.1	16.0	10.4	33.1
Amoxicilloic acid	42.9	18.4	11.0	9.1
Amoxicillin-2(s)-piperazine-2,5-dione	–	21.4	11.8	30.6
Amoxicillin-2(*R*)-piperazine-2,5-dione	–	20.9	11.7	30.1
p-Hydroxyphenylglycine	–	22.9	13.3	2.9
Amoxicillin-trimer	28.7	15.9	10.3	41.0

3.3.2. Specificity experiments by linking GFC and RPLC

As the chromatographic behavior of amoxicillin and its related impurities is affected by size exclusion and adsorption on the gel media, the elution order is not exactly according to molecular size. The molecular size of the impurities eluting before the amoxicillin peak may not be larger than amoxicillin. Some chromatographic information was obtained by the analysis of standards of the impurities. For the amoxicillin sample, the column switching technology was also used to study the specificity. The effluent of each peak on the gel chromatographic system was switched to the RPLC system and analyzed. The retention times of amoxicillin and its related impurity standards are shown in Table 3. The RPLC chromatogram of the polymerized amoxicillin sample obtained by linking GFC and RPLC are shown in Fig. 3.

It can be seen that peak 6 coincides with the peak of the ring-open dimer in the RPLC system. The ring-closed dimer was not determined in the effluent of peak 7 in the gel system as its -lactam cycle may be hydrolyzed during chromatographic elution. As a summary, the separation results of polymerized amoxicillin sample on the gel system and the RPLC system, peak 1 to peak 5, can be assigned to amoxicillin polymers having a lower polarity than the ring-open dimer and a larger size than the amoxicillin dimer. The degree of polymerization increased in analogy to the dimer. Peak 7 was contents mostly amoxicilloic acid. In this study, amoxicillin sodium, amoxicillin trihydrate and amoxicillin capsule were examined by the column switching system. The results show that peak 7 contains amoxicilloic acid and ring-closed dimer by comparing the results of the analysis of the reference standards

The separation of amoxicillin polymers on a Sephadex G-10 column was examined by linking GFC and RPLC. In the chromatogram of the RPLC system it can be seen that the effluent collected from the gel system in the retention time range of 35 to 45 minutes contains amoxicilloic acid and dimer, etc.

3.4. Assay of amoxicillin high molecular weight impurities by GFC

Amoxicillin trihydrate bulk materials were dissolved in 0.1 mol/l phosphate buffer (pH 7.0) and its high molecular weight impurities were detected by the GFCs. The results on the Superdex peptide accord to those obtained on the TSK G2000SW, but slightly differ from those obtained on the Sephadex G-10 (Table 4).

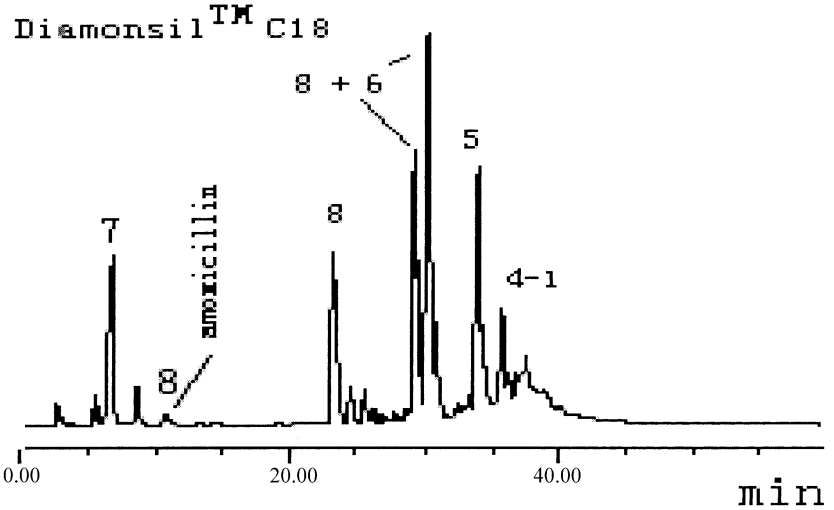

Fig. 3. Chromatogram of the polymerized amoxicillin using the C_{18} column. (The gradient was started after the elution of the amoxicillin peak. The markers of the chromatographic peaks in this figure are the same as in Fig. 2a.)

