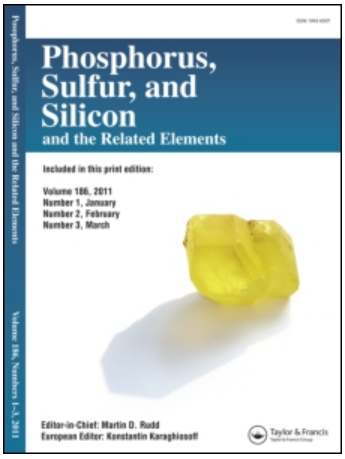

Phosphorus, Sulfur, and Silicon and the Related Elements

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713618290

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Peng Zhang[ab]; Mian Liu[ac]; Hongxia Liu[a]; Zhenhua Xie[a]; Feng Liu[a]; Zongwei Cai[d]; Yuyang Jiang[a]
[a] The Key Laboratory of Chemical Biology, Guangdong Province, Graduate School at Shenzhen, Tsinghua University, Shenzhen, P. R. China [b] Department of Chemistry and Chemical Engineering, Henan University of Technology, Zhengzhou, P. R. China [c] Chemistry Department, Key Laboratory of Chemical Biology and Organic Chemistry of Henan, Zhengzhou University, Zhengzhou, P. R. China [d] Department of Chemistry, Hong Kong Baptist University, Kowloon Tong, Kowloon, P. R. China

To cite this Article Zhang, Peng , Liu, Mian , Liu, Hongxia , Xie, Zhenhua , Liu, Feng , Cai, Zongwei and Jiang, Yuyang(2008) 'The Pharmacokinetics Analysis of the Phosphoryl Peptides in MCF-7/ADR Cells', Phosphorus, Sulfur, and Silicon and the Related Elements, 183: 2, 737 — 744
To link to this Article: DOI: 10.1080/10426500701807749
URL: http://dx.doi.org/10.1080/10426500701807749

Phosphorus, Sulfur, and Silicon, 183:737–744, 2008

ISSN: 1042-6507 print / 1563-5325 online
DOI: 10.1080/10426500701807749

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The Pharmacokinetics Analysis of the Phosphoryl Peptides in MCF-7/ADR Cells

Peng Zhang,[1,2] Mian Liu,[1,3] Hongxia Liu,[1] Zhenhua Xie,[1] Feng Liu,[1] Zongwei Cai,[4] and Yuyang Jiang[1,5]

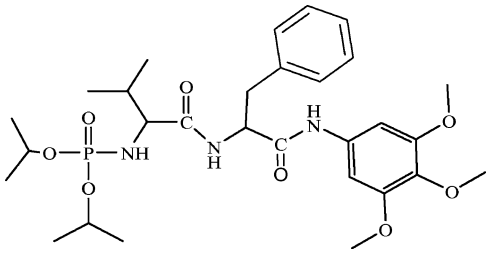
[1]The Key Laboratory of Chemical Biology, Guangdong Province, Graduate School at Shenzhen, Tsinghua University, Shenzhen, P. R. China
[2]Department of Chemistry and Chemical Engineering, Henan University of Technology, Zhengzhou, P. R. China
[3]Chemistry Department, Key Laboratory of Chemical Biology and Organic Chemistry of Henan, Zhengzhou University, Zhengzhou, P. R. China
[4]Department of Chemistry, Hong Kong Baptist University, Kowloon Tong, Kowloon, P. R. China
[5]School of Medicine, Tsinghua University, Beijing, China

We have developed and validated a practical high-performance liquid chromatography method to determine the concentration of N-diisopropyloxyphosphoryl-valine-phenylalanine-3, 4, 5-trimethoxy aniline (DIPP-Val-Phe-TMOA) in Adriamycin-resistant human breast adenocarcinoma (MCF-7/ADR) cells. First, DIPP-Val-Phe-TMOA was extracted from MCF-7 cells with perchloric acid, followed by a cleanup procedure with centrifugal separation, and then determinated by HPLC using a Waters C18 column with a gradient elution (solvent A: 10 mM ammonium acetate at pH 7.0, B: acetonitrile). The flow rate was maintained at 1.0 ml/min at room temperature. The detection wavelength was at 254 nm. Meanwhile, this validated method was successfully applied to analyze the concentration levels and the pharmacokinetics parameters of DIPP-Val-Phe-TMOA in MCF-7/ADR cells.

