Rapid and sensitive anthrone—sulfuric acid assay in microplate format to quantify carbohydrate in biopharmaceutical products: Method development and validation

Alberto Leyva a,*, Anelis Quintana a, Meily Sánchez a, Elias N. Rodríguez b, José Cremata c, Julio C. Sánchez a

a Process Control Department, Center for Genetic Engineering and Biotechnology, Ave 31 between 158 and 190, P. O. Box 6162, Havana 10600, Cuba
b Hepatitis B Department, Center for Genetic Engineering and Biotechnology, Ave 31 between 158 and 190, P. O. Box 6162, Havana 10600, Cuba
c Department of Physical Chemistry, Center for Genetic Engineering and Biotechnology, Ave 31 between 158 and 190, P. O. Box 6162, Havana 10600, Cuba

Received 20 May 2007; revised 10 September 2007; accepted 13 September 2007

Abstract

The need for an accurate, fast and reliable analysis of carbohydrate test is crucial for numerous biological processes. In that sense, anthrone—sulfuric acid assay is one of the most efficient quantification techniques successfully applied to carbohydrate determination. In this paper, a sensitive and accurate anthrone—sulfuric acid microplate assay was developed and validated for the quantitative estimation of yeast carbohydrates in the production of hepatitis B virus surface antigen, and the main component of the recombinant vaccine HEBERBIOVAC HB. A response surface methodology was applied to design and optimize the assay in order to maximize the differences on the expected effect and to minimize the number of experiments. The proposed method was linear over the concentration range from 10 to 120 μg/mL for glucose, with values for the coefficient of determination >0.99. Intra- and inter-assay variation coefficient ranged between 0.45–4.79% and 2.48–8.94%, respectively. The Student t-test used in the interference study, revealed good parallelism among curves (T obs /C20 ≤T 0.05), which indicates the lack of interference in the working range. Yields obtained in accuracy test for two concentration levels varied between 90 and 105%, confirming the assay’s reliability. In conclusion, the validated method, which has successfully been used for the process control monitoring of several samples generated from the production of hepatitis B vaccine, allows the quality and purity of the final product.

© 2007 The International Association for Biologicals. Published by Elsevier Ltd. All rights reserved.

Keywords: Carbohydrate; Hepatitis B surface antigen; Anthrone; Microplate; Validation

1. Introduction

Hepatitis B virus (HBV) is one of the world’s most widespread infectious agents. This virus can cause lifelong infection, liver cirrhosis, liver cancer and death [1,2]. Immunization with hepatitis B vaccine is the most effective method to prevent its infection and consequences. HEBERBIOVAC HB, Heberbiotec S.A, Cuba formulated with Pichia pastoris-derived hepatitis B surface antigen (r-HBsAg) has provided enough evidence about its safety and efficacy for the protection against hepatitis B infection [3]. Important features of active pharmaceutical ingredient (API) and the final formulate of recombinant vaccine are quality, strength and purity, as described by World Health Organization (WHO) requirements for vaccines [4]. In that sense, carbohydrates are of the most abundant living organism organic compounds that can be present physically associated or chemically bound to yeast-derived API.

Determination of carbohydrate content in a variety of samples is a basic analytical operation in many biotechnology processes. A large number of analytical procedures have been developed to measure its presence in water [5], fecal fat [6],

---

* Corresponding author. Tel.: +53 7 2716022x2110; fax: +53 7 2714764. E-mail address: alberto.leyva@cigb.edu.cu (A. Leyva).
plant extract [7], and yeast samples [8]. Among many colorimetric methods for carbohydrate determination, the anthrone—sulfuric acid [9] is one of the most commonly used techniques. This method has been used to measure the soluble sugars in samples of different vegetal tissues of apple trees [10], in maize plants [11] and spider hemolymph [12]. Other methods used to quantify carbohydrate are the phenol—sulfuric acid [13], the orcinol [14] and the resorcinol methods [15]. These assays must be validated first to demonstrate that they are useful for its intended purpose, either as an in-process analysis for the characterization of critical product/process attributes, or to support the documentation quality of API.

