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Optimisation for the separation of the oligosaccharide, sodium Pentosan Polysulfate by reverse polarity capillary zone electrophoresis using a central composite design

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Abstract

The separation by reverse polarity capillary zone electrophoresis of the therapeutically developed sodium salt of Pentosan Polysulfate was optimised through the analysis of response surface methodologies, modeled using a central composite design. The optimisation investigated injection pressure, injection time and voltage and the effect of the conditions on retention times, peak areas, separation efficiency and the method sensitivity. The overall goal was to develop the most sensitive results with no decrease in separation efficiency. The following results were obtained: (1) retention times generally decreased as injection pressure, injection time and voltage increased, injection time having the least effect; (2) as expected peak areas increased as injection pressure and injection time increased but decreased as voltage increased; (3) separation efficiencies generally increased as injection pressure and injection time decreased, with voltage having almost no effect. For the optimum condition, the sample was introduced at the inlet vial at the cathode hydrodynamically, at optimal setting of 44 s at 35 mbar. The optimal voltage was −20 kV. In comparison with other methods, the optimum showed increased sensitivity, resolution and separation efficiency. Repeatability studies were performed on the optimum parameter conditions. Relative standard deviation values obtained were between 0.9 and 5.4%.

Keywords: Capillary zone electrophoresis; Central composite design; Pentosan Polysulfate

1. Introduction

The oligosaccharide, Pentosan Polysulfate (PPS), has been investigated for pharmaceutical activity. PPS is a semisynthetic polyanionic sulfated polysaccharide that is structurally similar to glycosaminoglycans (GAGs) [1–4]. PPS is based on a natural product, extracted from the bark of the beechwood tree, as shown in Fig. 1 [5,6].

The identification of specific disaccharide sequences within GAGs is challenging due to the presence of isomeric forms and the complexity of

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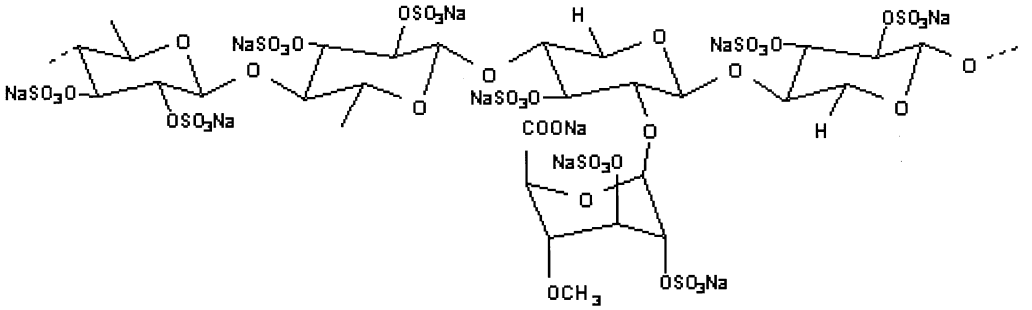

Fig. 1. The basic structure of NaPPS.

GAG polymeric structure [7–9]. Structural analysis of oligosaccharide has the potential of identifying structural conformation that may have an influence on bioactivity. It has been reported that the degree and distribution of sulfonation may be responsible for biological activity [10–13].

PPS has been investigated for its effectiveness against osteoarthritis [5,14,15], interstitial cystitis (chronic bladder inflammation) [16–18] and myocardial ischemia (obstruction of blood flow in the heart) [6,19]. It has been shown to increase capillary blood flow and as a result has been used as an anticoagulant and to be effective against brain paralysis (stroke) [5,6,14,15]. Furthermore, PPS, along with other sulfated polyanions, have shown beneficial effects in treatment of Alzheimer's, cerebral ischemia (obstruction of blood flow in the brain), human immunodeficiency virus (HIV)-related encephalopathy or prion diseases (degenerative brain diseases) [6,19–21]. The family of degenerative brain diseases includes bovine spongiform encephalopathies in cattle, scrapie in sheep, goats and rodents; and kuru, Creutzfeldt-Jakob disease and Gerstmann–Strussler–Scheinker syndrome (GSS) in humans [22,23].

