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Development and validation of an ion-pair reversed-phase high-performance liquid chromatographic method for the separation of phosphonodipeptides

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Abstract

The objective of this research was to develop a rapid, sensitive and reliable method for the separation of phosphonodipeptide prodrugs and parent compounds to facilitate the evaluation of cell permeation using in vitro cell culture models. Separation was accomplished isocratically within 10.0 min using a C_{18} (150×4.6 mm I.D., 3 μm) reversed-phase column. The mobile phase consisted of 5 m*M* tetrahexyl ammonium (ion-pair reagent) in 0.02 *M* phosphate buffer pH 6.5–acetonitrile (48.5:51.5, v/v). The flow-rate was 1.1 ml/min with detection at 221 nm. The standard curves were linear (r^2>0.999) over the concentration range 1–100 μ*M*. The method was reliable and reproducible, with the limit of quantitation being 1 μ*M* (25 ng on column).

Keywords: Phosphonodipeptides

1. Introduction

Investigation into the role of various protein kinases involved in signal transduction pathways is currently the focus of a number of laboratories. The Src family of non-receptor protein tyrosine kinases is one example. While the Src family of kinases have a role in the normal functioning of signal transduction pathways, they are involved in other responses such as changes in cell shape, cell contact and negative growth control upon deregulation or mutation [1]. Therefore, molecules that interfere with and/or inhibit signal transduction pathways may serve as useful agents in the treatment of various diseases such as cancer, osteoporosis and AIDS [2]. We are investigating dipeptide-based compounds, specifically phosphonodipeptides (PDPs) that possess Src SH2 domain-binding affinities [3,4].

One of the barriers to the successful delivery of these compounds is the inherently poor cellular penetration owing to the dianionic nature of the phosphonate moeity. To circumvent this obstacle, we have synthesized diethyl ester prodrugs of the most potent PDPs (Fig. 1) [5]. These prodrugs are expected to cross the cell membranes more efficiently than the parent and be reconverted to the active parent inside the cell [6]. Therefore, it was necessary to develop a HPLC method for quantitation of the

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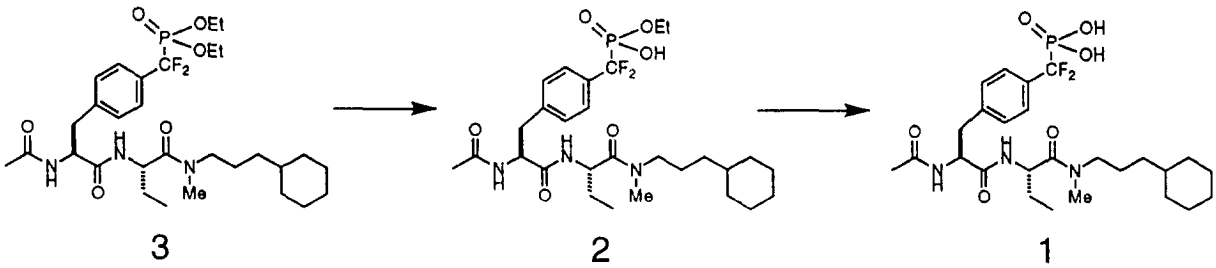

Fig. 1. Structures and proposed pathway of reconversion of 3 (diester) to 2 (monoester) and 1 (parent diacid).

diester prodrug, the intermediate monoester and the active diacid parent. The original method consisted of monitoring the diester prodrug and the diacid using a conventional reversed-phase column with a gradient of 0–100% acetonitrile in 20 min and a total cycle time of 40 min. Although separation was achieved, a relatively long analysis time per sample, high solvent consumption and poor sensitivity limited the use of this procedure for routine quantitative analyses.

The ion-pair reversed-phase chromatographic method developed here exploited the differences in the ionic nature of these compounds, i.e., ester prodrug (neutral), intermediate monoester (anionic) and the parent (dianionic). With the aid of tetrahexyl ammonium (THA) as an ion-pairing agent, efficient separation has been achieved. This method was rapid, reliable and reproducible, with a total cycle time of 12 min per sample and a limit of quantitation of 1 μM (25 ng on column).

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Spectra-Physics (Fremont, CA, USA) SP 8700 extended-range LC pump, SP 8780 autosampler with a 100-μl sample loop, and a Waters (Milford, MA, USA) variable-wavelength Lambdamax 481 detector set at 221 nm. The analytical column was a Supelcosil LC 18-DB (Bellefonte, PA, USA, 150×4.6 mm I.D. 3 μm) and an Alltech (Deerfield, IL, USA) C_{18} guard column. Peak recording and integration was accomplished with a Spectra-Physics Chromjet integrator.

