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Liquid chromatographic separation of phosphoramidate diastereomers on a polysaccharide-type chiral stationary phase

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Abstract

To improve the therapeutic potential of anti-HIV nucleoside analogues (d4T, AZT, 3TC and ddI), the delivery of the corresponding monophosphate from neutral, membrane-permeable prodrugs has been realised by the synthesis of lipophilic phosphoramidate triester prodrugs, such as the simple phenyl-L-alaninephosphate derivatives. However, the present non-stereoselective synthesis results in a mixture of 1:1 diastereomers, which differ from the configuration of the phosphorus atom asymmetric center. Since each diastereomer may have different biological activity and pharmacokinetic profile, analytical methods have to be developed for their separation. This work aims at showing the ability of a polysaccharide-type chiral stationary phase to resolve such diastereomers in reversed-phase high-performance liquid chromatography. The influence of operating parameters has been studied to optimise the separation; a thermodynamic approach has also been investigated to gain an insight in the retention mechanism of the prodrugs. Preliminary validation study (linearity, accuracy, repeatability) has yielded good results; in addition, the feasibility of HPLC–electrospray-mass spectrometry (HPLC–ESI-MS) coupling has been demonstrated and it is expected that this will lead to lower detection limits.
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Keywords: Chiral stationary phases, LC; Enantiomer separation; Phosphoramidates; Polysaccharides

1. Introduction

Several nucleoside analogues, particularly those with a 2′,3′-dideoxyribose moiety, have been approved in HIV therapy [1]. To be pharmacologically active, most antiviral nucleoside analogues [3′-azido-3′-deoxythymidine (AZT), 2′,3′-didehydro-3′-deoxythymidine (stavudine, d4T), L-2′,3′-dideoxy-3′-thiacytidine (lamivudine, 3TC) or 2′,3′-dideoxyinosine (didanosine, ddI)] must be phosphorylated to their 5′-triphosphate counterparts by cellular kinases [2–5]. In most cases, the first activation step (catalysed by nucleoside kinases), is the rate-limiting step; thus, to circumvent this poor metabolisation and deliver the monophosphate form directly into the target cells, a variety of masked nucleoside monophosphate analogues has been synthesised and reviewed [6,7], e.g. aryloxy phosphoramidates [8–10], substituted aryloxy phosphate heterocycles [11] and β-substituted dialkyl phosphates [12,13]. Among them, the lipophilic phosphoramidate triester prodrugs, such as the simple phenyl-L-alaninephosphate

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derivatives, which have been at the cornerstone of the pronucleotide or prodrug research field, exhibited marked antiviral activity at nontoxic concentrations [14]. Furthermore, the anti-HIV activity was fully retained in some kinase-deficient cells, suggesting that the phosphotriester prodrug efficiently released the phosphorylated metabolite into the infected HIV-cells. The probable mechanism of activation of those prodrugs first involves an esterase mediated hydrolysis of the carboxy ester, which could be a stereoselective mechanism [15]. Nucleosides are optically pure compounds; however, owing to a non stereoselective synthesis, introduction of a phosphorus asymmetric center yields a 1:1 mixture of diastereomers. Therefore, this chirality at the phosphorus atom (*R* or *S*) may induce some variations in terms of antiviral potency and pharmacokinetic profile, as reported for the prodrug of the anti-HIV approved 2-phosphonomethoxypropyl adenine (PMPA prodrug, GS-7340, Fig. 1), in which only one of the diastereomers (found to have a $P_{(S)}$ configuration), exhibited antiviral activity [16,17]. Therefore, efficient analytical methods for diastereomeric separation have to be developed, before considering the transposition to the preparative scale. In some cases, the resolution of the 1:1 diastereomeric mixtures resulting from the chirality at the phosphorus center, can be achieved by reversed-phase HPLC. However, several phosphoramidate derivatives have already been reported to be unresolved by this method [18]. To cope with this, the molecular imprinting approach has been investigated in HPLC, using a stationary phase imprinted with a single anti-HIV prodrug diastereomer; nevertheless, poor efficiency was observed. Thus, as part of our ongoing synthesis and bioanalytical research on anti-HIV agents [19–21], this paper reports the diastereomeric separation of phosphoramidate prodrugs of d4T, AZT, ddA and 3TC (Fig. 1), by HPLC on a polysaccharide-type chiral stationary phase (CSP). After a preliminary HPLC study on a C_{18} column which led to insufficient separation of the studied prodrugs, the influence of various operating parameters has been studied to optimise the HPLC separation on the CSP, before quantification and partial validation of the method. The feasibility of HPLC–ESI-MS coupling is also shown in this paper.

