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Stereospecific chemical and enzymatic stability of phosphoramidate triester prodrugs of d4T in vitro

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Abstract

The phosphoramidate triester prodrug approach is widely used to deliver nucleotide forms of nucleoside analogues into target cells. We investigated the stereoselective stability of a series of prodrugs of the anti-HIV agent 2′,3′-didehydro-2′,3′-dideoxythymidine (d4T). Chemical stability was evaluated in phosphate buffer at pH values of biological relevance (i.e. pH 2.0, 4.6, 7.4). Enzymatic stability was tested in human plasma, in Caco-2 cell homogenates and monolayers and in rat liver. The compounds were relatively stable to chemical hydrolysis. Between 50 and 70% of unchanged prodrug was recovered after 16 h incubation in human plasma, with no stereoselective preference for phosphate diastereoisomers. The *p*-OMe phenyl derivative, however, was an exception and only 5% of one diastereoisomer was recovered. In Caco-2 cells the stability and stereoselectivity largely depended on the experimental conditions: high enzymatic activity and stereoselectivity was observed in cell homogenates, but not in monolayers. In rat liver S9 fractions the stability profile was similar to that in Caco-2 cells and carboxyl ester cleavage appeared to be the sole mechanism of degradation in both media. The large and unpredictable differences in stereoselective metabolic rate of the pronucleotide series here presented suggest that in vivo circulating levels of intact prodrug could exert profoundly different activity or toxicity due to preferential body distribution of one diastereoisomeric form.

Keywords: Anti-HIV pronucleotides; d4T; Ester prodrugs; Caco-2; Antivirals

1. Introduction

The phosphoramidate triester approach is a prodrug methodology (“pronucleotide”) increasingly used to improve the pharmacological activity of existing nucleoside analogues (Wagner et al., 2000). The principal objective is to boost the activity of those nucleoside analogues that exhibit inefficient intracellular phosphorylation by host cell or virally-encoded nucleoside kinases to produce the active triphosphorylated form. The pronucleotide approach is based on the ability to deliver to the target cell the monophosphorylated form of a variety of nucleoside analogues. The phosphate group is masked with neutral lipophilic groups to obtain a lipophilic membrane-permeable derivative able to access intracellular target sites, wherein hydrolysis yields the free nucleoside monophosphate.

In the case of the phosphoramidate derivatives of the 2′,3′-dideoxy-2′,3′-didehydro analogue of thymidine, d4T (stavudine), the ability to release the free mononucleotide has been proposed to depend on carboxylesterase-mediated hydrolysis of the carboxylic ester function in the amino acid moiety (Balzarini et al., 1996). Spontaneous elimination of the phenol and an enzymatic cleavage of the P–N bond will then release d4TMP (Fig. 1) (Saboulard et al., 1999). For optimal pharmacological action the activation to d4TMP must occur within the target cell, but not during the process of absorption and distribution. Previous studies investigating the activation pathway of a series of d4T and AZT pronucleotides in different biological media (i.e. CEM cell extracts, human serum, mouse serum) have shown different degrees of stability with variation in the amino acid moiety, and the carboxyl ester group (Saboulard et al., 1999). The alaninyl phosphoramidate triester derivatives were efficiently converted to the free nucleoside monophosphate in intact cells (Balzarini et al., 1996). In this current work we have investigated the stability to chemical and enzymatic