2.2. Preparation of standard solutions and standard curves

Compounds (>99% purity) (3) (diethyl ester), (2) (monoester) and (1) (diacid) analogs of phospho(difluoromethyl)phenylalanine were used throughout the course of this study (Fig. 1). Stock solutions (20 mM) of the diester and monoester were prepared in analytical grade ethanol, whereas the diacid was in HPLC-grade water. A working stock solution (1 mM) of each compound in 50% (v/v) acetonitrile (ACN) in water was prepared fresh daily. Serial dilutions of the working stock solution with 50% (v/v) ACN in water were used to obtain the desired concentrations of the calibration standards (1–100 μM). Standard curves were constructed using peak areas of the various compounds. The linearity of the curves was verified using linear regression analyses with appropriate weighting factors.

2.3. Mobile phase

HPLC-grade acetonitrile and water were obtained from EM Separations (Gibbstown, NJ, USA). Phosphate buffer (20 mM) was prepared by adding 3.48 g of dibasic potassium phosphate (EM Science, Gibbstown, NJ, USA) to 1 l of HPLC-grade water. The solution was adjusted to pH 6.85 with phosphoric acid (EM Science). 2.25 g (5 mM) of tetrahexyl ammonium hydrogen sulfate (Cat No. 39692-3, Aldrich, Milwaukee, WI, USA) was added to 1 l of the phosphate buffer solution and the pH was adjusted to 6.50±0.05 with 1 M NaOH or phosphoric acid, as necessary. This mixture was filtered through a Nylon-66 membrane filter (0.45 μm) to eliminate any particulate matter. The mobile phase consisted of aqueous component (phosphate buffer

with THA) and acetonitrile (48.5:51.5, v/v), degassed with helium before introduction to the pump.

2.4. Validation (accuracy and precision)

Validation of the method was accomplished by assaying eight different concentrations of the three compounds (diethyl ester, monoester and the diacid) for five days (i.e., $n=5$). This enabled between-day comparison of accuracy and precision. On one of the days, three injections (i.e., $n=3$) of the eight standards were made to facilitate within-day comparison of accuracy and precision. The % relative error (R.E.), i.e., (true concentration−observed concentration)×100/(true concentration) served as an indicator of accuracy. The % relative standard deviation (R.S.D.), i.e., (S.D.×100)/(mean) of the observed concentrations served as an indicator of the precision.

2.5. Column care

Since conditioning of the column using ion-pair chromatography takes much longer (4–8 h) relative to conventional reversed-phase high-performance liquid chromatography (RP-HPLC), the column was continuously washed with recirculating mobile phase when samples were not being analyzed. Non-routine maintenance consisted of washing the column successively with 100% water, followed by 50% ACN in water and storage in 90% ACN in water. The guard column was routinely changed to avoid excessive pressure build-up in the HPLC system.

2.6. Cell uptake studies

The Balbc3T3 fibroblast cell line was obtained from ATCC (Rockville, MD, USA) and maintained with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, and pencillin–streptomycin. The cells were routinely grown in T-75 flasks, harvested with trypsin-EDTA and plated at a density of 2.5×10^5 cells/ml in 4.71 cm^2 6-well clusters. On the day of an uptake experiment, the medium was aspirated and the cell monolayer was rinsed twice with 1 ml of prewarmed (37°C) uptake buffer (5 m*M* KCl, 132.5 m*M* NaCl, 1.8 m*M* $CaCl_2$, 10 m*M* MOPS, pH 7.4). One ml of the incubation solution (100 μ*M* of the compound in the uptake buffer) was added to each well. The cluster was mounted on a plate shaker and then incubated for different time periods (0–180 min) at 37°C. At the end of the incubation period, each well was sampled (50 μl) to determine the concentration of the compound in the extracellular milieu. Subsequently, the dosing solution was aspirated, each well was rinsed with 2 ml of ice-cold uptake buffer, and the entire cluster was sonicated in 1.0 ml of water for 20 min to lyse the cells. After aliquoting a portion of this lysate for protein determination, 600 μl was transferred to an Eppendorf tube and centrifuged at 16 000 *g* for 10 min, and a 100-μl sample was then transferred to an autosampler vial. ACN (500 μl) was added to the remaining solution (500 μl) in the Eppendorf tube, vortexed for 1 min and centrifuged at 16 000 *g* for 10 min. A 100-μl sample of the supernatant was then combined with the 100-μl aliquot of the aqueous supernatant in the autosampler vial and assayed using the HPLC conditions outlined above. Blank lysates in uptake buffer devoid of the drug were prepared using the same method.