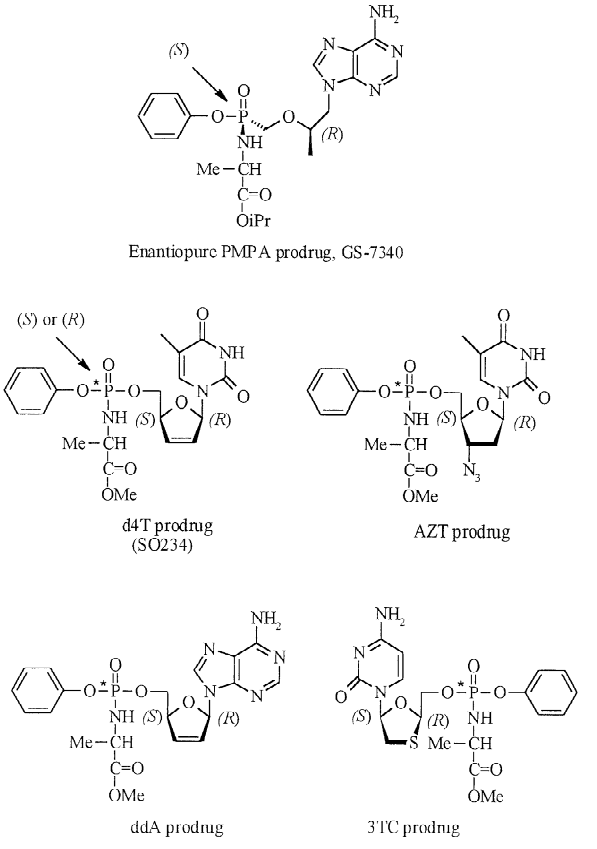

Fig. 1. Structure of the phosphoramidate triester anti-HIV pronucleotides.

2. Experimental

2.1. Apparatus

HPLC experiments were carried out on a Thermo Separation Products (Les Ulis, France) Model Spectra Series P-4000 quaternary pump, equipped with an on-line degasser, and a Rheodyne (Cotati, CA, USA) Model 7125 injection valve fitted with 20-μl loop. UV detection was carried out at 254 nm with a Kratos (Applied Biosystems, Courtaboeuf, France) Spectroflow 783 UV spectrophotometric detector and data were computer analyzed using EZChrom Elite software (version 2.5).

Two analytical columns were used in this work: a Nucleosil® C_{18} (Shandon, Eragny, France) column (100×2.1 mm I.D., particle size 3 μm), and a Chiralcel® OD-RH [cellulose tris(3,5-dimethyl-

phenylcarbamate)] (Chiral Technologies, Illkirch, France) column (150×4.6 mm I.D., particle size 5 μm). Typical flow-rates of 0.2 and 0.5 ml/min were applied, respectively. Temperature was regulated and controlled by a Gecko-cil (Cluzeau, France) oven.

HPLC–ESI-MS experiments were carried out using a Perkin-Elmer (Toronto, Canada) API 300 triple quadrupole mass spectrometer with IonSpray as ion source. The mass spectrometer was operated in the positive ion mode, and nitrogen was used as curtain and nebuliser gas. A secondary pump delivered a make-up (MeOH/water, 95:5+0.5% HCOOH) at a 0.5 ml/min flow-rate, to promote the formation of positive ions [22]. Junction with the HPLC mobile phase was ensured by a post-column mixing tee. A 1:10 split was used to avoid too high a flow-rate into the ion source. After optimisation, ionspray, orifice and ring voltages were set at 5.8 kV, 25 V and 250 V, respectively. A 1 s dwell-time was used for MS acquisition in selected ion reaction (SIR) mode.

