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# Suppression of peak tailing of phosphate prodrugs in reversed-phase liquid chromatography

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ABSTRACT

Peak tailing of phosphate prodrugs in acidic mobile phases was thoroughly investigated. The results indicated that both metal–phosphate interactions and silanophilic interactions contributed to the observed peak tailing. Column pretreatment with phosphate buffers was demonstrated to be an effective and robust approach in suppressing metal–phosphate interaction. Silanophilic interactions, such as hydrogen bonding interactions between protonated isolated silanol groups and partially deprotonated phosphate groups were mobile phase pH dependent. The combination of column pretreatment and volatile low pH mobile phase buffers can be used to mitigate peak tailing issues in developing MS compatible RPLC methods for phosphate prodrugs. The use of non-endcapped columns should be avoided in RPLC analysis for phosphate prodrugs due to large amount of residual silanol groups in the stationary phases.



## 1. Introduction

Prodrugs are pharmacological substances that are administered in an inactive form and then metabolized in vivo into the active compound. As new drug candidates in modern drug discovery, they have become increasingly popular in the last decade as an effective approach to improve drug solubility and bioavailability. Phosphate prodrugs are one type of prodrugs using functional group approach [1]. Peak tailing has been known to be one of the analytical challenges provided by phosphate compounds, such as nucleotides, phosphopeptides, phospholipids and phosphorylated sugars in reversed-phase liquid chromatography (RPLC) [2–7]. Peak tailing can hinder reproducible integration and accurate quantitation due to lower detection sensitivity and poor resolution of impurity peaks which elute on the tail of a phosphate compound. To ensure the quality of analytical data, tailing factors are often used as system suitability criteria for HPLC methods in pharmaceutical analysis. For example the European Pharmacopeia suggests default tailing factors of between 0.8 and 1.5 [8].

Most studies on peak tailing of phosphate compounds have focused on the effect of active metal ions existing on the surface of LC flow path, such as stainless tubing, column frits, stationary phases and eletrospray probes in MS detectors [2,6,7,9]. A generally accepted explanation is that peak tailing is attributed to the strong chelating complexation between phosphate moieties and active metal ions, such as Fe(III), on the surface of LC flow path. Immobilized metal-ion affinity chromatography has utilized this kind of complexation to achieve selective separation and concentration of phosphopeptide from non-phosphorylated peptides [10,11]. Though no direct evidence on such complex formation during reversed-phase LC separation has been reported, indirect evidence, such as observation of improved peak shape of nucleotides after replacing stainless steel tubing with PEEK tubing, suggest such interactions are a cause of peak tailing [7]. Meanwhile, such complexes can also be formed between phosphate compounds and metal ions in mobile phases. This has been identified in HPLC analyses of phospholipids and LC–MS analysis of phosphopeptides [5,12]. A few methods to improve peak shape of phosphate compounds in RPLC by suppressing metal–phosphate interaction have been reported. One common approach is to use competing buffers such as phosphates in the mobile phases however, this can lead to MS compatible issues [7,13]. Asakawa et al. reported that a volatile buffer, ammonium bicarbonate, was also effective at reducing peak tailing of phosphate compounds in neutral pH, with the additional advantage of being LC–MS compatible [2]. Wakamatsu et al. reported that peak shape of phosphate compounds could be

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dramatically improved by simply flushing stainless steel tubing and electrospray probe with phosphoric acid prior to sample analysis, and such treatment remained effective even when non-phosphate buffers were used for sample analysis [7]. Yet another approach was reported by Liu et al. that demonstrated LC–MS detection sensitivity of phosphopeptides could be greatly increased by adding EDTA to the sample solutions [5]. These results indicate that strong metal chelating reagents, such as phosphate and EDTA, can effectively shield active metal ions on the surface of LC flow path to prevent further interactions with phosphate compounds.

Most of previous work on phosphate compound peak tailing was conducted on bio-molecules, such as nucleotides, phosphopeptides. Peak tailing of smaller organic synthetic compounds, as such phosphate prodrugs, has not been studied as extensively. Recently during the development of a RPLC method for a phosphate prodrug, it was observed that peak tailing of the phosphate prodrugs increased when 0.1% TFA was replaced with 0.1% phosphoric acid in the mobile phases. This observation appeared to contradict what had previously been reported in the literature, prompting an investigation into other types of secondary interactions that could account for peak tailing observed other than complexation with active metal ions. One possible explanation that was theorized was silanol–phosphate interactions. Though silanophilic interactions, such as cation exchange and hydrogen bonding, between analytes and residual silanols on silica particle surface have been studies [14–17], there are very limited studies published on silanol–phosphate interactions in RPLC. Zakaria and Brown noticed that silanol–phosphate interactions contributed to the retention of nucleotides in RPLC with acidic mobile phases [18]. Kim et al. reported that addition of 0.1–1.0% phosphoric acid into sample solutions could significantly improve the detection sensitivity for phosphopeptides in RPLC–MS analysis [4]. Though the authors suggested that improvement in detection sensitivity was due to phosphoric acid blocking the residual silanol on both fused silica capillary and silica particle surfaces, no experiments were performed to evaluate the role of metal–phosphate interaction. As a result, metal–phosphate interactions cannot be ruled out as the source of the peak tailing observed.

