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Evaluation of liquid chromatography methods for the separation of ampicillin and its related substances

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Abstract

Two laboratories collaborated to examine the selectivity of four isocratic liquid chromatography (LC) methods for the separation of ampicillin and its related substances. The United States Pharmacopeia (USP) assay method gave the best selectivity. Similar selectivity was obtained on C18 columns as well as on C8 and poly(styrene–divinylbenzene) copolymer columns. A resolution test using cefradine was proposed to replace the test with caffeine prescribed by the USP. Based on the USP method, a gradient LC method was developed for the analysis of related substances in ampicillin. This LC method has been proposed for assay and purity control in the ampicillin monographs of the European Pharmacopeia.

Keywords: Ampicillin; Assay; Related substances test; Purity; Liquid chromatography

1. Introduction

Ampicillin is an important β-lactam antibiotic. The sodium salt of ampicillin especially is relatively unstable, both in aqueous solution and in storage, leading to the formation of a variety of degradation products. Fig. 1 shows the structures of ampicillin and its related substances that were available in the laboratory.

Many liquid chromatography (LC) methods have been described for the analysis of ampicillin. Some papers describe the determination of ampicillin in biological samples [1–6]. These methods highlight the separation of the antibiotic from the background of biological material. Some papers discuss the separation of ampicillin diastereoisomers [7] or the separation of ampicillin from other penicillins or other drugs [8,9]. Some papers deal with stability studies of ampicillin under different conditions [10,11]. Some papers report special de-

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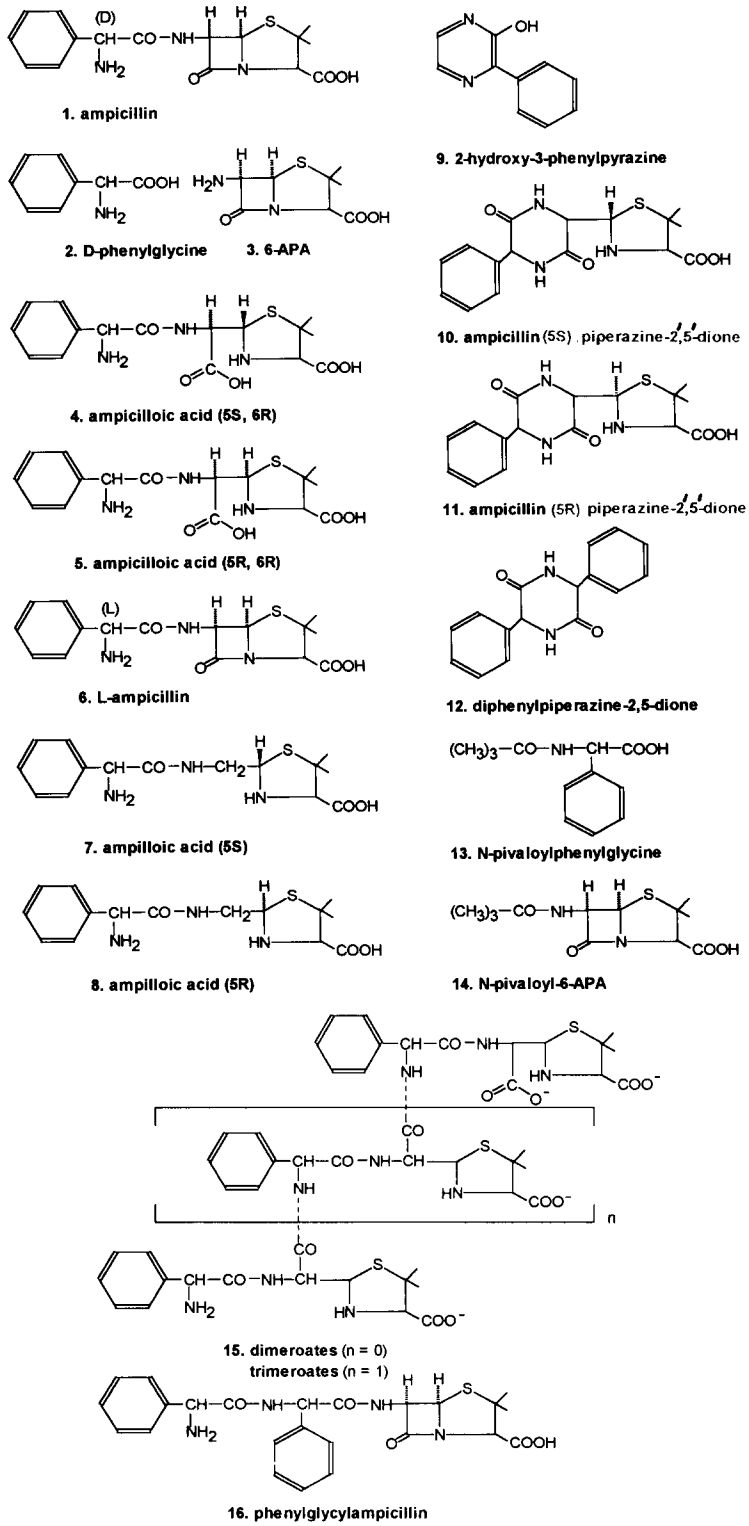
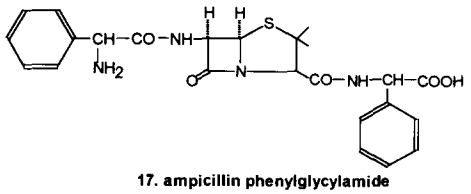
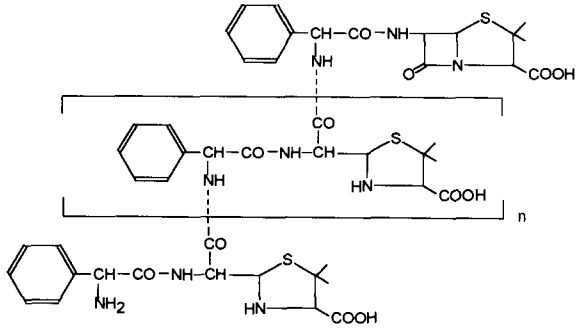


