Comparison of a Fast Liquid Chromatography Column with a Standard Column for the Rapid Isocratic Quantitation of ABA

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Abstract

The suitability of a small C_{18} column (35 mm, 3-µm diameter packing matrix) for the quantitation of the plant hormone 2-*cis*, 4-*trans* abscisic acid (ABA) is assessed. The small C_{18} column has a retention time (t_r) of 105 s and a minimum mass sensitivity of 500 pg, whereas a standard C_{18} column has a t_r of 1920 s and a minimum mass sensitivity of 2500 pg. The minimum mass sensitivity and t_r for the small C_{18} column appear to be the lowest reported figures currently available for the quantitation of ABA using a C_{18} column, and the small C_{18} column offers considerable advantages in terms of solvent consumption, reduced analysis time, and increased mass sensitivity. This type of column is ideally suited for the quantitation of ABA, in which the resolution requirements do not exceed approximately 5000 plates.

Introduction

2-*cis*, 4-*trans* Abscisic acid (ABA) is a sesquiterpenoid which exhibits geometrical isomerism. The 2-*cis*, 4-*trans* enantiomer occurs naturally within plants and has been widely implicated as a general "stress hormone" (1–3). During periods of stress, the effects of this hormone enable the plant to mediate short-term and long-term environmental pressures with metabolic demands, especially the regulation of water relations during periods of water stress (4–8). It is thought that ABA is able to mediate long-term stresses by transducing environmental stimuli into changes in gene expression (9–11). Recent experiments appear to verify this hypothesis, and there is now an increasing emphasis being placed upon understanding the detailed nature of the pathway(s) whereby ABA synthesis is able to affect gene expression (12).

The elucidation of this transduction behavior is reliant upon a precise and accurate quantitation method. Contemporary physico-chemical methodologies involving reversed-phase high-performance liquid chromatography–ultraviolet detection (RP-HPLC–UV) systems (13,14) typically use gradient elution programs in conjunction with C_{18} analytical columns capable of generating about 12,500 plates (15). However, the majority of ABA separations can be accomplished using an isocratic elution program (16). Furthermore, a consideration of the minimum plate-requirement equation (17) indicates that such ABA separations could be performed using a column with a substantially lower plate number. Therefore, quantitation of ABA could be performed using a column of considerably lower efficiency, subsequently affording such benefits as lower retention times (t_r) and a more gentle operating pressure (Δp).

Knox (18) examined the optimum combination of resolution (N), Δp, and t_r and concluded that the majority of chromatographic separations could be performed using a column 20–40 mm in length with a particle diameter (dp) of 1–2 µm for the packing bed matrix. If the analytes are eluted from these columns using a Δp of 200 bars, then a very fast elution time of 10 s for the unretained solute is obtained, although resolution decreases to 5000 plates. This loss of resolution is not necessarily a problem because the plate requirement for any given chromatographic separation is generally a function of the capacity factors of the critical pair, and most routine analyses can be adequately resolved with chromatographic efficiencies of approximately 1500–5000 plates (19,20).

For an optimum column design for the quantitation of ABA, there are two main contributing factors that need to be considered. First, there is a low plate requirement, warranting the use of an alternative column designed using parameters similar to those suggested by Knox (18). Second, the analysis of ABA, or any other plant hormone, is essentially a trace analysis, because the analyte concentration within the original sample is extremely small, and trace analyses are best performed using columns of short length and small dp (20). Such columns have been commercially available for over ten years (21), but do not appear to have been used for the analysis of plant hormones. A

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consideration of chromatographic theory indicates that such columns, commonly known as "fast liquid chromatography (LC)" columns (22), have numerous inherent benefits such as a low void volume, increased mass sensitivity, decreased t_r, lower solvent volume usage, and lower operating costs (23). The main disadvantages associated with the use of fast LC columns are a low dead volume tolerance, tendency of the smaller dp packing matrix to become clogged with particulate matter (19), increased inlet and outlet turbulence (20), and the development of significant thermal effects (18).