3. Results

Representative chromatograms obtained using the ion-pair RP-HPLC method are shown in Fig. 2. The three peaks of interest were well resolved from each other. Peak areas of each of the three compounds were plotted against concentration and least-squares linear regression analyses was performed. Table 1 summarizes the calibration curves for the three compounds. Plots were linear over the concentration range of 1–100 μ*M* with a correlation coefficient (r^2) >0.999. Statistical analyses showed that the slopes were significantly different from zero ($P<0.001$) and the intercepts were not significantly different from zero ($P>0.01$). The within-day and between-day accuracy and precision, as measured by %R.E. and %R.S.D., respectively, are summarized in Table 2 Table 3. These results confirmed that the method developed here was both reliable and reproducible. The limit of quantitation was 1 μ*M* using the criterion that the signal from the minimum quantifiable peak should be three or more times the baseline noise level.

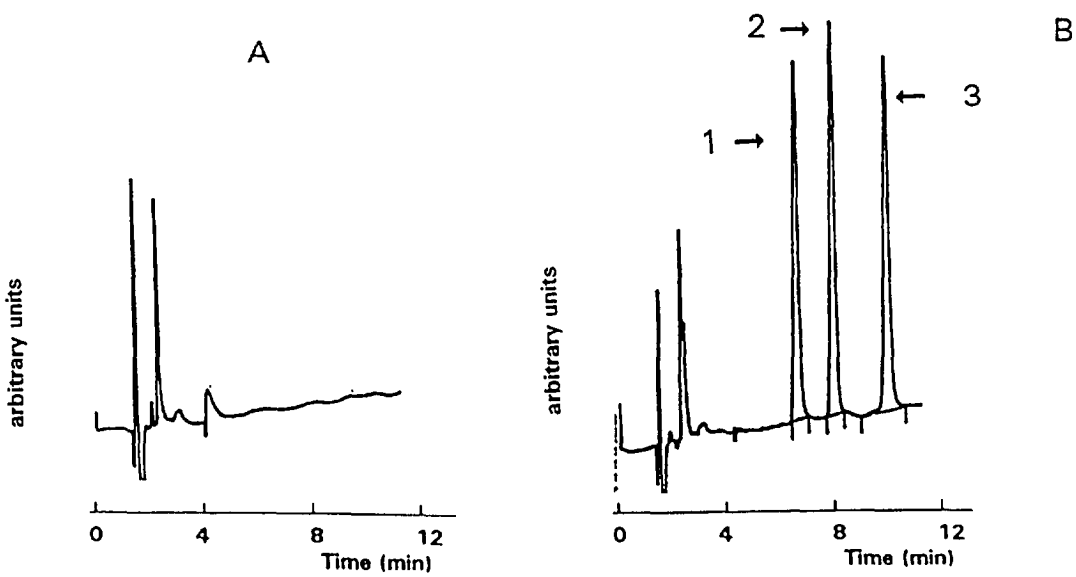

Fig. 2. Representative chromatograms of 1, 2 and 3 using the ion-pair reversed-phase HPLC method. (A) Blank matrix (50% ACN in water), and (B) a 5 μ*M* standard of each compound in the same matrix.

Table 1
Summary of calibration curves for 1, 2 and 3

Compound	Slope[a] (mean±S.E.)	Intercept[b]	*n*	r^2
1	0.57±0.03	−0.10	5	0.999
2	0.64±0.06	−0.03	5	0.999
3	0.68±0.07	−0.01	5	0.999

[a] Slopes were significantly different from zero at $P<0.001$.
[b] Intercepts were not significantly different from zero at $P<0.01$.

