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Analytical methods and stability assessment of liquid yeast derived sucrase

Kylie A. McIntosh [a], Susan A. Charman [a], Lowell A. Borgen [b], William N. Charman [a,*]

[a] *Department of Pharmaceutics, Victorian College of Pharmacy, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia*
[b] *Orphan Medical Inc., Minnetonka, MN 55305, USA*

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Abstract

Two independent analytical methods for determining the activity and stability profile of liquid yeast derived sucrase (YS) were established and validated in order to conduct preliminary stability studies as a function of temperature. The methods included a hexokinase-based (HK) enzymatic assay for determining the formation of glucose upon hydrolysis of sucrose by YS, and a direct polarimetric procedure to quantitate YS hydrolysis of sucrose. Both assays were validated with respect to YS dilution, incubation time, sucrose or glucose concentration, linearity of response and within- and between-day variability. A preliminary stability study was conducted over a 24 week period with liquid YS samples stored at −20, 4, 30, 40 and 50°C. Enzymatic activity was monitored as a function of time using both the HK and polarimetric assays. Liquid YS samples stored at −20, 4 and 30°C retained 100% activity after 24 weeks storage, while the samples stored at 40°C lost approximately 70% activity over the same storage period and samples stored at 50°C lost approximately 95% activity after 12 weeks storage. The two methods of analysis gave consistent results over the course of the study.

Keywords: Sucrase; Hexokinase; Polarimetry; Stability

1. Introduction

Congenital sucrase–isomaltase deficiency (CSID) is an inherited disaccharidase deficiency responsible for the malabsorption of sucrose. This relatively rare condition is typically manifested as diarrhoea, gas and abdominal pain. The current treatment for CSID patients involves a lifelong adherence to a sucrose-free diet [1]. Problems with poor dietary compliance, and the associated continuing presence of untoward gastrointestinal symptoms, have led to interest in an orally administered enzyme preparation. The successful use of liquid yeast-derived sucrase (YS) for the treatment of fourteen patients with CSID was reported in 1993 [2]. These clinical data extended earlier findings in which eight children treated with lyophilised and fresh *Saccharomyces cerevisiae*

* Corresponding author. Tel.: +61 3 99039519; fax: +61 3 99039560; e-mail: Bill.Charman@vcp.monash.edu.au

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(baker's yeast) reported either a complete loss, or at least a significant reduction, in the gastrointestinal symptoms [3]. It has been suggested that such treatment may also be useful in patients with secondary sucrase deficiency such as that caused by coeliac disease or acquired immunodeficiency syndrome [1].

A major advantage of using liquid YS for the treatment of young children is its palatability and ease of administration when compared to either lyophilised or fresh baker's yeast (each administered as a suspension of yeast cells in water). However, a significant barrier to the commercial and clinical acceptance of liquid YS therapy is the lack of adequate stability data to verify the product quality upon storage. It is therefore necessary to have available reliable method(s) for determining sucrase activity.

The objectives of this study were to establish and validate two independent analytical methods for determining the activity and stability profile of liquid yeast-derived sucrase (YS). The assays included an indirect hexokinase-based (HK) enzymatic assay method which monitors the formation of glucose following hydrolysis of sucrose by YS and a polarimetric technique which directly monitors the optical activity due to changes in the relative proportions of sucrose and the hydrolysis products, glucose and fructose. The advantage of using these two techniques was that they measured different and independent endpoints thus providing greater confidence when comparing the stability data generated using each of the methods. Following validation of the two methods, a preliminary stability assessment of a liquid YS product was conducted over a period of 6 months with storage at −20, 4, 30, 40 and 50°C.

2. Materials and methods

2.1. Materials

Liquid YS was provided by Orphan Medical Inc. (Minnetonka, MN) and was used without further purification. Sucrose, glucose, fructose and the enzyme standards sucrase, lactase, maltase, and isomaltase, in addition to the HK assay kit, were obtained from Sigma (St. Louis, MO). Water obtained from a Milli-Q water purification system (Millipore, Bedford, MA) was used throughout all the studies. All reagents were of analytical grade.

