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Selective on-line detection of boronic acids and derivatives in high-performance liquid chromatography eluates by post-column reaction with alizarin

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ABSTRACT

An on-line high-performance liquid chromatography (HPLC) method for the rapid and selective detection of boronic acids in complex mixtures was developed. After optimization experiments at an HPLC flow rate of 0.40 mL/min, the HPLC-separated analytes were mixed post-column with a solution of 75 μM alizarin and 0.1% triethylamine in acetonitrile, which was delivered at a flow rate of 0.60 mL/min. The reaction between alizarin and boronic acids occurred in a reaction coil of dimensions of 3.5 m × 0.25 mm at a temperature of 50 °C, resulting in fluorescent complexes that were detected as positive peaks by a fluorescence detector (λ_{exc} 469 nm and λ_{em} 610 nm). The method enabled the selective detection of various boronic acids and derivatives, with a limit of detection of phenylboronic acid of 1.2 ng or 1 μM. It could successfully monitor the progress of two organic reactions involving boronic acid-containing compounds, and provided useful insights into the course of the reactions.

1. Introduction

Boronic acids are key compounds with increasing relevance in both science and medicine [1]. Many boronic acids have been used because of their unique binding with sugars, in diverse fields of research such as glucose monitoring for diabetic patients [2], fluorescent imaging of cell surface carbohydrates for cancer diagnosis [3] and site-specific immobilization of antibodies for sensitive immunoassays [4]. Boronic acids have also been used as therapeutic agents, Bortezomib being the first commercially approved boron-containing drug [5]. In organic synthesis, boronic acids are well known as building blocks for the synthesis of complex molecules, for example via the famous Suzuki carbon–carbon coupling reaction [6].

Ahead of any application of novel boronic acids, these compounds need to be prepared. From our own experience, they prove to be difficult to synthesize, difficult to analyze and difficult to purify. Among others, one particular problem was the difficult interpretation of thin-layer chromatography (TLC) plates from complex reaction mixtures that contained boronic acids. It was hard to tell whether the much-desired product was formed, and how it could be isolated. To partially solve this issue, we developed a method to stain boronic acids on silica TLC plates in a selective and sensitive way, using the natural dye alizarin [7]. Alizarin shows little fluorescence by itself, but when it interacts with a boronic acid in suitable conditions, a strongly fluorescent boronic ester is formed (Fig. 1). When the alizarin-stained and dried TLC plate is placed under a UV lamp (366 nm), yellow fluorescent spots reveal the presence of boronic acids. This greatly facilitates the analysis of crude reaction mixtures in organic synthesis.

This method is limited, however, to TLC analysis on silica plates. TLC is a useful tool and the first line of approach in organic synthesis. However, its limited separation capacity renders it sometimes essential to analyze a complex reaction mixture by reversed-phase high-performance liquid chromatography (RP-HPLC). For the preparative separation of boronic acids, moreover, preparative RP-HPLC is superior to preparative TLC.

On-line HPLC post-column derivatization has received much attention, as it is a useful tool for the detection of specific analytes in complex mixtures [8]. In this approach an HPLC eluate and reagent solution are mixed together, and the resulting conversion of the analyte of interest allows its selective detection. Our

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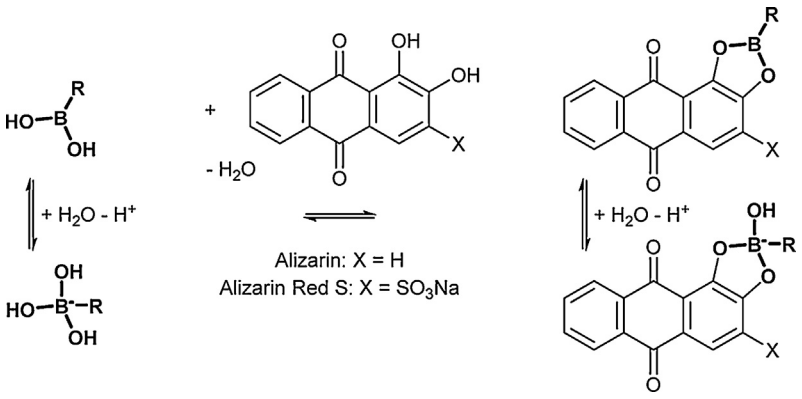

Fig. 1. Reaction of alizarin (or alizarin red S) with boronic acids to form a fluorescent complex.

group has developed different on-line HPLC detection methods for radical scavenging compounds [9]. Such compounds either react post-column with a stable free radical such as DPPH$^{\bullet}$ or ABTS$^{\bullet+}$ [10], or prevent the oxidation of a sensitive probe [11] resulting in a change in absorbance. Such methods proved versatile and could be used preparatively [12] and applied to complex mixtures [13].

Our experience with TLC detection of boronic acids using alizarin and on-line HPLC detection based on post-column reactions led to the idea of combining both techniques with the aim of selectively detecting boronic acids in HPLC eluates to facilitate analysis or subsequent preparative HPLC of reaction mixtures containing boronic acids. In this paper, the development and application of such on-line HPLC detection methodology for boronic acids are presented.