2.2. Chemicals

Acetonitrile and methanol of HPLC quality were obtained from J.T. Baker (Noisy le Sec, France); 18 MΩ deionised water was prepared using an Elgastat UHQ II system (Elga, Antony, France). Formic acid (98%) was purchased from Sigma–Aldrich–Fluka (St Quentin Fallavier, France). Phosphoramidate derivatives of AZT, ddA and 3TC were synthesised according to the literature [23]; d4T prodrug (SO324) was a kind gift from Pr. McGuigan. All prodrug stock solutions (1 mg/ml) were prepared in methanol before aqueous dilution to the working concentration (50 μg/ml).

3. Results and discussion

3.1. Preliminary study by reversed-phase HPLC

Preliminary experiments were carried out on a conventional C_{18}-bonded stationary phase, although attempts to resolve some diastereomeric phosphoramidate derivatives were already reported to be generally unsuccessful [18]. The operating parameters (mobile phase composition, temperature) were optimised in order to achieve the best separation of the phosphoramidate prodrug diastereomers. However, as illustrated in Fig. 2 for d4T and AZT prodrugs, only partial resolution was obtained under these conditions (the same results were observed for 3TC and ddA prodrugs), in spite of using a small particle size (3 μm). Therefore, since hydrophobic interactions were insufficient to separate the phosphoramidate diastereomers, the use of a chiral cellulose-based stationary phase was investigated to obtain faster and enhanced separations.

3.2. HPLC on a polysaccharide-type stationary phase

Among the different existing CSP, polysaccharide-based (cellulose, amylose) stationary phases have proved to be very efficient materials in HPLC (in normal and reversed-mode) for the resolution of either chiral drugs or diastereomers [24,25]. The discrimination power of these polysaccharide phases stems from complex interactions (which have not been fully elucidated yet) with the solutes. In summary, a combination of hydrophobic interactions, attractive forces (e.g. hydrogen bonding), dipole–dipole interactions and charge transfer (π–π) formation are believed to explain the molecule recognition process [25,26].

HPLC enantioseparations of organophosphorus compounds with a chiral phosphorus atom have already been reported on such polysaccharide CSP [27], and were carried out in normal-phase mode (unsuitable for MS coupling). Since these CSP demonstrate interesting selectivities for organophosphorus compounds, the diastereomeric resolution of the pronucleotides can be reasonably conceived in reversed-phase HPLC.

3.2.1. Effect of acetonitrile percentage of the mobile phase

The influence of the mobile phase composition (water/acetonitrile) on the prodrug diastereomeric separations was investigated at room temperature. For each pair of diastereomers, the effect on retention factor (k) and separation factor (α), efficiencies (N) and resolution (R_s) is reported in Table 1.