In a previous paper [16], the results of a preliminary investigation of the adaptation of anthrone—sulfuric acid method for 96-well microplate assay were given. However, the detectable range of this method is 50–400 µg/mL and it needs a longer reaction period. We described a simple and more sensitive microplate assay to quantify yeast carbohydrate using the same reaction for colorimetric determination of total carbohydrate in biopharmaceutical products.

In this paper we standardized and validated an anthrone—sulfuric acid adapted to microplate. This assay was able to quantify with efficient total carbohydrates from P. pastoris along the hepatitis B vaccine manufacturing process. This procedure has the highest sensitivity and is the simplest among the anthrone—sulfuric acid assay reported so far.

2. Material and methods

2.1. Materials

All chemicals of analytical grade were used as supplied. Concentrated sulfuric acid and anthrone were from Merck, Germany. Microplates (Nunc, Maxisorp®, Life Technologies) were from Roskilde, Denmark. Also microplates from Costar (Corning Inc., Corning, NY) were used. A microplate reader (Labsystem, Helsinki, Finland) and its accompanying software were employed as well.

2.2. Anthrone reagent

The anthrone reagent was prepared right before analysis by dissolving 0.1 g of anthrone (0.1%) in 100 mL of concentrated sulfuric acid (98%), protected from light and used within 12 h.

2.3. Assay procedure

Hundred and fifty microliters of anthrone reagent was added to each well of the microplate containing 50 µL of standard solutions, positive control, manufacturing samples dilutions and blank. Plates were then placed 10 min at 4 °C. Subsequently, plates were incubated 20 min at 100 °C. After heating, a cooling step treatment for 20 min at room temperature before reading absorbance at 620 nm triplicate in a microplate multiscan reader was performed to optimize the reaction conditions. Measures were taken in triplicate. Colorimetric response was compared to a standard curve based on glucose, and total carbohydrate content was expressed as µg/mL of glucose.

2.4. r-HBsAg source

A recombinant strain of P. pastoris (C-226) was grown in saline medium supplemented with glycerol, and its expression was induced by methanol; the r-HBsAg was submitted to initial purification steps as previously described [17–19]. Manufacturing process sample supernatant of cellular disruption (SCD), supernatant of acid precipitation (SAP), supernatant of semipurification by diatomaceous earth matrix (SSD), eluant of negative ion-exchange chromatography (NIEC), desalted eluant of immunoaffinity chromatography (DIAF), eluant of positive ion-exchange chromatography (PIEC), diafiltered eluant (DFE), eluant of size-exclusion chromatography (SEC), active pharmaceutical ingredient (API), and buffers corresponding to each production process sample used in this study were provided by the Hepatitis B Production Department of the Center for Genetic Engineering and Biotechnology in Havana.

2.5. Preparation of buffer and solutions used in the manufacturing process

All buffers were made in injection and purified grade water. The disruption (SCD), and precipitation buffers (SPA) (Tris 20 mM—EDTA 5 mM—NaCl 0.3 M, pH7 and pH8, respectively) were supplemented with concentrated potassium thiocyanate (KSCN). The semipurification (SSD) and negative ion-exchange chromatography (NIEC) buffers were prepared as following (Tris 20 mM—EDTA 3 mM—NaCl 250 mM and NaCl 1 M, respectively).

In the affinity purification step (IAF), elution of HBsAg occurs with the equilibration buffer (Tris 20 mM—EDTA 3 mM) containing 3 M of KSCN and then desalted. Positive ion-exchange chromatography (PIEC) was used after immunopurification step to further purify the HBsAg (Tris 20 mM—EDTA 3 mM—NaCl 0.4 M). The subsequently chromatography step (ED and SEC) containing the buffer (Tris 20 mM—NaCl 0.2 M—sodium deoxycholate 0.05%). The final formulation (API) contain phosphate buffer.