2.2. Glucose–hexokinase assay

Liquid YS was freshly diluted 1:5000 with water on the day of each experiment. A 50 μl aliquot of the diluted liquid YS was added to 200 μl of a 1.71 g dl^{-1} (50 mmol l^{-1}) sucrose solution. The resulting mixture was incubated at 37°C for 30 min and then analysed for glucose content using the commercial HK assay kit. A 10 μl aliquot of sample or standard glucose solution was added to 1.5 ml HK reagent, mixed by gentle inversion and then incubated at room temperature for 5 min. Blanks were prepared by adding water to the HK reagent instead of the glucose containing solutions. The absorbance at 340 nm was subsequently determined using a Cary 3 UV spectrophotometer (Varian Instruments) to enable calculation of the glucose concentration using Eq. (1):

$$\text{Glucose conc. (mg dl}^{-1}) = \frac{\Delta A \times \text{total volume (ml)} \times \text{MW}_{\text{glucose}} \times 100 \text{ ml dl}^{-1}}{6.22 \times \text{pathlength (cm)} \times \text{sample volume (ml)} \times 1000 \text{ μg mg}^{-1}} \quad (1)$$

where ΔA is the difference in the absorbance of the sample and the blank and 6.22 is the millimolar absorptivity of nicotinamide adenine dinucleotide (NAD) at 340 nm.

During the pre-study validation, glucose standard solutions in the concentration range of 100–800 mg dl^{-1} were analysed to determine assay linearity. The limit of quantitation (LOQ) for glucose was determined using Eq. (2):

$$\text{LOQ (mg dl}^{-1}) = (10 \times \text{SD}_{\text{blank}})/\text{slope} \quad (2)$$

where SD_{blank} was the standard deviation for the replicate blank measurements and the slope was obtained from the regression of absorbance versus glucose concentration. Within- and between-day assay reproducibility were evaluated by repeated analysis of the 200 mg dl^{-1} glucose standard

solution. During the course of the stability study, blanks and a 200 mg dl^{-1} external standard were included in each experiment and three separate determinations were performed for each YS sample. YS activity was expressed as the amount (in g) of sucrose hydrolysed per min by 1 ml of (undiluted) liquid YS at 37°C and was calculated taking into account the difference in the molecular weights for glucose and sucrose and the reaction volumes.

Assay conditions were optimised by evaluating the rate of formation of glucose using samples containing 1.71 g dl^{-1} sucrose and liquid YS at dilutions ranging from 1:2500 to 1:10000. The samples were incubated at 37°C and assayed at regular intervals using the HK assay to determine the concentration of glucose produced as a function of time. The effect of the YS dilution factor on the reaction rate was then studied over the dilution range 1:2500–1:80000 using a 30 min incubation followed by glucose determination using the HK assay. The effect of potential contaminating enzymes in the liquid YS samples which could interfere with the glucose–HK assay was also studied by spiking liquid YS (1:5000 dilution) with maltase, isomaltase and lactase at concentrations of approximately 2 μg ml^{-1} each prior to adding the enzyme preparation to the sucrose solution and incubating for 30 min. The amount of glucose produced was determined as described above.

2.3. Polarimetry assay

Liquid YS was freshly diluted 1:500 with water on the day of each experiment. The YS solutions, 11 g dl^{-1} sucrose solution buffered to pH 4.6, and water (blank) were pre-equilibrated in a 25°C water bath for approximately 10 min. A 2.5 ml aliquot of the YS solution or water was then added to 25 ml of the buffered sucrose solution, mixed and incubated at 25°C for 20 min. The reaction was quenched by the addition of 50 μl 25% (w/v) NaOH. The samples and blanks were subsequently placed in a 10 cm cell of a JASCO DIP-370 digital polarimeter (Japan Spectroscopic Company, Tokyo) and the angle of rotation (α) was recorded over a 5 min period (readings taken at 1 min intervals) for each solution. The angle of rotation was taken as the mean of the five readings.