A typical reversed-phase behaviour is observed for

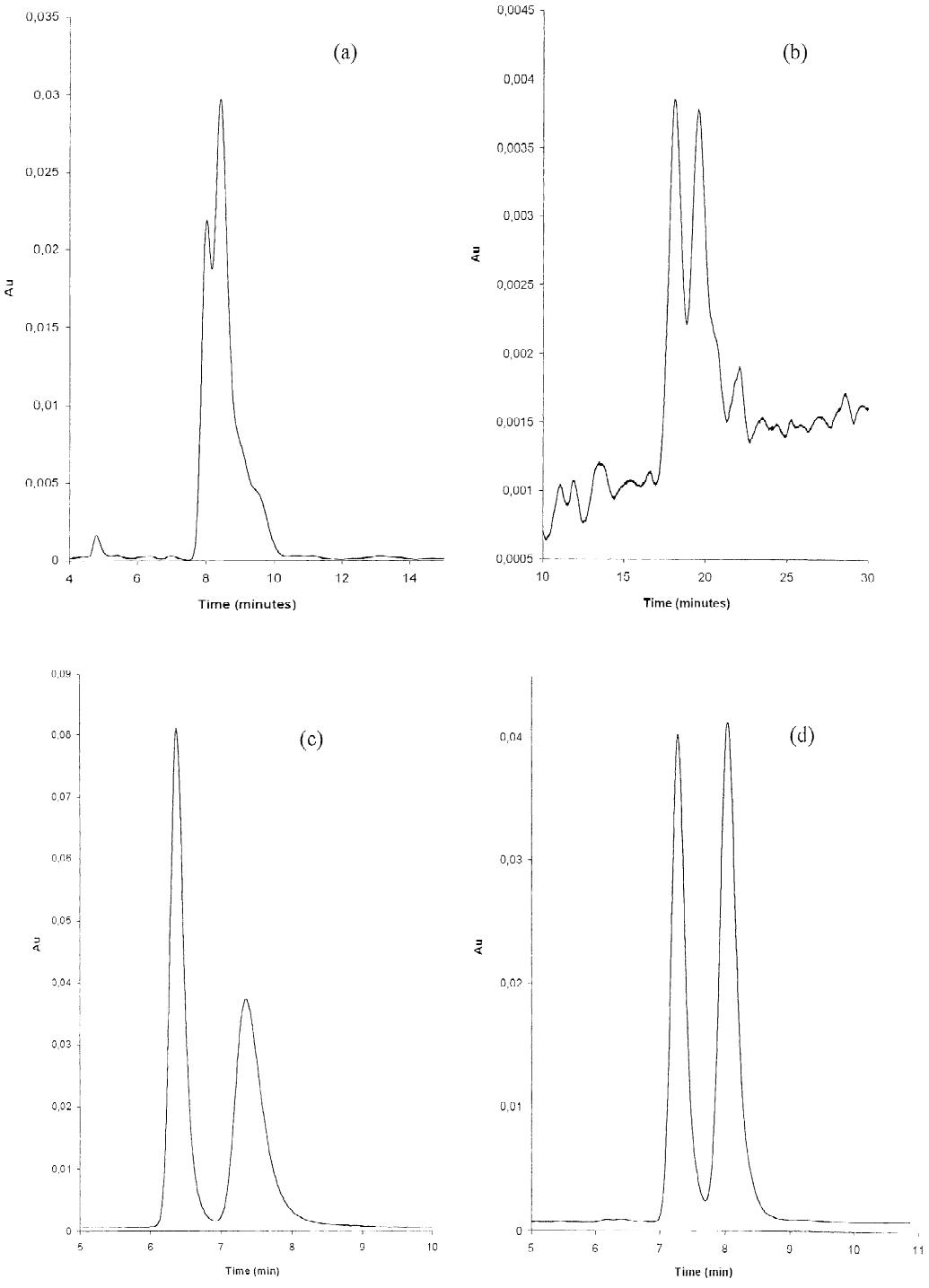

Fig. 2. Comparative reversed-phase HPLC–UV (254 nm) of the studied phosphoramidates: Nucleosil C_{18}: (a) d4T prodrug (MeCN/water 23:77, v/v) and (b) AZT prodrug (MeCN/water 23:77, v/v). Chiralcel OD-RH: (c) d4T prodrug (MeCN/water 30:70, v/v) and (b) AZT prodrug (MeCN/water 40:60, v/v).

Table 1
Influence of the acetonitrile content (%) of the mobile phase (MeCN/water) on the retention (k), separation factor (α), efficiency (N) and resolution (R_s) of the prodrug diastereomers

Compound	Eluent	k_1	k_2	α	N_1	N_2	R_s
d4T prodrug	A	5.61	7.50	1.34	2164	1437	2.79
	B	1.16	1.52	1.31	4155	2115	2.04
	C	0.38	0.50	1.31	4620	2665	1.19
AZT prodrug	A	27.68	34.12	1.23	2557	2037	2.81
	B	4.00	4.91	1.23	4351	3596	2.59
	C	1.00	1.22	1.22	4869	4279	1.22
3TC prodrug	A	3.25	4.24	1.31	1389	1344	1.93
	B	0.83	1.02	1.22	1933	1657	1.02
	C	0.22	0.27	1.22	2570	2123	0.27
ddA prodrug	A	2.81	3.15	1.12	2193	2860	1.08
	B	0.58	0.63	1.07	2234	2916	0.50
	C	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.

