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HPLC and mass spectrometry analysis of the enzymatic hydrolysis of anti-HIV pronucleotide diastereomers

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Abstract

In one current strategy to develop membrane-soluble pronucleotides, the phosphoramidate derivatives of the approved anti-HIV nucleosides 2′,3′-didehydro-3′-deoxythymidine (d4T), 3′-azido-3′-deoxythymidine (AZT), (−)-β-L-2′,3′-dideoxy-3′- thiacytidine (3TC), and 2′,3′-dideoxyadenosine (ddA) exhibit promising antiviral activity. However, the non-stereoselective synthetic route results in a mixture of diastereoisomers, which differ in the configuration of the phosphorus chiral center. Since it is believed that enzymatic ester hydrolysis is the first step in the intracellular activation of these prodrugs and that this process could be dependent on the stereochemistry at the phosphorus center, analytical methods must be developed. In the present work, in vitro evaluation of the selectivity of pig liver esterase (PLE) towards each diastereomer of d4T, AZT, 3TC, and ddA prodrugs has been investigated, applying our recently published HPLC-MS procedure using a polysaccharide-type chiral stationary phase [1]. This method has been used to analyze the products of the PLE-catalyzed hydrolysis of the pronucleotides. It was found that both diastereomers of the four prodrugs were substrates for PLE.

Keywords: Phosphoramidates; Prodrugs; HIV; Pig liver esterase; Mass spectrometry; Cellulose chiral column

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1. Introduction

The nucleoside analogues have been widely explored as potential antiviral and antitumoral chemotherapeutic agents [2]. Among them, AZT (3′-azido-3′-deoxythymidine **1**), d4T (2′,3′-didehydro-3′-deoxythymidine **2**), 3TC ((−)-β-L-2′,3′-dideoxy-3′-thiacytidine **3**), and ddA (2′,3′-dideoxyadenosine **4**) (Fig. 1) are currently used in the HIV therapy. Recently, nucleoside phosphoramidates have been designed [3] to penetrate the cell membrane and bypass the first phosphorylation step, which is known to be rate limiting. These membrane-permeable prodrugs, once into the cell, are submitted first to an enzyme-mediated hydrolysis, followed by a chemical step, which results in the intracellular release of the monophosphate nucleoside (Scheme 1).

The non-stereoselective synthetic route produces the phosphoramidate prodrugs as mixtures of diastereomers (in which the phosphorus chiral center could be *R* or *S*). One reason for the lack of antiviral activity of these compounds may be due to the fact that the two diastereomers are processed at different rates. Hence, the reactivity of the diastereomeric mixture of phosphoramidates in an esterase reaction is important and informative for several reasons. For example, it offers a simple

