Instrument parameters controlling retention precision in gradient elution reversed-phase liquid chromatography

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ABSTRACT

The precision of retention time in RPLC is important for compound identification, for setting peak integration time windows and in fundamental studies of retention. In this work, we studied the effect of temperature (T), initial (ϕ_o) and final mobile phase (ϕ_f) composition, gradient time (t_G), and flow rate (F) on the retention time precision under gradient elution conditions for various types of low MW solutes. We determined the retention factor in pure water (k'_w) and the solute-dependent solvent strength (S) parameters of Snyder's linear solvent strength theory (*LSST*) as a function of temperature for three different groups of solutes. The effect of small changes in the chromatographic variables (T, ϕ_o, ϕ_f, t_G and F) by use of the *LSST* gradient retention equation were estimated. Peaks at different positions in the chromatogram have different sensitivities to changes in these instrument parameters. In general, absolute fluctuations in retention time are larger at longer gradient times. Drugs showed less sensitivity to changes in temperature compared to relatively less polar solutes, non-ionogenic solutes. Surprisingly we observed that fluctuations in temperature, mobile phase composition and flow rate had less effect on retention time under gradient conditions as compared to isocratic conditions. Overall temperature and the initial mobile phase composition are the most important variables affecting retention reproducibility in gradient elution chromatography.

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1. Introduction

The precision of chromatographic retention time is an important analytical metric [1–7]. Irreproducible retention times have many possible sources including irreproducible flow rates, fluctuations in the system temperature and variations in eluent composition (when controlled by the instrument). The potential for fluctuations in retention time under gradient as compared to isocratic elution may be increased by the added complexity of the eluent program. Flow rate, column temperature and the eluent program are all chromatographic variables set by the user and the accuracy and precision of these parameters are dependent on instrument performance. The effect of these parameters was studied in this work. We first looked at the equations governing the relationship between fluctuations in the gradient retention time and fluctuations in various instrumental variables such as flow rate, initial and final eluent composition, gradient time and temperature. We next measured the actual fluctuations in a specific instrument and

computed the contribution to the retention times for three sets of solutes. Other instrumental factors impacting retention precision such as electronic noise in the peak signal [8–11], and long term drift [12] have been studied elsewhere.

There are number of critical issues in chromatography that mandate good retention time precision. In automated quantitation, the integration start and stop times are specified within defined limits to assure that the peak is properly identified. These limits vary broadly depending on how narrow the peak is and how close it may be to neighboring peaks [13]. Perhaps the most rigorous application requiring extremely precise retention time data are multivariate chemometric curve resolution methods where data from two or more chromatographic runs are combined. In the most severe cases (e.g. using chemometric methods that combine two or more chromatographic runs with univariate spectral information) retention precision better than 0.2–0.3 times the peak half-width is required [13]. When more advanced methods are employed additional information (e.g. PARAFAC2 [14]) relaxes the requisite retention precision to approximately 0.7–1.4 times the peak half-width. Thus assuming a plate count of 10,000 and a retention time of 2 min, chemometric techniques may require retention times to be precise to 0.001–0.01 min. Retention precision is also a figure

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of merit to prove that an instrument is qualified for use [5,15]. Another use of retention time is to chemically verify the presence or absence of a compound [16–18]. In this regard the more precisely the retention time can be defined the fewer will be the number of compounds that can be confused with the analyte of interest. Additionally precise measurement of retention times allows the prediction of separations by using gradient elution training data through methods described by Snyder [19]. There have been a number of studies of the reproducibility of retention time in liquid chromatography that emphasize the importance of column equilibration [12,20–32]. It is possible to achieve run to run gradient elution retention reproducibility to ±0.002 min (standard deviation) in a bit more than two column volumes of re-equilibration (so-called "repeatable equilibrium") [20]. Given a stable column and a good signal to noise ratio [8] the parameters that control retention time precision are: temperature (T), initial (ϕ_o) and final (ϕ_f) mobile phase composition, gradient time (t_G) and flow rate (F).

To maintain retention repeatability care has to be taken to ensure that the instrument is functioning as intended. For example, several studies reported difficulties in precise column temperature control in liquid chromatography especially when the instrument is operated at higher temperatures [33–39]. Zhu and coworkers pointed out that if the source of the problem is the column thermostat the oven set-point temperature may differ from the actual temperature by several degrees. There have been a few reports on the stability of the column temperature which is what controls retention reproducibility [33]. Peasen et al. explored thermal effects that can induce baseline fluctuations. They reported that temperature can cycle with time [40]. Another source of problems is the differences between the actual column temperature and oven temperature set point, which may not be the same when using different equipment for the same method [41,42]. In prior work we showed that at flow rates of 1 mL/min or less temperature control of the column was not as tight as at higher flow rates (2–3 mL/min). This was attributed to quite evident changes in the duty cycle of the oven column compartment temperature control system [20].