Fig. 1. Structures of ampicillin and its related substances.



17. ampicillin phenylglycylamide



18. dimer (n = 0)
trimer (n = 1)

Fig. 1. (contd.).

tection techniques such as laser-based polarimetry and chemiluminescence [12,13]. A number of papers discuss the determination of ampicillin [14–17]. An LC method for the assay of ampicillin is prescribed by the United States Pharmacopeia (USP) [18]. However, the selectivity towards the related substances and the reproducibility of the selectivity on different columns have not been sufficiently discussed.

In the present study, the selectivity of four isocratic LC methods for assay has been examined. Two were taken from the literature [17,18] and the other two were made available by manufacturers of ampicillin. All methods use C_{18} stationary phases. Table 1 shows the LC conditions for the four methods. The prescribed conditions were slightly adapted in our study, as shown. The aim of our study was to examine whether an existing assay method was sufficiently selective and whether it could be adapted in order to be suitable as a related substances test. This required the availability of the related substances, some of which were kindly donated by manufacturers, but most had to be prepared in the laboratory.

2. Experimental

2.1. Samples

Related substances originate from semi-synthesis and from degradation. The structures of the available related substances are shown in Fig. 1. D-Phenylglycine (**2**) and 6-aminopenicillanic acid (6-APA, **3**) are the basic constituents of ampicillin which are commercially available. L-Ampicillin (**6**), diphenylpiperazine-2,5-dione (**12**), *N*-pivaloylphenylglycine (**13**), *N*-pivaloyl-6-APA (**14**), phenylglycylampicillin (**16**) and ampicillin phenylglycylamide (**17**) can arise from the semi-synthesis of ampicillin. The other related substances are decomposition products. Related substances **6**, **12**, **13**, **14** and **17** were obtained from Antibioticos and Biochemie S. A., Barcelona. Ampicilloic acid (5*S*, 6*R*) (**4**), ampicilloic acid (5*R*, 6*R*) (**5**), ampilloic acid (5*S*) (**7**) and ampilloic acid (5*R*) (**8**) were prepared as described by Munro et al. [19]. 2-Hydroxy-3-phenylpyrazine (**9**) was prepared as described by Lebelle et al. [20]. Ampicillin (5*R*) piperazine-2′,5′-dione (**11**) was prepared as described by Bundgaard and Larsen [21]. Epimerization of **11** to ampicillin (5*S*) piperazine-2′,5′-dione