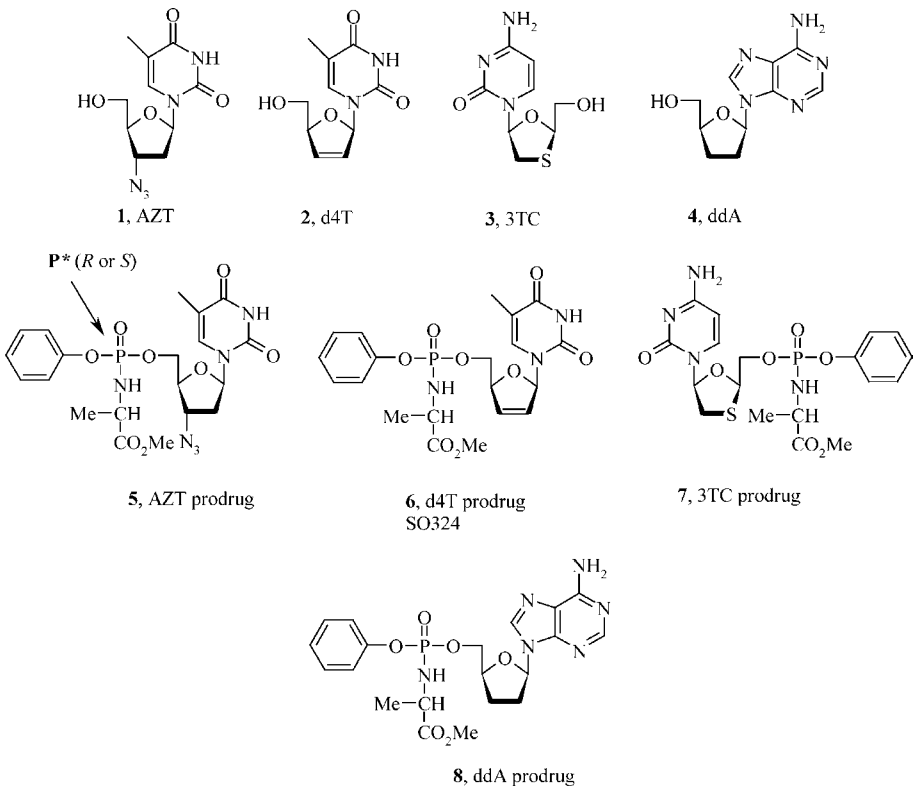
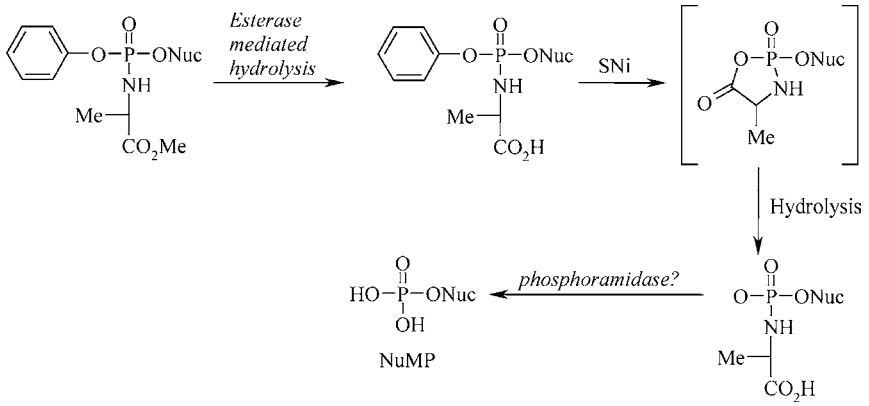


Fig. 1. Chemical structures of anti-HIV nucleosides and of their phosphoramidate derivatives.



Scheme 1. Mechanism of decomposition of aryl phosphoramidates diester pronucleotides.

prediction of the metabolism of phosphoramidate nucleosides and a rationale of the precise role of the stereochemistry at the phosphorus chiral center. Pig liver esterase (PLE) has been widely used as a model for intracellular enzymes [4–6], and as a model for the enzymatic-mediated hydrolysis of pronucleotides [4,6–8]. Therefore, as part of our on-going anti-HIV research, the present work addresses the differential reactivity of the PLE-mediated hydrolysis of (aryloxy)phosphoramidate derivatives of AZT (**5**), d4T (**6**), 3TC (**7**), and ddA (**8**), using recently developed HPLC assay and mass spectrometry [1]. In order to determine whether both diastereomers of each prodrug are substrates of PLE (and thus to investigate the possible role of the configuration of the phosphorus center), the diastereomeric prodrugs **5**–**8** were subjected to the PLE-catalyzed reaction and the products analyzed. Mass spectrometry allowed us to identify the unreacted phosphate triesters as well as their hydrolyzed products.

2. Materials and methods

Acetonitrile, methanol, acetone, and dichloromethane of HPLC quality were obtained from J.T. Baker (Noisy le Sec, France). Deionized water (18 MΩ) was obtained from an Elgastat UHQ II System (Elga, Antony, France). Pig liver esterase (PLE, activity 19 units/mg) and TRIS (tris-hydroxyaminomethane) buffer were purchased from Sigma-Aldrich-Fluka (St Quentin Fallavier, France). Phosphoramidate derivatives of AZT (**5**), 3TC (**7**), and ddA (**8**) were synthesized according to a literature procedure [3]. The d4T prodrug (**6**, SO324) was obtained as a gift from Professor Chris McGuigan (Welsh School of Pharmacy, University of Wales, Cardiff, UK).

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 performed at 254 nm with a Kratos (Applied Biosystems, Courtaboeuf, France) Spectroflow 783 UV spectrophotometric detector. The data were collected and analyzed using EZChrom Elite software (version 2.5). A Chiralcel OD-RH [cellulose tris(3,5-dimethylphenylcarbamate)] (Chiral Technologies, Illkirch, France) analytical column (150 × 4.6 mm i.d., particle size 5 μm) was used for HPLC separation of the prodrug diastereomers. The flow rate was 0.5 mL/min.