A wide range of analytical methods have previously been employed to characterise GAGs. Polyacrylamide gel electrophoresis [7,10] and strong anion exchange HPLC (SAX-HPLC) [10,13] have been used in the quantitative and qualitative analysis of oligosaccharides. Both these methods were limited by poor sensitivity and were unable to elucidate the polymeric structure. High-performance size-exclusion chromatography was shown to differentiate mass and size distributions [3,17,24]. However to date, CE has provided superior resolution when compared to other methods.

Gas chromatography (GC) has also been used for oligosaccharide characterisation but a time consuming derivatisation must be performed [25]. Many saccharides cannot be derivatised successfully as sugar groups of interest decompose during the hydrolysis step [26,27]. GC coupled with mass spectrometry had been considered, but it was unable to discriminate between all possible linkages and this is the major disadvantage of this technique [26]. Derivatisation for micellar electrokinetic chromatography has limited applications [27].

Fourier transform infrared spectroscopy and ^{1}H and ^{13}C NMR spectra provide specialised information on GAG structure. Specific positioning and chain linkages can be identified in this manner [17,27–29]. All the techniques require large sample volumes and specific instrumental set up [12,30]. Raman spectroscopy has also been found to be a powerful detection technique. CE analysis coupled with Raman spectroscopy as the detection mode allows for oligosaccharide characterisation [29,30].

Slight structural changes within GAGs are detectable by CE and as a result, oligosaccharide compositional analysis is possible [10]. CE is a sensitive technique providing high resolution and resolving power while requiring only small amount of sample [10,30,31]. CE has the capability of resolving complex GAGs structures that differ only by the position of a sulfate group. The high resolving power of CE can provide a separation

over a wider mass range than LC with smaller sample volumes. Additionally, stereoisomers can be resolved by CE [10,31].

GAGs are highly anionic and exhibit very little chromophoric activity. These characteristics render normal polarity capillary zone electrophoresis (CZE) unsuitable. However, these properties can be advantageously used with indirect UV reverse polarity CZE [27,29,32]. For the analysis of the therapeutically developed sodium salt of PPS (NaPPS), the background electrolyte (BGE) acts as the chromophore for UV detection. Reverse polarity CZE was applied to this problem in order to provide oligosaccharide compositional analysis.

The separation of high molecular mass oligosaccharides species by CE has been difficult to achieve, as fragments tend to migrate at similar retention times [10,11]. The CE parameters may be altered in such a way as to achieve acceptable resolution. The application of a voltage gradient has been explored [33].

The separation of NaPPS by reverse polarity CZE was optimised. The goals were to achieve the greatest resolution, maximum number of peaks and highest sensitivity. This was achieved through the application of response surface methodologies, modeled using a central composite design (CCD). Additionally, the optimum for each parameter could then be acquired [34]. This optimisation study investigated injection pressure, injection time and voltage. The chromatographic criteria investigated were retention times, peak areas and separation efficiency.

2. Experimental

2.1. Chemicals and reagents

All chemicals used were of HPLC analytical grade. The NaPPS sample was supplied courtesy of Asst. Prof. Peter Ghosh of Royal North Shore Hospital, Sydney, Australia. Chemicals used in this study were Benzene-tricarboxylic acid (BTC) (Aldrich, NSW, Australia) and NaOH (Ajax, NSW, Australia). Water used was of HPLC-grade (Milli-Q, Millipore, WI). Syringe filters were used to filter samples and standards were 0.45 μm (Bonnet equip., NSW, Australia).

