ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
22 (2000) 661–666

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Simultaneous determination of α, β and γ cyclodextrins by LC[☆]

M.S. Kinalekar, S.R. Kulkarni, P.R. Vavia *

Pharmaceutical Division, Department of Chemical Technology (Autonomous), University of Mumbai, Nathalal Parikh Marg, Matunga, Mumbai-400 019, India

Received 26 November 1998; received in revised form 6 August 1999; accepted 24 August 1999

Abstract

Cyclodextrins (CDs) can be synthesized from starch by cyclodextrin glycosyltransferase (CGTase). This enzyme produces α-, β- and γ-CDs in varying proportions. In the production of cyclodextrins, purity as well as yield are important factors. A precise and reproducible method was developed and validated for the simultaneous determination of α-, β-, and γ-CDs. Optimum separation between the three CDs was achieved using a Finepak amino column with a mobile phase consisting of acetonitrile-water (70:30, v/v) at a flow rate of 1 ml/min. Detection was carried out using a differential refractive index detector. The developed method gave good chromatographic resolution of the three components with retention times of 13.16, 16.83 and 21.74 min for α-, β- and γ-CDs, respectively. The polynomial regression data for the calibration plots exhibited good linear relationship (coefficient of correlation $r = 0.9987$ for α, $r = 0.9986$ for β and $r = 0.9998$ for γ-CDs) over a concentration range of 2–10 mg/ml. Statistical analysis proves that the proposed LC method is precise, reproducible and accurate for the estimation of α-, β- and γ-cyclodextrins. The method can be employed for determination of percent purity as well as estimation of process yields of the cyclodextrins during the enzymatic production. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: LC analysis of Cyclodextrins; Separation of α-, β- and γ-cyclodextrins

1. Introduction

Cyclodextrins (CDs) are cyclic and non-reducing oligosaccharides joined by α-1,4 glucosidic linkage and designated as α-, β- and γ-cyclodextrins according to the number of glucose units [1–3]. The most remarkable molecular feature of the CDs is their ability to form inclusion complexes with numerous guest molecules without a covalent bond being formed [4]. Many organic compounds form inclusion complexes with the CDs, modifying properties and making them valuable for pharmaceutical research and industry [4–11].

The synthesis of the CDs is possible through enzymatic route [3,12]. The increasing importance of CDs due to versatility of their applications has

[☆] Presented at the III International Symposium and Short Course on Separation Sciences (SSS-98), Bhopal, India November, 1998.

* Corresponding author.

0731-7085/00/$ - see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0731-7085(99)00299-X

created a need to study and understand the enzymatic conversion. This has necessitated the development of a suitable method for the analysis of CDs during the course of their enzymatic synthesis. Various chromatographic methods for the separation of CDs are already cited in literature such as adsorption chromatography on charcoal [13], Sephadex G-15 [14], gel chromatography on polyacrylamide [15], TLC method [16] and LC method [17,18].

The objective of the present work was to develop an online and simultaneous LC method for the separation and analysis of CDs in the reaction mixture. The method employed for synthesis of CDs involved enzymatic conversion of starch with simultaneous separation of the products using an ultrafiltration system (molecular weight cut off-20 kDa) to avoid interfering effects of various other byproducts of the process in detection of CDs.

2. Experimental

2.1. Materials

Cyclodextrin glycosyltransferase and glucoamylase were gifted by Amano pharmaceutical, Japan. α-, β- and γ- cyclodextrin were gifted by Nihon Shokuhin Kako, Japan. β-D-Glucose was purchased from Ranbaxy Chemicals, India. HPLC grade solvents were purchased from Spectrochem, India. All other chemicals used were of analytical grade and demineralised, double distilled water was employed.

2.2. Instrumentation

The liquid chromatograph comprised of a Jasco-PU 980 pump (Jasco, Japan) and a differential refractive index detector (Perkin Elmer LC-25, USA) with a sensitivity of 5×10^{-5} RIU. Data integration was done using Borwin Software package V1.21 for LC integration. The column used was a Finepak-NH_2 (5 μm, 250 × 4.6 mm) (Jasco, Japan). Injections were carried out using a 20 μl sample loop at 25°C.