Mass spectrometry (MS) experiments were performed using a Perkin–Elmer (Foster City, CA, USA) API 300 triple quadrupole electrospray ionization (ESI) mass spectrometer. The mass spectrometer was operated in both positive and negative ion modes, and nitrogen was used as carrier (0.95 L/min) and nebuliser gas (1.44 L/min). Samples were directly infused into the ESI source by a Harvard Model 22 syringe pump at a flow rate of 5 μL/min. After optimization of the MS parameters, ion spray, orifice, and ring voltages were, respectively, set at 5.8 kV, 40, and 400 V in the positive mode, and at −5.4 kV, −70, and −310 V in the negative mode. A 2 ms dwell-time and a 2.0 ms pause time were used for MS acquisition, which were computer controlled using LC2 Tune Software.

2.2. Treatment of phosphoramidates (*5*–*8*) with pig liver esterase

The PLE-mediated hydrolysis of **5**–**8** was performed according to the procedure reported elsewhere [9], but included an extra centrifugation step. Phosphoramidate derivatives of AZT, d4T, ddA, and 3TC (about 9 μmol) were separately introduced into conical centrifuge tubes and dissolved in a mixture of acetone (0.1 mL) and Tris buffer pH 7.6 (1 mL). Pig liver esterase (31.6 mg) was added into each tube and the reaction mixture was incubated for 24 h at 37 °C. It should be noted that a time course (20, 40, 60 min, 2, 4, 6.5 h incubation) of the hydrolysis of 3TC prodrug **7** was carried out using the same protocol, for a more accurate evaluation. After incubation, each enzymatic solution was diluted with water (10 mL) before centrifugation to precipitate PLE. The supernatants were then transferred into fresh tubes for liquid–liquid extraction (LLE). Subsequently, the solutions were extracted with dichloromethane (2 × 10 mL). Organic layers were combined and extracted with water (10 mL), and the aqueous layers were combined. Both aqueous and organic layers were then evaporated to dryness under nitrogen gas and stored at −20 °C until analysis. Organic fractions were expected to contain the unreacted phosphate triesters whereas the aqueous fractions were expected to contain the hydrolysis products [9]. Sample solutions were filtered before analysis through 0.22 μm membrane filters (Prolabo, France).

3. Results and discussion

The only reported resolution of the diastereomers of d4T phosphoramidate derivative (**6**, SO324) has been carried out using a molecularly imprinted stationary phase