The concentration of sucrose present in the samples was determined by comparison to a standard curve of optical rotation versus sucrose concentration. The standards contained sucrose, glucose, and fructose to reflect the varying degrees of hydrolysis of sucrose to equimolar concentrations of glucose and fructose since the hydrolysis products also contribute to the optical activity of the solution. Standard concentrations ranged from 10:0:0 g dl^{-1} sucrose–glucose–fructose (representing the initial sucrose concentration) to 8.5:0.79:0.79 g dl^{-1} sucrose–glucose–fructose (reflecting a 15% decrease in sucrose concentration with the concomitant formation of equimolar concentrations of glucose and fructose). Since the activity resulted in a decrease in the sucrose concentration from 10 g dl^{-1}, the limit of quantitation for sucrose was calculated by Eq. (3):

$$\text{LOQ (g dl}^{-1}) = (10 \times \text{SD}_{\text{ext std}})/\text{slope} \quad (3)$$

where $SD_{ext\ std}$ was the standard deviation for replicate measurements for the 10 g dl^{-1} external standard and the slope was obtained from the regression of the optical rotation versus sucrose concentration with the standards prepared as described above. Within- and between-day reproducibility of the polarimetry measurements were determined using replicate determinations for the standard solutions. During the course of the stability study, a blank and a 10 g dl^{-1} external standard were included on each analysis day. YS activity was expressed as the amount of sucrose (in g) hydrolysed per min by 1 ml of (undiluted) liquid YS at 25°C and was calculated taking into account the reaction volumes.

Assay development included an evaluation of the time dependent changes in the optical rotation of standard solutions containing sucrose, glucose and fructose at 25°C over a 4 h period. The effect of the incubation time on the extent of sucrose hydrolysis by YS was evaluated over a period of 120 min with incubation at 25°C. The YS dilution factor in these studies was varied from 1:1000 to 1:250. The effect of potential

contaminating enzymes in the liquid YS samples was studied by spiking liquid YS (1:500 dilution) with maltase, isomaltase and lactase at concentrations of approximately 20 μg ml^{-1} each prior to adding the enzyme preparation to the sucrose solution and incubating for 20 min. The amount of glucose produced was determined using the polarimetry assay as described above.

2.4. Stability assessment

The stability of the liquid YS product was assessed over 24 weeks storage at −20, 4, 30, 40 and 50°C. The glucose–HK and polarimetry assays were employed for determination of the sucrase activity throughout the study. A given analytical run was accepted if the measured concentration of either glucose or sucrose in the external standard was within ±20% of the nominal value. The activity data were expressed as a percentage of the initial activity (at time zero) as determined by each assay in order to allow a direct comparison between the two analytical methods. The physical appearance of the samples was also monitored as a function of storage time.

3. Results and discussion

3.1. The HK assay

A HK-based assay for determination of blood glucose concentrations, using hexokinase and glucose-6-phosphate dehydrogenase, was previously adapted for use in determining the activity of sucrase by Harms et al. [3]. The assay is an indirect method which involves several enzymatic steps. Glucose, produced by the sucrase-mediated hydrolysis of sucrose over a given time period, is phosphorylated by hexokinase to produce glucose-6-phosphate, which is subsequently oxidised to 6-phosphogluconate in the presence of NAD. During this coupled oxidation process, an equimolar amount of NAD is reduced to NADH which is monitored spectrophotometrically at 340 nm. Under carefully controlled conditions, the consequent increase in absorbance at 340 nm is directly proportional to the glucose concentration. A variation on this method has recently been reported for the evaluation of sucrase kinetics whereby sucrase activity was continuously monitored using HK, mutarotase and glucose 6-phosphate dehydrogenase as ancillary enzymes [4].

Using the optimised conditions, the assay was found to be linear up to approximately 400 mg dl^{-1} as shown in Fig. 1. The LOQ for glucose was calculated to be 33 mg dl^{-1}. Reproducibility studies showed both within- and between-day variability were less than 5% for the blank and the 200 mg dl^{-1} glucose standard solution. Using the HK assay for glucose determination, the incubation of fresh liquid YS (1:5000 dilution) with 1.71 g dl^{-1} sucrose for 30 min at 37°C typically resulted in the formation of 163 mg dl^{-1} glucose which translated to an activity of 2.58 g sucrose hydrolysed per min for 1 ml (undiluted) YS. Replicate activity measurements using the HK assay varied by less than 3% both within- and between-days. The glucose LOQ of 33 mg dl^{-1} corresponded to a minimum quantifiable activity of 0.52 g min^{-1} ml^{-1} or approximately 20% of the initial activity. The potential contaminating enzymes maltase, isomaltase and lactase were found to have no effect on the HK assay or the amount of glucose produced by liquid YS.