2.3. Optimization of the solvent system and separation of CDs

Varying compositions of acetonitrile-water viz. 60:40, 65:35, 70:30, v/v were evaluated as mobile phase to achieve good resolution of α-, β- and γ-CD. The pH of the above solvent systems was found to be between 5.5–6.0.

2.4. Validation

Evaluation of the LC method was based on proportionality (linearity assay), precision and accuracy, limit of detection, limit of quantitation and ruggedness.

2.4.1. Linearity

Standard α-, β- and γ-CDs were separately run on the amino column, using acetonitrile-water (70:30, v/v) as the solvent system. The retention times of all the three CDs and glucose were determined. These CDs were then run as mixture and the base-line separation of the peaks of all the three CDs was obtained. A set of mixtures of CDs containing 2–10 mg of each CD was prepared in water. Each mixture was run and the chromatograms were obtained. A correlation between the peak-area and concentration of CDs was established and the calibration curves were obtained. The calibration curve for glucose was not determined as it was not significant in this study. The concentrations of unknown samples of CDs were determined from these calibration curves using model $y = mx + c$.

2.4.2. Precision and accuracy

A standard solution of 4 mg/ml, within the linearity range, was selected and analysed six times. This assay was repeated six times.

2.4.3. Limit of detection and limit of quantitation

In order to determine the limit of detection and limit of quantitation demineralised, double distilled water was injected six times. The noise level was determined. The limit of detection was estimated to be three times the standard deviation and limit of quantitation was ten times the standard deviation value.

2.4.4. Ruggedness

Ruggedness of the proposed method was carried out by using reagents from different lots and different manufacturers.

2.5. Application of validated method for determination of cyclodextrins in continuous process

2.5.1. Production of Cyclodextrins

It is reported that during batch production the CDs formed insitu inhibit the enzyme cyclodextrin glycosyltransferase (CGTase) [19] and this necessitates the continuous removal of CDs from the proximity of the enzyme as soon as they are formed. Moreover, the determination of the process yields of CDs in the reaction mixture during the course of production is difficult due to the numerous byproducts, in the form of linear dextrins of variable lengths and the enzyme present in the reaction mixture. CDs were produced in three distinct steps viz. liquefaction of starch with CGTase to reduce the viscosity of the solution, conversion of liquefied starch to CDs and simultaneous separation of these CDs from the reaction mixture through ultrafiltration unit. The ultrafiltration system comprised of a cross-flow module, the schematic representation of the ultrafiltration unit is depicted in the Fig. 1. The ultrafiltration unit was modified by using a low molecular weight cut off polysulfone membrane (20 kDa) to avoid as many impurities (linear dextrins) as possible from passing through it. The filtrate thus obtained was a mixture of the three CDs, glucose and various other linear dextrins (those which were small enough to permeate through the ultrafiltration membrane).

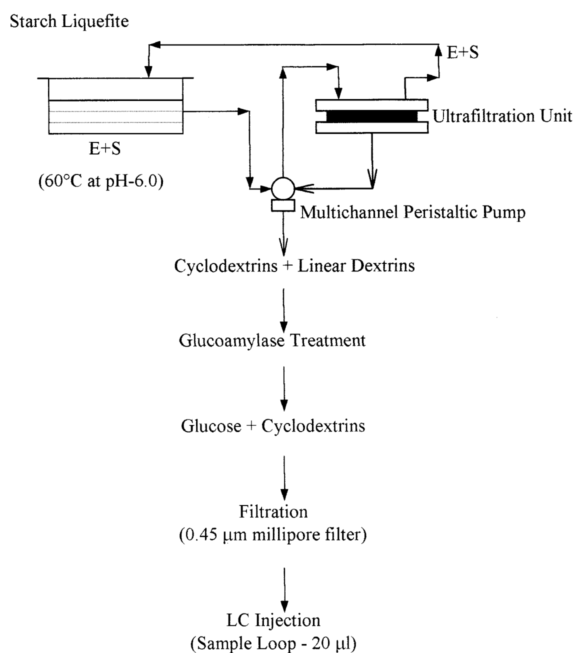

Fig. 1. Schematic representation of ultrafiltration unit for the production of cyclodextrins.