2.2. Instrumentation

The analysis was preformed on the Hewlett Packard ^{3D}CE Instrument, equipped with a UV diode-array detector. A polyimide coated fused-silica capillary (Agilent Technologies, Vic, Australia) (ID = 50 μm), with an effective length of 48 cm (total length 56 cm) was used throughout this study. The wavelength of the UV detector was set at 320 nm (reference wavelength set at 217 nm). Chemstation (Agilent Technologies) installed on a personal computer allowed for instrumental operation and integration strategies. MINITAB statistical software (Minitab Inc. Release 13.1) was used to achieve surface response methodology information.

2.3. Sample preparation

2.3.1. Background electrolyte

367.8 mg of benzene-1,2,4-tricarboxylic acid (BTC) was dissolved into 50.0 ml Milli-Q® water. Approximately 42 ml of freshly prepared 0.1 M NaOH was added to obtain a pH of 4.9. This solution was then made to 200.0 ml with Milli-Q® water. The final concentration of 8.75 mmol l^{-1} was obtained for the BTC solution.

2.3.2. Sample

Ten milligram of PPS was made up to 10.0 ml with Milli-Q® water. The final concentration of 1-mg ml^{-1} was obtained.

2.4. Method development

2.4.1. CE method

All solutions were filtered through a 0.45 μm syringe filter. The sample was introduced at the cathode hydrodynamically, at an optimal setting of 44 s at 35 mbar. The optimal voltage applied was −20 kV. The temperature for the optimum analysis was held at 25 °C. Before its first use, the capillary was flushed with freshly prepared 1.0 M NaOH for 1 h. Equilibration was preformed for 1 h by flushing with the BGE at 25 °C. This has the

effect of protonating the silanol sites on the capillary surface. The capillary was rinsed between each injection for 10 min with the BGE. After each sequence or where the capillary was not used for extended periods, the capillary was rinsed with Milli-Q® water for 10 min and then dried by flushing with air for 10 min.

2.4.2. Response surface methodology

Through the use of MINITAB, polynomial equations, response surface and contour plots for a particular response were produced. Each response surface plot, detailed in multi-dimensions, the experimental conditions for the combination of every electropherogram obtained from the CCD [35,36]. The coded polynomial equation employed for each response were as described by Eq. (1) [37–40].

$$Y = c_0 + c_1x_1 + c_2x_2 + c_3x_3 + c_4x_1^2 + c_5x_2^2 + c_6x_3^2 + c_7x_1x_2 + c_8x_1x_3 + c_9x_2x_3 \quad (1)$$

Within Eq. (1), x_1–x_3 are the variable parameters, and c_0–c_9 are the coefficient values obtained through MINITAB. The response surface plots were obtained through a statistical process that described the design and the modeled CCD data [35,36,41]. Response surface methodologies graphically illustrate relationships between parameters and responses and are the only way to obtain an exact optimum [36,38–41].

The raw data from MINITAB allows equations for each set of results to be formulated. For each response that was analysed, three categories of confidence values, P, were generated. Confidence values are the confidence limits that show the significance of the data. The first category is the confidence limit of 95%, giving a value of P equal to $1.00-0.95$, thus $P = 0.05$. Each term that bears a confidence value of less than or equal to 0.05 is said to be 'significant' within the equation [42]. When the term is equal to or less than 0.25 but greater than 0.05, is then classified into the second category as 'not insignificant'. When the confidence value of a term exceeds 0.25, it is placed in the third category, classified as 'insignificant'.

The confidence values depict the dominance of each term within an equation for a particular response. Thus conforming to the graphical relationship between the parameters and responses for each response within the CCD. The repeatability of a method can be assessed using the optimum obtained through a CCD.

2.4.3. Central composite design

A sequence of experiments for the CCD was generated by MINITAB (Table 1). The design was blocked to ensure randomisation and consistency from run to run. This was necessary as it was not possible to run the complete design in one session. Each experiment involved a different combination of experimental data points for the three variables. For ease of data handling and data volume control, only 15 of the most prominent peaks were integrated and studied.