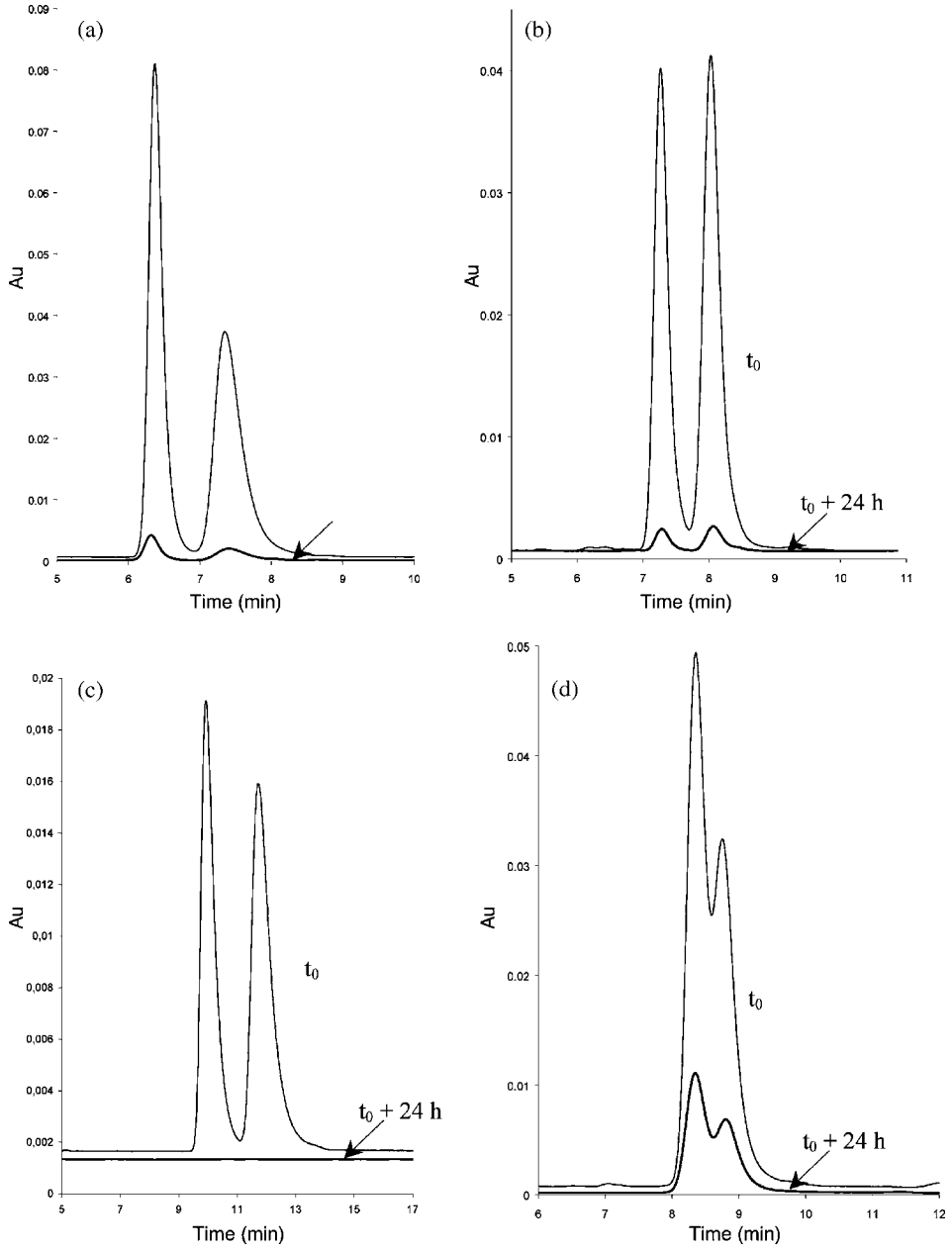


Fig. 2. HPLC-UV diastereomeric separation of the studied phosphoramidates, before and after PLE-mediated hydrolysis: (a) d4T prodrug (SO324), (b) AZT prodrug, (c) 3TC prodrug, and (d) ddA prodrug. *Conditions*: Chiralcel OD-RH stationary phase; mobile phase: (a) 30/70, (b) 40/60, (c) and (d) 20/80 (v/v) MeCN/water.

in HPLC [10], but with poor efficiency. To circumvent this problem, we recently developed an HPLC analytical method using a chiral cellulose-based stationary phase, which was demonstrated to resolve the phosphoramidates **5**–**8** with better resolution [1]. This method could be used to assess the enzymatic-mediated hydrolysis of the four pairs of diastereomers. In the organic fractions, which are likely to contain the unreacted prodrugs, one would expect that either both peaks would decrease at the same rate or that one would decrease faster than the other. Fig. 2 illustrates the HPLC analysis of d4T, AZT, 3TC, and ddA prodrugs before and after a 24-h treatment with PLE. A simultaneous decrease of the peak area of each diastereomer was observed, suggesting that both diastereomers are substrates for PLE. However, with regard to the 3TC prodrug, no signal was available from the organic fraction (e.g., after 24 h incubation), showing that enzymatic kinetics was much faster for the 3TC prodrug than for the other phosphoramidates. Since no conclusion could be made from this experiment, a more accurate study was carried out on the 3TC prodrug, in which the analyte was applied to PLE for different incubation times (from 20 min to 6.5 h). As shown in Fig. 3, the hydrolysis rate was different for each diastereomer. A plot of peak area ratio versus the incubation time was found to be a decreasing curve, indicating that phosphorus configuration has an effect on the esterase activity. Nonetheless, since pure diastereomers were not available, a determination of which isomer is processed faster could not be performed. Finally, after 6.5-h