2.5.2. Sample preparation

The filtrate obtained from the ultrafiltration unit was then treated with glucoamylase to convert the linear dextrins into glucose. This was then filtered through a 0.45 μm cut-off microfilter (Millipore) and injected (sample-loop size = 20 μl) in the LC system to determine concentrations of α-, β- and γ-CDs.

3. Results and discussion

3.1. Optimization of solvent system and separation of CDs

When acetonitrile-water in 60:40, v/v and 65:35, v/v proportions were employed as the mobile phase, poor resolution of CDs was obtained. The mobile phase consisting of acetonitrile-water (70:30, v/v) was found to be a suitable solvent system for developing the chromatograms of CDs. The retention times of glucose, α-CD, β-CD and γ-CD significantly differed from each other and the samples containing these compounds could be effectively separated under the conditions used (Fig. 2). The elution sequence of CDs was; α-followed by β-, followed by γ-CD, respectively. The retention times of glucose and CDs are given in Table 1.

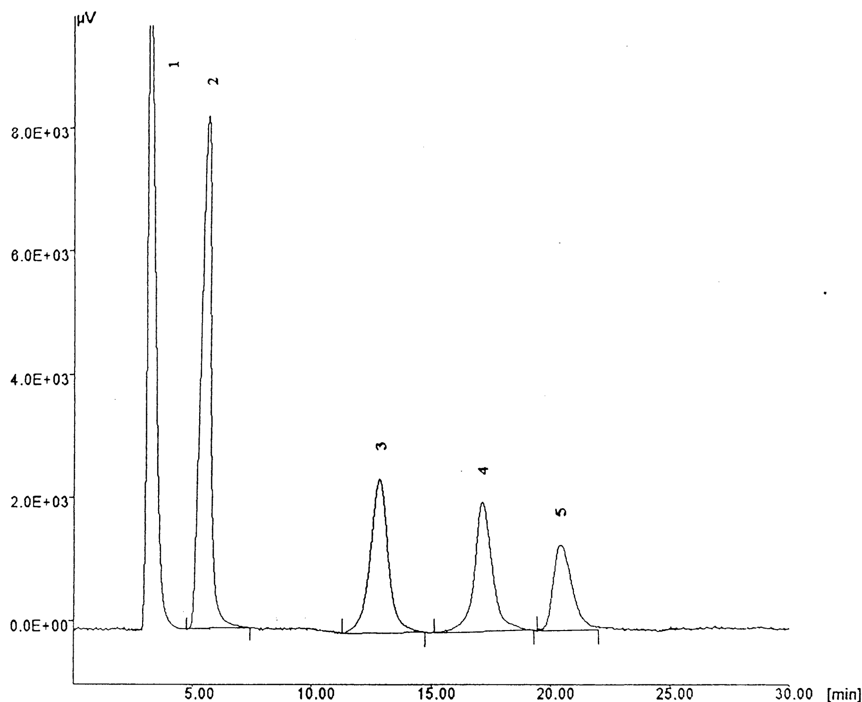

Fig. 2. LC chromatogram of the separation of glucose and cyclodextrins after the ultrafiltration and glucoamylase treatment. Key: 1, solvent peak; 2, glucose (15 mg/ml); 3, α-CD (8 mg/ml); 4, β-CD (6 mg/ml); 5, γ-CD (4 mg/ml).

3.2. Validation

3.2.1. Linearity

Three standard curves for α-, β- and γ-CDs were prepared by plotting the peak area versus the individual cyclodextrin concentration. The polynomial regression for the calibration plots showed good linear relationship over a concentration range of 2–10 mg (Table 2). The mean values of intercept, slope and correlation coefficient were 151.79 ± 0.420, 124753.0 ± 0.246 and 0.9987 ± 0.0024 for α-CD; 1261.16 ± 0.234, 10246.69 ± 0.742 and 0.9986 ± 0.032 for β-CD; 639.028 ± 0.222, 13927.72 ± 0.178 and 0.9998 ± 0.0010 for γ-CD (each concentration of the mixture was injected six times) over the concentration range studied.

3.2.2. Precision and accuracy

Precision and accuracy were tested at 4 mg/ml level for all the three CDs. The relative standard deviations were found to be 1.33, 1.21 and 1.27% for α-, β- and γ-CD, respectively.