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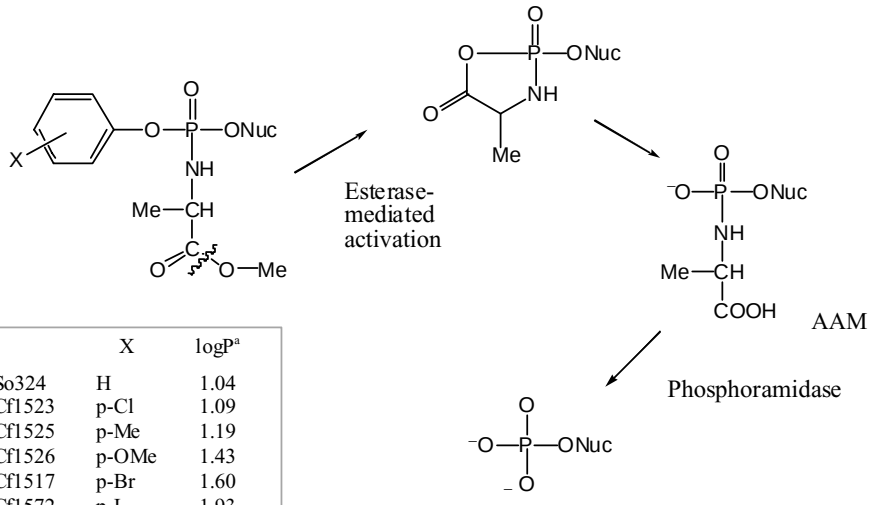

	X	logP[a]
So324	H	1.04
Cf1523	p-Cl	1.09
Cf1525	p-Me	1.19
Cf1526	p-OMe	1.43
Cf1517	p-Br	1.60
Cf1572	p-I	1.93

Fig. 1. The aryloxy phosphoramidate prodrugs (pronucleotides) of d4T examined in this study and the proposed activation pathway. The cleavage of the methoxy group is considered the first step in the enzymatic hydrolysis of the compounds and is followed by spontaneous cyclization and displacement of the aryloxy group and formation of the amino acyl metabolite (AAM); the last activation step is catalyzed by intracellular phosphoramidase. Chirality at the P atom of the pronucleotide generates two diastereoisomers. Taken from Siddiqui et al. (1999).

ester hydrolysis of a series of pharmacologically active alaninyl phosphoramidate analogues of d4T (Fig. 1) in human plasma, rat liver and in different Caco-2 cell preparates, since esterase mediated degradation in the GI tract can be particularly critical in limiting oral absorption of ester prodrugs (Van Gelder et al., 2000a,b, Annaert et al., 1998). The phosphoramidates are chiral at phosphorus and prepared as roughly 50:50 mixtures of phosphate diastereoisomers. The aspect of phosphate stereochemistry on the process of activation was also, for the first time, herein considered.

2. Materials and methods

2.1. Materials

All reagents used were of analytical grade. Caco-2 cells were obtained from the European Collection of Animal Cell Cultures (ECACC). Phosphate buffered saline pH 7.4 (PBS), Dulbecco's modified Eagle's medium (DMEM) and penicillin/streptomycin were from Life Technologies (Paisley, UK). Tissue culture plastics were from Corning–Costar (Bucks, UK). D4T phosphoramidate prodrugs were synthesised according to previously published procedures (McGuigan et al., 1996). Pronucleotides solutions were prepared by spiking DMEM with a concentrated stock of prodrug dissolved in DMSO. The final concentration of DMSO within solutions used for experiments was always adjusted to 1%.

2.2. Chemical stability

Pronucleotide stability to chemical hydrolysis was studied in 0.05 M NaH_2PO_4 at pH 2.0, 4.6, and 7.4. An accurately weighted amount of the investigated compounds was dissolved in DMSO, and then diluted with the respective phosphate solution to final concentration of 0.2 mM of pronucleotide. Samples were incubated in closed vials at 37 °C for 20 h in the dark. Solutions were withdrawn at the end of the time period and stored at −80 °C or immediately analyzed by HPLC. The storage at −80 °C for up to 1 month was found not to alter pronucleotide stability.