2. Experimental

2.1. Chemicals and their abbreviations

For the analytical experiments, the following chemicals were used: alizarin (97%) and triethylamine (TEA) (99%) from Acros Organics; acetonitrile (ACN) (HPLC-S) from Biosolve BV (Valkenswaard, The Netherlands); methanol (MeOH) (CHROMASOLV®, for HPLC, ≥99.9%), acetic acid (ACS Reagent ≥99.7%), phosphate-buffered saline pH 7.4 (PBS) (cat. no P3813), citric acid, 2-(cyclohexylamino)ethanesulfonic acid (CHES), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), sodium hydroxide, alizarin Red S (ARS) (certified by Biological Stain Commission), phenylboronic acid (PBA) (purum, ≥97.0%), phenylboronic acid pinacol ester, phenylboronic acid *N*-methyliminodiacetic acid (MIDA) ester (97%), potassium phenyltrifluoroborate (95%), 4-trifluoromethylphenylboronic acid (≥95.0%), 4-carboxyphenylboronic acid, 4-methoxyphenylboronic acid (≥95.0%), 3-aminophenylboronic acid monohydrate (98%), butylboronic acid (97%), potassium butyltrifluoroborate (95%), benzoic acid (ACS reagent, ≥99.5%), nitrobenzene (ACS reagent, ≥99.0%), acetophenone (puriss. p.a., ≥99.0%), 4-hydroxybenzamide (98%), chlorobenzene (puriss. p.a., ACS reagent, ≥99.5%), bromobenzene (≥99.5%), *p*-toluenesulfonic acid monohydrate (ACS reagent, ≥98.5%), aniline (ACS reagent, ≥99.5%), phenol (unstabilized, ReagentPlus®, ≥99%) and 4-methoxybenzonitrile (99%) from Sigma–Aldrich; boric acid from an unknown source, sodium borate made in situ from boric acid and sodium hydroxide; benzaldehyde (≥98%) from Fisher Scientific and 4-fluorobenzaldehyde (98%) from VWR International. Ultrapure water (="water") was produced using a Milli-Q Integral 3 system from Millipore (Molsheim, France).

For the esterification reaction, the following chemicals were used: 4-carboxyphenylboronic acid (CPBA) from Sigma–Aldrich, absolute ethanol (AnalaR NORMAPUR® ACS) from VWR International and hydrochloric acid (37%) from Acros Organics.

For the thiol–ene reaction, the following chemicals were used: (4-allylaminocarbonyl)phenylboronic acid (AACPBA) (97%) and *N*-α-*t*-butyloxycarbonyl-L-cysteine (Boc-Cys) (99%) from ABCR (Karlsruhe, Germany), 2,2-dimethoxy-2-phenylacetophenone (DMPA) (99%) from Sigma–Aldrich, HPLC-grade tetrahydrofuran (unstabilized, HPLC-S) from Biosolve BV.

2.2. Off-line fluorescence spectrometry

Solutions containing alizarin (or ARS) and/or boronic acids were placed in quartz cuvettes and analyzed with an Edinburgh Instruments FLS900 Fluorescence spectrometer. A Xe900 lamp was used for excitation and an R928 photomultiplier tube was used for detection. Excitation spectra were measured with λ_{em} 610 nm and emission spectra were measured with λ_{ex} 469 nm.

Measurements were performed about 15 minutes after mixing alizarin (or ARS) with boronic acid solutions. Detailed composition of these solutions can be found in the corresponding figure captions.

2.3. On-line HPLC instrumental set-up

The instrumental set-up is depicted in Fig. 2. The HPLC delivery system consisted of a WellChrom HPLC Pump K-1001 and a WellChrom Solvent Organizer K-1500 (Knauer, Germany). The manual injector was a Multiport Streamswitch MUST HP 6 (Spark Holland, Emmen, the Netherlands) with a loop of 10 μL. Separations were carried out on an Alltima C18 HPLC column (5 μm, 150 mm × 3 mm i.d., 100 Å pore size, Alltech Associates Inc., Deerfield, IL), and the HPLC flow rate was set at 0.40 mL/min. UV detection was carried out using a 2487 Dual λ Absorbance Detector (Waters, Milford, MA) set at a detection wavelength of 254 nm. Post-column delivery of the alizarin solution was achieved as follows: the alizarin solution was displaced from a 150 mL Superloop™ (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) by infusing water into the opposite end of the Superloop™ by means of a Gynkotek High Precision Pump Model 300. The flow rate of this HPLC pump is further described as "alizarin flow rate". The tested reaction coils were made of PEEK tubing (VICI AG International, Schenkon, Switzerland). Post-column fluorescence detection was done using an FP-1520 Intelligent Fluorescence Detector (JASCO, Tokyo, Japan), λ_{ex} 469 nm and λ_{em} 610 nm. Components of the set-up were connected with tubing of various materials and dimensions (details in Table S1).