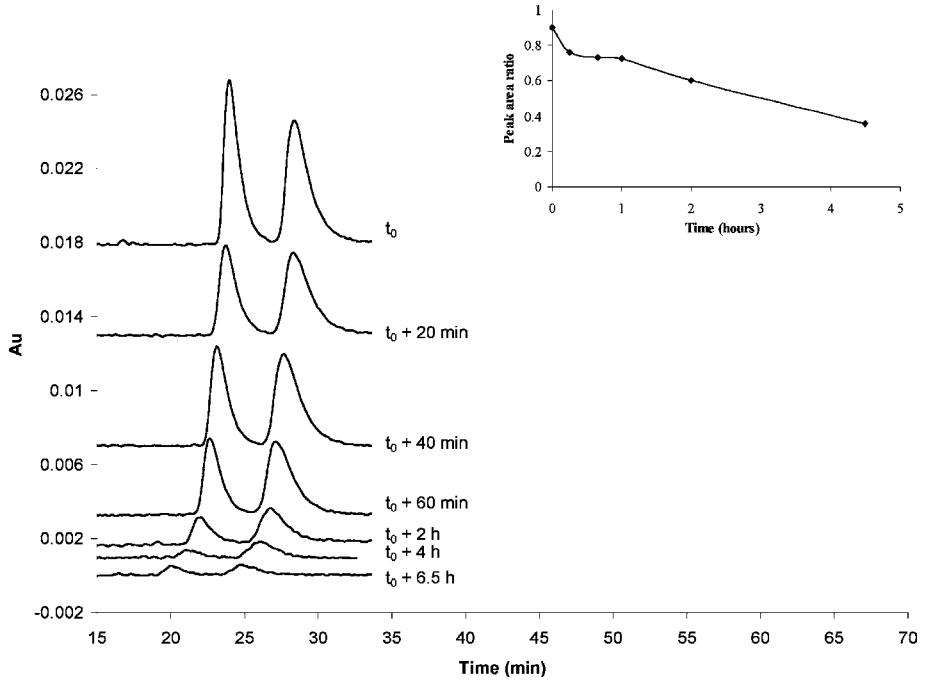


Fig. 3. Influence of incubation time on 3TC prodrug diastereomers. The conditions are the same as those described in Fig. 2.