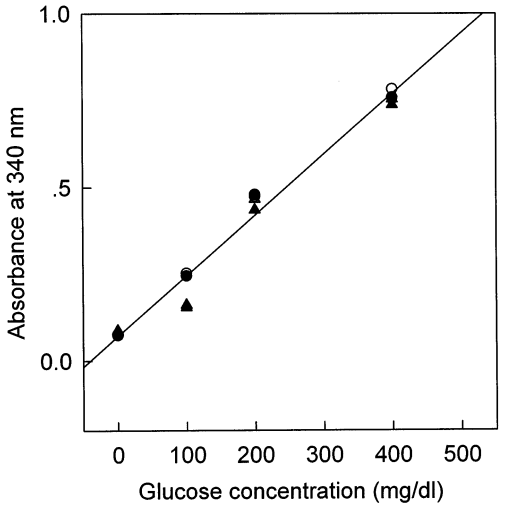

Fig. 1. UV absorbance at 340 nm vs. glucose concentration determined using the HK assay ($n = 4$ measurements at each concentration).

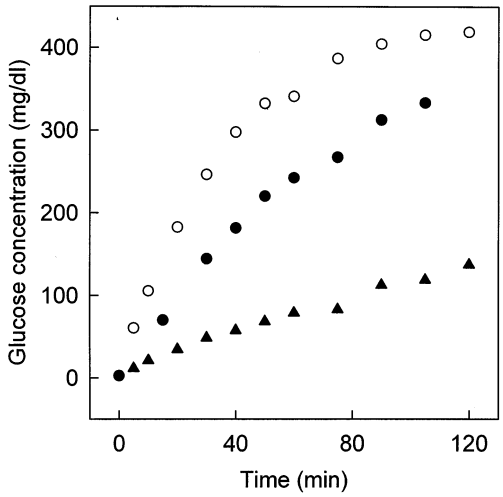

Fig. 2. Glucose production as a function of incubation time at 37°C for YS dilutions of 1:2500 (○), 1:5000 (●) and 1:10000 (▲). Data were obtained using the HK assay.

A number of studies were conducted to optimise the assay and determine the potential sources of variability. Early experiments showed that the mixing of the 10 μl sample with the HK reagent was critical and a standard mixing technique was subsequently employed. The effect of incubation time on the HK reaction was determined following the addition of the HK reagent to standard glucose solutions. The resulting absorbance at 340 nm was monitored for an additional 60 min after the initial 5 min incubation period. The absorbance values remained unchanged during this time confirming that the reaction had reached completion within the first 5 min (data not shown). This experiment was repeated for the samples containing YS, sucrose and glucose in order to determine the effect of residual sucrase and the presence of sucrose on the HK reactions. The absorbance readings were comparable to those obtained for a similar concentration of glucose in the absence of YS and sucrose and were again constant for the 60 min period.

The rate of formation of glucose produced by YS was studied in order to evaluate the kinetics of the reaction and to confirm the suitability of the 30 min incubation time. Fig. 2 is a graphical representation of the glucose produced for three YS dilutions as a function of incubation time. The glucose concentration increased in a near-linear manner over the first 30 min for each of the three YS dilutions.

The effect of liquid YS dilution was investigated over the range 1:2500–1:80000 in order to evaluate the choice of the 1:5000 dilution factor for the assay. Fig. 3 depicts the relationship between the rate of hydrolysis of sucrose and the dilution factor for liquid YS. The symbols represent data from three separate experiments illustrating the high degree of reproducibility. For dilution factors of 1:5000 and greater (reciprocal dilution factors up to 20×10^{-5}), the rate increased in proportion to the increase in YS concentration suggesting that the substrate (sucrose) concentration sufficiently exceeded the concentration of the enzyme. Under these conditions, the initial rate is equal to the maximum rate which is proportional to the total enzyme concentration according to the classical Michaelis–Menten relationship. At higher concentrations of YS, the deviation from linearity indicated that the substrate was no longer in excess and the initial rate depended upon both the substrate and enzyme concentrations.