Keywords Determination; high-performance liquid chromatography; MCF-7/ADR cells; N-diisopropyloxyphosphoryl-valine-phenylalanine-3,4,5-trimethoxyaniline

This work was supported by the National Natural Science Foundation (China, 20472043, 20572060, 20672068), the Project of Science and Technology of Guangdong Province (2005A11601008), Mega-Project of Science Research of Ministry of Science and Technology (China, 2005CCA03400), and the Natural Science Foundation of Guangdong Province (China, 06028200).

Address correspondence to Dr Yuyang Jiang, the Key Laboratory of Chemical Biology, Guangdong Province, Graduate School at Shenzhen, Tsinghua University, Shenzhen 518055 P. R. China. E-mail: Jiangyy@sz.tsinghua.edu.cn



SCHEME 1 The structure of dipp-val-phe-tmoa.

INTRODUTION

It is well known that more N-phosphoryl peptides and their analogues have been becoming a class of novel antineoplastic drugs.[1–3] The modification of amino acids and peptides, such as phosphorylation, methylation, acetylation, and glycosylation, might have incurred a great improvement in their physiochemical property.[3–5] For example, N-[(a-rh-aminopyranosyloxy) hydroxyphosphinyl]-L-Leu-L-Trp and its analogues were inhibitors of endothelin converting enzyme.[6] Now we have synthesized a series of N-(O,O-diisopropyl) phosphoryl peptide derivatives in our laboratory. Our previous studies demonstrated that these compounds would possess higher hydrophobicity, and better bioavailability and pharmacological activity. N-diisopropyloxyphosphoryl-valine-phenylalanine-3,4,5-trimethoxyaniline(DIPP-Val-Phe-TMOA; Scheme 1) is a new potential pre-drug that was found to induce the apoptosis of multiple tumur cells.[7] Meanwhile, the previous results indicated that the pharmaceutical efficiency of this compound mainly depends on the intracellular concentration levels. For further investigation of its pharmaceutical effect, it is necessary to establish a practical approach to observe and measure the concentration variation of DIPP-Val-Phe-TMOA in cells.

So far, several methods, such as ion chromatography (IC),[8] gas chromatography,[9] high-performance capillary electrophoresis (HPCE),[10–11] and high-performance liquid chromatography (HPLC)[12–14] have been frequently used to analyze peptides and their derivatives. For the past half century, HPLC has been proven to be the most prevalent technique because of its rapidity, precision, accuracy, simpleness, and automation. Thus, in the present article, HPLC was the preferential method for the analysis of DIPP-Val-Phe-TMOA. The aims of this article included: (1) to develop a practical extraction and cleanup procedure prior to the analysis of DIPP-Val-Phe-TMOA in the MCF-7/ADR cells; (2) to develop a practical HPLC method for

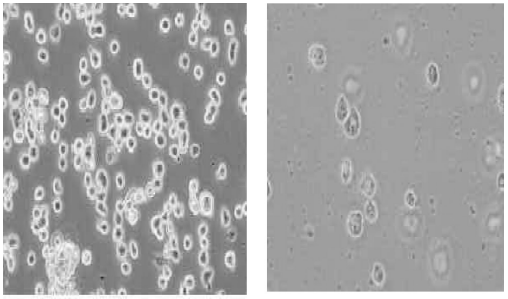


FIGURE 1 The microscope spectra of MCF-7/ADR cells in culture medium. (a) MCF-7/ADR cells in RPIM-1640 culture medium, (b) MCF-7/ADR cells treated by 0.5% perchloric acid.

measurement and evaluation of the concentration level of DIPP-Val-Phe-TMOA in MCF-7/ADR cells; and (3) to obtain the pharmacokinetics parameters of DIPP-Val-Phe-TMOA in MCF-7/ADR cells.