2.3. Degradation of pronucleotides in plasma

Pronucleotide stability was tested in plasma preparations obtained from healthy volunteers. The incubation mixture contained 160 μl of plasma, 20 μl of drug stock solution at the concentration of 5 mM and 20 μl of $MgCl_2$ (25 mM) in phosphate buffer (pH 7.4). At the end of each incubation period (0, 3 or 16 h at 37 °C) 200 μl of ice-cold methanol/acetonitrile 1/1 (v/v) was added, the tubes were vortex-mixed for 20 s to precipitate proteins, left on ice for 30 min and centrifuged for 5 min at 15,000 × *g*. The supernatant was immediately analyzed by HPLC or stored at −80 °C until analysis. The storage at −80 °C for up to 1 month was found not to alter pronucleotide stability.

2.4. Cell culture

Caco-2 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The medium was changed every 48 h. Cells were seeded at 50,000/cm^2 in tissue culture flasks and cultured until confluent at 37 °C in 90% relative humidity and 5% CO_2. Cells between passage numbers 30 and 40 were used for degradation experiments. The

cells were detached from the flasks by washing with PBS, followed by 0.5% trypsin and 0.2% EDTA in PBS.

2.5. Degradation in Caco-2 cell homogenates, suspensions and monolayers

Caco-2 cells were grown on culture flasks until confluence, detached, resuspended in DMEM and counted. Suspended cells were pelleted by centrifugation, resuspended in PBS and washed two more times. Cell pellets were stored at −80 °C. On the day of the experiment cells were diluted in PBS containing $MgCl_2$ (10 mM) to a density of 2×10^6 cells per 100 μl and sonicated in iced water for 60 s. The incubation mixture contained 100 μl of cell homogenate and 100 μl of drug stock solution 1 mM in PBS containing $MgCl_2$ (10 mM). Reaction mixtures were incubated at 37 °C and at fixed time-points 200 μl of ice-cold methanol/acetonitrile 1/1 (v/v) was added, the tubes were vortex-mixed for 20 s, left on ice for 30 min and centrifuged for 5 min at 15,000 × *g*. The supernatant was immediately analyzed by HPLC or stored at −80 °C until analysis. The storage at −80 °C for up to 1 month was found not to alter pronucleotide stability. For inhibition studies homogenates were preincubated for 30 min at 37 °C in the presence of different concentrations of the carboxylesterase inhibitor phenylmethylsulfonyl fluoride (PMSF) before the addition of the pronucleotide solution.

For the preparation of cell suspensions Caco-2 cells were grown on culture flasks until confluence, harvested, washed twice in PBS, counted and aliquoted at the concentration of 2×10^6 cells per 100 μl media. After the addition of 100 μl of 1 mM pronucleotide stock solution the vials were placed on an orbital shaker and left at 37 °C. The incubation reaction was stopped by adding 200 μl of ice-cold methanol/acetonitrile 1/1 (v/v), followed by sonication and centrifugation at 15,000 × *g* for 10 min. The supernatant was analyzed by HPLC. When not analyzed immediately, the supernatant was stored at −80 °C. Under these conditions pronucleotides were found to be stable for up to 1 month.

Uptake and degradation was examined in Caco-2 cells cultured for 17 days in plastic wells. Before the experiments, the culture media was removed, the monolayers were rinsed and preincubated with 1 ml warm DMEM. After 15 min the media was removed and replaced with 1 ml DMEM containing the pronucleotide at a concentration of 0.5 mM. At 0, 1, 4, or 16 h time point media samples were collected and stored. Monolayers were washed twice with ice-cold PBS and then exposed to ice-cold methanol/acetonitrile 1/1 (v/v), the cell lysate harvested, sonicated and centrifuged for 10 min at 15,000 × *g*. The supernatant was analyzed by HPLC.