To obtain an optimum, response criteria were selected. The criteria involved:

- Sum of all the retention times within an electropherogram (sum retention)
- Sum of all the areas within an electropherogram (Sum area)
- Plate count (N)
- Normalised peak areas with respect to retention time (area/retention)

The response surface plots evaluated the influence of each variable on the selected response. High, middle and low values within the CCD, tabulated in Table 2, were used to assess the response surface plot data.

3. Results and discussion

3.1. Central composite design results

Several criteria were investigated and the most significant information generated was for sum of the retention times, sum of the peak areas, plate count (N) for peaks 1, 2, 3 and 7 and peak area normalised with respect to retention time (area/retention) for peaks 1, 2, 3 and 7. Of the 15 peak studied, 4 peaks were selected (1, 2, 3 and 7) for plate count and normalised peak areas, again for ease of data handling.

Table 1
A sequence of experiments for the CCD was generated by MINITAB

Experiment	Block	Injection pressure (mbar)	Injection time (s)	Voltage (−kV)
1	2	45	45	23
2	2	35	35	20
3	2	25	45	17
4	2	45	25	17
5	2	25	25	23
6	2	35	35	20
7	1	35	35	20
8	1	35	35	20
9	1	25	45	23
10	1	45	45	17
11	1	45	25	23
12	1	25	25	17
13	3	35	35	20
14	3	19	35	20
15	3	35	35	20
16	3	35	19	20
17	3	35	35	25
18	3	35	35	15
19	3	50	35	20
20	3	35	50	20

Table 2
High, middle and low values for the variables within the CCD

	High	Middle	Low
Injection pressure (mbar)	50.0	34.5	19.0
Injection time (s)	50.0	34.5	19.0
Voltage (−kV)	25.0	20.0	15.0

For the plate count (N) for peak 2, an equation was constructed from the MINITAB data by the substitution of each constant and coefficient into the coded equation. Eq. (2) was the resulting equation.

$$N = 172085 - 4304P - 2532T + 162V + 25P^2 + 25T^2 + 35V^2 + 31PT + 29PV - 58TV \quad (2)$$

The information concerning the coefficient values supported by the confidence values were then used to examine the series of response surfaces generated.

For this response, the equation and the confidence values found that the injection pressure (P), injection time (T), pressure^2 (P^2), time^2 (T^2), pressure × time (PT) were significant, while time × voltage (TV) were 'not insignificant'. Also, voltage (V), voltage^2 (V^2) and pressure × voltage (PV) were found to be insignificant.

One of the features that was explored in the response surface plots was the shape of the curves present. Since a polynomial equation was derived, quadratic curvatures, such as parabolas, were expected. Fig. 2(a) shows a surface plot for the plate count (N) for peak 2 response when injection time was held at 50.0 s. Parabolic curvature was observed for injection pressure, as P^2 was significant. V^2, being insignificant, showed an almost entirely linear curve for voltage.

Each of these term, including the insignificant terms, were utilised in the equation. For example, Fig. 2(b) shows a surface plot for the sum of the retention times response when voltage was held at −20.0 kV. The confidence values showed that V was significant and V^2, T and TV were not insignificant. Thus, voltage displayed parabolic curvature and injection pressure and injection time were linear when injection pressure or injection time were held at set values, as P^2 and T^2 were insignificant. Since voltage was the single most influential variable, parabolic curvature for injec-