RESULTS AND DISCUSSION

Sample Preparation

Many methods could be used to extract the compounds from live cells, such as ultrasonic extraction, enzymatic degradation, organic solvent extraction, acidic extraction, and so on. In this assay, two different extraction solvents, methanol and perchloric acid, were used to treat live cells. The shapes of the cells' exposure after 1 min were investigated visually under an Olympus IX-50 microscope at 40-fold magnification. It is clearly seen from Figure 1 that cells treated with perchloric acid were hardly visible in the matrix, whereas cells treated with methanol showed no obvious change in shapes and numbers, which need more than 15 min for the complete release of compounds.

To test the stability of DIPP-Val-Phe-TMOA in perchloric acid, the standard solutions of the same concentration were prepared with methanol and perchloric acid, respectively, and then DIPP-Val-Phe-TMOA were measured by described HPLC method. The closer peak areas were observed for both DIPP-Val-Phe-TMOA–tested solutions. Therefore, perchloric acid was seleted to extracting DIPP-Val-Phe-TMOA from MCF-7/ADR cells.

HPLC Separation

With consideration of the structure and lipophilic of DIPP-Val-Phe-TMOA, the reversed phases HPLC was first applied as the separation

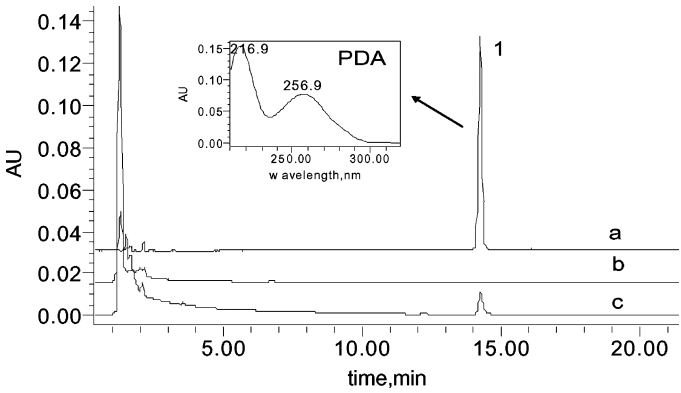


FIGURE 2 Chromatogram and UV spectrum of Dipp-Val-Phe-TMOA. (a) Standard solution of Dipp-Val-Phe-TMOA, (b) culture blank, and (c) the extraction of Dipp-Val-Phe-TMOA in MCF-7/ADR cells. HPLC conditions: the separation column was Waters C_{18} column (Symmetry, 150 × 4.6 mm, 3.5 μm), the mobile phases consisted of 10 mM ammonium acetate (NH_4Ac) at pH 7.0 (A) and acetonitrile (B). The elution program was as follow: 30% B was increased to 70% from 1–20 min; and then 70% B was decreased quickly to 30% at 21 min. The column was re-equilibrated for 15 min prior to the next injection. The flow rate was at 1.0 ml/min at room temperature. The detection wavelength was at 254 nm. The sample injection volume was 50 μl. Peak 1: Dipp-Val-Phe-TMOA.

mode with the linear gradient program to achieve the better resolution. Meanwhile, based on the photodiode array detector (PAD) results from the main peak of standard amperometric detection (Figure 2), the detection wavelength for the determination of DIPP-Val-Phe-TMOA was set at 254 nm, which corresponds to the peptide bond and phenyl in its structure. Under these conditions, the typical chromatograms for standard solution and extractions from cell incubation with or without DIPP-Val-Phe-TMOA were shown in Figure 2, indicating that the DIPP-Val-Phe-TMOA could be baseline separated within 20 min without interference from the background matrix.