2.6. Statistical analysis

Calibration curves were obtained with different standard concentrations (glucose). Linearity was determined using the least-squares method, and the criterion for acceptance linearity was 10% recovery. Sensitivity was defined as the capacity of the method to detect smaller changes in the sample concentration. Regression coefficient (r²), y-intercept, slope of the regression line, and residual sum of squares were also analyzed. Working range was established between the highest and lowest concentration values with satisfactory accuracy and precision. Quantification limit was the smallest amount of the analyte that can be quantitatively measured in a sample with acceptable accuracy and precision. We accepted the
3. Results

3.1. Assay optimization

When carbohydrates react with the anthrone reagent, a green color is produced. The reaction product could be measured at different wavelengths [20–25]. The absorbance spectrum of that reaction was recorded in a wide range of wavelengths (from 500 to 800 nm). The highest absorbance value is reached at 622 nm as reported before by other authors [21,25]. Therefore, all the experiments were followed at 620 nm, due to its proximity to the commercially available filters of microplate reader.

A user-specified surface response design was performed to determine optimal reaction conditions. In this study, the effect of three parameters [temperature (a), incubation time (b) and carbohydrate concentration (c)] over the anthrone–glucose colorimetric reaction was evaluated. To determine the statistical significance of experimental factors, a multifactor analysis of variance (ANOVA) was conducted divided into separate pieces for each effect. All effects were p-values < 0.05, indicating that they are significantly different from zero at the 95% confidence level.

When a surface response analysis was conducted, an empirical third order polynomial equation, which quantified a mathematical relationship among observed data and meaningful variable with the experimental points was developed and defined as:

\[
OD_{620} = -0.598 + 0.0058a + 0.0198b + 0.0069c - 0.0002ab - 0.0001b^2 - 0.00003bc
\]

Fig. 1 shows the relationship of absorbance at 620 nm with the reaction conditions. The model describes the analytical conditions at which absorbance reaches a maximum value at 100 °C in 20 min. The R-squared statistic calculated indicates that the model fitted explains 97.1% of the variability at 620 nm.

3.2. Method validation

3.2.1. Edge effect and signal homogeneity

Analysis of the mean OD of the edge wells versus the mean OD of the interior wells was studied. Although the assay was carried out at 100 °C during 20 min, these conditions did not affect the absorbance in peripheral wells versus interior ones (Fig. 2). Plates were also able to resist the high temperatures without deformation. Similar results were observed in Costar plates (data not shown). On the other hand, the plate signal homogeneity was tested. Three different concentration levels of the glucose standard solution were applied in three assays using a pre-established design (see Section 2). The CV in each solution at high, medium and low concentrations was 8.7, 6.5 and 7.6%, respectively, confirming the signal homogeneity along the microplate under the fixed experimental conditions.

Fig. 1. Surface response curve in a three-dimensional plot shows the absorbance relationship of the colorimetric reaction under experiment conditions. Height of the surface represents the value of observed optical density (OD) at 620 nm.

Fig. 2. Temperature effect in the assay. Behavior of the edge effect between the interior and peripheral wells at low, medium, and high levels of standard concentration. Values in the chart represent the relation between interior wells and peripheral wells at each concentration level.
3.2. Linearity and working range

The linearity of an analytical method symbolizes its ability to elicit test results that are either directly, or by a well defined mathematical transformation, proportional to the concentration of analyte in samples within a given range [26]. To determine the linear range of the microplate format assay, including the quantification limit (LOQ), standard curves ranging 10–120 µg/mL of glucose were prepared and least-squares linear regression analyses were calculated using the data observed for absorbance at 620 nm. Determination coefficient ($r^2$) was over 0.99 in all cases. Concentration values experimentally determined at each point of the standard curve were precise and accurate (Table 1). The calculated $F$ Snedecor associated probability was <0.01, confirming assay linearity in the studied range. The LOQ was 10 µg/mL.

3.2.3. Interferences

When the anthrone reaction is used to quantify carbohydrates in the presence of other substances, possible interferences should be detected [10]. Samples of r-HBsAg in nine different buffers from the manufacturing process were tested to detect possible endogenous interferences. This parameter was examined by comparing slopes using Student’s $t$-test. Samples were diluted according to its working dilution. Student’s $t$-test used in this study revealed good parallelism among curves ($T_{obs} \leq T_{0.05}$). Besides, we determined the concentration of the positive control in each assay and the coefficient of variation of all values was lower than 10%. In addition, the analysis to the obtained values of other important parameters was within the limits of acceptance of the test (Table 2). These results corroborate the lack of interference in the working range.

