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Liquid chromatographic separation of hexopyranosylated cytosine nucleosides from their degradation products

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Abstract

Development of a liquid chromatographic method which can separate each of a series of hexopyranosylated cytosine nucleosides from their degradation products formed at acid, neutral and basic pH is described. Both silica-based reverse-phase and polymer columns were examined. Influence of the mobile phase pH, ion-pairing agent, concentration of the buffer and type and concentration of organic modifier were systematically investigated. The concentration of the ion-pairing agent and the buffer were found to have a major effect on selectivity. Samples were finally analyzed on a poly(styrene-divinylbenzene), PLRP-S 100 Å (8 μm) 250 × 4.6 mm I.D. column at 60°C and with a mobile phase consisting of acetonitrile–sodium octanesulphonate (pH 2.5; 0.02 M)–potassium phosphate buffer (pH 2.5; 0.2 M)–water (X:25:50:25 − X, v/v, where X is variable). © 1997 Elsevier Science B.V.

Keywords: Liquid chromatography; Ion-pairing; Hexopyranosylated cytosine nucleosides

1. Introduction

Nucleosides play a central role in the biochemistry of living organisms and chemists have been interested in modifying structures of naturally occurring nucleosides in order to obtain therapeutic agents. Nucleoside analogues are in clinical use as antiviral and antitumour drugs. Research focussing on new drugs for the treatment of acquired immunodeficiency syndrome (AIDS) has intensified and a variety of nucleoside analogues with activity against human immunodeficiency virus (HIV) have been discovered, of which zidovudine, zalcitabine, didanosine, stavudine, and lamivudine have been released for clinical use. Recently, the interest in nucleosides with a six membered carbohydrate moiety has risen due to the discovery of anhydrohexitol nucleosides as potent antivirals [1]. Compounds **1**–**6** (Fig. 1) are nucleosides which have a cytosine base and a deoxygenated hexopyranose sugar instead of the naturally occurring pentofuranose sugar (ribose or deoxyribose). They were synthesized as potential antiviral agents but on evaluation none of them had biological activity [2–4]. However, it was found interesting to carry out stability studies on **1**–**6** because such knowledge is useful for derivatization reactions and because compounds

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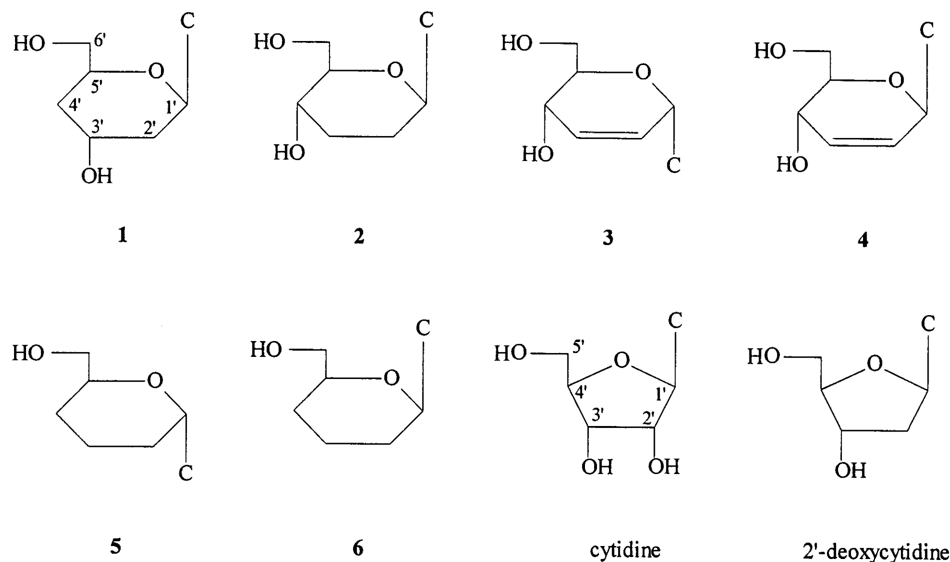

Fig. 1. Structures of compounds **1**–**6**, cytidine and 2′-deoxycytidine. **1**: 1-(2,4-dideoxy-β-D-*erythro*-hexopyranosyl)cytosine, **2**: 1-(2,3-dideoxy-β-D-*erythro*-hexopyranosyl)cytosine, **3**: 1-(2,3-didehydro-2,3-dideoxy-α-D-*erythro*-hexopyranosyl)cytosine, **4**: 1-(2,3-didehydro-2,3-dideoxy-β-D-*erythro*-hexopyranosyl)cytosine, **5**: 1-(2,3,4-trideoxy-α-D-*glycero*-hexopyranosyl)cytosine, **6**: 1-(2,3,4-trideoxy-β-D-*glycero*-hexopyranosyl)cytosine.