Evaluation of HPLC Method

We dissolved DIPP-Val-Phe-TOMA in methanol to make 2000 μg/ml stock solution. The standard solutions were prepared by diluting stock solution in the mobile phase to form the final concentrations of 1, 5, 10, 20, 200, and 2000 μg/ml. Each determination was performed triplicately. Under the optimal HPLC conditions, the linear relationship between the amount of DIPP-Val-Phe-TMOA and the peak area monitored

TABLE I Recovery of Dipp-Val-Phe-TMOA

Component	Added amount (ng)	Measured amount (ng)	Recovery (%)	Mean (%)	RSD (%)
DIPP-Val-Phe-TMOA	5000	5116.82	102.34	100.32	1.90
		4927.55	98.55		
		5003.62	100.07		
	1000	939.11	93.91	94.82	1.56
		965.23	96.52		
		940.17	94.02		
	250	247.75	99.10	98.65	0.42
		245.72	98.29		
		246.39	98.55		

at 254 nm was tested. The representative calibration equation was $y = 546.55x–518.15$ ($n = 6$), with the regression correlation coefficient $R^2 = 0.9998$ over the concentration range from 10 to 20000 ng. The limit of detection (LOD) for DIPP-Val-Phe-TMOA was 2.73 ng at a signal/noise ratio of 3:1.

The recovery and relative standard deviation (RSD) were tested at three concentrations by spiking the sample with 250 ng, 1000 ng, and 5000 ng standard in culture medium, respectively. Then, DIPP-Val-Phe-TMOA was extracted using sample extraction procedure. The recoveries were calculated by comparing the measured amount with the injected amount. The results were shown in Table 1. From the table, it could be seen that the recoveries ($n = 3$) were between 94.82% and 100.32%, with RSD ranging from 0.42% to 1.90%.

The intra-day and inter-day reproducibility were estimated by calculating the RSD of retention times and peak areas of the standard solution (200 μg/ml). The intra-day RSDs ($n = 5$) of retention times and peak areas were 0.05% and 0.7%, respectively. The inter-day RSDs ($n = 3$) of retention times and peak areas were 0.55% and 1.2%, respectively.

Pharmacokinetics Analysis of DIPP-Val-Phe-TOMA in MCF-7 Cell Lines

Determination of the concentration of DIPP-Val-Phe-TMOA in MCF-7/ADR cells was conducted by the proposed HPLC method. The concentration-time curve was shown in Figure 3. It can be seen that the amounts of DIPP-Val-Phe-TMOA in the cells reached the maximum value within 4 h and went rapidly down to a moderate level within 8 h. After 8 h it maintained at a moderate level for more than 10 h. The pharmacokinetics calculation showed that the peak

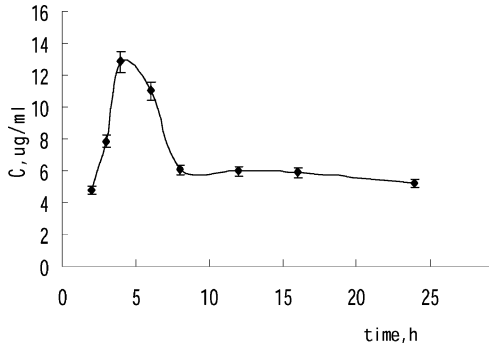


FIGURE 3 The concentration of Dipp-Val-Phe-TMOA in MCF-7/ADR cells HPLC conditions as Figure 2.