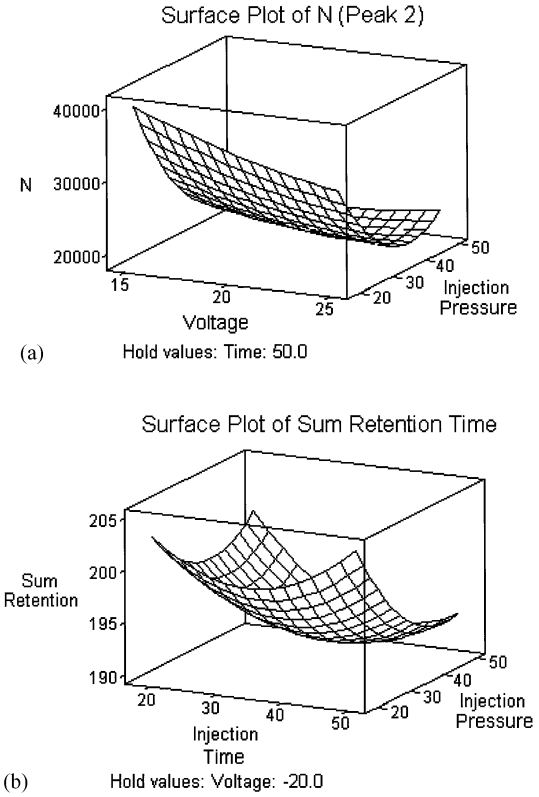

Fig. 2. (a) The surface plot for the plate count (N) for peak 2 response when injection time was held at 50.0 s. (b) The surface plot for the plate count (N) for peak 2 response when voltage was held at -20.0 kV.

tion pressure and injection time was observed when V was held at set values.

For each series of held values for each response, an interpretation of the influence of each variable on the response was made.

Similarly, interpretations were carried out, though not described in detail here, for the other response criteria introduced earlier.

All interpretations of the CCD and response surface data for a single response illustrated how the equation and the computed confidence values were represented by the theoretical response surfaces. The dominance of a particular term could then be identified.

3.2. Optimisation

From the response surface interpretation, a relationship between each variable and response was established. The information gathered from the equations and the graphs could then be summarised as follows,

- Retention time generally decreased as P, T, and V increased, T having the least effect overall.
- Sum area increased as P and T increased but decreased as V increased.
- Plate count (N) generally increased as P and T decreased. At high and middle held values of P and T, V had no effect on N.

The goals for the optimisation of this method included: minimising retention time, maximising the sum of the peak areas, maximising plate count and maximising the normalised peak. These can be summarised as in Table 3.

For the optimisation goal to be attained, the manipulation of the variables to obtain the ideal responses was preformed. For example, to minimise the retention time, it was desirable to have all the variables at high ranges. Ideally, voltage should not be excessively high, as degradation of

Table 3
Tabulation of how injection pressure, injection time and voltage could be altered to achieve ideal response values

Response	Ideal value	Achievable by		
		Injection pressure	Injection time	Voltage
Retention time	Minimum	↑	↑	↑
Sum area	Maximum	↑	↑	↓
Plate count	Maximum	↓	↓	↑
Area × retention	Maximum	↑	↑	↓

Table 4
Results of the repeatability study, tabulating the average of the normalised peak data (area × retention) for each duplicate peak set and the %RSD values

Peak #	Average area/retention	S.D.	%RSD
1	2.3055	0.05	2.01
2	11.2213	0.11	0.62
3	2.1037	0.07	2.89
4	2.9385	0.08	2.97
5	4.4811	0.09	1.93
6	5.7429	0.19	3.40
7	5.6950	0.09	1.74
8	5.5796	0.16	3.00
9	3.7463	0.20	5.38
10	2.9437	0.04	1.81
11	1.7978	0.05	2.29
12	1.0434	0.05	4.60
13	0.7633	0.03	4.18
14	1.1089	0.05	3.90
15	0.5571	0.02	4.38

the capillary may occur. With respect to the above goals, the optimum was obtained.

Pressure × time, retention time and sum area were used for the initial optimum condition set. These responses were particular to each electropherogram. Two more optimum condition sets were obtained by introducing plate count and the normalised peak area. All three optimum conditions were found to be similar, resulting in the final optimum conditions, rounded to the nearest whole number. These were reported as: injection pressure: 35 mbar, injection time: 44 s and voltage: −20 kV.