The consistency of the mobile phase composition is another important parameter that influences retention precision. When a fresh batch of eluent is prepared a small change in composition can have a significant effect on the absolute value of the retention time. In gradient elution, the retention reproducibility may suffer if the instrument is not consistently delivering the same eluent profile (e.g. gradual changes in solvent composition with time) [25]. Extremely good isocratic retention reproducibility can be achieved by gravimetric metering of the eluent [20]. When an improperly functioning pump is used disturbances in mobile phase composition or flow rate and changes in gradient time have a direct impact on the "gradient steepness" and will change the retention time [43–45].

Herein we attempt to better understand the effect of the various instrumental parameters on retention time precision using three different, hopefully representative, test groups of solutes: the first is a group of 14 of the more retained standard solutes of the 16 solutes used by Snyder and Dolan in their hydrophobic subtraction method [46,47], the second is a set of 18 common pharmaceuticals, and the third is a set of 22 simple derivatives of benzene which have been used extensively to study retention mechanisms in RPLC [48–50]. Based on measured characteristics of these compounds we can compute their retention times under a wide variety of gradient conditions. We then investigated the effect of small changes in the chromatographic parameters (temperature, initial and final eluent strength, gradient time and flow rate) on the precision in retention time.

2. Theory and computational methods

2.1. Predicting gradient elution retention time

The logarithmic retention factors of analytes ($\ln k'$) in RPLC generally decrease in a *quasi*-linear fashion as the volume fraction of organic modifier (ϕ) in the eluent is increased under isocratic conditions. This behavior is approximated by the basic retention equation of linear solvent strength theory (*LSST*) [51].

$$\ln k' = \ln k'_w - S\phi \tag{1}$$

where S is the solute's sensitivity to changes in ϕ and $\ln k'_w$ is its extrapolated retention in pure water. Other relationships could be used but we feel they would simply complicate the mathematics below and not do much to enhance our understanding of the principles involved.

We assume that the above equation is accurate and thus used the linear solvent strength theory to predict gradient retention times (t_R). Schoenmakers and coworkers derived the following equation for predicting the retention time of a solute under linear gradient elution conditions [52]:

$$t_R = t_o + t_D + \frac{t_o}{b} \ln \left(b \left(k'_o - \frac{t_D}{t_o} \right) + 1 \right) \tag{2}$$

where t_o is the column dead time, t_D is the system dwell time, and k'_o is the retention factor in the initial eluent. The gradient steepness (b) is defined as:

$$b \equiv \frac{S\Delta\phi V_m}{Ft_G} \tag{3}$$

where $\Delta\phi$ is the change in the initial (ϕ_o) and final (ϕ_f) eluent strength, V_m is the column dead volume (mL), F is the flow rate (mL/min) and t_G is the gradient time (min). It is important to note that Eq. (2) is only valid for those peaks eluting between $t_o + t_D$ and $t_o + t_D + t_G$.

To utilize Eq. (2) and predict the retention times of many solutes under gradient elution conditions, training runs are used to obtain values of S and k'_w as a function of column temperature. In principle one needs only two gradient runs to determine S and $\ln k'_w$ where only t_G is varied, but three runs provide more reliable results [53]. Thus, we collected solute retention time data using gradient times of 10, 30 and 60 min under otherwise fixed conditions. Further, we measured retention times in this fashion at three different column temperatures (35, 50 and 65 °C). In principle we could then obtain S and $\ln k'_w$ values at each temperature studied by using the Solver add-in function of Excel to minimize the sum of the squares of the residuals of t_R, which were predicted against the experimental values (i.e. $\sum_{i=1}^{n}(t_{R,predicted} - t_{R,experimental})_i^2$) [53,54]. This procedure would entail determining 6 parameters (3 values of S and $\ln k'_w$) from a total of 9 measurements. In plotting $\ln k'_o$ vs. temperature we always observed quite good straight lines as expected when using three values of T spanning a range of 35–65 °C. Thus, we made the reasonable approximations that over this narrow range in temperature both S and $\ln k'_w$ would be reasonably linear functions of temperature:

$$S = a_s + b_s T \tag{4}$$

$$\ln k'_w = a_w + b_w T \tag{5}$$

We then used Solver with the 9 data points (3 values of t_G at 3 temperatures) to obtain the best values of the 4 parameters of Eqs. (4) and (5). This is statistically more satisfying and in our experience the error in using Eqs. (4) and (5) is almost certainly no greater than incurred in the use of Eq. (1) at each temperature.