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A simple way to configure on-line two-dimensional liquid chromatography for complex sample analysis: Acquisition of four-dimensional data

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ABSTRACT

An on-line comprehensive two-dimensional liquid chromatography (HPLC × UPLC-TOF MS) was set up just using the injection valve of the ultra performance liquid chromatography (UPLC) as the interface through which the effluent of high performance liquid chromatography (HPLC) was injected automatically to UPLC coupled to time-of-flight mass spectrometry (TOF MS). As a demonstrative application, a complex sample of Traditional Chinese Medicine, Qingkailing was analyzed. As a result, a four-dimensional (4D) data containing 2D retention times, peak intensity and m/z ratios was plotted, where 398 peaks were counted and low concentration components were distinguished from the high concentration ones with a total peak capacity of 1090. Comparing with traditional 3D data acquired by HPLC × HPLC, the 4D data generated by HPLC × UPLC-TOF MS can increase the number of recognized components by three times, reduce the analysis time by 75%. Such a configuration of HPLC × UPLC-TOF MS can realize easily upon commercial chromatographs while exhibited enhanced separation efficiency, high sensitivity, huge peak capacity and great potential in complex sample analysis.

1. Introduction

High-resolution analytical techniques are essential for dealing with complex samples [1], because single technique can hardly be used to fully handle samples like those encountered in proteomic [2] and metabolomic research [3]. Comprehensive two-dimensional liquid chromatography (LC × LC) is attracting increasing attention with its dramatically high resolving power. In principle, LC × LC can greatly increase the peak capacity of highly optimized one-dimensional HPLC [4]. In recent researches, LC × LC systems have been used to separate molecules in biological systems [5–7] polymers [8], natural products [9–15] and other complex mixtures [16].

The peak capacity of LC × LC can be calculated by the following formula: $n_c = n_1 \times n_2$ [17,18], in which n_1 is the peak capacity of the first dimension, and n_2 is the peak capacity of the second dimension. Li [4] has introduced many corrected equations; however, increasing peak capacity of the first and the second dimension (n_1, n_2) will raise the total peak capacity (n_c) in all corrections. In addition, the LC × LC system combines two columns with different separation mechanisms, for example, normal phase (NP), reverse phase (RP), size exclusion chromatography (SEC), iron exchange chromatography (IEX) and hydrophilic interaction chromatography (HILIC), to achieve optimal separation. The speed of the second dimension is a key feature of a successful separation. Shen [19] developed an off-line approach which achieved a considerably high peak capacity (> 10,000) in the separation of peptide digests from the human plasma proteome but required a rather long analysis time (2 day). The results confirm the great potential of application of off-line comprehensive two-dimensional HPLC in the analysis of highly complex samples. The off-line comprehensive two-dimensional HPLC also underlines the considerable cost of time that these analyses might entail. Monolithic columns and parallel columns have been used as the second dimension to improve the throughput of the multiple dimensional analyses [20–26]. Short columns, high column temperature and fast gradient elution have been applied in the second dimensional separation which maintains one of the best performances ever achieved in on-line comprehensive two-dimensional HPLC separation with a peak capacity of 1350 and an analysis time of 20 minutes only [27,28].

At pressures of up to 15 000 psi and flow rates as high as 2 mL/min, the application of UPLC with small particles (< 2 μm) could produce up to 8-fold improvement in sensitivity, a 1.4-fold increase in resolution, and a 9-fold increase in sample throughput [29–31]. Ultra performance liquid chromatography combined with mass spectrometry (UPLC-MS and UPLC-MS/MS) offers

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unprecedented on-column resolving power, sensitivity, speed of analysis, and mass selectivity [32]. UPLC coupled to time-of-flight mass spectrometers (UPLC-TOF MS) has been applied in the analysis of complex samples, such as metabolites[33–35], pollutants [19], polymers [36] and pharmaceutical [32]. Ultra performance liquid chromatography as the second dimension is another possibility for multidimensional liquid chromatography analysis (HPLC × UPLC). An elevated column temperature, short columns and a high flow rate can improve the resolution of HPLC × UPLC. Peak capacity of HPLC × UPLC will be much higher than that of conventional HPLC × HPLC in the same chromatographic conditions. Recently, UPLC as the second dimension in an off-line comprehensive two-dimensional liquid chromatography has been investigated for the screening of pharmaceutical samples [37]. However there remain challenges in the exploitation of on-line HPLC × UPLC system, for example, the limitation of the ultra high pressure interface of HPLC and UPLC.

Natural products are very complex mixtures containing hundreds or even thousands of constituents of different structural types and concentrations. Only a few of them have been elucidated as being responsible for their pharmacological activity and/or toxicity. Derived from Traditional Chinese Medicine, *Qingkailing* injection is prepared from eight herbs, including *Radix Isatidis*, *Flos Lonicerae*, *Fructus Gardenise*, *Cornu Bubal*, *Concha Margaritifera*, *aicalinum*, *Acidum Cholicum*, and *Acidum Hyodesoxy-cholicum*. It has excellent curative effects on circulation system disease, phlogistic disease, virosis and some inexplicable fever. The main chemical components of *Qingkingling* injection are bile acids, amino acids, flavonoids, organic acids, nucleotides, iridoid glycoside, pigments, volatile compounds, inorganic compounds, etc. [38,39]. Due to such complex components, *Qingkailing* injection can be suitably analyzed and assayed by the comprehensive two-dimensional liquid chromatography system.