Table 1
LC conditions for four isocratic LC methods

Method	Source	Mobile phase prescribed	Column temperature (°C) prescribed	Flow rate (ml min $^{-1}$) prescribed	Detection UV wavelength (nm) prescribed	Column temperature (°C) used	Flow rate (ml min^{-1}) used	Detection UV wavelength (nm) used
I	Manufacturer 1	Methanol –phosphate buffer (pH 7.0, 19 mM) (2:8, v/v)	50	2	220	50	1	254
II	Manufacturer 2	Methanol –phosphate buffer (pH 5.0, 20 mM) (25:75, v/v)	40	1.5	210	40	1	254
III	Lauback	SDS (0.035 M) + formic acid (2.0 M) –acetonitrile –water (100:350:up to 1000)	Ambient	3	254	30	1	254
IV	USP	Acetic acid (1.0 M)–potassium dihydrogen phosphate (1.0 M)–acconitrile –water (1:10:80:909)	Ambient	2	254	30	1	254

(**10**) was performed in a similar way as described by Haginaka and Wakai [22] for amoxicillin. Related substance **16** was made as described by Grant and Alburn [23]. The oligomeroates (**15**) and oligomers (**18**) were prepared as described by Bundgaard and Larsen [24] and Roets et al. [25]. Samples of ampicillin trihydrate (94%) and ampicillin sodium (85%) were available.

2.2. Solvents and reagents

Acetonitrile (HPLC grade) was from Rathburn (Walkerburn, UK). Methanol (Roland, Brussels) was distilled before use. Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, formic acid, acetic acid and sodium dodecyl sulfate (SDS) were from Acros (Beerse, Belgium). Water was distilled twice.

2.3. LC apparatus and operating conditions

The equipment consisted of an L-6200 pump (Merck-Hitachi, Darmstadt, Germany), a Model CV-6-UHPa-N60 Valco injector (Houston, TX) with a 20 or 50 μl loop, a Model D 254 nm fixed-wavelength UV monitor (LDC/Milton Roy, Riviera Beach, FL) and an integrator Model 3396 Series II (Hewlett-Packard, Avondale, PA). The columns (25 cm × 0.46 cm i.d.) used in this study are reported in Table 2. Most of the experiments were carried out in laboratory A, but a number of experiments was repeated in laboratory B, to examine reproducibility. The columns used in laboratory B are identified with an asterisk.

2.4. Mobile phases

The mobile phases were prepared as described in Table 1. For some columns, the content of organic modifier was slightly adapted in order to obtain similar retention times for ampicillin. Mobile phases were degassed by ultrasonication.

2.5. Sample preparation

Samples used for the selectivity study were prepared in mobile phase at the following concentrations: ampicillin, 1 mg ml^{-1}; **2** and **3**, 0.1 mg ml^{-1}; **4**, **5** and **6**, 0.2 mg ml^{-1}; **9**, 0.02 mg ml^{-1}; other related substances, 0.5 mg ml^{-1}. 20 μl of solution was injected.

3. Results and discussion

In preliminary work, three different C_{18} columns were used in laboratory A for the study of the selectivity. The capacity factors of ampicillin and of its related substances were determined. The experiments were repeated on one column in laboratory B. Ampicillin phenylglycylamide (**17**), the trimeroates (**15**, $n = 1$) and the oligomers (**18**) were always eluted far after ampicillin and therefore they are not reported in the results. For method I, the results are shown in Table 3. The amount of methanol in the mobile phase for each column was: A = 2, B* = 1.9, C = 1.5, E = 1. Ampicillin was not separated from its related substances on columns A, C and E. For method II, the results are shown in Table 3. The amount of methanol in the mobile phase for each column was: A = C = E = 20, D* = 24. Ampicillin was separated from its related substances on columns A, D* and E but not on column C. For method III, the results are shown in Table 3. The amount of acetonitrile in the mobile phase for each column was: A = C = E = 350, D* = 385. Ampicillin was separated from its related substances on columns A and E but not on columns C and D*. Moreover, for methods I, II and III, differences in elution order of ampicillin and its related substances were observed on all columns.