A practical application of this method was to study the uptake of these three compounds in Balbc3T3 cells. Representative chromatograms using the ion-pair RP-HPLC method developed here are shown in Fig. 3. The peak of interest was observed to be well resolved from the endogenous compounds in the cell lysates. The peak areas obtained by incubation of each of these compounds for each time period was converted to their respective concentrations using a

Table 2
Between-day variability for the assay of 1, 2 and 3

Concentration	1		2		3	
(μ*M*)	R.E. (%)	R.S.D. (%)	R.E. (%)	R.S.D. (%)	R.E. (%)	R.S.D. (%)
1	−0.51	3.47	−0.72	3.72	−1.94	10.90
5	2.55	2.75	4.10	1.48	4.65	5.54
10	1.82	0.95	1.48	2.60	2.11	3.69
20	1.58	2.02	1.98	2.50	1.63	2.18
30	0.18	1.92	0.42	1.48	0.02	0.96
40	−0.69	1.72	−0.86	2.19	−0.66	1.86
50	−2.09	3.33	−2.00	3.74	−2.09	3.64
100	−3.47	1.12	−4.13	1.19	−4.78	1.75

%R.E. indicates accuracy and %R.S.D. indicates the precision, $n=5$.
%R.E.=(True concentration−observed concentration)×100/(true concentration).
%R.S.D.=(S.D.×100)/(mean).

Table 3
Within-day variability for the assay of 1, 2 and 3

Concentration	1		2		3	
(μM)	R.E. (%)	R.S.D. (%)	R.E. (%)	R.S.D. (%)	R.E. (%)	R.S.D. (%)
1	−1.80	3.72	−1.63	1.47	−1.39	4.61
5	8.47	2.77	6.55	1.63	5.48	2.40
10	2.25	1.57	4.19	1.71	3.48	0.66
20	0.79	0.22	1.29	0.70	0.86	1.02
30	−2.06	0.87	−1.71	0.66	−1.26	0.67
40	−0.80	0.75	−0.68	0.25	−0.61	0.29
50	−2.76	0.80	−2.57	0.43	−0.37	3.92
100	−4.40	0.54	−5.52	0.53	−6.57	0.80

%R.E. indicates accuracy and %R.S.D. indicates the precision, $n=5$.
%R.E.=(True concentration−observed concentration)×100/(true concentration).
%R.S.D.=(S.D.×100)/(mean).

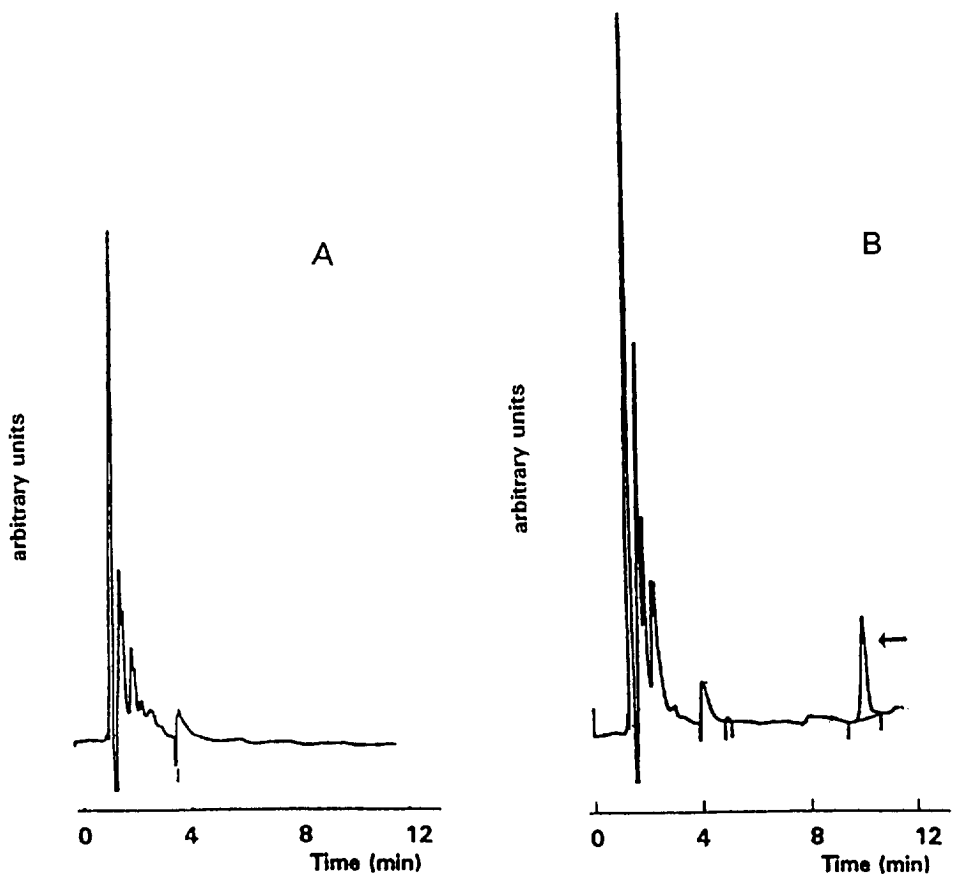

Fig. 3. Representative chromatograms of uptake studies. (A) Blank lysate, and (B) a sample of intracellular uptake of 3 in Balbc3T3 cells after incubation with this compound for 3 min. The arrow refers to the elution position of 3. No reconversion to 1 or 2 was observed.