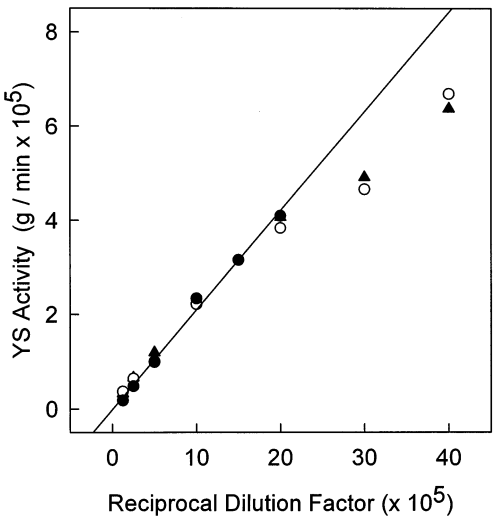

Fig. 3. The relationship between the rate of sucrose hydrolysis and the dilution factor for liquid YS. Data were obtained using the HK assay for glucose. The symbols represent data from three separate experiments.

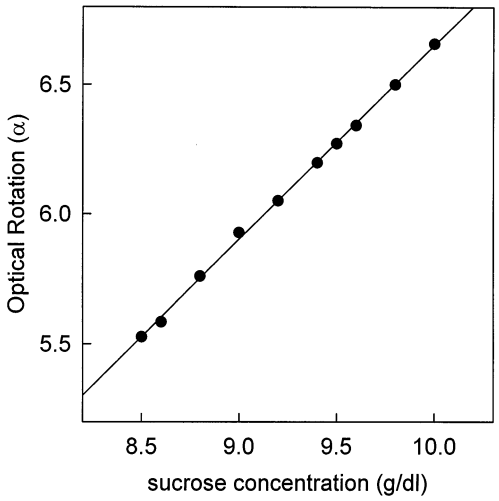

Fig. 4. Calibration curve for standards containing sucrose, glucose and fructose reflecting the varying degrees of hydrolysis of a 10 g dl^{-1} sucrose solution. Data were obtained using the polarimetry assay. The solid line represents the regression line for the data.

3.2. The polarimetry assay

A polarimetry assay for the determination of invertase (sucrase) activity is described in the Food Chemicals Codex. The polarimetric assay involves the measurement of the change in the optical rotation of a sucrose solution as it is hydrolysed by liquid YS to produce equimolar concentrations of glucose and fructose. The decrease in sucrose concentration and the corresponding increase in concentrations of glucose and fructose is reflected by a change in the optical rotation of the solution. The polarimetric analysis of liquid YS samples can therefore provide a direct and simple means of determining sucrase activity.

The optical activity at 25°C was linearly related to the concentration of sucrose over the range of 1–20 g dl^{-1}. Fig. 4 shows a calibration curve obtained using standards containing sucrose, glucose and fructose reflecting varying degrees of hydrolysis of a 10 g dl^{-1} sucrose solution. The presence of the hydrolysis products glucose and fructose in the standards was required since they each contributed to the optical rotation of the sample. The variability for replicate polarimetric measurements over a 5 min period for the standard solutions was less than 1% both within- and between-days. The LOQ for sucrose (the smallest decrease which could be quantitated) was calculated to be 0.037 g dl^{-1}. Typical sucrose concentrations at the end of a 20 min incubation with YS (1:500) at 25°C were 8.89 g dl^{-1}. These values corresponded to an activity of approximately 2.72 g sucrose hydrolysed per min for 1 ml (undiluted) YS. Replicate activity measurements using the polarimetry assay varied by less than 2% both within- and between-days. Using the LOQ for sucrose of 0.037 g dl^{-1}, the minimum quantifiable activity using the polarimetry assay was 0.093 g min^{-1} ml^{-1} or 3.4% of the initial activity. The potential contaminating enzymes were found to have no effect on the activity determined using the polarimetry assay.

Several studies were conducted to confirm the suitability of the chosen conditions for the determination of YS activity. The time dependence of the optical rotation of the standard solutions (containing sucrose, glucose and fructose) was measured after 30 min, 1.5 and 4 h incubation (Table 1). There was essentially no difference between the measurements taken after the 1.5 and the 4 h incubation. As the proportional concentration of glucose and fructose in the standard solutions was increased, there was an increase in the time-dependent change in the optical rotation which most likely reflected the time required to establish an equilibrium between the two cyclic structures of glucose, α-D-glucose and β-D-glucose, and the straight chain form. Despite the time dependency of the optical rotation, routine measurements were made immediately after the reaction was quenched so as to reduce the time required for analysis.