Table 2
General information on columns

Column	Stationary phase and manufacturer	Particle size (μm)
A, B*	Hypersil ODS (Shandon, Runcorn, UK)	5
C, D*	Bio-sil C18 (Bio-Rad, Nazareth, Belgium)	5
E, F*	Spherisorb ODS-1 (Phase Separations, Queenferry, UK)	10
G*	Lichrospher C18 (Merck, Darmstadt, Germany)	5
H	RoSil C8 (Altech, Deerfield, IL)	8
I	Chromspher C8 (Chrompack, Middelburg, The Netherlands)	5
J	Zorbax C8 (Du Pont Co., Wilmington, DE)	7
K	PRP-1 (Hamilton, Reno, NV)	7–9
L, M*	PLRP-S (Polymer Laboratories, Church Stretton, UK)	8

For method IV, after preliminary work on three C_{18} columns in laboratory A, it was clear that this method gave a satisfactory and repeatable selectivity. This was confirmed by laboratory B, using three C_{18} columns. Thereafter C_8 columns (H, I, J) and even poly(styrene–divinylbenzene) copolymer columns (K, L, M*) were also examined. Columns K and L were used at 50°C. The results are shown in Fig. 2; on all columns ampicillin was separated completely from all its related substances. Table 4 shows general information on method performance using method IV. According to the USP monograph [18], the resolution between the caffeine and the ampicillin peaks should be not less than 2.0, the capacity factor of ampicillin not more that 2.5, the symmetry factor not more than 1.4. With the exception of the symmetry factor on column K, all parameters comply with the requirements of the USP method.

In the present study, a resolution test using cefradine is proposed to replace the prescribed test with caffeine. Caffeine was replaced because its structure is different from that of β-lactam antibiotics, and therefore its chromatographic behavior is not related to that of ampicillin. Table 4 shows the results of the resolution tests and the selectivity towards the separation of ampicillin and its epimer L-ampicillin.

Quantitative aspects of this method have been examined. It was decided to inject an amount of 30 μg of ampicillin in 50 μl of mobile phase. At

Table 3
Capacity factors (k') of ampicillin and its related substances on C18 columns acording to methods 1, 2 and 3

Related substances	Method 1				Method 2				Method 3			
	Column				Column				Column			
	A	B*	C	E	A	C	D*	E	A	C	D*	E
1	5.43	5.01	2.68	4.96	2.18	1.39	2.06	1.31	9.03	4.47	6.97	5.39
2	0.26	0.19	0.47	0.55	0.28	0.23	0.26	0.30	3.21	1.82	3.03	2.16
3	0.25	0.22	0.18	0.27	0.21	0.24	0.16	0.20	3.48	2.11	3.11	2.52
4	0.40	0.39	0.20	0.43	0.43	0.35	0.29	0.30	5.84	2.62	4.36	3.36
5	0.52	0.50	0.20	0.43	0.71	0.50	0.53	0.44	6.89	3.22	5.26	3.97
6	5.46	4.73	2.35	4.45	1.08	0.77	0.97	0.73	8.38	4.15	6.80	4.91
7	4.62	3.96	2.69	5.01	3.06	2.05	2.85	1.82	18.01	6.35	14.83	8.73
8	6.49	5.79	3.45	6.91	4.34	2.68	4.01	2.82	>20	7.78	14.83	9.86
9	7.63	5.74	8.23	16.26	7.48	5.66	8.75	4.91	1.30	1.51	1.31	1.39
10	2.39	2.02	1.11	2.08	1.89	1.26	1.30	0.85	0.91	0.92	0.76	0.87
11	3.53	2.99	1.28	2.65	2.64	1.39	1.80	0.95	1.12	1.01	0.95	0.98
12	7.16	5.38	5.53	11.90	5.29	2.50	5.75	2.97	1.27	1.24	1.10	1.41
13	6.63	5.71	1.88	3.72	5.45	2.40	3.66	1.61	2.96	1.92	2.60	1.88
14	17.12	14.63	4.48	10.25	11.40	4.45	7.57	3.21	3.45	3.59	6.27	3.75
15	3.80	3.45	0.89	3.29	6.52	2.28	3.57	2.01	>20	7.59	17.40	10.96
16	12.62	>20	14.25	>20	11.68	4.46	9.09	3.87	18.55	7.66	13.96	9.48

about 22°C this solution remained stable for at least 22 h. The repeatability was checked by analysing the same solution of ampicillin six times (RSD = 0.15%). A linearity test was performed (y = peak area, x = amount injected in micrograms): $y = 21351x + 2966$, $S_{y,x} = 400$, $r = 0.9999$, number of concentrations = 3, total number of analyses = 14, range of x covered by the experiments = 21–39 μg.