1–**6** are also potential candidates for incorporation into oligonucleotides which may serve as therapeutic agents against viral infections and/or cancers. Stability studies required an analytical method that could separate each of compounds **1**–**6** from its degradation products formed at acid, neutral and basic pH, but such a method did not exist. Indeed, to our knowledge no liquid chromatographic work has been published about hexopyranosyl nucleosides. On the other hand ample literature is available about chromatography of nucleoside structures [5,6], be it established antiviral [7] or anticancer [8] drugs, or drugs under development [9], or nucleosides used as biological markers in disease states [10]. Most of these methods either use (ion pair) reversed-phase or ion exchange chromatography.

The purpose of this work was to develop a suitable liquid chromatographic (LC) method to support stability studies on hexopyranosylated cytosine nucleosides.

2. Experimental

2.1. Samples and reagents

The synthesis of compound **1** has been published elsewhere [2] and so has that of **2**, **3** and **4** [3], and of **5** and **6** [4]. Cytosine, uracil, sodium octanesulphonate and methanol were purchased from Acros Chimica (Geel, Belgium). Methanol was distilled before use. LC-grade tetrahydrofuran and acetonitrile from Rathburn (Walkerburn, Scotland) were used. Water was distilled twice in glass apparatus. All other reagents were of pro-analysis quality (Acros Chimica).

2.2. Liquid chromatography

Liquid chromatographic apparatus consisted of an SP 8700 XR solvent delivery system (ThermoSeparation Products, Fremont, CA, USA), used at a flow rate of 1 ml/min, an injector Model CV-6UH-Pa-N-60 (Valco, Houston, TX, USA) equipped with a 20 µl loop, a Merck-Hitachi (Darmstadt, Germany) Model L-4000 variable UV detector set at 270 nm and a Hewlett-Packard Model 3396 Series II integrator (Avondale, PA, USA). The stationary phases examined were RSil C18 LL 5 µm (Alltech Associates Inc., Laarne, Belgium), PRP-1 7–9 µm (Hamilton Company, Reno, NV, USA), Rogel 80 Å 8 µm (Bio-Rad Laboratories, Eke, Belgium), PLRP-S 100 Å 8 µm and PLRP-S 1000 Å 8 µm (Polymer Laboratories, Church Stretton, Shropshire, U.K.), all in 250 × 4.6 mm I.D. columns. The mobile phase finally used consisted of acetonitrile–sodium octanesulphonate (pH 2.5; 0.02 M)–potassium phosphate buffer (pH 2.5; 0.2 M)–water (X:25:50:25 − X, v/v, where X is variable). Validation of the liquid chromatographic method was performed by checking the linearity of the detector signal for all six compounds, by evaluating the repeatability for compound **1** and by determining the limits of detection and quantitation for compound **1** (see Section 3). Samples of compound **1** used for kinetic studies were prepared by diluting 0.1 ml of a 5×10^{-3} M stock solution with 4.9 ml of 0.01 M sodium dihydrogen citrate buffer at pH 1.22 or with 0.01 M sodium phosphate buffer at pH 6.75 or at pH 11.99. The ionic strength of the buffers was adjusted to 0.07 with KCl before measuring the final pH. Aliquots (0.5 ml) of this solution were put in vials, capped and degraded at 100°C in a Memmert (Schwabach, Germany) oven. The vials were removed and quenched with 0.5 ml of 0.093 M KOH, water or 0.01 M HCl to give a final nucleoside concentration of 5×10^{-5} M (amount injected was 0.25 µg).

Table 1
Influence of mobile phase pH on the chromatographic parameters of separation of compound **1** and cytosine at pH 1.22 and 100°C

X	Capacity factor		Resolution	Peak symmetry	
	Cytosine	**1**	Cytosine/**1**	Cytosine	**1**
1.5	1.5	2.2	3.5	1.1	1.3
2.0	1.4	2.2	5.0	1.2	1.2
2.5	1.2	2.2	6.2	1.2	1.1
3.0	1.1	2.2	7.5	1.2	1.2

Mobile phase: tetrahydrofuran–sodium octanesulphonate (pH X; 0.02 M)–potassium phosphate buffer (pH X; 0.2 M)–water (1.5:15:45:38.5, v/v). Flow: 1 ml min^{-1}. Column: PLRP-S 100 Å 8 µm. Column temperature: 60°C. Detection: UV at 270 nm.