Conditions: Chiralcel OD-RH column (150×4.6 mm, 5 μm), 25 °C, 254 nm. Eluent: A: MeCN/water 20:80 (v/v); B: MeCN/water 30:70 (v/v); C: MeCN/water 40:60 (v/v); n.r., non resolved.

all compounds: an increase in the organic modifier amount results in a decrease in retention (k) and separation (α) factors. This can be explained by a decrease in hydrophobic interactions of the solutes with the stationary phase, as well as π–π competing interactions of the solvent towards the substituted phenyl moieties of the CSP. The diastereomeric resolution of the prodrugs occurs owing to a difference in the magnitude of interactions of each diastereomer with the CSP. In brief, each diastereomer fits stereogenically in a different way into the chiral grooves of the stationary phase, and develops π–π interactions of different extent with the CSP [28,29]. As a result, Fig. 2 illustrates the diastereomeric separation of d4T and AZT prodrugs, obtained after optimisation of the mobile phase composition.

3.2.2. Influence of the temperature

Table 2 reports the influence of temperature on the different parameters (retention and separation factors,

Table 2
Effect of temperature on the retention (k), separation factor (α), efficiency (N) and resolution (R_s) of the prodrug diastereomers

Compound	Temperature (°C)	k_1	k_2	α	N_1	N_2	R_s
d4T prodrug	25	1.06	1.37	1.30	4363	2507	2.00
	30	1.02	1.29	1.27	4750	3049	1.94
	35	0.98	1.22	1.24	5521	4033	1.88
	40	0.95	1.16	1.22	5965	4836	1.82
AZT prodrug	25	3.71	4.55	1.22	3488	3614	2.48
	30	3.45	4.15	1.20	4564	3911	2.36
	35	3.19	3.78	1.18	5414	4497	2.20
	40	2.99	3.49	1.16	5609	5099	2.11
3TC prodrug	25	3.98	5.13	1.29	1308	1299	1.77
	30	3.56	4.48	1.26	1657	1320	1.73
	35	3.29	4.08	1.24	1867	1494	1.71
	40	2.89	3.51	1.22	2071	1694	1.61
ddA prodrug	25	2.80	3.15	1.12	1837	1606	0.96
	30	2.71	3.03	1.12	1925	1762	0.88
	35	2.58	2.86	1.11	2128	1900	0.85
	40	2.52	2.79	1.10	2580	2278	0.91

Conditions: Chiralcel OD-RH column (150×4.6 mm, 5 μm); UV detection: 254 nm. Mobile phase: MeCN/water: 30:70 (v/v) (d4T prodrug), 40:60 (v/v) (AZT prodrug), 20:80 (v/v) (ddA and 3TC prodrugs).

efficiencies, resolution). An increase in temperature leads to a general decrease in the retention factors and resolution, and to a slight increase in efficiencies. Therefore, 25 °C temperature seems suitable to perform good diastereomeric separations.

To gain an insight in the retention mechanism of the prodrugs, the thermodynamic Van't Hoff relationship was studied by plotting ln *k*-values versus $1/T$. Indeed, in HPLC, the partition coefficient (K) of a solute decreases when increasing the temperature according to [30]:

$$\ln K = -\Delta G^\circ/RT = -\Delta H^\circ/RT + \Delta S^\circ/R \quad (1)$$

where ΔG° is the Gibbs free energy for the solute-stationary phase interaction, R the gas constant (8.314 J mol^{-1} K^{-1}), ΔH° and ΔS°, the enthalpy and entropy of transfer of the solute from the mobile to the stationary phase.

The distribution constant K is directly linked to the retention factor k, following:

$$k = \Phi \times K \quad (2)$$

with Φ, the stationary to mobile phase volume ratio.