2.6. Preparation of S9 fractions

Male WISTAR rats weighing 200–250 g were sacrificed. Their livers were removed and washed in PBS. Tissue was finely minced with scissors, transferred to glass homogenizing vessel on ice and homogenized with a motor-driven Teflon pestle in thee volumes of cold phosphate buffer 50 mM pH 7.6. The homogenate was centrifuged at 9000 × *g* for 30 min in a refrigerated (2 °C) Sorvall RC-5B centrifuge (Dupont, Newtown, CT). The supernatant (S9), containing the cytosolic and microsomal fractions was isolated. Protein concentration in the S9 fractions was measured using a Bio-Rad RC DC colorimetric assay (Bio-Rad, Hercules, CA) using absorbance at 750 nm. Each incubation mixture contained 1 mg protein diluted in phosphate buffer 50 mM pH 7.6 containing $MgCl_2$ 10 mM and NADPH 1.0 mM. Standard solutions in DMSO were then added at the final concentration of 0.25 mM of pronucleotide and less than 1% DMSO. Controls were prepared with no NADPH added and immediately extracted with acetonitrile. For inhibition studies samples were preincubated for 30 min at 37 °C in the presence of different concentrations of PMSF before the addition of the pronucleotide solution. The samples were incubated at 37 °C for different time-points. The reaction was terminated by placing the flasks on ice followed by the addition of an equal volume of ice-cold methanol/acetonitrile 1/1 (v/v). Samples were spun at 5000 × *g* for 10 min at 20 °C and the supernatant was stored at −20 °C until HPLC analysis.

2.7. HPLC Analysis of d4T and phosphoramidate prodrugs

The concentration of phosphoramidate prodrugs in the incubation media was determined by HPLC. A Thermo Separations Products HPLC system was used, consisting of P4000 Quaternary Pump, autosampler, column oven and a diode array detector. The column used was a Merck LiChoCart Lichospher C18 (250 mm × 4 mm). Elution was performed at 30 °C isocratically using a mobile phase water/methanol/acetonitrile 60/25/15 (v/v/v) and at flow rate of 1 ml/min; the injection volume was 50 μl. Detection wavelength was 266 nm. The compounds were identified according to peak retention times and UV spectra. The concentrations of test compounds were determined using calibration graph of each compound: standard curves, obtained by linear regression, were linear ($r^2 > 0.99$). The relative standard deviation for within-day and between-day precision was assessed by analyzing each day for 5 days ($n = 5$) a set of two concentration levels, and was less than 5% for each compound. The accuracy was determined by subtracting the measured concentration from its theoretical value; the mean relative error (M.R.E.) of the difference from theoretical was less than 10%.

2.8. Statistical analysis

Comparisons between two groups were made by non-paired student *t*-test with level of significance at $P < 0.05$. Comparisons for more than two groups were made by ANOVA and Duncan's multiple range test with $P < 0.05$ significance level.

Table 1
Stability of pronucleotides in phosphate buffer: recovery (%) of drug after 20 h incubation at 37 °C

	pH 2.0		pH 4.6		pH 7.4	
	FE[a]	SE[a]	FE	SE	FE	SE
H	99.8 ± 2.4	99.9 ± 2.9	99.6 ± 7.3	99.7 ± 3.1	99.7 ± 8.0	99.7 ± 12.7
p-OMe	93.1 ± 1.0	87.5 ± 3.7	91.1 ± 3.7	89.0 ± 2.0	91.2 ± 5.6	90.3 ± 5.3
p-Me	94.7 ± 3.0	93.8 ± 3.1	98.3 ± 2.9	98.3 ± 3.9	93.8 ± 3.9	94.6 ± 1.8
p-Cl	95.1 ± 5.0	96.5 ± 2.6	99.6 ± 2.1	99.1 ± 2.0	87.9 ± 2.9	83.3 ± 3.1
p-Br	85.9 ± 3.8	86.1 ± 3.8	99.8 ± 11.1	99.1 ± 10.9	80.7 ± 6.0	77.3 ± 5.8
p-I	80.2 ± 3.0	80.3 ± 3.0	86.0 ± 3.0	87.1 ± 3.8	79.1 ± 5.0	73.3 ± 5.5

Values are mean ± S.D. of triplicate assays.
[a] FE and SE refer to the two diastereoisomers with stereochemical variation on the phosphorus atom (fast eluting, FE and slow eluting, SE).