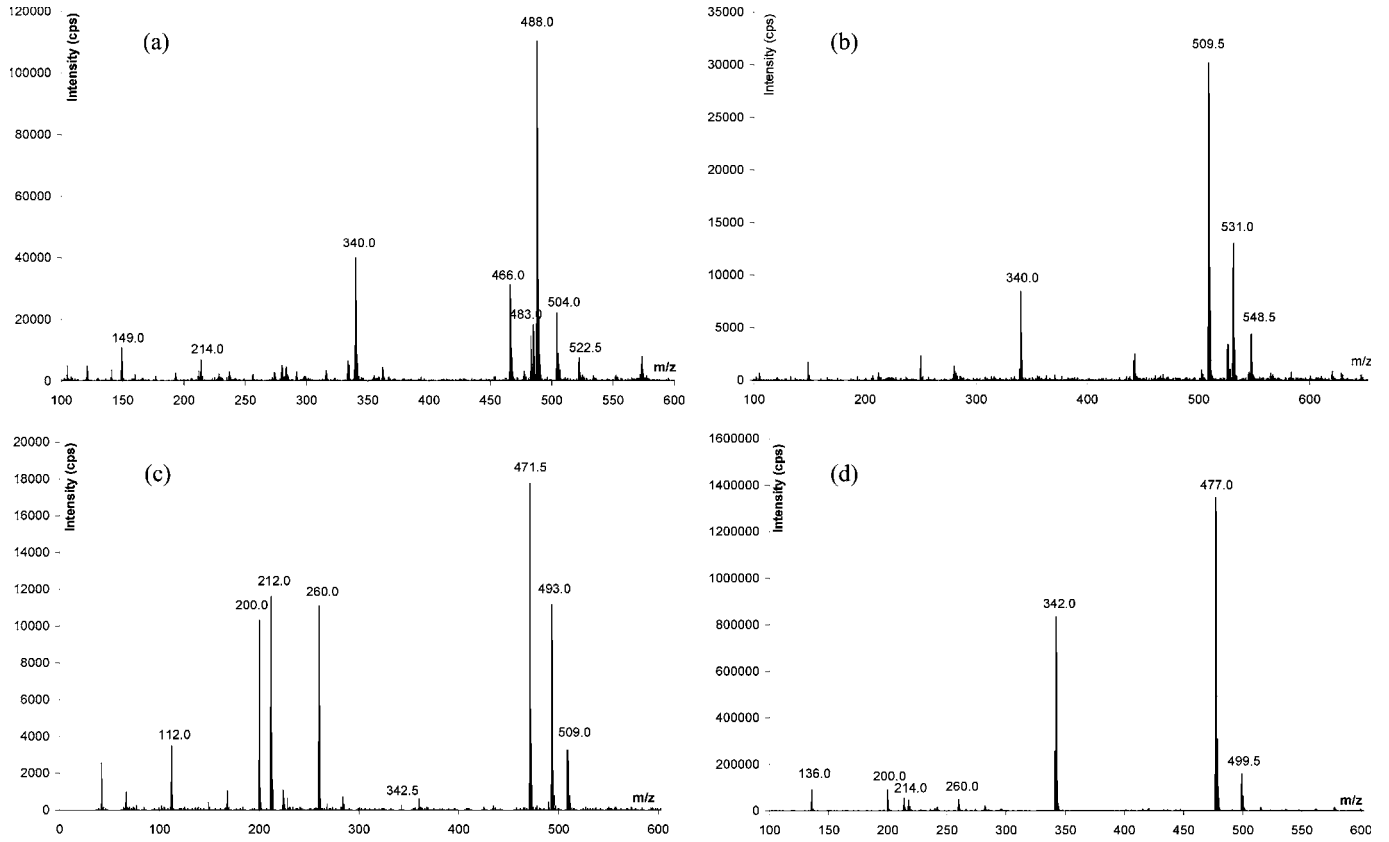


Fig. 4. MS spectra of phosphoramidates standard solutions: (a) d4T prodrug (m/z 466.0), (b) AZT prodrug (m/z 509.5), (c) 3TC prodrug (m/z 471.5), and (d) ddA prodrug (m/z 477.0).

incubation, hydrolysis of both diastereomers of 3TC prodrug was almost complete, indicating that both diastereomers were substrate of PLE.

3.1. Mass spectrometry characterization

Mass spectrometry is an efficient analytical tool, which provides unique selectivity, improved sensitivity, and valuable structural information. Hence, both organic and aqueous fractions (resulting from PLE assay) were characterized by direct injection into the mass spectrometer. Preliminary experiments on standard solutions of the four prodrugs were performed to determine the optimal electrospray ionization parameters (ionspray, orifice, and ring voltages). The analytes were found to give greater response in the positive mode; therefore, the positive mode was selected for MS analyses of standards and organic fractions. With regard to the aqueous fractions, they were analyzed in both positive and negative modes. However, since PLE-mediated hydrolysis results in a loss of a methyl and/or a phenyl moiety, the negative mode gave a better signal for all degradation products.

Fig. 4 illustrates the MS spectra of the prodrugs standard solutions (e.g., before treatment with PLE). For each compound, the molecular ion $[M + H]^+$ and its sodium adduct $[M + Na]^+$ can be observed at m/z values reported in Table 1. As expected, the same m/z values are observed in the MS spectra of the organic fractions (Fig. 5), reflecting the small amount of unreacted phosphoramidates (after 24-h incubation except for 3TC prodrug, which was incubated for 6.5 h). With regard to the aqueous fractions, the main hydrolysis product corresponds, for all compounds, to the loss of both phenyl and methyl moieties, in agreement with previous reported work [6,9]. Indeed, this is explained by the initial esterase action on the methyl ester followed by an intramolecular ring closure with removal of the phenoxy group by the carboxylate anion to form a five-membered ring mixed anhydride. Subsequently, this anhydride is hydrolyzed to give the final PLE-hydrolysis product (Scheme 1). Therefore, the ions corresponding to the single loss of either the methyl or the phenyl groups are not observed. Thus, the in vitro PLE-catalyzed hydrolysis of (aryloxy)phosphoramidate pronucleotides leads to the amino acyl phosphoramidates. This enzymatic assay gives important information with regard to the in vitro metabolism and the potent antiviral action. Indeed, according to this enzyme-mediated hydrolysis, the amino acyl phosphoramidate intermediates seem to be needed in the pronucleotide approach for phosphoramidate compounds to act as nucleotide prodrugs.

Table 1
m/z Values of molecular ions and their sodium adducts (positive mode) and of PLE-mediated hydrolysis products (negative mode)

Compound	m/z				
	$[M + H]^+$	$[M + Na]^+$	$[M\text{-phenyl}]^-$	$[M\text{-}CH_3]^-$	$[M + H\text{-phenyl-}CH_3]^-$
d4T prodrug	466.0	488.5	388.0	450.0	374.0
AZT prodrug	509.0	531.5	431.5	493.5	417.5
3TC prodrug	471.5	493.0	393.5	455.5	379.5
ddA prodrug	477.0	499.5	399.5	461.0	385.0