Therefore, expression (1) becomes:

$$\ln k = -\Delta H^\circ/RT + \Delta S^\circ/R + \ln \Phi \quad (3)$$

Experimentally, Van't Hoff plots were made for each diastereomer of the four prodrugs, varying temperature from 30 to 40 °C (e.g. from 303 to 313 K). A good linear correlation was observed over all the studied range ($r > 0.98$), indicating that the retention mechanism is independent of a change in temperature in the studied range (Table 3), and that the CSP does not undergo a change in conformation [31]. From these linear plots, the standard enthalpy ΔH° and entropy ΔS° were calculated, using the slope ($-\Delta H^\circ/R$) and the intercept ($\Delta S^\circ/R + \ln \Phi$). The enthalpy variations are related to the transfer of the solutes from the mobile to the stationary phase: the smaller the ΔH° (negative values), the more efficient is this transfer. The negative values of entropies reflect a slight increase in order when the solutes are transferred from the mobile to the stationary phase. As a result, according to ΔG° negative values, the solute transfer is enthalpically driven ($\Delta H^\circ > T\Delta S^\circ$) in this temperature range and can be described as a replacement of weak solute–solvent interactions by strong solute–stationary phase interactions [32].

4. Preliminary validation study

4.1. Linearity, limit of detection (LOD) and limit of quantification (LOQ)

For each phosphoramidate, linearity was assessed using five standard solutions (each injected in triplicate) in a 10 to 50 μg/ml concentration range. The five-point calibration curves were found to be linear (Table 4) since least squares regression gave good correlation coefficients ($r > 0.9998$) for each diastereomer; relative standard deviations (RSD) on the slope ranged from 0.2 to 1.0%. An extreme RSD value on the intercept was observed for ddA prodrug, indicating that the intercept is significantly different from the origin, from a statistical viewpoint, and that

Table 3
Thermodynamic results and correlation coefficients of Van't Hoff plots for each prodrug diastereomer. Same conditions as in Table 2

Compound		ΔH° (J mol^{-1})	ΔS° (J mol^{-1})	ΔG° (J mol^{-1})[a]	r
d4T prodrug	Diastereomer 1	−5505	−7.3	−3328	0.999
	Diastereomer 2	−8969	−16.7	−3977	0.999
AZT prodrug	Diastereomer 1	−11 274	−16.2	−6443	0.999
	Diastereomer 2	−13 872	−23.2	−6945	0.999
3TC prodrug	Diastereomer 1	−16 600	−33.5	−6612	0.988
	Diastereomer 2	−19 112	−39.8	−7246	0.990
ddA prodrug	Diastereomer 1	−5629	−0.4	−5516	0.977
	Diastereomer 2	−6483	−1.5	−6032	0.975

[a] $T = 298$ K.

Table 4
Results of linearity studies for each prodrug diastereomer

Compound		Slope	RSD (%)	Intercept	RSD (%)	*r*
d4T prodrug	Diastereomer 1	20 122	0.4	−29 320	9.7	0.9999
	Diastereomer 2	18 095	0.7	−33 422	12.6	0.9999
AZT prodrug	Diastereomer 1	16 004	0.3	−28 217	5.3	0.9999
	Diastereomer 2	19 351	0.2	−31 860	3.1	0.9999
3TC prodrug	Diastereomer 1	15 386	0.8	−72 996	5.4	0.9999
	Diastereomer 2	17 168	1.0	−84 607	6.9	0.9998
ddA prodrug	Diastereomer 1	25 430	0.8	7568.9	88.2	0.9999
	Diastereomer 2	16 945	0.6	−2809.1	125.2	0.9999

Conditions: Chiralcel OD-RH column (150×4.6 mm, 5 μm); 25 °C, UV detection: 254 nm. Mobile phase: MeCN/water: 30:70 (v/v) (d4T prodrug), 40:60 (v/v) (AZT prodrug), 20:80 (v/v) (ddA and 3TC prodrugs).

the calibration curve cannot be forced to the origin. However, those results may stem from the partial resolution of this compound, whose separation could be improved using a longer column.

