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Short communication

Using chromatogram averaging to improve quantitation of minor impurities

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ABSTRACT

Averaging of chromatograms can lead to enhancement of signal to noise ratio (S/N) in proportion to the square root of the number of measurements. Although the general principle has been known for decades, chromatogram averaging is almost never used in current pharmaceutical research. In this study we explore the utility of this approach, showing it to be a simple and easily accessible method for boosting sensitivity for quantification of minor components and trace impurities, where current techniques deliver insufficient S/N.

1. Introduction

Detection and quantification of minor impurities is an ongoing challenge in pharmaceutical analysis. In addition to longstanding difficulties relating to the accurate quantitation of trace level impurities, potential mutagenic impurities (PMIs) have gained recent attention from health authorities and the pharmaceutical industry alike as a special class of compounds requiring low level detection [1–4]. The identification and control of PMIs derived from drug substance synthesis and degradation poses a significant challenge [5–7], often requiring measurement at ppm levels [8,9]. This often necessitates the replacement of conventional HPLC methods with alternative analytical techniques or detection tools that provide enhanced sensitivity. Improved chromatographic instruments and columns that afford sharper, more efficient chromatographic peaks have been applied to PMI detection and quantification, as have a variety of derivatization and enhanced MS detection approaches [10–12], but switching to a new analytical method to obtain sufficient sensitivity can often require considerable time and effort for method discovery and optimization.

Signal averaging is routine in many fields of spectroscopy, such as NMR, IR, *etc.* By averaging a set of independent measurements, areas of signal remain strong, while areas of noise cancel, resulting in an overall increase of the signal to noise ratio (S/N) in proportion to the square root of the number of measurements. Averaging a number of individual chromatograms of the same sample to obtain a composite chromatogram with improved S/N has been known for decades [13–19] but plays almost no role in contemporary chromatographic investigations in the pharmaceutical industry.

This lack of adoption of chromatogram averaging during the early days of HPLC may have arisen from practical limitations relating to the slow speed of chromatographic separations [14], poor retention time reproducibility [20] and lack of an ability to conveniently digitize and average chromatographic data. Modern HPLC instrumentation addresses all of these obstacles, affording fast separations, highly reproducible retention across multiple chromatographic runs and convenient access to digital data. Consequently, the practical implementation of this long known but rarely utilized approach becomes highly attractive.

Realizing that even simple chromatogram averaging approaches could potentially be valuable for quantifying trace impurities at the edge of detection, we investigated the utility of chromatogram averaging for improving the S/N for the analysis of minor components in pharmaceutical samples.

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2. Materials and methods

2.1. Instrumentation

High performance liquid chromatography (HPLC) experiments were performed on an Agilent 1100 system (Agilent Technologies, Palo Alto, CA, USA) with a G1312A binary pump, a G1367A WPALS autosampler and a G1315 B diode-array detector and an Agilent 1290 Infinity II system, equipped with a G7104A quaternary pump, a G7167 B multisampler, a G7117 B diode array detector and a 6120 B Quadrupole LC/MS detector with electrospray ionization.

LC multiplexing experiments were performed on an Agilent 1290 Infinity system equipped with a high-performance auto sampler model HiP-ALS SL and a G4212A photodiode array detector (DAD). All instruments were controlled by the Agilent ChemStation software. Binary modulation sequences were applied to the auto sampler using a macro that can be read by ChemStation. For a detailed description of the macro see reference [21]. To start a multiplexing run the macro containing information about sample positions, injection order, injection intervals, and run time was executed using a pre-run command.

2.2. Chemicals, reagents and stationary phases

Acetonitrile (HPLC grade) was purchased from Fisher Scientific. Ethanol (HPLC grade), formic acid (HCOOH), ammonium formate ($HCOONH_4$), D-Alanine (99%, 99% *ee*) and L-Alanine (99%, 99% *ee*) were purchased from Sigma–Aldrich. Ultrapure water was obtained from a Milli-Q Gradient A10 water purification system from Millipore.

Separation of the PMI containing API was conducted on an Ascentis express C18 column (2.7 mm, 4.6 mm × 100 mm, ACN/H_2O ($HCOONH_4$) 90/10 and H_2O ($HCOONH_4$) 50:50, 1 mL/min). D/L-Alanine were separated on an Astec Chirobiotic™ T column (5 μm, 4.6 mm × 250 mm, EtOH/H_2O (0.1% formic acid) 65:35, 0.75 mL/min).