standard curve generated on the day of the experiment. The uptake rate (i.e., the slope of the plot of the amount taken up versus time) of compound (3) in these cells was calculated to be 0.4 nmol/min cm^2. No uptake of compound (1) or compound (2) was observed (data not shown).

4. Discussion

The objective of this research was to develop a rapid reliable and reproducible HPLC assay for the separation of PDP analogs in order to evaluate the cell permeation of these compounds using an in vitro cell monolayer model. In addition, as a part of drug discovery support, it was necessary to develop a relatively simple method with high throughput and the ability to be extended to the separation of other PDP-based novel analogs. Initially, quantitative analyses for this set of compounds was accomplished by adapting the conditions used in preparative RP-HPLC. Although separation and quantitation were achieved, this method suffered from certain drawbacks such as relatively long sample analysis time and poor sensitivity. The method reported here is a significant improvement over the original procedure and was successfully applied to the study of the uptake of PDPs in Balbc3T3 cells. Salient features of this method include better separation, 12 min cycle time per sample and a limit of quantitation of 1 μM.

The rationale for choosing the ion-pair mode for the separation of the three compounds in this report was based on the differences in their ionic nature. While the parent diacid is dianionic, the monoester is monoanionic and the diethyl ester prodrug is neutral. Therefore, with the aid of a suitable ion-pair reagent of opposite charge, separation can be achieved based on the differences in the interaction of the paired complex with the stationary phase. Initial success with tetrabutyl ammonium phosphate confirmed the utility of this approach. However, THA was finally chosen due to the better retention and the selectivity offered by the hexyl over the butyl side chain of the quarternary amine. This is in accordance with the theory of ion-pair chromatography where it has been shown that the addition of each methylene group to the counter-ion can potentially result in a change in the k' (capacity factor) values by a factor of up to 2.5 for a 1:1 ion pair, whereas the relationship is more complex for a 1:2 ion-pair [7]. Although the determination of the stoichiometry of the ion-pair formation is beyond the scope of this study it was encouraging to note that in this case the retention time of the diacid (1) moved from about 2.5 min with the butyl to about 6.5 min with the hexyl side chain.

In order to better characterize the method, the dependence of k' on the mobile phase variables i.e., (i) % organic fraction, and (ii) concentration of the ion-pair reagent was explored. With regard to the fraction of the organic component, optimum separation was observed only for the conditions described in Section 2. Even a 2% increase or a decrease in the organic component of the mobile phase caused poor resolution of the diacid and the monoester. If retention was totally governed by the nature of the phosphonate substituent, and, hence, the ion-pair formed with the counter-ion, one would expect the monoester to elute prior to the diacid, whereas an opposite behaviour is noticed. This suggests that although retention of the diacid and the monoester were primarily governed by the paired complex, the dipeptide backbone also contributed to the interaction (therefore retention) of these compounds to the stationary phase. While elucidation of the exact mechanism of retention for these compounds (i.e., ion-pair in the mobile phase vs. dynamic ion-exchange vs. dynamic complex exchange etc.) is beyond the scope of this study, it is clear that multiple interactions are responsible for the retention behaviour observed for these compounds. Peptide-based compounds are known to be more sensitive to the fraction of organic component in the mobile phase and elute within a narrow window of aqueous-to-organic phase volume ratio [8]. Thus, the sensitivity in the retention of the diacid and monoester exhibited for even a small change in the organic content is suggestive of a distinct role for the dipeptide backbone in the overall retention of these compounds.

With regard to the dependence of k' on the concentration of the ion-pair reagent (tetrabutyl ammonium phosphate), only slight increases ($<10\%$) in the retention of the diacid and monoester were observed over a ten-fold range of the concentration of the ion-pair reagent. This result was not surprising since at the pH of the mobile phase (6.50 ± 0.05), more than 85% of the phosphonate

groups were ionized as the pK_as of these groups are <2 and 5.7 [9]. Therefore, attempts to increase the solvent strength by increasing the concentration of the ion-pair reagent can be expected to result in only moderate changes in retention. This observation was in good agreement with that of Snyder et al. [7]. It should be mentioned that compound (3) was unaffected by changes in the concentration of the ion-pair reagent used.