In this study, UPLC coupled to time-of-flight mass spectrometry was investigated as the second dimension of the comprehensive HPLC × UPLC system. The UPLC injection valve was used as the interface of HPLC and UPLC as it solved problems caused by pressure difference in the two dimensions and avoided the use of additional high pressure valve. HPLC × UPLC-TOF MS system was used to analyze *Qingkailing* injection, a complex herbs extraction. A 4D data containing HPLC and UPLC retention time, peak intensity and m/z was plotted, which indicated higher resolution than the conventional 3D data. Low concentration components were identified in the 4D data. The total peak capacity of HPLC × UPLC-TOF MS system was calculated and compared with conventional off-line HPLC × HPLC.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile and methanol of HPLC grade were purchased from J. T. Baker (NJ, USA). Formic acid, ammonium formate, leucine-enkephalin, uridnine, guanosine, adenosine, phenylalanine, tryptophane and caffeic acid were obtained from Sigma-Aldrich (MO, USA). Genipin-1-gentiobioside, geniposide, baicalin, shanzhiside, neochlorogenic acid, gardenoside, scandoside methyl ester, wogonoside, hyocholic acid and cholic acid were standards (NICPBP, China). Ultrapure water (18.2 MΩ) used in all experiments was prepared with a Milli-Q water purification system (Millipore, France). *Qingkailing* injection was prepared by a Chinese pharmaceutical factory and filtrated by the microvoid filter film (0.22 μm).

2.2. Construction of HPLC × UPLC system

The first dimension HPLC consists of Jasco HPLC system equipped with a binary pump (Jasco PU-980, Japan), an injection valve (Rheodyne 7725i, USA), and a UV detector (Jasco UV-975, Japan). A PEAKSIMPLE chromatography data system (SRI Instruments Inc., USA) was used to record the detector signal as well as to calculate the retention time and peak area. The data was recorded at the rate of 1 Hz. The flow rate range of PU-980 pump is from 0.001 mL to 10 mL per minute. The volume of sample loop is 20 μL. The packed-column (200 mm × 2.1 mm i.d.) was used in the first dimension. The packing material is Toyopearl HW-40 S (TOSOH, Japan), a semi-solid globoid which is polymerized by vinyl alcohol and esters of methacrylic acid for size exclusion chromatography. This packing material has many advantages such as high physical and chemical stability, high column pressure, high flow rate, etc. Besides, with many hydroxides and ether bonds on its surface, it is highly hydrophilic and able to separate components of water-solubility well. SEC column packed with Toyopearl HW-40 S can separate components whose molecular weights are between 100 and 10,000. Due to the hydrophobic group, ion exchange and hydrogen bond, deviation of size exclusion chromatographic behavior of some components will be found. The mobile phase of the first dimension is 10 mmol/L ammonium formate and the flow rate range of the first dimension should be 0.005–0.1 mL/min.

Chromatographic separations of the second dimension were performed on a 100 × 2.1 mm ACQUITY 1.7 μm column (Waters Corp, USA) in a Waters ACQUITY UPLC system equipped with a binary solvent delivery system, and an autosampler. The mobile phase was water with 0.1% (by volume) formic acid (A) and acetonitrile (B). The column was maintained at 80 °C and eluted at a flow rate of 0.7 mL/min. The column effluent was directed to mass spectrometer. The gradient conditions of mobile phase were shown in Table 1. The total analysis time was 5 min. The data of mass spectrometry was recorded from 0 to 4.5 min and column equilibration and injection were finished from 4.5 min to 5.0 min.

As shown in Fig. 1, the UPLC two-position six-port injection valve (dash and solid lines) controlled by the UPLC software was used as the interface of SEC and UPLC. During one injection circle, 100 μL sample loop was used to collect the effluent of SEC column

Table 1
Parameters of comprehensive HPLC × UPLC chromatographic conditions.

Chromatographic conditions	First dimension HPLC	Second dimension UPLC
Columns	Toyopearl HW-40S 200 × 2.1 mm	ACQUITY UPLC RP C_{18} SB 100 × 2.1 mm 1.7 μm
Packing material	Toyopearl HW-40S	ODS C18
Chromatographic column	SEC	RPLC
Mobile phase	Ammonium formate 0.01 mol/L, pH 6.9	A: 0.1% formic acid–water B: acetonitrile
Flow rate	0.02 mL/min	0.7 mL/min
Eluting mode	Isocratic elution	0-1-3-3.5-4.5-5 min 5-5-30-50-5-5%B
Column temperature	25 °C	80 °C
Wave length	254 nm	254 nm
Sample size	20 μL	100 μL elution of SEC
Total separation time	250 min	5 min