The crude API sample was dissolved in ACN (0.1% HCl)/H_2O ($HCOONH_4$) 50:50 with a concentration of 1 mg/mL. D/L-Alanine samples were dissolved in MeOH/H_2O (80/20).

2.3. Preparation of buffer solutions

Solutions containing 2 mM ammonium formate ($HCOONH_4$) in water (pH 3.5) and 2 mM ammonium formate in acetonitrile (pH 3.5): 12.6 g ammonium formate ($HCOONH_4$) and 7.9 mL formic acid (HCOOH) were dissolved in 1 L of Millipore water. A 100-fold dilution of this stock solution was performed in either pure water or a 90:10 acetonitrile–water mixture to prepare the 2 mM solutions.

2.4. Data acquisition and processing

UV data for averaging and multiplexing experiments were collected at a wavelength of 210 nm with an acquisition rate of 20 or 40 Hz, MS data with a rate of 2.5 Hz. Data was exported as CSV file for further processing. For averaging experiments CSV files of multiple runs were imported to Microsoft Excel and an average chromatogram generated using the average function. These chromatograms were imported into ACD Spectrus Processor software and exported as CDF file, that allow for further evaluation with Agilent ChemStation software. For multiplexing data, construction of pseudorandom binary sequences and inverse Hadamard transformation of the convoluted chromatograms was performed by an executable program written in Pascal (Delphi RAD, Embarcadero Technologies, South San Francisco, U.S.A.) running under 32- and 64-bit Windows. The modulation sequences were constructed using virtual shift registers [21]. Evaluation of the data was carried out with the same program.

3. Results and discussion

An example illustrating the challenges of PMI analysis is depicted in Fig. 1, where the PMI, chloromethyl-pyridazinone (CMP, red arrow) can be observed in an API recently studied in these laboratories. Due to the projected dose of the API, the threshold for toxicological concern for CMP was determined to be 33 ppm, meaning that the level of CMP would need to be controlled to less than 10 ppm, well below the quantification limit of the HPLC-UV method. While 10 ppm CMP is observable with this chromatographic method (Fig. 1a), the S/N is insufficient (1.25) for reliable quantitation, being well below the prescribed values for limit of detection (S/N > 3) and limit of quantification (S/N > 10). Many avenues are available for improving S/N, including increased sample injection volume and/or concentration, change in detection wavelength, use of a more sensitive detector such as MS, or use of a comparable column having superior chromatographic efficiency. While insufficient S/N can often be overcome using one or the other of these approaches, some cases prove especially difficult, even when employing combinations of these techniques. Simply averaging the chromatograms obtained from 5 injections of the same sample afforded the composite chromatogram shown in Fig. 1b, where a greater than twofold improvement in S/N is obtained. Averaging 10 (Fig. 1c) or 20 (Fig. 1d) chromatograms afforded corresponding increases in S/N in good agreement with the theoretical increase as the square root of the number of chromatograms averaged. It should be noted that due to the high noise and relatively low signal for the CMP peak in the original chromatogram, the exact S/N ratio is difficult to determine precisely, which may account for the small difference between the actual and theoretical S/N enhancement provided by chromatogram averaging. The process of chromatogram averaging is fairly straightforward, with transfer of each of the chromatographic data files to Microsoft Excel and subsequent simple averaging across data for each time point to create a composite average chromatogram file, which can then be exported (to ACD Spectrus Processor) and saved in a file format that allows integration and analysis by a variety of chromatographic data processing software programs.

The number of chromatograms that must be averaged to afford a given S/N is easily determined, allowing prospective users a convenient way to judge whether or not this approach can afford the requisite S/N improvement. Clearly, the technique is best suited for situations where S/N is relatively close to the required value, and a manageable number of chromatograms can be collected and averaged. Considering the fact that nowadays a trend towards faster and faster separations has developed, with many separations being possible in less than a minute [22–24], collecting even multiple chromatograms can often be performed in a reasonable time.The technique would obviously not be well suited to situations where the relative amount of the PMI within the sample degrades or changes over the course of the chromatographic runs, or where the retention times of the peaks within the individual chromatograms show poor reproducibility. To avoid peak broadening due to changes in elution times of multiple chromatograms, it is important that retention times are very uniform. For modern (U)HPLC instruments an excellent reproducibility can be usually achieved. For example the impurity peak in Fig. 1 shows a total variability in retention time of about 400 ms. Averaging of 10 chromatograms leads to a slightly increased peak width (0.028 *vs* 0.026 min) whereas it has to be noted that the number does not increase further as more chromatograms are averaged. In cases where it is important not to lose resolution like closely eluting