Limits of detection (LOD) and quantification (LOQ) were defined, respectively, as signal-to-noise ratio equal to 3 and 10; LOQ lower than 6 μg/ml and LOD beyond 1.8 μg/ml were achieved by UV detection and experimentally checked.

4.2. Accuracy

Standard solutions ($n=5$) from 10 to 50 μg/ml were injected in triplicate to assess the accuracy of

Table 5
Results of recovery studies for each phosphoramidate diastereomer

Compound	Concentration (μg/ml)	Diastereomer 1		Diastereomer 2	
		Average recovery (%)	Confidence interval	Average recovery (%)	Confidence interval
d4T prodrug	10	99.35	1.67	101.30	3.07
	20	100.64	1.41	99.65	1.64
	30	99.59	1.94	99.11	1.91
	40	100.31	2.09	100.58	0.86
	50	99.87	2.22	99.96	2.10
AZT prodrug	10	100.53	3.33	100.08	3.72
	20	99.40	2.51	100.19	1.34
	30	100.27	1.15	99.80	2.17
	40	99.96	1.86	99.94	0.37
	50	100.00	3.08	100.08	2.87
3TC prodrug	10	98.75	1.00	98.92	0.52
	20	101.53	0.67	99.98	1.31
	30	99.66	2.06	101.35	2.01
	40	99.47	3.66	99.09	1.75
	50	100.27	2.12	100.14	2.04
ddA prodrug	10	101.86	1.11	101.23	2.45
	20	98.81	1.02	99.44	4.02
	30	99.51	0.58	100.13	1.19
	40	100.67	0.84	99.42	2.20
	50	99.87	0.92	100.37	3.21

Same conditions as in Table 4.

