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Effect of Temperature, Wavelength, pH, Ion Pair Reagents and Organic Modifiers' Concentration on the Elution of Cystatin C. Stability of Mobile Phase

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Abstract

Robustness of an analytical chromatographic method for separation of cystatin c has been verified. Changes in many parameters were carried out, such as, wavelength, column oven, mobile phase composition, chromatographic column.

Imperative changes have altered the efficiency of the chromatographic separation; such changes include pH alteration of the mobile phase as well as alkyl sulfonate molarity changing.

All robustness conditions showed no major effect on the chromatographic separation of the analyte except with the changes related to TFA and alkyl sulfonate ion pair reagents. Peak area RSD, asymmetry and No. of theoretical plates were < 0.7%, < 1.2 and > 10,000, respectively. Results obtained using mobile phase after 6 months of storage have proven its stability and possibility of use. Gradient elution mode was utilized to elute cystatin c with a UV detection of 224 nm. Ace and Waters C8 (150 x 4.6 mm i.d., 5 µm) as chromatographic columns were used.

Keywords: Cystatin C; Robustness; TFA; Stability of mobile phase

Introduction

Cystatin C (CC) is composed of 120 amino acid residues with a molecular weight of about 14 KDa and two intra-chain disulfide bonds. CC has an isoelectric point (PI) equal to 9.2; accordingly, and due to protonation of the acid group it has a positive charge at physiological pH. [1-5].

Ion pair liquid chromatography technique (Cation analysis) has been used for separation and quantification of CC, incorporating trifluoroacetic acid (TFA) and 1-hexane sulfonic acid sodium salt in the mobile phase.

Two ion pair reagents were included in the mobile phase. CC has a leader hydrophobic sequence which enhances the binding with the hydrophobic surface of the stationary phase. 1-hexane sulfonic acid sodium salt reagent was used to bind with the stationary phase instead of the analyte [5]. To accomplish an efficient separation, using of both ion pair reagents is deemed necessary. Typical working concentrations of both reagents were incorporated in the mobile phases [6].

Different factors render the use of TFA, it has low volatility at low pH values (in the present work pH 2.4), it sharpens the peak shape where the ionic interaction chance between the silanol groups of the stationary phase and CC is reduced and finally it has a low absorption at the working wavelength.

Organic modifiers can also compete with the ion pair for the stationary phase and then decreasing the effective capacity of the column. [7-9].

Experimental

Chemicals

CC protein was purchased from Scipac (UK), HPLC grade acetonitrile, methanol, 1-hexane sulfonic acid sodium salt, trifluoroacetic acid (TFA) and acetone were purchased from Merck (Germany).

Chromatographic conditions

Shimadzu HPLC system (Shimadzu, Japan) with a degasser, low pressure gradient pump, column oven, an autosampler, a UV detector was used. Data acquisition was performed with LC-Solution 1.21 software. A reversed phase Ace C8 (150 x 4.6 mm i.d., 5 μm) column was placed in the column oven at 25°C.

Mobile phase A of 0.01M 1-hexane sulfonic acid sodium salt plus 0.05% TFA, pH 2.4 (filtered through 0.45 μm Teflon filter) and mobile phase B (acetonitrile: methanol: mobile phase A) (300: 300: 225, v/v/v), pH 2.5) [10].

Stability of mobile phase solutions

Mobile phase has been stored at 25°C for six months. RSD were determined from 5 consecutive injections of calibration urine containing CC at its pathological concentration.

Robustness

It is defined as per the ICH Guideline Q2 (R1) [11], a measure

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of the method to remain unaffected by deliberate changes in the parameters of the method.

Eight chromatography parameters were altered; these parameters were found to contribute significantly to the method: acetonitrile and methanol content in the mobile phases, column oven temperature, wavelength, TFA and 1-hexan sulfonic acid sodium salt content in the mobile phases which corresponds to the pH value and the buffer molarity, respectively. Chromatographic column from another supplier with the same specification, in addition, to another alkyl sulfonic acid sodium salt ion pair reagent were also used. Analysis was carried out using a calibration urine containing CC at its pathological concentration (Table 1).