concentration (C_{max}) of DIPP-Val-Phe-TMOA in MCF-7/ADR cells was up to 12.32 μg.mL^{-1} at 3.15h. The absorption half-life ($T_{1/2Ka}$) and elimination half-life ($T_{1/2Ka}$) of DIPP-Val-Phe-TMOA was 2.67 ± 0.05 h and 10.24 ± 0.07 h, respectively. The area under the cell concentration time curve (AUC) was 203 ± 0.456 μg/h/mL^{-1}. It suggested that DIPP-Val-Phe-TMOA possessed higher hydrophobicity and could easily penetrate into the membrane of MCF-7/ADR cells. The results also revealed that DIPP-Val-Phe-TMOA has better biological activity and longer half lifetime. These are consistent with the results of biological experiments.[15]

EXPERIMENTAL

Chemicals

Adriamycin-resistant human breast adenocarcinoma cell (MCF-7/ADR) was purchased from Shanghai Institute of Cell Biology of Academy of Chinese Sciences. HPLC-grade Acetonitrile(ACN) and methanol(MeOH) were purchased from Fisher Scientific. Phosphate-buffer saline (PBS) stock solution (pH = 7.2 ~ 7.4) was prepared with 8 g/l NaCl, 0.2 g/l KCl, 3.6 g/l Na_2HPO4, and 0.2 g/l KH_2PO4. Water was purified using Milli-Q purification equipment. DIPP-Val-Phe-TOMA was synthesized in our laboratory (which was characterized using a Bio-Rad FTS-40 FTIR, elemental analysis, Bruker DPX-400 NMR, and Esquire 3000 ion-trap ESI-MS^n). Unless specified, all chemicals and solvents were of analytical reagent grade and were obtained from Beijing Chemical Factory (Beijing, China). All solvents and sample solutions used for HPLC were filtered through a 0.22 um membrane.

Cell culture

RPMI-1640 medium (Gibco, USA) was supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, USA), 100 units/ml of penicillin, 100 μg/ml of streptomycin, 2 mmol/l L-glutamine. MCF-7/ADR cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C. Within the following days, the cultures were generated continually with fresh RPMI–1640 medium until a density of 1×10^7 cells per ml was obtained. Cells were counted using a hematocytometer under the microscope (Olympus IX-50, Japan). The final volume of cultures was 100 ml.

Sample extraction procedure

Cells in logarithmic growth phase were incubated with DIPP-Val-Phe-TMOA (195 μg/ml) in eight culture tubes (each with 10 ml culture medium) at 37°C, respectively. Unless otherwise noted, 1×10^7 cells were used for per incubation. The culture times were 2, 3, 4, 6, 8, 12, 16, and 24 hours, respectively. At the end of the different incubation period, culture medium was removed from incubator, then washed the cells twice with ice-cold phosphate-buffered saline (PBS) and harvested with a cell scraper. The cells were transferred to a microfuge tube and centrifuged (Beckman Allegra 12R) at 2000 rpm for 4 min at 4°C. The pelleted cells were then lysed and protein precipitated by the addition of 1-ml 0.5% cold perchloric acid (PCA) for 1 min. The samples were vortexed and centrifuged (Beckman Allegra 60R) at 10000 rpm for 10 min at 4°C. The suspensions were stored at –80°C until analysis. All the extraction steps were performed on ice.

HPLC conditions

The HPLC measurements were performed on a Waters Allilance HPLC system (Waters Co. Ltd, USA) consisted of a 2695 delivery pump and 2996 photodiode array detector (PAD). Separation was carried out on a Waters C18 column (Symmetry, 150×4.6 mm, 3.5 μm, USA) in combination with a guard column caritridge (XTerra Sentry, 5.0×4.6 mm, 3.5 μm, Waters). The mobile phases consisted of 10 mM ammonium acetate (NH_4Ac) at pH 7.0 (A) and acetonitrile (B). The elution program was as follow: 30% B was increased to 70% from 1–20 min; and then 70% B was decreased quickly to 30% at 21 min. The column was re-equilibrated for 15 min prior to the next injection. The flow rate was maintained at 1.0 ml/min at room temperature. The sample injection volume was 50 μl. The detection wavelength was at 254 nm. These conditions were used for all experiments unless specified otherwise.

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