Although a fast LC column may be applicable to ABA analysis, there are numerous contributions to band broadening that may significantly impair resolution. This paper reports the comparative performance of a fast LC column and a conventional column for quantitation of 2-*cis*, 4-*trans* ABA using a standard ABA analysis procedure. Subsequent differences in quantitative performance are explained using kinetic test parameters.

Experimental

Assessment of column suitability for ABA analysis

Column design

The geometrical parameters for the fast LC and standard columns were 35 mm × 4.6-mm i.d. (3-µm dp) and 250 mm × 4.6-mm i.d. (5-µm dp), respectively.

Chromatographic conditions

An ion-suppression retention mechanism was used by running ABA (pK_a = 4.8) in a mobile phase of acetonitrile–H_2O (20:80, v/v) acidified to pH 3.1 with 1% glacial acetic acid and incorporated into an isocratic elution program. Samples of synthetic ABA were injected from a Waters (Milford, MA) WISP model 712 auto-injector and detected at 260 nm on a Waters 490E detector. Flow rates for each column were optimized so that the reduced velocity (v) was approximately 5 (24).

Evaluation parameters

Parameters of greatest practical importance for an ABA analysis are minimum mass sensitivity, retention time, solvent volume usage, and an estimate of variability. Minimum mass sensitivity was obtained from the mass of solute detectable at a peak height equivalent to twice the noise level (25). Solvent volume consumption was calculated using a methodology similar to that of Vonk et al. (26). Briefly, the optimum linear flow rate at the minimum reduced plate height (h) was converted to the effective volumetric flow rate (f_v) using:

$$f_v = \pi r^2 \varepsilon l u_o \qquad \text{Eq 1}$$

where $\pi r^2 l$ is the total column volume, ε is the fraction of the total column occupied by the mobile phase (≈ 0.75) (18,27), and u_o is the linear velocity.

This flow rate was then converted to an estimate of the solvent used (S) per ABA run using:

$$S = t_r f_v \qquad \text{Eq 2}$$

where t_r is the retention time of ABA for each column.

An overall estimate of variability was obtained from the coefficient of variation (CV) for column efficiency over the ten trial injections using:

$$CV\% = \frac{S}{\bar{\bar{x}}} \qquad \text{Eq 3}$$

where s is the standard deviation for the h measurements and $\bar{\bar{x}}$ is the grand mean for h.

Qualitative performance

The test procedures suggested by Bristow and Knox (24) were used to evaluate the kinetic performance of the two columns over a wide range of capacity factor (k') solutes at varying flow rates. The test conditions are given in Table I, and the reduced parameters h and v, together with the flow resistance parameter (ϕ) and the separation impedance factor (E), were calculated (24). In addition to these kinetic parameters, an assessment of dead volume tolerance was performed by analyzing various peak asymmetry parameters (16,24).

Table I. Test Conditions for Assessment of Kinetic Performance and Tolerance of Extra-Column Dead Volume

Column temperature	25°C
Detection	254 nm
Mobile phase	MeOH–H_2O (60:40, v/v)
Flow rate	$v \approx 5$ to $v \approx 100$

Test solutes	Retention	Concentration (g/L)
Uracil	unretained	0.005
Acetophenone	retained 1	0.01
Anisole	retained 2	0.25
Toluene	retained 3	0.70

Table II. Suitability of the Fast LC Column and the Standard Column for Quantitation of 2-*cis*, 4-*trans* ABA when Using an Isocratic Elution Program

Minimum h		
Parameter	**Fast LC column**	**Standard column**
f_v (mL/min)	0.30	0.50
t_r (sec)	699	4080
S (mL/run)	3.5	34
pressure (bars)	3.5	61
mass sensitivity (pg)	500	2500
CV (%)	3.9	1.8
Maximum flow velocity		
Parameter	**Fast LC column**	**Standard column**
f_v (mL/min)	2.0	1.0
t_r (sec)	105	1920
S (mL/run)	3.5	32
pressure (bars)	65	136

Results and Discussion

Assessment of column suitability for ABA analysis

At optimum flow velocities, the t_r of the ABA solute on the fast LC column was nearly six times smaller than the standard column, indicating considerable savings in running costs, especially solvent consumption (Table II). Additional characterization of the response of reduced plate height and operating pressure to an increase in flow velocity for the fast LC and standard columns indicated that quantitation of ABA was satisfactory at the increased flow velocities of 2.0 and 1.0 mL/min, respectively (Table II). This resulted in a further reduction in t_r and solvent consumption for both columns without sacrificing adequate resolution. The t_r (105 s) for the fast LC column using the increased flow velocity appears to represent the fastest analysis time yet available for quantitation of ABA (14,15,28).