Fig. 5 displays the time dependency of the hydrolysis reaction at 25°C evaluated at three different YS dilutions. For the two higher dilutions, the sucrose concentration decreased in a linear manner for at least 30 min indicating an excess of sucrose over this time period. For the lowest dilution, a deviation from linearity was evident after the 20 min time point.

Experiments were then conducted to confirm the stability indicating nature of the polarimetry

Table 1
Optical activity as a function of time for standard solutions containing sucrose, glucose and fructose reflecting varying degrees of hydrolysis of a 10 g dl^{-1} sucrose solution

Sucrose concentration (g dl^{-1})	Optical rotation (°) after 30 min	Optical rotation (°) after 90 min	Optical rotation (°) after 240 min
7.0	4.389 ± 0.010	4.138 ± 0.002	4.103 ± 0.004
8.0	5.189 ± 0.010	4.983 ± 0.001	4.957 ± 0.003
8.5	5.590 ± 0.008	5.402 ± 0.001	5.376 ± 0.001
9.0	5.928 ± 0.005	5.849 ± 0.001	5.823 ± 0.001
9.5	6.273 ± 0.004	6.228 ± 0.001	6.210 ± 0.002
10.0	6.658 ± 0.003	6.659 ± 0.002	6.655 ± 0.003

Values are mean ± SD, $n = 5$.

assay. Diluted YS samples were intentionally degraded by heating at 80°C for 15 min, by adding 25% NaOH (to mimic the quenching method), or by adding 1 M HCl. The polarimetry assay was carried out on the degraded samples as well as a control (undegraded) sample. Both heating and addition of NaOH induced a complete loss of activity, while addition of HCl produced an apparent 58% loss of activity.

3.3. Preliminary stability study

During the course of the stability study, external standards were included in each analytical run to confirm the acceptability of the results. The inclusion of full calibration curves with each run was not practical due to the analysis time and the cost associated with the commercial HK assay kit. Analytical runs were accepted if the external standards (containing glucose for the HK assay or sucrose for the polarimetry assay) were within ± 20% of the nominal value. Table 2 shows the results for the external standards over the course of the stability study. In all cases, the external standards were within ± 10% of the nominal value.

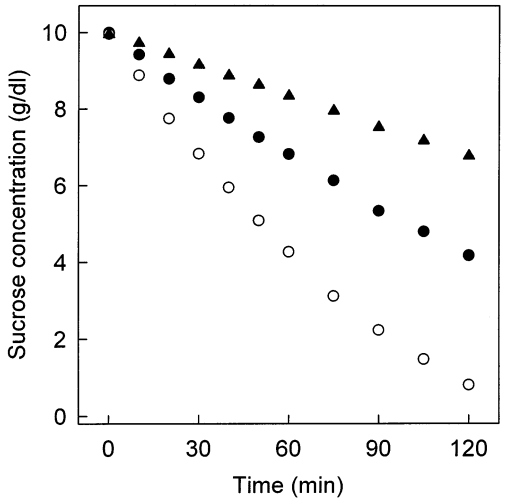

Fig. 5. Time dependency of the sucrose hydrolysis reaction at 25°C evaluated at YS dilutions of 1:250 (○), 1:500 (●) and 1:1000 (▲) monitored using the polarimetry assay.

The liquid YS samples stored at 4 and 30°C retained the original appearance of a clear, pale yellow, viscous liquid throughout the 24 week storage period. Upon equilibrating to room temperature, the − 20°C sample also returned to its original appearance. By week 4, the samples stored at 50°C had separated into two layers, with the lower layer comprising a cloudy, white precipitate which dispersed on shaking, and the upper layer being a clear yellow liquid. The same phenomenon was observed, although to a much lesser extent, in the 40°C samples by week 12. The 40 and 50°C samples also exhibited a strong odour characteristic of yeast after 12 and 4 weeks storage, respectively. Cultures were conducted at week 12 on the 4, 30 and 50°C samples to test for the presence of bacteria, yeast and fungi. As all cultures were negative, it was concluded that microbial contamination of the liquid YS had not occurred.