Parameter	-	0	+
Methanol content in mobile phase, constant Acetonitrile (mL)	285	300	315
Acetonitrile content in mobile phase, constant Methanol (mL)	285	300	315
Mobile Phase A (mL)	210	225	240
Wavelength (nm)	229	224	221
Column Oven Temperature (°C)	20	25	30
TFA Content (mL)	0	0.5	1.0
Alkyl Sulfonic Acid Sodium Salt (M)	0	0.01	NP

*0: Original condition; - and + are lower and upper limits, respectively

Table 1: Selected parameters and their variabilities*.

Injection #	Peak Area Stability Mobile Phase (X 10^{-6})	Peak Area Fresh Mobile Phase (X 10^{-6})
1	6.24	6.65
2	6.24	6.64
3	6.26	6.63
4	6.31	6.60
5	6.27	6.54
Average	**6.27**	**6.61**
RSD%	**0.47**	**0.67**
No. of Theoretical Plates	**10407**	**12726**
Asymmetry	**1.12**	**1.14**
Retention time (t_r)	**10.4**	**11.0**

Table 2: CC Using Stability Mobile Phase.

Injection #	Peak Area (219 nm) (X 10^{-6})	Peak Area (229 nm) (X 10^{-6})
1	6.54	3.17
2	6.55	3.18
3	6.54	3.18
4	6.54	3.18
5	6.52	3.17
Average	**6.54**	**3.18**
RSD%	**0.17**	**0.17**

Table 3: CC at Different Wavelengths (± 5 nm).

Injection #	Peak Area (+ 5 °C) (X 10^{-6})	Peak Area (- 5 °C) (X 10^{-6})
1	4.97	4.80
2	5.00	4.84
3	5.01	4.84
4	5.01	4.85
5	5.01	4.85
Average	**5.00**	**4.84**
RSD%	**0.35**	**0.43**

Table 4: CC at Different Column Oven Temperatures (± 5°C).

Injection #	Peak Area Using1-Pentane ion pair Reagent (X 10^{-6})	Peak Area Using1-Hexan ion pair Reagent (X 10^{-6})
1	6.23	6.65
2	6.26	6.64
3	6.29	6.63
4	6.27	6.60
5	6.25	6.54
Average	**6.26**	**6.61**
RSD%	**0.36**	**0.67**

Table 5: Using of 1-Pentane Sulfonic Acid Sodium Salt in the Mobile Phase.

Injection #	Peak Area Using Ace Column (X 10^{-6})	Peak Area Using Waters Column (X 10^{-6})
1	5.76	6.65
2	5.82	6.64
3	5.86	6.63
4	5.87	6.60
5	5.87	6.54
Average	**5.84**	**6.61**
RSD%	**0.81**	**0.67**
No. of Theoretical Plates	**13982**	**12726**
Asymmetry	**1.10**	**1.14**
Retention time (t_R)	**10.8**	**11.0**

Table 6: CC Using Waters Column.

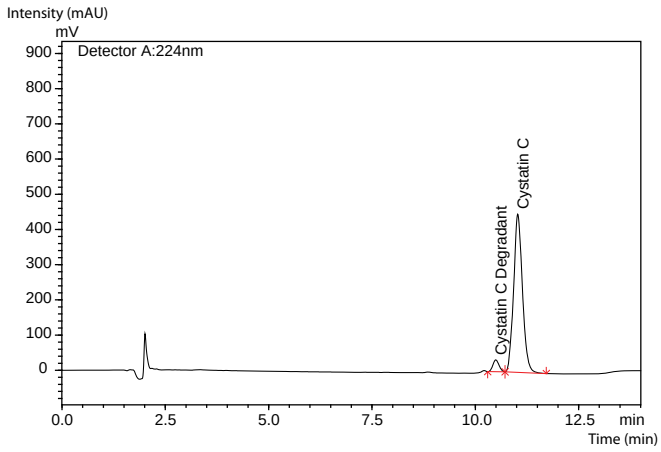

Figure 1: Chromatogram of CC at 224 nm and at 25°C Column Oven.