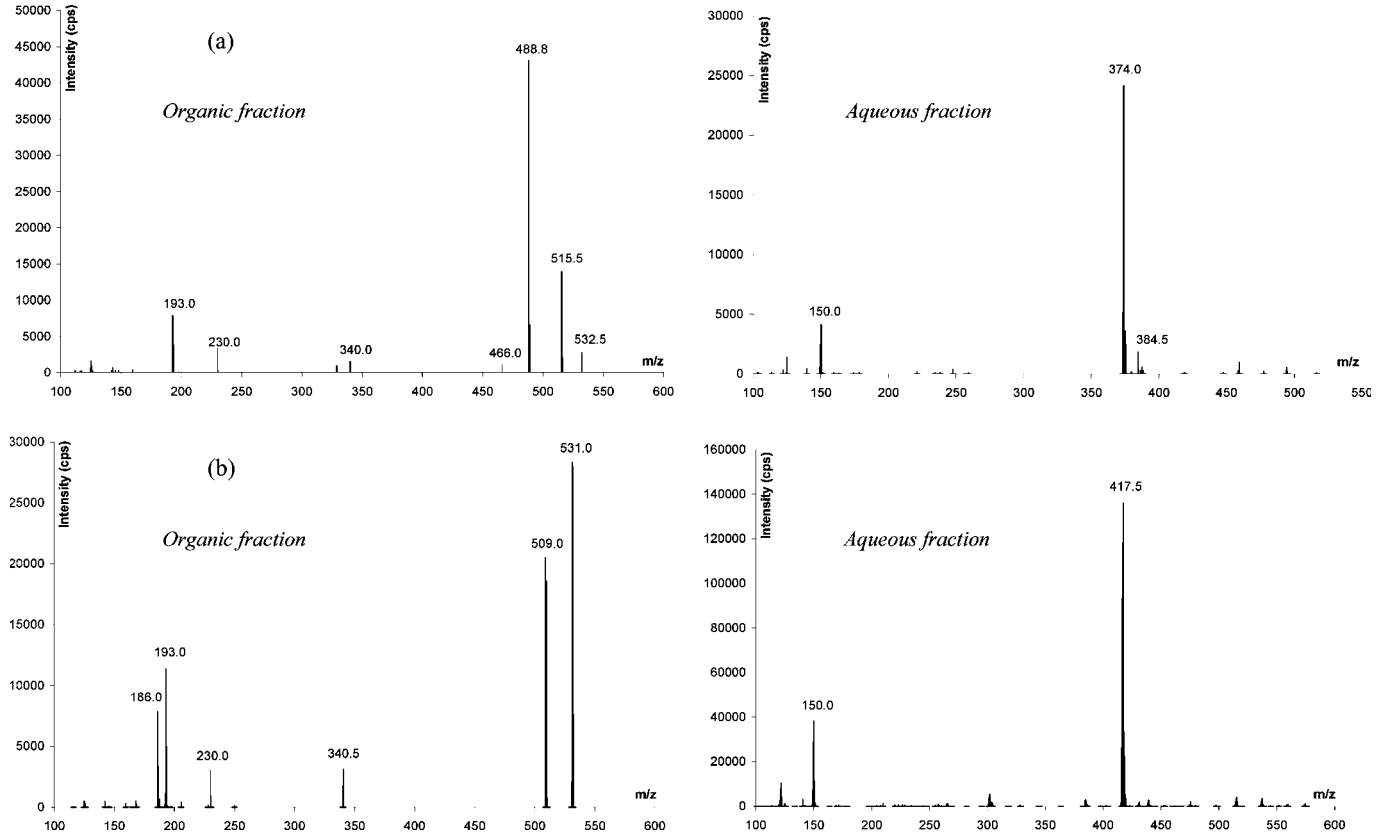


Fig. 5. MS spectra of organic and aqueous fractions after PLE-hydrolysis: (a) d4T prodrug, (b) AZT prodrug, (c) 3TC prodrug, and (d) ddA prodrug.