Table 4
Assay of amoxicillin high molecular weight impurities by GFC

Produce	High molecular weit impurity content (%)				
	Superdex peptide		TSK G200SW		G-10
	1	2	1	2	
1	0.27	0.29	ND	ND	0.10
2	0.24	0.26	ND	ND	0.05
3	0.14	0.17	ND	ND	0.11
4	0.21	0.23	ND	ND	0.22
5	0.28	0.30	ND	ND	0.19
6	0.09	0.35	0.08	0.32	0.17
7	0.06	0.29	0.09	0.36	0.14
8	0.07	0.28	0.07	0.34	0.14

1: Impurities eluting prior to amoxicillin (not including the impurities next to amoxicillin); 2: impurities eluting prior to amoxicillin.

4. Discussion

4.1. Comparison of three gel media used for the separation of amoxicillin polymers

As the chromatographic behavior of amoxicillin and its related impurities is affected by size exclusion and adsorption on gel media, they did not elute exactly according to the distribution of molecular size. However, size exclusion still plays a dominant role when using 0.01 mol/l phosphate buffer (pH 7.0) as a mobile phase. Amoxicillin polymers elute prior to amoxicillin. When applying the Superdex peptide and TSK G2000SW chromatographic systems, amoxicillin polymers with different degrees of polymerization were separated within a short analysis time, but amoxicilloic acid and the ring-closed dimer interfere with each other. Amoxicilloic acid seems to play an important role in the generation of amoxicillin polymers, therefore the limitation of amoxicilloic acid and amoxicillin high molecular weight impurities should be taken into consideration. As amoxicillin-2(*R*)-piperazine-2,5-dione can be separated from amoxicillin and was excluded prior to amoxicillin on the TSK G2000SW column, the separation method with the TSK G2000SW column is not advantageous over the method with the Superdex peptide column for determining amoxicillin polymers.

Due to the adsorption of the ring-closed dimer on the Sephadex G-10 column the peak with a narrow peak width, completely excluded, contains ring-closed dimer. Therefore this method is not suitable for detecting all amoxicillin polymers. But this method has an advantage in that all amoxicillin polymer peaks elute as one single peak and can be integrated for quantification.

4.2. Comparison of GFC and RPLC

Amoxicillin polymers are generated by polymerization between amoxicillin and amoxicilloic acid or between amoxilloic acid themself and exhibit a lower polarity than amoxicillin. The retention times of the polymer compounds are longer than the retention time of amoxicillin. The elution order of the polymer peaks is in accordance with the polarity of the compounds in the RPLC system. Since the degradation products also contain impurities with a lower polarity and smaller size, such as amoxicillin-2-piperazine-2,5-dione with a retention time close to that of the dimer amoxicillin, they may interfere with the dimer peak. The relative retention times of the impurities differ from the retention time of amoxicillin, which was tested on different columns and on different days to ensure accurate identification of the impurities. In addition, the gradient elution method enables the elution of all amoxicillin polymers within a moderately short time. Usually, as the baseline noise is much more intensive than the signals produced by polymers in low concentrations using the gradient elution method, quantification of the polymers is limited. In the gel chromatographic system based mostly on size exclusion, all polymer peaks coeluted at the front of the amoxicillin peak and the polymers peak are easily identified. Gel filtration chromatography is a versatile separation mode for the determination of polymers. In addition, the gel system is advantageous with regard to environmental protection, as no organic solvents are used as mobile phase components.

5. Conclusion

For analysis of high molecular weight impurities of amoxicillin in quality control laboratories, although some RPLC methods [3,4] and HPLC-MS technique [4] have been reported, it do not always feel convenience in a routine analysis as it is difficult to judge the polymer peaks if not using the reference substances. However the high molecular weight impurity peaks could be easily confirmed at GFC system. So it is an easy way to choice a GFS method for a routine polymer control. A novel gel filtration chromatographic system based on the interaction between cephalosporins and Sephadex G-10 has been developed [6]. But there was no GFS method reported using in amoxicillin polymer control. In this study, the separation of high molecular weight impurities in amoxicillin has been examined by three methods of GFC. The result shows that the gel filtration chromatography is a rather simple separation mode as compared to RPLC. Regarding the three gel filtration chromatographic methods, the separation on the Superdex peptide column is advantageous over other methods of determining amoxicillin polymers.

In addition, the gel system is advantageous with regard to environmental protection, as no organic solvents are used as mobile phase components.

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