### Table 2

Comparison of curves in different buffer used in the production process

<table>
<thead>
<tr>
<th>Curve/Buffer</th>
<th>Slope</th>
<th>y-Intercept</th>
<th>Background</th>
<th>$r^2$</th>
<th>Control (µg/mL)</th>
<th>$T_{obs}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCD</td>
<td>0.0065</td>
<td>0.066</td>
<td>0.045</td>
<td>0.9958</td>
<td>100.20</td>
<td>−1.05</td>
</tr>
<tr>
<td>SAP</td>
<td>0.0065</td>
<td>0.063</td>
<td>0.056</td>
<td>0.9923</td>
<td>93.17</td>
<td>−1.97</td>
</tr>
<tr>
<td>SSD</td>
<td>0.0063</td>
<td>0.055</td>
<td>0.049</td>
<td>0.9968</td>
<td>119.44</td>
<td>−0.68</td>
</tr>
<tr>
<td>NIEC</td>
<td>0.0061</td>
<td>0.058</td>
<td>0.042</td>
<td>0.9945</td>
<td>108.18</td>
<td>−1.03</td>
</tr>
<tr>
<td>IAF</td>
<td>0.0060</td>
<td>0.067</td>
<td>0.061</td>
<td>0.9918</td>
<td>105.64</td>
<td>−0.16</td>
</tr>
<tr>
<td>PIEC</td>
<td>0.0066</td>
<td>0.075</td>
<td>0.048</td>
<td>0.9971</td>
<td>101.88</td>
<td>−0.92</td>
</tr>
<tr>
<td>DFE</td>
<td>0.0064</td>
<td>0.062</td>
<td>0.054</td>
<td>0.9938</td>
<td>91.50</td>
<td>−1.23</td>
</tr>
<tr>
<td>SEC</td>
<td>0.0055</td>
<td>0.045</td>
<td>0.060</td>
<td>0.9884</td>
<td>117.48</td>
<td>0.67</td>
</tr>
<tr>
<td>API</td>
<td>0.0062</td>
<td>0.072</td>
<td>0.045</td>
<td>0.9928</td>
<td>105.69</td>
<td>−1.43</td>
</tr>
<tr>
<td>Acceptance limits</td>
<td>(0.0078 – 0.0055)</td>
<td>(0.080 – 0.021)</td>
<td>&lt;0.100</td>
<td>≥0.98</td>
<td>(122.07 – 90.66)</td>
<td>$T_{0.05, (10)}$ (2.23)</td>
</tr>
</tbody>
</table>

All assays were performed under the same conditions.

### 3.2.4. Precision and accuracy

The precision of an analytical method is the homogeneity of values within a series of individual measurements of an analyte when the analytical procedure is repeatedly applied to multiple aliquots of a single volume of a biological matrix [27]. In this case, the intra-assay variability was determined by comparing the concentration values of each type of manufacturing process samples within its corresponding replica. Variation coefficient obtained from duplicates by sample within a plate ranged 0.45–4.79%, indicating the low variation within a plate. Intra-assay variation was calculated by the comparison of total carbohydrate concentrations obtained from sample ($n = 9$) runs in three separate assay performed by three analysts. CV ranged from 2.48 to 8.94% (Table 3). Accuracy was assessed by the recovery test, measuring the difference between the endogenous concentration of the sample and a known concentration of the analyte added. This spike was developed at a known concentration given by the standard curve central region. This procedure yields two types of samples, which represent both low and high quantification range. Recovery values obtained for each sample analyzed are shown in Table 4. Yields calculated in accuracy test for these concentration levels ranged between 90 and 105%, confirming the reliability of the assay.