3. Results

The pronucleotides were quantified by reverse phase HPLC-UV detection. The phosphate diastereoisomers were efficiently separated and were assigned as fast eluting (FE, less lipophilic) and slow eluting (SE, more lipophilic), according to the retention times.

Chemical stability was studied at experimental conditions of biological relevance, i.e. at pH 2.0, 4.6, and 7.4 (Table 1). Stability was only slightly affected by the pH over the 20 h incubation period. The *p*-Cl, *p*-Br and *p*-I derivatives were more stable at pH 4.6 as compared to pH 2.0 and 7.4 ($P < 0.05$). Differences in stability between the FE and SE diastereoisomers were not statistically significant for any pronucleotide examined.

The stability of the d4T pronucleotides was also studied in human plasma (Table 2). The amount of unchanged prodrug remaining after incubation in plasma samples for up to 20 h was calculated against a set of controls ($n = 3$) extracted from plasma samples at t_0 in order to account for any possible loss of sample during analysis due to protein binding. The mean extraction efficiency of the pronucleotides from plasma samples was approximately 95% with a C.V. never greater than 13%. Statistically significant ($P < 0.05$) differences in stability in plasma between diastereoisomers were only observed for the *p*-OMe (CF1526) derivative, with a half-life of 25.9 and 3.8 h for FE and SE, respectively. The SE diastereoisomer of CF1526 was significantly ($P < 0.05$) less stable than any other compound analyzed. The addition of PMSF (1.0 mM) inhibited, although not completely, the degradation of this pronucleotide: after 20 h incubation the percent recovery of the FE diastereoisomer was 58.9 ± 0.8% (mean ± S.D.), while no SE diastereoisomer could be detected in the incubation media. In the treatments containing PMSF, 82.3 ± 1.7% and 78.4 ± 1.7% of FE and SE diastereoisomers, respectively, were recovered. The degradation profile for this compound after 3,16, and 20 h incubation is shown in Fig. 2.

In Caco-2 cell homogenates (Fig. 3A) all of the phosphoramidate prodrugs underwent substantial degradation over the 1 h incubation period with significant differences between the phosphate diastereoisomers. Both diastereoisomers of the *p*-I derivative were completely degraded, as were the SE diastereoisomers of the *p*-Br and *p*-Cl derivatives. When incubating for a shorter time (i.e. 30 min), the FE and SE recovered were (mean ± S.D.) 35.13 ± 7.1%, 10.90 ± 2.1% for the *p*-I derivative, and 32.30 ± 0.7, 11.4 ± 1.5% for the *p*-Br derivative, respectively.

In order to assess the dependence of the degradation in Caco-2 upon esterase activity, the prototype prodrug So324 (final concentration 500 μM) was incubated for up to 4 h with Caco-2 homogenates. In this media So324 was extensively metabolized, half-life was 1.5 and 0.26 h for the FE and SE diastereoisomer, respectively; 16% of FE diastereoisomer could be recovered unchanged at the end of the incubation period, while no detectable SE diastereoiso-

Table 2
Stability of d4T and phosphoramidate prodrugs in plasma: percentage recovery of drug after 3 and 16 h incubation at 37 °C

	3 h (100.19 ± 0.72)[a]		16 h (85.52 ± 3.72)[a]	
	FE[b]	SE[c]	FE	SE
H	85.6 ± 5.3	90.2 ± 6.1	73.1 ± 3.8	71.1 ± 5.8
p-OMe	97.2 ± 3.7	58.6 ± 2.3	70.2 ± 2.3	5.5 ± 0.8
p-Me	94.8 ± 4.9	94.1 ± 5.0	58.6 ± 3.9	55.5 ± 5.0
p-Cl	92.1 ± 1.3	81.3 ± 5.1	65.2 ± 2.1	57.5 ± 4.0
p-Br	93.0 ± 8.5	89.8 ± 8.0	61.9 ± 2.1	68.1 ± 14.2
p-I	93.8 ± 1.4	91.8 ± 1.9	53.1 ± 4.0	53.5 ± 4.4

Values are mean ± S.D. of triplicate assays.
[a] d4T.
[b] Fast eluting diastereoisomer (FE).
[c] Slow eluting diastereoisomer (SE).