3. Results and discussion

The LC method was developed using a sample of compound **1** which had been partially degraded at 100°C and at pH 1.22. The main emphasis in optimizing the method was on obtaining good resolution between compound **1** and its degradation products (mainly cytosine) and good peak symmetry of **1**, because kinetic calculations were based on the peak areas of **1**–**6**. During preliminary studies, various columns were investigated using a mobile phase containing tetrahydrofuran, sodium octanesulphonate (pH 2.5; 0.02 M) and potassium phosphate buffer (pH 2.5; 0.2 M). The PLRP-S 100 Å 8 µm column was chosen for further work because it was the only column that resolved compound **1** from both cytosine and from its second degradation product, while the other columns only separated **1** from cytosine. Besides, the RSil C18 LL 5 µm column gave

Table 2
Influence of concentration of ion-pairing agent on the chromatographic parameters of the separation of compound **1** and its degradation products at pH 1.22 and 100°C

X	Capacity factor			Resolution		Peak symmetry	
	Cytosine	**DP2**	**1**	Cytosine/**DP2**	**DP2**/**1**	Cytosine	**1**
10	1.0	a	1.8	a	a	1.3	1.4
15	1.2	a	2.2	a	a	1.2	1.1
20	1.5	2.3	2.6	4.2	1.2	1.4	b
25	1.6	2.2	2.8	3.1	2.6	1.3	1.0
30	1.9	2.2	3.1	2.0	3.9	b	1.1

Mobile phase: tetrahydrofuran–sodium octanesulphonate (pH 2.5; 0.02 M)–potassium phosphate buffer (pH 2.5; 0.2 M)–water (1.5:X:45:53.5$-X$, v/v). Flow: 1 ml min^{-1}. Column: PLRP-S 100 Å 8 µm. Column temperature: 60°C. Detection: UV at 270 nm.

DP2, degradation product 2.
a, No separation of **DP2** from **1**.
b, No separation from **DP2** at 0.05 of peak height.

broad peaks, the Rogel 80 Å 8 µm column showed a lot of fronting, while the PLRP-S 1000 Å 8 µm column had very poor retention of the compounds. Only the PRP-1 7–9 µm column gave peaks that were as sharp and symmetrical as the PLRP-S 100 Å 8 µm one. Kinetic studies were carried out over a long time and so a polymer column also offered the advantage of long life as compared to silica-based reversed-phase columns. Previous studies on nucleosides showed that there was an overall decrease in retention, but no change in selectivity when temperature was increased [11] and therefore column temperature was not investigated. However, the column was maintained at 60°C by means of a water-bath to increase efficiency and to reduce back-pressure. An ion-pairing agent (sodium octanesulphonate) was added to the mobile phase because compounds **1**–**6** were too hydrophilic and were eluted quickly even with low amounts of organic modifier. An acidic buffer was used to ensure protonation of the cytosine base so that the compounds could interact with the ion-pairing agent. The mobile phase was optimized by systematic investigation of the influence of pH, concentration of the ion-pairing agent, concentration of the

Table 3
Influence of concentration of buffer on the chromatographic parameters of the separation of compound **1** and its degradation products at pH 1.22 and 100°C

X	Capacity factor			Resolution		Peak symmetry	
	Cytosine	**DP2**	**1**	Cytosine/**DP2**	**DP2**/**1**	Cytosine	**1**
40	1.9	2.2	3.1	1.5	4.1	a	1.0
45	1.9	2.3	3.2	2.2	3.7	1.3	1.0
50	1.7	2.2	2.9	2.9	3.0	1.2	1.0
55	1.7	2.2	2.8	3.4	2.5	1.2	1.0
60	1.6	2.2	2.6	3.8	2.0	1.2	1.1

Mobile phase: tetrahydrofuran–sodium octanesulphonate (pH 2.5; 0.02 M)–potassium phosphate buffer (pH 2.5; 0.2 M)–water (1.5:25:X:73.5$-X$, v/v). Flow: 1 ml min^{-1}. Column: PLRP-S 100 Å 8 µm. Column temperature: 60°C. Detection: UV at 270 nm.

DP2, degradation product 2.
a, No separation from **DP2** at 0.05 of peak height.