This optimum showed increased resolution, decreased retention times and overall greater efficiency. An optimised electropherogram of NaPPS is illustrated in Fig. 3.

3.3. Repeatability

Repeatability studies were performed on the optimum parameter conditions. The NaPPS solution was repeatedly injected under the optimum conditions six times. Each peak in the electropherograms were normalised (area/retention) and the relative standard deviation (RSD) was calculated (Table 4).

This low range of RSD values (0.9–5.4%) yielded a low average (3.0%) indicating high repeatability within the method.

A linearity study between the concentration ranges of 2.5–0.25 mg ml^{-1} of NaPPS was preformed (Table 5). Although the coefficients of variation are not ideal, they were considered acceptable due to the low concentration and the minimal separation for this type of application.

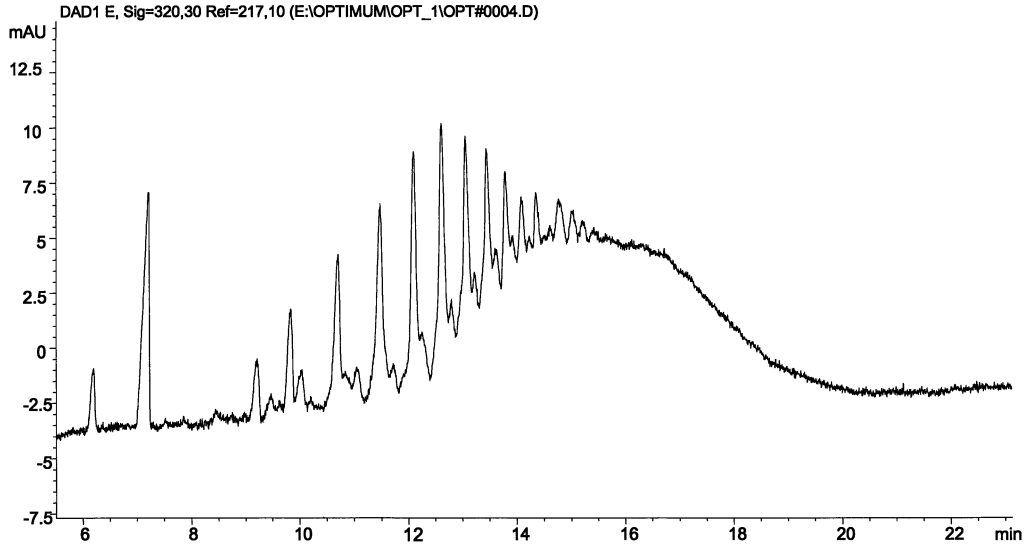

Fig. 3. An optimised electropherogram of NaPPS.

Table 5
Results of the linearity study between the concentration ranges of 2.5–0.25 mg ml^{-1} of NaPPS

Peak	R^2	Intercept	Intercept error	Slope	Slope error
2	0.9901	0.1397	0.6604	1.0375	0.0520
5	0.9961	−0.1116	0.1559	0.3902	0.0123
7	0.9979	−0.2408	0.1470	0.5024	0.0116

The robustness of the method was also established by utilising a Plackett-Burman design. Five factors were tested using a seven factor reflected Plackett-Burman design. Each factor was varied approximately 5% above and below the optimum value. Results showed the method to be robust.

4. Conclusion

This study has shown an improved separation of NaPPS by reverse polarity CZE incorporating experimental designs. The optimisation of NaPPS was successfully completed employing response surface methodologies from the data obtained from a CCD. Response surface methodologies produced an optimum for injection pressure (35 mbar), injection time (44 mbar) and voltage (−20 kV). As a result, this brought about increased resolution, decreased retention times and overall greater efficiency than past methods in a minimum number of experiments performed. The low RSD indicated high repeatability within the method.

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