Table 3 presents the results of the HK and polarimetry assays for the activity of liquid YS

Table 2
External standard determinations using the hexokinase and polarimetry assays over the course of the stability study

		Measured concentration (g dl^{-1})	Deviation from nominal concentration (%)
Initial	HK[a]	198 ± 3	−1.0
	Pol.[b]	9.98	−0.2
Week 1	HK	200 ± 1	0
	Pol.	9.96	−0.4
Week 2	HK	196 ± 6	−2.0
	Pol.	9.96	−0.4
Week 4	HK	204 ± 11	+2.0
	Pol.	9.97	−0.3
Week 6	HK	197 ± 1	−1.5
	Pol.	9.95	−0.5
Week 8	HK	199 ± 10	−0.5
	Pol.	9.95	−0.5
Week 12	HK	218 ± 11	+9.0
	Pol.	9.96	−0.4
Week 24	HK	200 ± 1	0
	Pol.	9.97	−0.3

[a] The nominal concentration of glucose in the HK assay was 200 mg dl^{-1}; the measured concentration represents the mean of 2–4 measurements.
[b] The nominal concentration of sucrose in the polarimetry assay was 10 g dl^{-1}; the measured concentration represents the mean of 5 polarimetry measurements made on a single sample. The SDs for these measurements were below 0.01 in all cases.

samples stored for up to 24 weeks. The activity was virtually unchanged after 24 weeks storage at −20, 4 and 30°C. At a storage temperature of 40°C, the activity gradually decreased so that approximately 30% of the initial activity remained at the conclusion of the 24 week stability study. In contrast, only 5% of the initial activity remained after 12 weeks storage at 50°C. The activity data showed that the HK and polarimetry assays were in good agreement with regard to the time-dependent loss of YS activity, although the lower LOQ for the polarimetry assay allowed for additional data points which could not be obtained using the HK assay.

The activity data obtained using the two assay methods were expressed as a percentage of the initial activity measured by each method. It was not possible to compare the absolute YS activity (g sucrose hydrolysed per min per ml) using the polarimetry and HK-based procedures as there were differences in incubation temperatures, periods of incubation, solution pH, and the required dilutions of YS. Both assays employed a pH which was within the optimal pH range for sucrase activity which is reported to be 3.5–5.5 [5,6].

The liquid YS product was shown to retain its activity and physical appearance upon storage for up to six months at temperatures below 30°C. It is possible that the stability demonstrated by the sucrase enzyme may be attributed, at least in part, to its extensive glycosylation. Sucrase contains approximately 50% mannose and 3% glucosamine [6]. There is evidence to suggest that its glycoprotein nature is responsible for maintaining its functional structure and activity profile under a variety of conditions [7–9].

4. Conclusions

This study validated the use of both a HK-based assay and a polarimetric assay for the determination of the activity of liquid YS. Both assays demonstrated comparable activity data during the time-course of the preliminary stability study. The liquid YS product investigated demonstrated excellent stability upon storage with samples stored at or below 30°C retaining full activity for a period of at least 6 months. These data appear promising for future liquid YS oral formulation development strategies.

Table 3
Activity of liquid YS as a function of storage temperature over a 24 week period determined using the hexokinase and polarimetry assays

		Relative activity of liquid YS				
		−20°C	4°C	30°C	40°C	50°C
Initial activity (%)[a]		100	100	100	100	100
Week 1	HK[b]	—	—	91 ± 4	93 ± 5	74 ± 3
	Pol.[c]	—	—	98	96	74
Week 2	HK	99 ± 6	95 ± 3	92 ± 9	93 ± 7	48 ± 5
	Pol.	102	99	96	97	61
Week 4	HK	94 ± 6	96 ± 12	96 ± 4	88 ± 8	39 ± 4
	Pol.	102	100	98	94	38
Week 6	HK	98 ± 4	102 ± 4	107 ± 5	91 ± 10	21 ± 2
	Pol.	100	98	95	90	21
Week 8	HK	106 ± 2	110 ± 7	106 ± 2	91 ± 8	<LOQ
	Pol.	104	103	94	86	15
Week 12	HK	93 ± 7	99 ± 6	101 ± 5	81 ± 6	<LOQ
	Pol.	96	100	100	88	5
Week 24	HK	96 ± 6	107 ± 2	96 ± 7	29 ± 1	<LOQ
	Pol.	104	103	99	28	<LOQ

[a] Activity of liquid YS expressed as a percentage of initial activity as measured by each assay.
[b] Activity determined using the HK method. Values represent the mean ± SD for independent samples analysed in triplicate.
[c] Activity determined using the polarimetry method for a single sample.

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