3.2.3. Limit of detection and limit of quantitation

The limit of detection for α-, β- and γ-CDs was found to be 0.0249 mg/ml, 0.0462 mg/ml and 0.0258 mg/ml, respectively. The limit of quantitation for α-, β- and γ-CDs was 0.083 mg/ml, 0.154 mg/ml and 0.086 mg/ml, respectively.

3.2.4. Ruggedness

The analytical method was found to be rugged with respect to the solvents employed from different lots and manufacturers. The RSD for precision was found to be 1.23, 1.08 and 1.43% for α-, β- and γ-CD, respectively, and RSD for accuracy

Table 1
The retention time of glucose and CDs

Components	Retention time (min)
Glucose	6.02 ± 0.070
α-CD	13.16 ± 0.045
β-CD	16.83 ± 0.070
γ-CD	21.74 ± 0.060

Table 2
A correlation between concentration of CDs and peak areas

Concentration of CDs (mg/ml)[a]	Peak area (average ± S.D.)		
	α-CD	β-CD	γ-CD
2	29609.60 ± 112.3	24067.375 ± 364.12	25370.00 ± 95.5
4	53946.60 ± 242.50	40923.80 ± 177.30	53832.60 ± 168.90
6	85625.20 ± 266.20	64874.70 ± 143.95	74662.40 ± 234.24
8	111214.30 ± 467.80	82032.35 ± 243.66	103917.80 ± 356.32
10	137857.00 ± 451.84	104597.65 ± 221.63	126027.60 ± 667.96

[a] Each of the concentrations of each CD was injected six times.

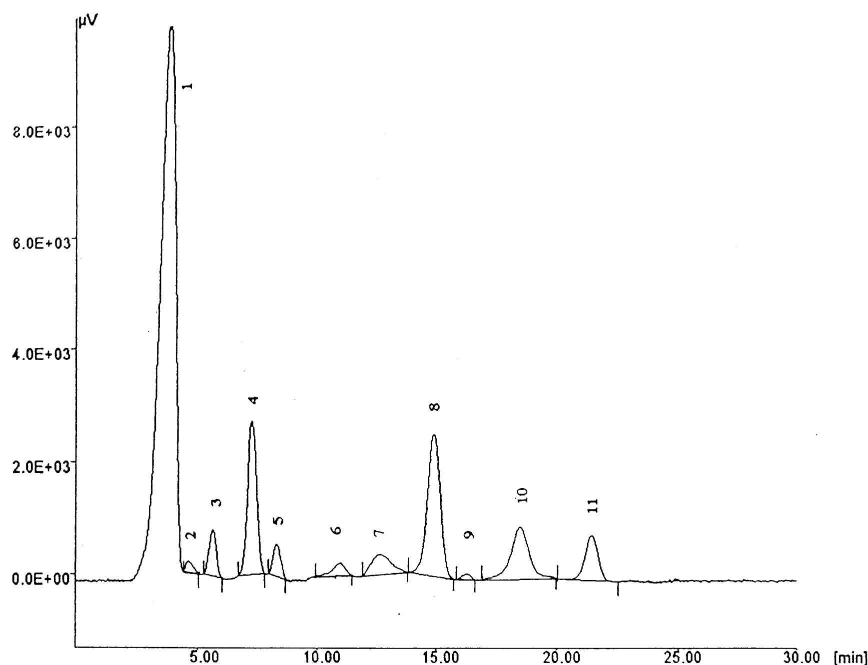

Fig. 3. LC chromatogram before the ultrafiltration and glucomaylase treatment. Key: 1, solvent peak; 3, glucose; 7, α-CD; 10, β-CD; 11, γ-CD; 2; 4; 5; 6; 8; 9, linear dextrins impurities. The sample injected was from the reaction mixture after 4 h of enzyme addition without ultrafiltration and glucoamylase treatment.

of α-, β- and γ-CD was found to be 1.65, 1.52 and 1.23%, respectively.