A related substance test based on this assay method was also developed. Considering the retention times of the potential impurities it was necessary to use gradient elution. The chromatographic procedure was carried out with mobile phases A: acetic acid (1 M)–potassium dihydrogen phosphate (1 M)–acetonitrile–water (1:10:50:939, v/v/v/v) and B: acetic acid (1 M)–potassium dihydrogen phosphate (1 M)–acetonitrile–water (1:10:400:589, v/v/v/v). A freshly prepared test solution containing 150 μg in 50 μl was injected. The elution was started isocratically with a mobile phase ratio A:B of 85:15. After 10 min a linear gradient elution was started to reach a mobile phase ratio A:B of 0:100 over a period of 30 min. The chromatography was continued with mobile phase B for 15 min. Then the column was equilibrated with a ratio A:B of 85:15 for 15 min. Results of this related substances test for an old sample of ampicillin sodium using three C18 columns (A, C, E) are shown in Table 5 (k′ values for related substances). The results show the good selectivity of the gradient method for related substances. It is seen that in the gradient part the sequence of related substances may vary on the three C18 columns. Column E, with the 10 μm particles, gave a worse separation of related substances than columns A and C. It was decided to continue to work with stationary phases with a particle size of 5 μm. Fig. 3 shows a typical chromatogram. The use of 254 nm as detection wavelength has the advantage that the slope of the baseline during gradient elution is not too steep.

With the conditions described, the limit of detection (LOD) with a signal-to-noise ratio of 14 was 0.02%, expressed as ampicillin. The limit of quantitation (LOQ) was 0.04% ($n = 6$, relative standard deviation (RSD) = 6.2%).

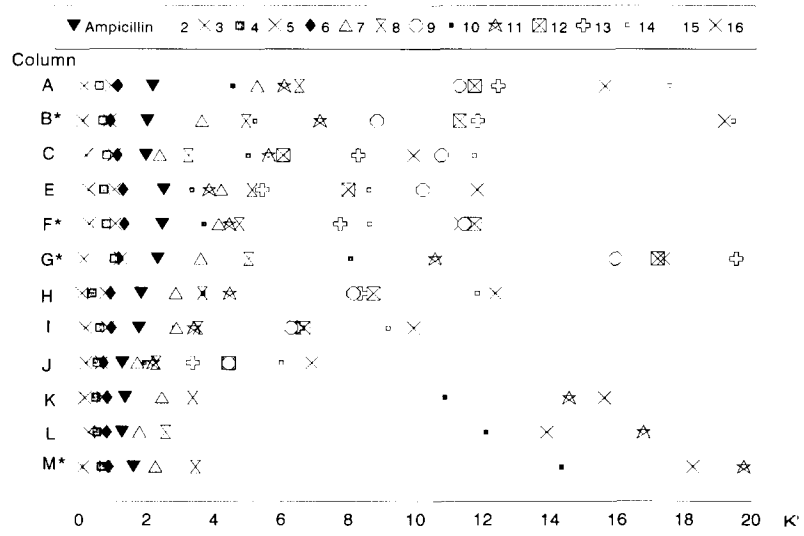


Fig. 2. Capacity factors of ampicillin and its related substances on different columns following the USP method.

4. Conclusions

It can be concluded that the USP method is the only isocratic method that is sufficiently selective in a reproducible way to be used for the assay of ampicillin. Caffeine could be replaced by cefradine in the resolution test. The gradient elution method based on the USP method is suitable as a related substances test. The performance of this method for assay and purity testing of ampicillin will be further examined in a collaborative study.

Acknowledgements

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Table 4
General information on performance of the USP method[a]

Column	Amount of acetonitrile in mobile phase	k' Ampicillin	S Ampicillin	n Ampicillin	Rs Ampicillin Caffeine	Rs Ampicillin Cefradine	α
A	110	2.17	0.70	4420	2.9	7.6	1.90
B*	80	2.01	1.16	2540	2.3	5.1	2.23
C	100	1.97	1.05	6340	22.0	4.2	1.76
E	100	2.50	1.06	2640	13.2	3.4	1.94
F*	120	2.44	1.12	2640	16.7	3.1	1.85
G*	102	2.31	1.0	4190	15.9	8.5	2.01
H	100	1.82	0.81	3780	6.0	4.1	2.00
I	100	1.76	0.85	3360	5.1	3.8	1.87
J	70	1.27	1.27	2440	6.5	3.4	1.79
K	80	1.34	1.64	2500	13.0	5.9	1.65
L	80	1.25	1.20	4270	17.7	6.6	1.56
M*	74	1.60	1.08	5280	16.9	8.0	1.88

[a] k' = capacity factor; S = symmetry factor; n = number of theoretical plates; Rs = resolution; $\alpha = k'$D-ampicillin/k'L-ampicillin.