The goal of this work was to gain a better understanding of the mechanism of phosphate prodrug peak tailing in RP HPLC separations and to develop a general approach to improve peak shape with MS compatible mobile phases. While the study focused on the silanophilic interaction, both metal–phosphate and silanophilic interactions have been explored to differentiate the influence that these two interactions had on the observed peak tailing. Endcapped and non-endcapped columns, as well as mobile phase additives/pHs were evaluated. Based on the study results, a mechanism for the cause of phosphate prodrug peak tailing was proposed, and an approach to suppress peak tailing was established.

## 2. Experimental

### 2.1. Chemicals

Acetonitrile (MeCN) and trifluoroacetic acid (TFA) were purchased from J.T. Baker (PA, USA). Sodium phosphate dibasic ($Na_2HPO_4$), ammonium acetate ($NH_4OAc$), phosphoric acid ($H_3PO_4$), hydrochloric acid (HCl), citric acid, and methyl sulfonic acid (MSA), were purchased from EMD (NJ, USA). All chemicals were reagent grade. Phosphate prodrug A and phosphate ester prodrug B (abbreviated structures shown in Fig. 1) were synthesized in house by the Chemical Development Department of Bristol-Myers Squibb (NJ, USA). Shown in Fig. 2 is the structure of the parent compound BMS-582949 [19] for the prodrug A. Three additional prodrug compounds containing the same phosphate moiety as prodrug A were also examined in this work to further expand the scope of the study, however only the results of prodrug A are presented to avoid redundancy in the discussion since all four compounds followed the same trend.

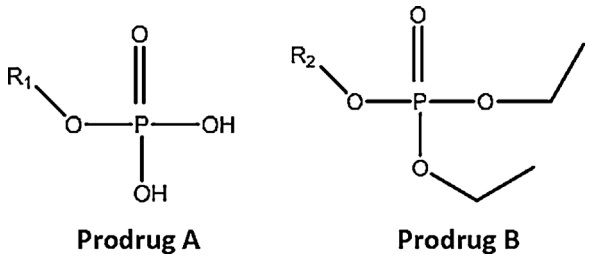


**Fig. 1.** Schematic structures of the prodrugs. R1 and R2 correspond to different parent compound moieties. Prodrug A is a phosphate compound; prodrug B is a phosphate ester.

### 2.2. Instruments

All HPLC separations were performed on a Waters Acquity UPLC system (Milford, MA, USA) that was equipped with a Photodiode Array detector and a binary pump. Data collection and analysis were performed using Empower software. A pH meter (Orion, MA, USA) was used to measure pH of mobile phase A. The majority of the chromatographic separations were conducted using endcapped Acquity UPLC BEHC18 columns of 1.7 μm particle size in dimension 2.1 mm × 100 mm (Waters, Ireland). As a comparison, YMC UltraHT Pro C18 of 2.0 μm particle size at 2.0 mm × 100 mm (YMC, Japan), and a non-endcapped C18 column, Agilent Zorbax SB C18 of 1.8 μm particle size at 2.1 mm × 100 mm (Agilent, USA), were also used in the study.

### 2.3. HPLC conditions

Mobile phase A consisted of aqueous buffer of varying concentrations and mobile phase B was 100% MeCN. The pH values reported in the following experiments and figures refer to the pH value of mobile phase A, which was adjusted with different acidic buffers as described in the following section. A simple linear gradient was used in the HPLC elution starting with 25% B, increasing to 80% B in 7 min, then returning to initial conditions and equilibrating for 4 min prior to the next injection. The flow rate was set at 0.4 mL/min and the column temperature was controlled at 40 °C. Samples were prepared in water:MeCN (60:40) at concentrations of 0.2 mg/mL and 1.2 μL was injected. All UV chromatograms were collected at 239 nm.

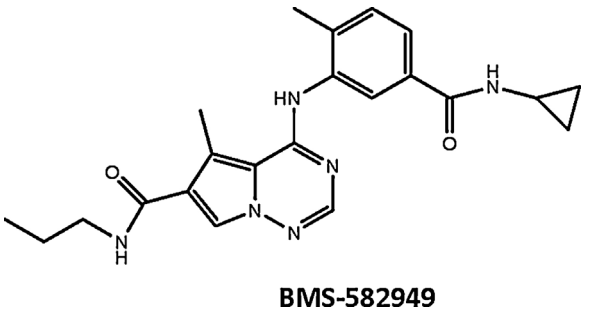


**Fig. 2.** Chemical structures of BMS-582949, the parent compound for prodrug A.