Results and Discussion

Stability of mobile phase

Close retention time of CC peak, RSD < 0.7%, No. of theoretical plates was > 10,000 and the asymmetry factor was < 1.2, all prove the stability of mobile phase after six months of room temperature storage. (Table 2).

Robustness

No. of theoretical plates, asymmetry factors, RSD of peak areas and retention times were used to evaluate the effects on the qualitative responses (Table 3-6).

No major changes were observed as a result of the alterations made except when TFA and alkyl sulfonate ion pair reagents were eliminated from the mobile phases (Figure 1-3).

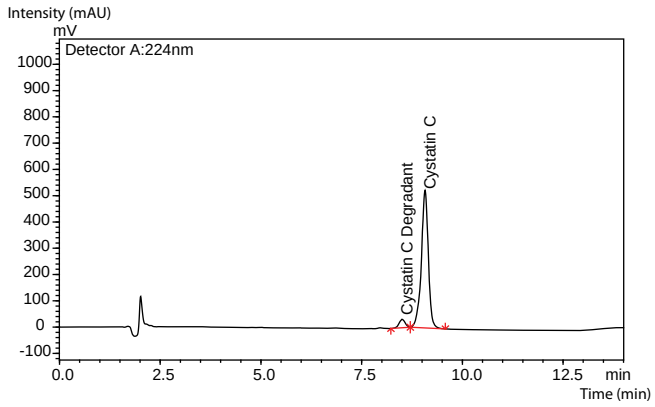

Figure 2: Chromatogram of CC, Using 1-Pentane Sulfonic Acid Sodium Salt.

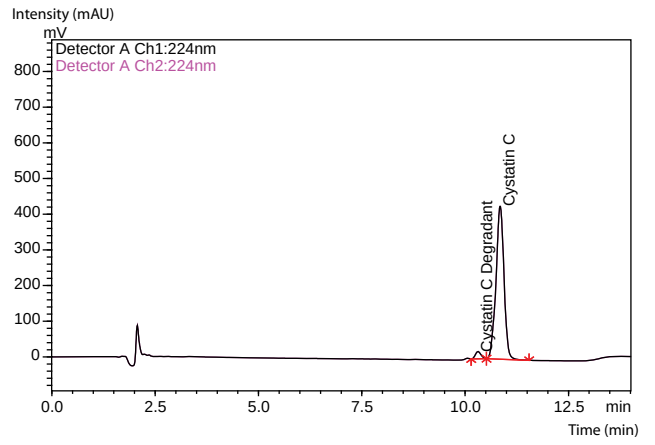

Figure 3: Chromatogram of CC, Using Waters C8 (15 cm*4.6 mm), 5µm Column.

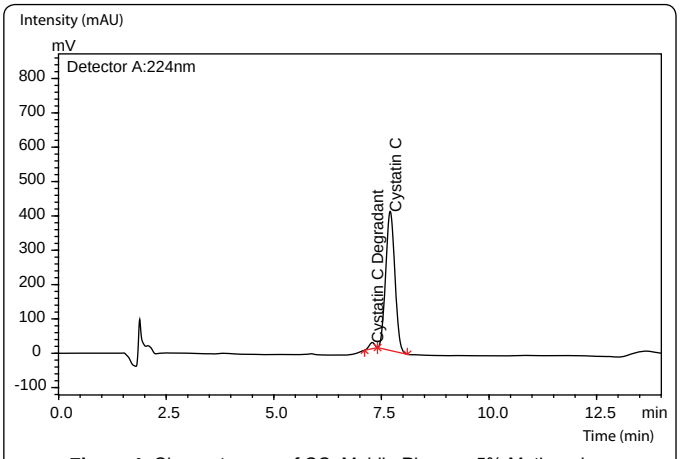

Figure 4: Chromatogram of CC, Mobile Phase + 5% Methanol.

Alteration of organic modifiers content (acetonitrile and methanol) has affected only the retention time of CC peak. Nevertheless, the obtained RSD values for CC peak area did not exceed 0.6% (Figure 4,5) (Table 7,8).

Interestingly, CC peak was distorted when no TFA is added to the mobile phase and the peak area of CC was reduced by about 67%. This phenomenon can be attributed to the silanol groups in the stationary phase that have been activated as a result of high pH value (7.0). Moreover, CC protein might dissociate at high pH values due to denaturation process. [6,12].