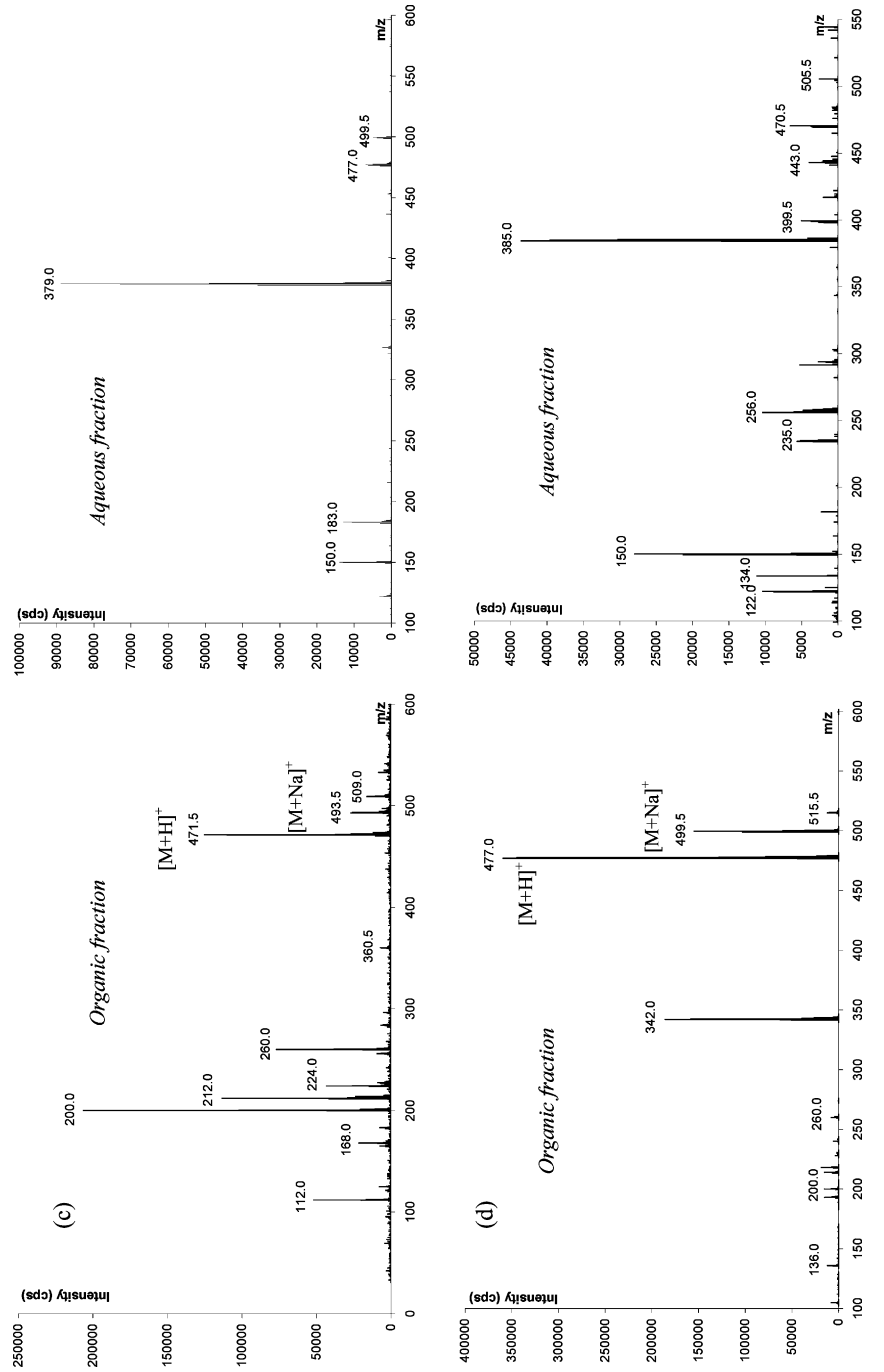


Fig. 5. (*continued*)

4. Conclusion

Pig liver esterase-mediated hydrolysis of phosphoramidate derivatives of d4T, AZT, 3TC, and ddA has been used as a model of the first step of the intracellular esterase hydrolysis. After liquid–liquid extraction, both organic and aqueous layers were characterized by high performance liquid chromatography (HPLC) and mass spectrometry (MS). Diastereomeric separations were achieved by an HPLC method, which was also used to monitor the residual unreacted prodrugs (found in the organic fraction). The presence of unreacted phosphoramidates was confirmed by mass spectrometry. Mass spectrometry also allowed the identification of the hydrolysis products in the aqueous fractions. Both diastereomers of the prodrugs were found to be substrates for PLE. A more precise study on the phosphoramidate derivative of 3TC suggested that it is a better substrate which is processed more quickly. Finally, for the four prodrugs, the hydrolysis products corresponded to the loss of both phenyl and methyl moieties. These HPLC and MS analytical methods may represent useful tools for future pharmacokinetic studies on different enzymes such as those found in human peripheral blood mononuclear cell extract.

Acknowledgments

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