### 3.3. Practical evaluation of the validated procedure

The validated assay in the present work was used to determine the total carbohydrate values in different manufacturing process samples (see Section 2). Fig. 3 shows the results from three batches evaluated using this method. The graphic demonstrates that carbohydrate contaminants were efficiently removed in the immunoaffinity purification step. Also, due to the sensitivity of this method, it is possible to detect the low carbohydrate levels in the rest of the standard chromatographic steps and in the final product, which fulfill the requirements...
according to the internal manufacturer to total carbohydrates contaminant in each process sample.

To demonstrate this behavior in regular use, the specification limits for the positive control used in the assay were determined. Fig. 4 shows a little variance between 30 individual runs for a period of 3 months, demonstrating the assay’s consistency. The concentration values of the entire control fell within ±2SD; indicating that this method was run with accuracy and precision.

4. Discussion

The importance of carbohydrate quantification in recombinant vaccines and pharmaceutical products to verify their quality, and purity, is mandatory by many regulatory agencies such as the United States Food and Drug Administration (FDA) and WHO [4,28–30]. With this purpose, we developed, and validated a microplate anthrone—sulfuric acid microassay to quantify total carbohydrates during the production process of the Cuban hepatitis B vaccine (HEBERBIOVAC HB, Heberbiotec S.A, Cuba), formulated with \textit{P. pastoris}-derived HBsAg [17].

Analytical assays used to tightly control biopharmaceutical manufacturing processes are generally optimized to demonstrate its suitability for its intended purpose, as an in-process analysis for the characterization of critical process/product attributes or final product characteristics. However, such analyzes are neither often modeled nor controlled using advanced statistical methods as response surface methodologies (RSM) [31]. RSM includes factorial designs and regression analysis and allows the multifactorial experiments

Table 3

<table>
<thead>
<tr>
<th>Samples</th>
<th>CV (%)</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analyst 1</td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>SCD</td>
<td>15,688.84</td>
<td>16,480.42</td>
</tr>
<tr>
<td>SAP</td>
<td>9588.91</td>
<td>10,475.94</td>
</tr>
<tr>
<td>SSD</td>
<td>6897.32</td>
<td>6555.29</td>
</tr>
<tr>
<td>NIEC</td>
<td>3720.06</td>
<td>4067.21</td>
</tr>
<tr>
<td>IAF</td>
<td>24.43</td>
<td>25.69</td>
</tr>
<tr>
<td>PIEC</td>
<td>115.21</td>
<td>110.24</td>
</tr>
<tr>
<td>DFE</td>
<td>1295.95</td>
<td>1213.05</td>
</tr>
<tr>
<td>SEC</td>
<td>302.66</td>
<td>298.68</td>
</tr>
<tr>
<td>API</td>
<td>87.57</td>
<td>81.31</td>
</tr>
</tbody>
</table>

Table 4

<table>
<thead>
<tr>
<th>Samples</th>
<th>Statistics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>CV (mg/mL)</td>
</tr>
<tr>
<td>SCD</td>
<td>60.32</td>
</tr>
<tr>
<td>SAP</td>
<td>54.54</td>
</tr>
<tr>
<td>SSD</td>
<td>56.42</td>
</tr>
<tr>
<td>NIEC</td>
<td>52.79</td>
</tr>
<tr>
<td>IAF</td>
<td>57.30</td>
</tr>
<tr>
<td>PIEC</td>
<td>54.70</td>
</tr>
<tr>
<td>DFE</td>
<td>58.10</td>
</tr>
<tr>
<td>SEC</td>
<td>70.19</td>
</tr>
<tr>
<td>API</td>
<td>55.18</td>
</tr>
</tbody>
</table>

$T_{0.05, (2)} = 4.30.$
without requiring a large number of runs. In this kind of experimental design, factor levels should be selected from extreme conditions in order to maximize the differences on the expected effect, minimizing the number of experiments. They are usually done in random order to avoid the possibility of time-related effects altering the results. This statistical processing allows the estimation of the optimal reaction conditions to determine the total carbohydrate concentration as a reaction time for 20 min at 100°C for routine analysis of several manufacturing process samples at the same time. In these conditions, the maximal development of reaction between anthrone and sulfuric acid, and the total degradation to free sugars and their glycosides were obtained. This result allowed the performance of this assay in only two steps analyzing several samples at the same time. The simplicity of this method avoids any inconvenience produced by samples handling and improves in five-fold the sensitivity obtained with the assay reported by Laurentin in 2003 [16]. Under fixed working conditions, the linear range 10<sup>e</sup>–120<sup>e</sup> mg/mL was obtained with a seven-point standard curve.