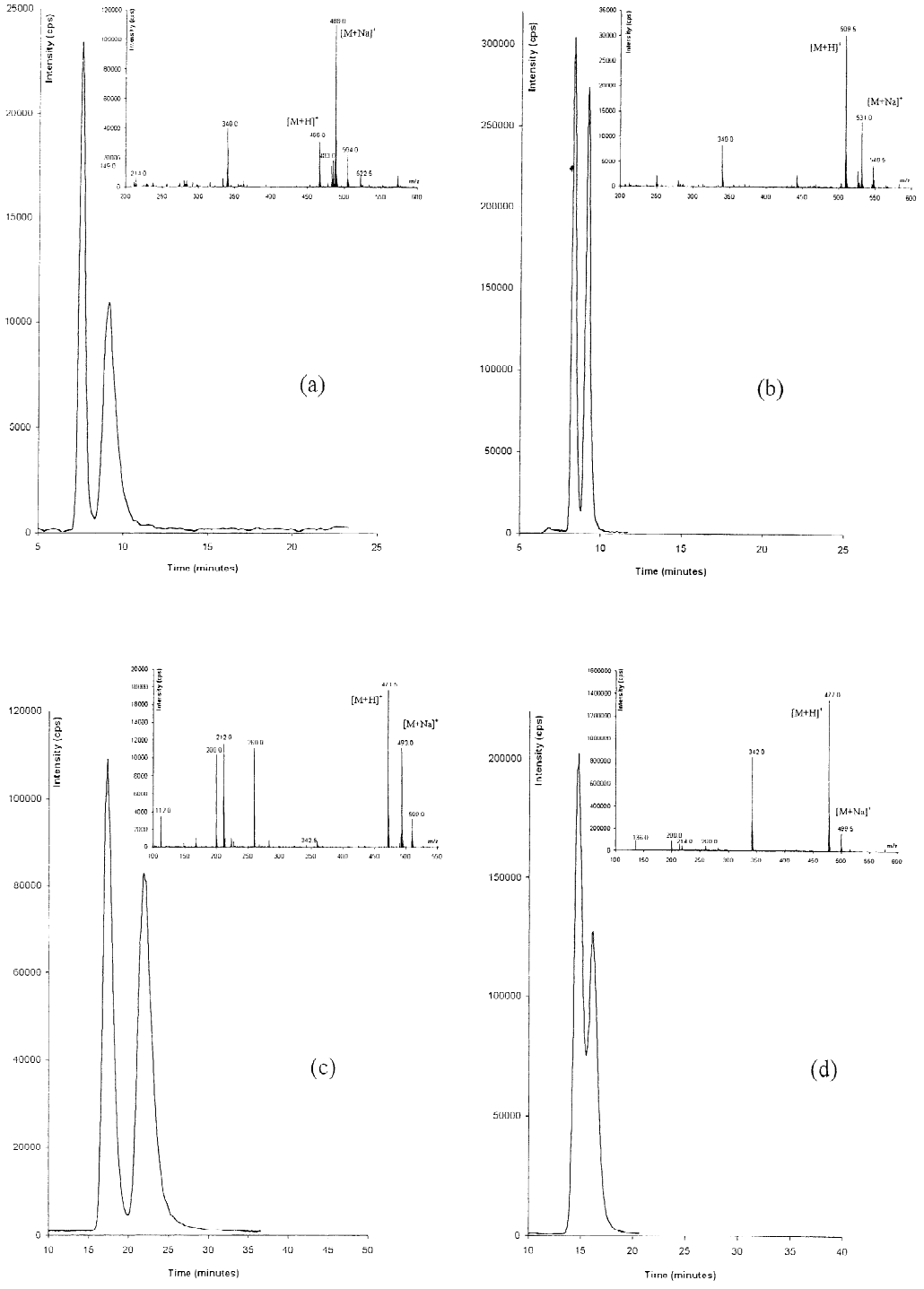

Fig. 3. HPLC–ESI-MS (SIR) chromatograms of phosphoramidates on Chiralcel OD-RH: (a) d4T prodrug (m/z 466.0); (b) AZT prodrug (m/z 509.5), (c) 3TC prodrug (m/z 471.5) and (d) ddA prodrug (m/z 477.0). Mobile phases: (a) MeCN/water 30:70 (v/v), (b) MeCN/water 40:60 (v/v), (c,d) MeCN/water 20:80 (v/v).

the method, which was expressed as a percentage of the theoretical concentration. The recovery study results are shown in Table 5.

4.3. Repeatability

Standard solutions (20 μg/ml) of each prodrug were injected 10 times to evaluate the intra-day repeatability. RSD values were found to be generally less than 1% on retention times and inferior to 4% on peak areas; however, repeatability may be efficiently improved by calculating the retention times and peak area ratios: in these conditions, RSD values are lowered to 0.4 and 2%, respectively.

5. HPLC–ESI-MS coupling

HPLC is needed to separate the diastereomers which give the same MS spectra. Improved detection sensitivity is provided using MS in selected ion reaction (SIR) mode: indeed, at least a fivefold enhancement is observed (LOQ$<$1.3 μg/ml, LOD$<$ 0.4 μg/ml), compared to UV detection. The feasibility of such HPLC–ESI-MS coupling, after optimisation of ESI parameters for each prodrug (10 μg/ml), is illustrated in Fig. 3. Such a method may be useful either to determine low concentrations in plasma and cells, or to provide valuable structural information (enzymatic hydrolysis products, for instance).

6. Conclusion

The polysaccharide-type chiral stationary phase (Chiralcel OD-RH) has been demonstrated to be a very useful material to carry out HPLC separation of phosphoramidate diastereomers. The thermodynamic study has shown that the solute transfer is enthalpically driven; however, increasing temperature was detrimental to the resolution.

Quantitative analysis has shown good results in terms of linearity, accuracy and repeatability, in spite of passable LOD and LOQ values. Nonetheless, the viability of HPLC–ESI-MS coupling enables to enhance the detection sensitivity and provides a unique selectivity. Further HPLC–ESI-MS–MS experiments would enable to reach greater sensitivity limits.

Finally, the next step of this work would be the isolation of each pure diastereomer (preparative scale) as this would be essential for HPLC identification and further pharmacological studies.

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