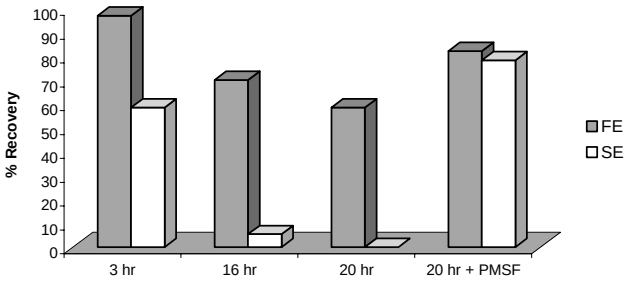

Fig. 2. The *p*-OMe derivative's fast eluting (FE) and slow eluting (SE) diastereomers recovered after incubation in human plasma at 37 °C for 3, 16, and 20 h in absence and presence of PMSF. Bars are mean of two experiments performed in triplicate, with C.V. <5%.

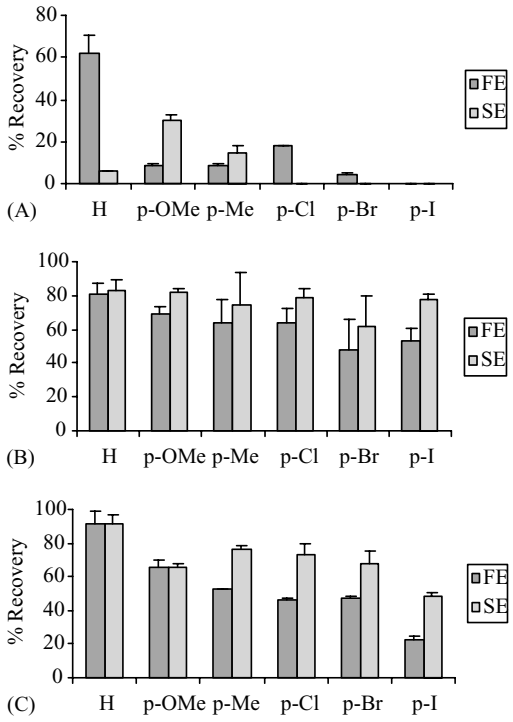

Fig. 3. Percent recovery of pronucleotides incubated with (A) 2×10^6 Caco-2 cell homogenate, 1 h incubation time at 37 °C. (B) 2×10^6 Caco-2 cell suspension over 4 h. Between groups differences of averages were not statistically significant ($P > 0.05$). (C) Caco-2 cell monolayers, 4 h incubation time at 37 °C in six-well plates. Bars represent the average cumulative recovery of pronucleotides in the free incubation media and the intracellular fraction. The standard deviations (error bars) are means of two to four experiments performed in triplicate.

mer could be recovered. In contrast, when the cell extract was incubated in the presence of the carboxylesterase inhibitor PMSF, the amount of unchanged prodrug recovered after 4 h was approximately 85% for each diastereoisomer. PMSF concentrations in the range from 1.0 to 10 mM were used, and provided similar results. For example, when 10 mM PMSF concentration was used, 85.1 ± 0.8 and 87.5 ± 0.6% of the FE and the SE diastereoisomer, respectively, were recovered. This is taken as direct and clear support for the notion that carboxyesterase-mediated cleavage is a key step in the activation of the nucleotide analogue. However, the fact that it was not possible to reach 100% inhibition suggests that other PMSF-insensitive hydrolases may be involved in the hydrolysis of the methyl ester bond.