The phenomenon known as “edge effect”; usually due to several factors that might affect the optical densities measured in the peripheral wells and the central wells in a microwell are described in immunoassay type—ELISA [32]. In the present work, this event was mainly studied because of the extreme condition used in the assay. However, the high incubation temperature used in the assay did not affect the optical density between all wells along the microplate; neither deformed the plates (data not shown). In a previous work related to the adaptation of anthrone—sulfuric acid reaction to 96-well plates, it was demonstrated that those polystyrene microplates can resist high temperature [16], but did not show details of edge effect studies.

Precision of the assay was calculated according to the International Conference on Harmonization (ICH) guidelines [33,34]. For our experimental conditions, the repeatability and intermediate CV precision percent ranged between 0.45<e>–8.28% and 2.48<e>–8.94%, respectively, thus indicating low intra- and inter-assay variation and also aimed at high sample stability. These values were similar to those reported by other researchers [15].

The presence of proteins often can cause interferences in carbohydrate estimation. Some researchers have also reported that the anthrone—sulfuric acid procedure is subjected to interference with several ions such as NO<sub>3</sub>⁻, Fe, and Cl<sup>e</sup>⁻ [35–37]. In this study, the solution used in the process and pollutant proteins in crude samples did not interfere in the quantification of total carbohydrate in each sample analyzed. In the r-HBsAg production process high concentration of thiocyanate is used to promote total refolding of r-HBsAg.

Purification efficiency of production process steps was evaluated by measuring the total carbohydrate concentration with the anthrone—sulfuric acid microassay. The results obtained demonstrate that the immunoaffinity purification was an efficient step removing around 99% of carbohydrates contaminant. The quantity of r-HBsAg carbohydrate in this stage was of 2.3, 2.6 and 2.7 µg carbohydrate per 20 µg of this protein in the three analyzed batches (Fig. 3). These values are in

![Fig. 3. Determination of carbohydrate concentration through each step of the downstream production process.](image-url)
agreement with the limit established for recombinant vaccines under WHO guidelines [4], and the reported by Pentón in 1994 [3] (≤3/20 µg of r-HBsAg).

The validation of a procedure confirms that the method is suitable for the intended purpose. For this reason, pharmaceutical validation and process controls are important in spite of the problems that may be encountered [38]. Therefore, validation of this assay should be regarded as part of an integral concept to ensure the quality, safety and efficacy of r-HBsAg vaccine.

According to the results for analyzing r-HBsAg manufacturing process obtained with this method, we recommend it to process control of biopharmaceutical products as erythropoietin (EPO), and epidermal growth factor (EGF) obtained by recombinant way.

The standardized and validated assay above is simple and can be adapted and performed in any research laboratory. In such sense, this assay employs 20 volumes less of anthrone–sulfuric acid reagent than the traditional assay, reduces the assay time, decreasing the cost and the hazards of the assay. Besides, traditional methods for quantifying total carbohydrate are complex and time consuming [9].

The anthrone–sulfuric acid microplate assay, described in this paper, has been used for several times in our laboratory and has been found to be suitable to quantify total yeast carbohydrates through the production process of hepatitis B vaccine. Finally, the use of microplate assay allows total carbohydrates determination in numerous samples quickly and easily.

Acknowledgments

The authors wish to thank Dr. Leonardo Canaan-Haden for assistance and technical advice. We are also grateful to Ba. José Luis Fernández Sierra, and Dr Ricardo Silva for reviewing the English.

References

[34] ICH. International conference on harmonization of technical requirements for registration of pharmaceuticals for human use, validation of analytical procedures; 1996. pp. 1–8.