Table 4
Influence of concentration of tetrahydrofuran on the chromatographic parameters of the separation of compound **1** and its degradation products at pH 1.22 and 100°C

X	Capacity factor			Resolution		Peak symmetry	
	Cytosine	**DP2**	**1**	Cytosine/**DP2**	**DP2**/**1**	Cytosine	**1**
0.8	2.4	2.9	4.9	2.0	6.3	1.3	1.2
1.0	2.2	2.6	4.1	2.2	5.1	1.2	1.1
1.5	1.7	2.2	2.9	2.9	3.0	1.2	1.0

Mobile phase: tetrahydrofuran–sodium octanesulphonate (pH 2.5; 0.02 M)–potassium phosphate buffer (pH 2.5; 0.2 M)–water (X:25:50:25 − X, v/v). Flow: 1 ml min^{-1}. Column: PLRP-S 100 Å 8 μm. Column temperature: 60°C. Detection: UV at 270 nm. **DP2**, degradation product 2.

buffer and the type and concentration of the organic modifier.

The pK_a of **1** is expected to be similar to that of cytidine, which is 4.2 [12]. The capacity factor (k') of compound **1** is constant between pH 1.5 and 3 (Table 1) presumably because the compound is protonated in this pH region. All chromatographic parameters were calculated using equations from the European Pharmacopoeia [13]. As the pH decreases, the capacity factor of cytosine, the main degradation product of **1** increases. This is unexpected because the pK_a of cytosine is 4.7 [6] and so it should be protonated in this region. pH 2.5 was chosen for further studies because it gave good resolution and the best peak symmetry of compound **1**.

Increasing the concentration of sodium octanesulphonate allowed separation of a second degradation product (**DP2**) which was produced in much smaller quantities (about 3% of total chromatographic area) than cytosine and could not be identified. The ion-pairing agent had a significant effect on the retention of **1** and cytosine, but not on that of **DP2**. The peak symmetry of **DP2** could not be calculated because the peak was not separated from cytosine at 0.05 of the peak height. The best resolution and peak symmetry for compound **1**, cytosine and **DP2** were obtained using 25% of sodium octanesulphonate (pH 2.5; 0.02 M) (Table 2).

In preliminary studies it was noticed that samples of **1** which had been degraded using buffers

Table 5
Chromatographic parameters of compounds **1**–**6**, cytidine and 2′-deoxycytidine partially degraded at pH 11.99 and 100°C

Compound	Capacity factor				Resolution	Peak symmetry
	Degradation Products			Nucleoside		Nucleoside
1[a]	0.4[c]	0.8[d]	1.7[e]	3.2	3.6	1.1
2[a]	0.4[c]	0.8[d]	1.7[e]	3.5	4.0	1.1
3[a]	0.4[c]	0.8[d]	1.7[e]	4.0	4.8	1.2
4[a]	0.4[c]	0.8[d]	1.7[e]	3.0	3.3	1.1
5[b]	0.3[c]	0.7[e]	1.6[d]	3.4	4.5	1.1
6[b]	0.3[c]	0.7[e]	1.8[d]	3.9	4.4	1.0
Cytidine[a]	0.4[f]	—	—	2.0	8.7	1.0
2′-Deoxycytidine[a]	0.5[g]	—	—	2.9	8.6	1.2

Mobile phase: acetonitrile–sodium octanesulphonate (pH 2.5; 0.02 M)–potassium phosphate buffer (pH 2.5; 0.2 M)–water (X:25:50:25 − X, v/v, where X = 5 and 10 for a and b, respectively). Flow: 1 ml min^{-1}. Column: PLRP-S 100 Å 8 μm. Column temperature: 60°C. Detection: UV at 270 nm.

c, uracil; d, the deamination product (different for each nucleoside); e, cytosine; f, uridine; g, 2′-deoxyuridine. Resolution refers to that of nucleoside and nearest degradation product.