One of the major problems associated with fast LC columns is the potentially short column life that may result from deterioration of the packing bed matrix. An excessively high operating pressure will result in the development of a proportionately larger void volume within a fast LC column in comparison with a standard column, resulting in a greater decrease in the chromatographic efficiency of the fast LC column. Because the pressure drop across the fast LC column was 65 bars (Table II), it was unlikely that significant void volumes would develop.

The minimum mass sensitivity obtained for ABA with the standard C_{18} column (2500 pg, Table II) was typical of conventionally designed C_{18} columns in RP-HPLC–UV systems (29). Use of the fast LC column gave a fivefold increase in mass sensitivity (i.e., 500 pg) and was equivalent to the most sensitive RP-HPLC–UV system currently available for quantitation of ABA (30). This increase in sensitivity was mostly attributable to the smaller degree of dilution of the analyte during the course of migration from the injector to the detector (26).

The variability in efficiency for the fast LC column was twice that of the standard column. However, this variability was still very small (3.9%) and was unlikely to seriously impair the performance of the column for quantitation of ABA.

Table III. Assessment of the Kinetic Performance of the Two Columns under Ideal Conditions*

Parameters	Fast LC column	Standard column
h, $v \approx 5$	2.1	4.4
$v \approx 100$	6.0	13.4
ϕ	303	1263
E	1174	6800

* NB-toluene was used as the test solute.

Table IV. Assessment of Susceptibility of the Two Columns to Extra-Column Variance*

Parameters	Fast LC column	Standard column
k' for solute 1	0.64	1.15
k' for solute 2	1.35	2.54
k' for solute 3	2.87	5.53
Asymmetry (at $h \approx 5$)	1.11	1.20
Asymmetry gradient	0.04	–0.11

* NB-toluene (solute 3) was used for asymmetry measurements.

Kinetic performance

Consideration of the reduced plate height response to variations in reduced velocity indicated that both columns were well-packed and had acceptable mass transfer characteristics (Table III).

The fast LC column had an exceptionally low ϕ value, which was attributable to the short column length and low optimum flow rate of 0.3 mL/min. The standard column ϕ value was typical for a column 250-mm long (16,24).

Optimization of all of these kinetic parameters within any chromatographic separation is ultimately concerned with minimizing E, which is essentially a consideration of all the factors contributing to chromatographic efficiency (16). The E value for the fast LC column was very low, indicating that this type of column was well-suited for the quantitation of ABA. The standard column had a moderately high E value, which was mostly attributable to the increased flow resistance of the longer column.

Dead volume tolerance

Table IV shows the expected trend of smaller capacity factors for each of the test solutes eluted from the fast LC column, indicating that extra-column effects may have significantly contributed to a lowering of efficiency for this column type (22). This susceptibility to extra-column variance can be assessed by measuring peak asymmetry. Both columns had slightly elevated asymmetry ratios, which were attributable to either extra-column variance or thermodynamic peak spreading (16). It is possible to assess which process is contributing to peak asymmetry by regressing the asymmetry ratio over a range of reduced velocities (24). The fast LC column had a positive gradient, indicating poor dead volume tolerance, whereas the standard column had a negative gradient, indicating a probable thermodynamic origin for the marginal asymmetry ratio.

Conclusion

Both columns had similar, acceptable levels of inherent performance in terms of packing bed structure and mass transfer properties. However, quantitation of ABA was optimized by incorporating a fast LC column into an isocratic elution program, resulting in the main benefits of a faster analysis time, increased mass sensitivity, and decreased solvent consumption. These benefits are attributable to the optimization of plate height and permeability to flow, as indicated by the low separation impedance factor.

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