CC peak was distorted when no alkyl sulfonates reagent is added to the mobile phase in addition to retention time shifting was also

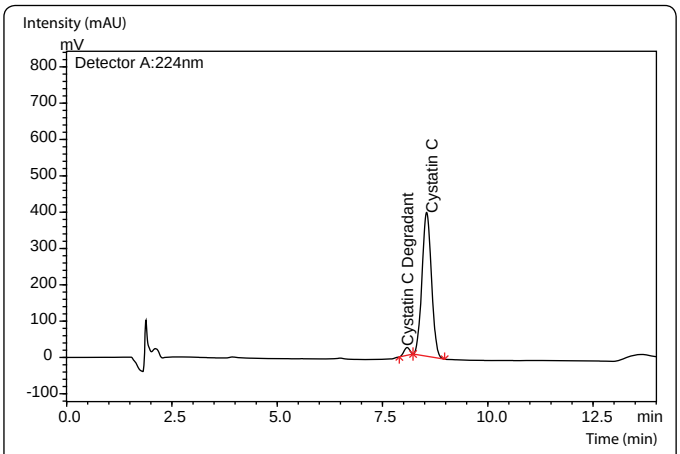

Figure 5: Chromatogram of CC 0.2 mg/mL, Mobile Phase - 5% Methanol.

Injection #	Peak Area (+ 5 %) (X 10^{-6})	Peak Area (- 5 %) (X 10^{-6})
1	6.15	6.08
2	6.21	6.14
3	6.22	6.16
4	6.20	6.14
5	6.18	6.14
Average	**6.19**	**6.13**
RSD %	**0.45**	**0.49**

Table 7: ± 5 % Acetonitrile Added to Mobile Phase, Constant Methanol.

Injection #	Peak Area (+ 5%) (X 10^{-6})	Peak Area (- 5 %) (X 10^{-6})
1	6.00	6.10
2	6.08	6.18
3	6.06	6.19
4	6.06	6.18
5	6.07	6.18
Average	**6.05**	**6.17**
RSD %	**0.52**	**0.60**

Table 8: ± 5 % Methanol Added to Mobile Phase, Constant Acetonitrile.

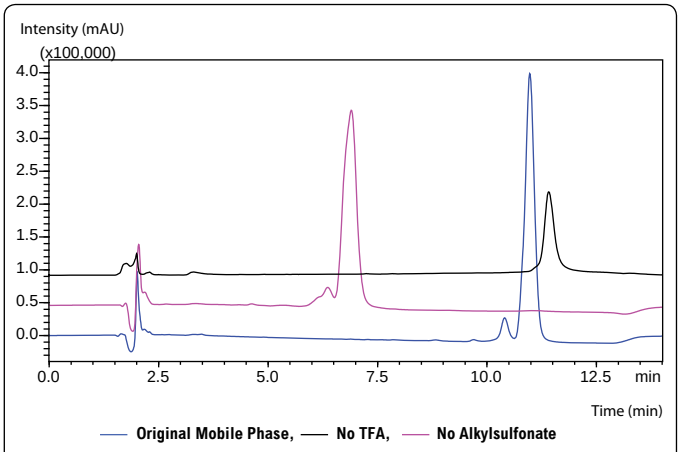

Figure 6: Overlay Chromatograms of CC, Comparison Among Three Mobile Phases.

observed. The interaction between the hydrophobic leader of CC sequence and the hydrophobic stationary phase is considered as a reason behind the observed distortion, whereas, in case of alkyl sulfonates-containing mobile such interaction occurs with the alkyl sulfonate reagent. The reduction in the retention time (6.9 minutes instead of 10 minutes) can be also attributed to the low concentration of the alkyl sulfonate in the mobile phase (Figure 6).

Conclusion

The present study has investigated the effect of varying some of the chromatography parameters of the previously developed and validated HPLC method. The obtained results proved the robustness of the method in addition to the stability of the mobile phase. According to the obtained results, the presence of the both ion pair reagents, TFA and the alkyl sulfonate, is extremely important to achieve the maximum separation and sensitivity.

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