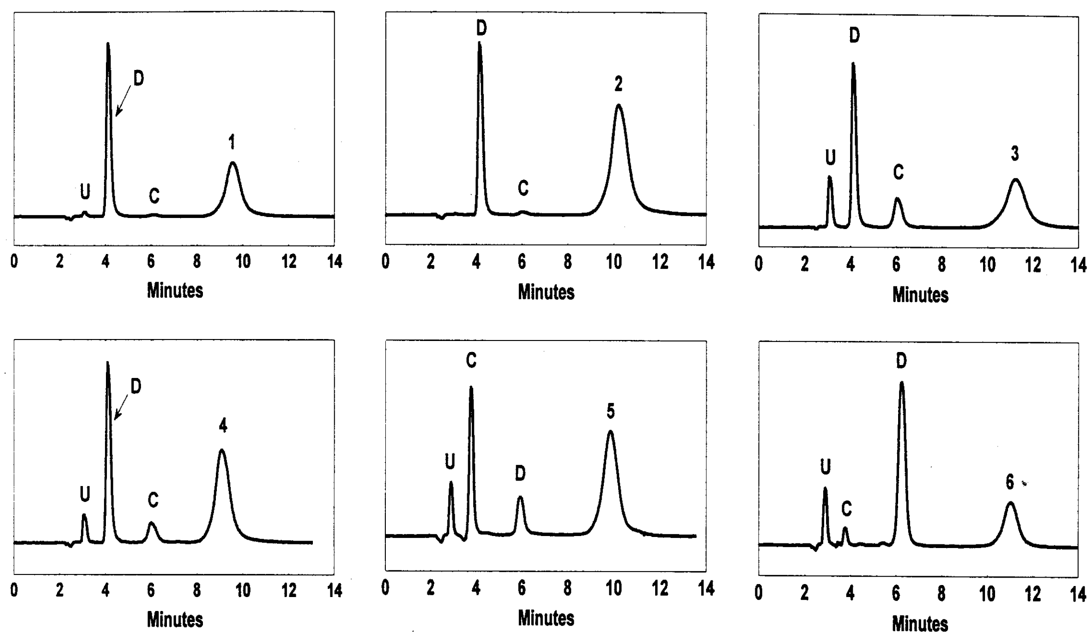

Fig. 2. Liquid chromatograms of partially degraded samples of **1**–**6** at pH 11.99. Mobile phase: acetonitrile–sodium octanesulphonate (pH 2.5, 0.02 M)–potassium phosphate buffer (pH 2.5, 0.2 M)–water (X:25:50:25 − X, v/v, where X is 5 for **1**–**4** and 10 for **5** and **6**). Flow: 1 ml/min. Column: PLRP-S 100 Å 8 μm. Column temperature: 60°C. Detection: UV at 270 nm. C, cytosine; D, deamination product (different for each nucleoside); U, uracil.

of acid and basic pH, both of which had been neutralized to pH 7, had different retention times (11.1 and 8.2 min) using a mobile phase consisting of tetrahydrofuran–sodium octanesulphonate (pH 3.0; 0.02 M)–potassium phosphate buffer (pH 3.0; 0.2 M)–water (1.5:15:5:78.5, v/v). The difference in retention was due to insufficient buffering capacity and was observed only when this mobile phase contained less than 40% buffer. During optimization of the method, use of 50% of potassium phosphate buffer (pH 2.5; 0.2 M) gave sufficient buffering, good resolution and the best peak symmetries of compound **1** and **DP1** (Table 3).

Finally, different concentrations of different organic modifiers were investigated. Tetrahydrofuran was initially used because it gave sharp and symmetric peaks in previous work on aminated nucleosides [11]. Decreasing the amount of tetrahydrofuran improved resolution between **1** and **DP2**, but not that between cytosine and **DP2** (Table 4). Peaks were more asymmetric when methanol was used instead of tetrahydrofuran. Acetonitrile improved the resolution between **DP2** and both cytosine and **1** and its concentration was adjusted to suit each compound in the final mobile phase (able 5). The best LC system for analysis of **1**–**6** consisted of acetonitrile–sodium octanesulphonate (pH 2.5; 0.02 M)–potassium phosphate buffer (pH 2.5; 0.2 M)–water (X:25:50:25 − X, v/v, where X is variable).

The system developed was also suitable for analysis of partially degraded samples of cytidine and 2′-deoxycytidine, resolving them from their acid degradation products, cytosine and uridine for the former and cytosine alone for the latter. For analysis of a sample of cytidine degraded at pH 1.22, the amount of acetonitrile was reduced to 1% in order to resolve cytosine from cytidine. At alkaline pH, the degradation products of compounds **1**–**6** were uracil, cytosine and the respective deamination products (which have uracil in place of cytosine base). The alkaline degradation products of cytidine and 2′-deoxycytidine were

Table 6
Regression relationships for the linearity of detector response for compounds **1**–**6**

Compound	y	R	Standard error of y estimate
1	$(5.05\times10^{10})x-6983$	0.9992	39 723
2	$(6.94\times10^{10})x-11\,905$	0.9997	36 627
3	$(6.79\times10^{10})x-37\,591$	0.9996	36 467
4	$(7.63\times10^{10})x-23\,942$	0.9997	36 111
5	$(5.36\times10^{10})x-1818$	0.9998	22 744
6	$(5.18\times10^{10})x+19\,317$	0.9987	54 470

y, peak area; x, concentration injected in M.

uridine and 2′-deoxyuridine, respectively. All these nucleosides were well resolved from their degradation products. Table 5 gives some chromatographic parameters of partially degraded samples of **1**–**6**, cytidine and 2′-deoxycytidine at pH 11.99 using the above described system and ig. 2 shows typical chromatograms.