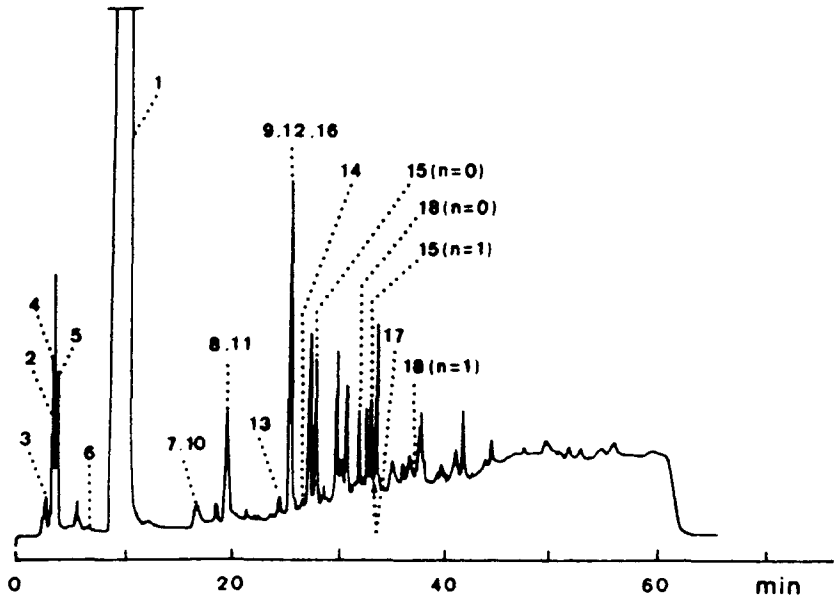

Fig. 3. Chromatogram of ampicillin sodium on Hypersil ODS obtained with gradient elution. Mobile phase: (A) acetic acid (1 M)–potassium dihydrogen phosphate (1 M)–acetonitrile–water (1:10:50:939, v/v/v/v); (B) acetic acid (1M)–potassium dihydrogen phosphate (1 M)–acetonitrile–water (1:10:400: 589, v/v/v/v). Gradient elution: 0–10 min, isocratic elution, with a ratio A:B of 85:15, 10–40 min, a linear elution with a ratio A:B of 0:100, 40–55 min, isocratic elution with a ratio A:B of 0:100, 55–70 min isocratic elution with a ratio A:B of 85:15.

Table 5
Capacity factors of ampicillin and its related substances on three C18 columns as obtained by gradient elution[a]

	k'																			
Column	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15 $n=0$	15 $n=1$	16	17	18 $n=0$	18 $n=1$
A	3.1	0.2	0.1	0.4	0.6	1.2	5.6	6.9	9.0	5.6	6.9	9.0	8.6	9.5	10.0	11.8	9.0	12.1	11.6	15.0
C	2.2	0.3	0.5	0.9	1.1	1.6	2.7	3.6	8.6	5.1	5.1	8.1	7.0	8.9	9.2	11.7	7.5	9.9	10.7	13.1
E	2.5	0.5	0.2	1.0	1.2	1.6	3.6	4.4	8.3	2.6	2.6	7.0	4.7	7.5	11.4	14.1	8.7	13.5	13.5	15.9

[a] Mobile phase: (A) acetic acid (1 M)–potassium dihydrogen phosphate (1 M)–acetonitrile–water (1:10:50:939, v/v/v/v); (B) acetic acid (1 M)–potassium dihydrogen phosphate (1 M)–acetonitrile–water (1:10:400:589, v/v/v/v). Gradient elution: 0–10 min, isocratic elution with a ratio A:B of 85:15, 10–40 min a linear elution with a ratio A:B of 0:100, 40–45 min, isocratic elution with a ratio A:B of 0:100m 55–70 min, isocratic elution with a ratio A:B of 85:15.

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