The stability of the pronucleotides was also evaluated following exposure to intact cells in suspension (4 days post-seeding), and as such probed the accessibility of the pronucleotides to metabolizing enzymes in the degradation process. Both diastereoisomers of So324 incubated with Caco-2 cell suspensions had a half-life of approximately 10 h; the fraction of unchanged pronucleotide measured after 16 h was 35.61 ± 4.7 and 37.30 ± 5.7% for the FE and SE diastereomers, respectively. The process was not stereospecific at any time-point ($P > 0.05$). The pronucleotide series was then compared in a 4 h incubation experiment (Fig. 3B); no statistical difference between either SE diastereoisomers within the group, or the FE diastereoisomers within the group were noticed. Comparisons of the respective FE and SE diastereoisomers showed that for only the *p*-OMe and *p*-I derivatives was there any statistically significant difference, with the FE diastereoisomer less stable in both cases.

The pronucleotides were also exposed to Caco-2 monolayers at 17 days post seeding to assess if differences in enzymatic expression of differentiated monolayers would affect the decomposition profile. In a 4 h incubation experiment differences between FE and SE diastereoisomers of the respective *p*-Me, *p*-Cl, *p*-Br, *p*-I derivatives were statistically significant. The half-life of So324 calculated from the concentration of pronucleotide recovered in the incubation media and intracellularly was 14.9 and 16.5 h, for the FE and SE diastereoisomer, respectively. After 4 h the amount recovered was 91.6 ± 7.0 and 91.3 ± 4.8% (Fig. 3C). At all time-points the fraction of So324 recovered intracellularly was less than 2% of the total amount recovered.

Comparative stability data between the cell suspensions and the cell monolayers showed no clear trend for the stability of the pronucleotides. Interestingly, opposite stereoselective degradation of the *p*-Cl, *p*-Br and *p*-I derivatives resulted in the monolayer and suspension incubations as compared with the homogenates (Fig. 3).

The stability of the prototype prodrug So324 was further evaluated in rat hepatic S9 fraction to assess the substrate specificity for CYP450 and hepatic hydrolases. The degradation profile in rat preparations was similar to that observed in Caco-2. The FE diastereoisomer was more stable than the SE diastereoisomer (Fig. 4). Half-lives were calculated at 17 and 0.6 min for FE and SE, respectively. Incubation in the presence of 1 mM PMSF significantly inhibited the degradation. For example, at the 90 min time-point the amount of So324 recovered in samples treated with PMSF was 92.9 ± 7.6 and 87.4 ± 3.3% of the initial concentration of the FE and SE diastereoisomer, respectively,

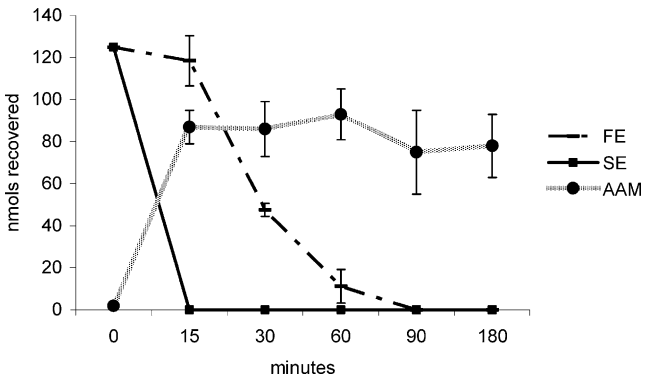

Fig. 4. Enzymatic degradation profile of the FE and SE diastereoisomers (0.125 mM) of So324 in rat liver S9 fractions at 37 °C. Disappearance of starting material corresponded with the appearance of the amino acyl metabolite (AAM). Points are average (±S.D.) of the determinations.

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