3.3. Application of validated method for determination of cyclodextrins in continuous process

In the continuous process, in order to avoid the product inhibition of the enzyme it is necessary to know the amount of CDs present in the proximity of the enzyme. The applicability of the proposed LC method finds extensive importance in the analysis of the CDs during the continuous production of CDs. The chromatogram in Fig. 3 shows the interference of the linear dextrins when the sample was not pretreated by ultrafiltration process and glucoamylase enzyme treatment, in the detection and estimation of the CDs. The interference of the linear dextrins in the chromatogram was eliminated by using ultrafiltration and glucoamylase treatment of the reaction mixture as can be seen in Fig. 2. The process yields of

Table 3
The percent yields of cyclodextrins

Cyclodextrin (CD)	Percentage yield of cyclodextrins
α	14.56
β	30.41
γ	9.72

CDs formed in the continuous process were optimized by using the proposed LC method. The results are depicted in the Table 3. Periodic analysis of the reaction mixture during the course of continuous production of CDs is of immense value with respect to the optimization of the rates of the reaction. The sensitivity of the presented method enabled the periodic quantification of the synthesised CDs within 30 min of the reaction.

4. Conclusion

We have described a rapid, precise and reliable method for the estimation of α-, β-, and γ-CDs. The chromatographic method described here provides the method for the analysis of the CDs obtained during their enzymatic synthesis from starch. The data based on process yields can be effectively utilized for the optimization of batch and continuous processes of CD production. LC method provides a better resolution, accuracy and precision for the identification and estimation of CDs. The simplicity of the technique should make it the method of choice for routine analysis.

Acknowledgements

The authors are grateful to Nihon Shokuhin Kako, Japan for gifting α-, β- and γ-cyclodextrins and Amano Pharmaceutical, Japan for gifting the enzymes cyclodextrin glycosyltransferase and glucoamylase. The sponsors of the project, the Department of Biotechnology (Govt. of India) are gratefully acknowledged.

References

[1] D. French, Adv. Carbohydr. Chem. 12 (1957) 189–260.
[2] S. Pedersen, B.F. Jensen, H. Dijkhuizen, S.T. Jorgensen, B.W. Dijkstra, Chemtech. 25 (1995) 19–25.
[3] J. Szejtli, Chem. Rev. 98 (1998) 1743–1753.
[4] J. Szejtli, in: V.F. Smolen, L. Ball (Eds.), Molecular Entrapment and Release Properties of Drugs by Cyclodextrins in Controlled Drug Bioavailability, vol. 3, John Wiley, New York, 1985, pp. 365–420.
[5] J. Szejtli, in: G.O. Philips, D.J. Wedlok, P.A. Williams (Eds.), A new food additive: The β-cyclodextrin in Gums an Stabilizers in Food Industry-3, Elsevier, London, 1986, pp. 351–362.
[6] N. Nakamura, K. Horikoshi, Chem. Econ. Eng. Rev. 14 (1981) 32–38.
[7] S. Nagatomo, Chem. Econ. Eng. Rev. 17 (1985) 28–34.
[8] J. Szejtli, B. Zsadon, T. Cserhati, in: W.L. Hinze, D.W. Armstrong (Eds.), Cyclodextrin use in separations in Ordered Media in Chemical Separations, American Chemical Society, 1986, pp. 200–217.
[9] K. Uekama, F. Hirayama, T. Irie, Chem. Rev. 98 (1998) 2045–2076.
[10] A. Hedges, Chem. Rev. 98 (1998) 2035–2044.
[11] R. Breslow, S. Dong, Chem. Rev. 98 (1998) 1997–2011.
[12] A. Tonkova, Enzyme Microbiol. Technol. 22 (1998) 678–686.
[13] J.N.J.J. Lammers, J. Chromatogr. 41 (1969) 462–466.
[14] J.H. Carter, E.Y.C. Lee, Anal. Biochem. 39 (1971) 521–542.
[15] R.D.P. Khanolkar, B.D. Hosangadi, Indian J. Chem. 21 (1982) 799–802.
[16] N. Wiedenhof, J. Chromatogr. 15 (1964) 100–102.
[17] B. Zsadon, K.H. Otta, F. Tudos, J. Chromatogr. 172 (1979) 490–492.
[18] K. Hokse, J. Chromatogr. 189 (1980) 98–100.
[19] T.J. Kim, Y.D. Lee, H.S. Kim, Biotech. Bioeng. 41 (1993) 88–94.