Although ion-pair RP-HPLC for the separation of PDPs is not well-established, attempts have been made to separate diastereomers of protected and unprotected PDPs using ion-exchange-, normal- and reversed-phase HPLC using octadecyl, nitrile and aminopropyl sorbents [10,11]. While these authors have successfully separated diastereomers of protected and unprotected peptides individually, no attempt was made to separate a mixture of protected and unprotected PDPs.

Finally, as shown in Fig. 4, this method has been successfully used in our laboratory to separate and quantitate two novel analogs of the parent PDP, thereby demonstrating the versatility of this method. Although significant uptake of compound (3) was realized (Fig. 3), no reconversion of the diethyl ester to the intermediate monoester or the parent diacid was observed. Our current focus is to develop prodrugs with bioreversible progroups; i.e., groups that not only enhance transport across the cell monolayer but are also capable of yielding the active parent subsequent to internalization of the prodrug molecule.

Acknowledgments

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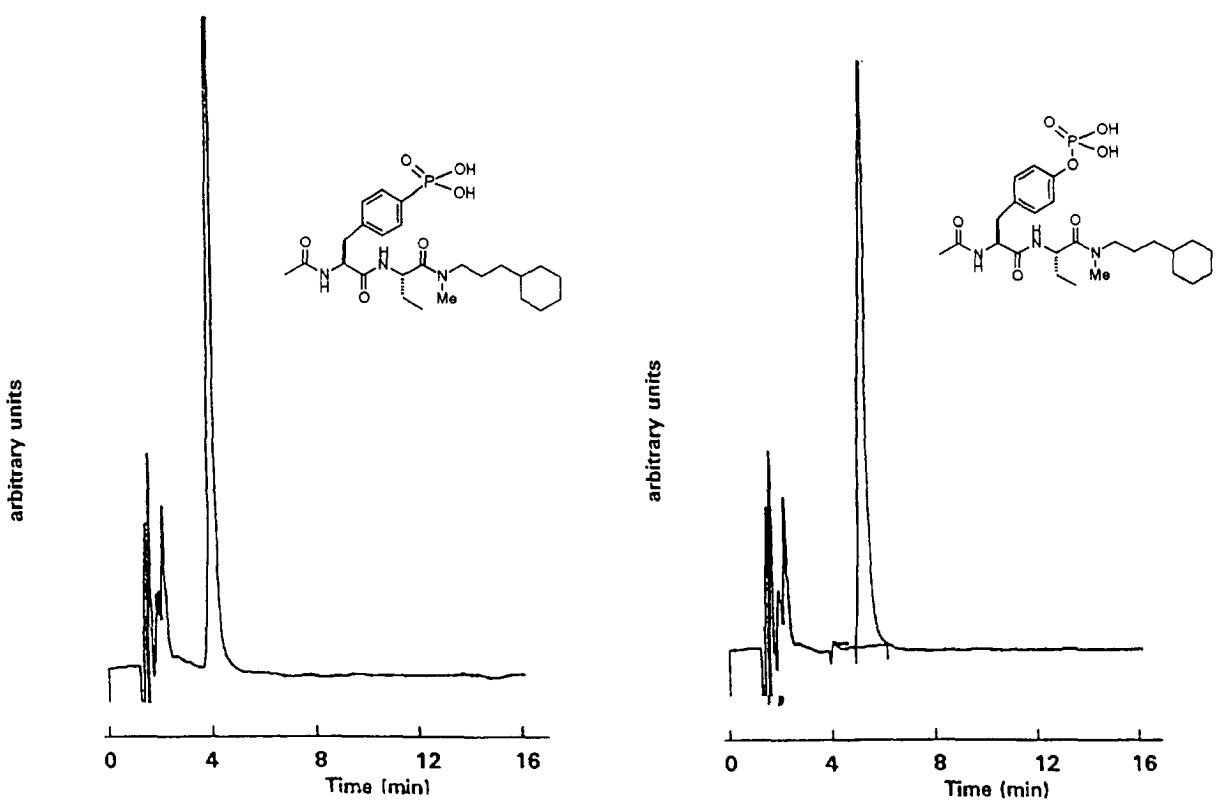

Fig. 4. Representative chromatograms and structures of two novel PDP analogs separated using the outlined method.

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