Cytosine, cytidine and 2′-deoxycytidine elute slower than uracil, uridine and 2′-deoxyuridine, respectively, because the cytosine compounds are ion-paired at pH 2.5 while the uracil ones are not. Uridine is well resolved from 2′-deoxyuridine by use of 1% acetonitrile in the mobile phase, but uracil and uridine are not well resolved. Cytidine is the most hydrophilic (three sugar –OH groups) of these nucleosides and it was eluted fastest, while the least hydrophilic nucleosides (**5** and **6**) were the slowest and they required higher amounts of acetonitrile. 2′-Deoxycytidine is eluted faster than **1**–**4**, implying that pentofuranosyl nucleosides elute faster than corresponding hexopyranosyl ones containing the same number of sugar –OH groups. Cytosine elutes slower than the deamination products of **1**–**4**, but faster than those of **5** and **6**, showing that the number of sugar hydroxyl groups have a big influence on retention. The compounds show no relationship between the type of anomer and retention (**3** vs. **4** and **5** vs. **6**). The mobile phase developed can be used for analysis of similar nucleosides, with or without minor modifications. It can also be used as a model to predict retention of other nucleosides based on the type of sugar (hexose or pentose), the number of sugar hydroxyl groups and the basicity of the base moiety.

The linearity of detector response was tested at 270 nm for compounds **1**–**6** using the following concentrations: 5×10^{-6} M, 10^{-5} M, 2×10^{-5} M, 3×10^{-5} M, 4×10^{-5} M, 5×10^{-5} M and 6×10^{-5} M (21 data points). Table 6 shows the regression relationships obtained. These results also indicated the good repeatability of the method (for compound **1**: R.S.D. = 0.4%; $n=3$, concentration 6×10^{-5} M), the limit of detection was 5 ng (concentration 10^{-6} M) at a signal to noise ratio of 3 and the limit of quantitation was 25 ng (R.S.D. = 11%; $n=5$; concentration 5×10^{-6} M).

References

[1] I. Verheggen, A. Van Aerschot, S. Toppet, R. Snoeck, G. Janssen, J. Balzarini, E. De Clercq, P. Herdewijn, J. Med. Chem. 36 (1993) 2033–2040.
[2] K. Augustyns, J. Rozenski, A. Van Aerschot, G. Janssen, P. Herdewijn, J. Org. Chem. 58 (1993) 2977–2982.
[3] P. Herdewijn, A. Van Aerschot, J. Balzarini, E. De Clercq, Nucleosides Nucleotides 10 (1991) 119–127.
[4] P. Herdewijn, A. Van Aerschot, Bull. Soc. Chim. Belg. 99 (1990) 895–901.
[5] P.R. Brown, A.M. Krstulovic, R.A. Hartwick, in: J.C. Giddings, E. Grushka, J. Cazes, P.R. Brown (Eds.), Advances in Chromatography, vol. 18, Marcel Dekker, New York, 1980, pp. 101–138.
[6] J.R. Miksic, in: P.R. Brown (Ed.), HPLC in Nucleic Acid Research, Methods and Applications, Chromatographic Science Series, vol. 28, Marcel Dekker, New York, 1984, pp. 317–338.
[7] N. Bleyzac, R. Boulieu, J. Chromatogr. B 658 (1994) 173–176.
[8] F. Albertioni, B. Pettersson, V. Reichelová, G. Juliusson, J. Liliemark, Ther. Drug Monit. 16 (1994) 413–418.
[9] D.B. Whigan, A.E. Schuster, J. Chromatogr. B 664 (1995) 357–363.

[10] A. Werner, J. Chromatogr. 618 (1993) 3–14.
[11] G. Thoithi, A. Van Schepdael, P. Herdewijn, E. Roets, J. Hoogmartens, J. Chromatogr. A 689 (1995) 247–254.
[12] J. Clauwaert, J. Stockx, Z. Naturforsch. 23B (1968) 25–30.
[13] European Pharmacopoeia, Maisonneuve, Sainte-Ruffine, 2nd ed., 1987, V.6.20.4.