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# Quantitative analysis of natural cyclodextrins by high-performance liquid chromatography with pulsed amperometric detection: Application to cell permeation study

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ABSTRACT

Simple HPLC-PAD methods were developed for quantitation of cyclodextrins (CDs) in aqueous matrices from *in vitro* cell permeation studies. C-18 solid-phase extraction was used for sample pretreatment. Samples were analysed using acetonitrile–water mobile phase with post-column alkalization by 0.5 M NaOH. Zorbax SB-Aq (for α-CD) and Zorbax SB-Phenyl (for β-CD and γ-CD) columns gave excellent peak shape and sufficient resolution of CD to glucose (2.7–3.2). The methods showed good concentration–response relationship ($r \geq 0.999$), precision (RSD% 0.7–5.1), repeatability (RSD% 3.4–13.7) and accuracy (87–107%). The limits of quantitation were 0.78, 0.46 and 0.52 μg/ml for α-CD, β-CD and γ-CD (RSD% of 10.6, 8.1 and 16.3, respectively).



## 1. Introduction

Cyclodextrins (CDs) are pharmaceutical excipients, which have been widely used to modify the physicochemical and biopharmaceutical properties of drugs [1]. The CD molecules consist of α(1,4)-linked glucopyranose units which form a cyclic structure. Natural CDs (i.e. chemically unmodified CDs) include α-CD, β-CD and γ-CD which consist of 6, 7 and 8 glucopyranose units, respectively. The inner cavity of a CD molecule provides a lipophilic microenvironment for the complexation of lipophilic drugs, while the outer surface of the molecule is highly hydrophilic due to the free hydroxyl groups facing the openings of the cavity. Since the CD molecules do not contain chromophores or fluorophores, their direct detection has been limited to refractive index (RI), evaporative light scattering (ELSD), pulsed amperometric (PAD) or mass spectrometric (MS) methods. In general, the RI and ELSD detectors are of low sensitivity and therefore, they have been applied for the analysis of relatively high concentrations of CDs [2–4]. However, a high sensitivity (from micromolar to nanomolar level) for the analysis of CDs has been obtained by using a MS detection [5,6] or PAD detection techniques [7–12].

In contrast to the direct detection methods, the use of photometric or fluorescence-based detection of CDs requires a complexation reaction with a specific UV–vis-absorptive of fluorescent reagent prior to the detection [13–23]. In general, with these indirect methods, sensitivities up to micromolar levels have been obtained. However, the method sensitivity may be highly dependent on the stability of the complexes (as the poor complexation efficiency decreases the observed response) and the degree of complexation (as the uncomplexed reagent may increase the background noise) [18,21]. Furthermore, it should be assured that the complex stability is not altered due to the possible environmental changes during the analysis [22] or due to the properties of CD itself [19].

The aim of the present study was to develop a simple and sensitive quantitative analysis method for determination of CDs in an aqueous sample matrix used in cell permeation studies. From the sake of simplicity, an HPLC application with a direct detection method was preferred. Pulsed amperometric detection (PAD) was chosen because of its relatively high sensitivity and its overall applicability to carbohydrate (including CD) analyses. However, the published applications of HPLC-PAD for the quantitative analysis of CDs were not directly applicable for our purposes due to divergent sample matrices, reasonably long analysis times, unsatisfactory chromatographic performance and/or relatively low sensitivity.

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Shortly, the previously published HPLC-PAD methods involve the analysis of mixtures of natural and/or branched CDs in biological samples [8,10,12,24] or in water [9,11], and the trace analysis of natural CDs [25]. However, to our knowledge, the quantitative methods for the cell permeation studies of CDs have been unavailable to date. Thus, there was a need to develop a novel CD analysis method for that purpose, including the specificity of natural CDs over high concentrations of glucose and inorganic salts in the matrix.

In the present study, novel quantitative HPLC-PAD methods were developed for the analysis of α-CD, β-CD and γ-CD in aqueous matrices from Calu-3 cell permeation studies of CDs.

## 2. Experimental

### 2.1. Chemicals

α-Cyclodextrin (Cavamax W6 Pharma; mw 972) was purchased from Wacker-Kemi AB (Solna, Sweden). β-Cyclodextrin (Cavamax W7 Pharma; mw 1135) and γ-cyclodextrin (Cavamax W8 Pharma; mw 1297) were purchased from Wacker Chemie GmbH (Burghausen, Germany). Anhydrous D(+)-glucose was purchased from ICN Biochemicals Inc. (Aurora, Ohio), HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) from Sigma–Aldrich Chemie GmbH (Steinhem, Germany), HBSS (Hank's balanced salt solution without phenol red; 10× concentrate) from Gibco (Paisley, Scotland) and sodium hydroxide concentrate (NaOH; HPLC grade, 49.4 g/100 g, 1.512 kg/l) from Fluka Chemika (Buchs, Switzerland). Acetonitrile and methanol were of HPLC grade and all chemicals were used as received.

The HBSS-Hepes solution (10 mM HEPES in 1× HBSS solution, pH 7.4) was prepared in sterile water and used as the cell permeation medium. The solution contained glucose (1 mg/ml) and inorganic salts ($CaCl_2$ 0.14 mg/ml, KCl 0.04 mg/ml, $KH_2PO_4$ 0.06 mg/ml, $MgCl{\cdot}6H_2O$ 0.1 mg/ml, $MgSO_2{\cdot}7H_2O$ 0.1 mg/ml, NaCl 8 mg/ml and $Na_2HPO_4{\cdot}7H_2O$ 0.09 mg/ml) which were regarded as the interfering compounds of the sample matrix.

### 2.2. Equipment

Agilent 1100 series high-performance liquid chromatography system (Agilent Technologies Inc., Waldbronn, Germany) consisted of a binary pump, vacuum degasser, autosampler and thermostated column compartment. NaOH solution was delivered via a post-column t-piece by LKB Bromma 2150 HPLC pump (LKB, Stockholm, Sweden) which was connected to a pulse dampener. The HPLC system was connected to Esa Coulochem III detector (ESA Inc., Chelmsford, MA, USA) with a pulsed amperometric cell equipped with a gold-plated working electrode (Esa 5040 analytical cell). The data was analysed using Agilent Technologies ChemStation for LC (Rev. A.08.03).

### 2.3. Method description

α-CD analyses were performed with a Zorbax SB-Aq reversed-phase column (5 μm, 4.6 mm × 150 mm, Agilent Technologies, USA). β-CD and γ-CD analyses were performed with a Zorbax SB-Phenyl column (4.6 mm × 150 mm, 5 μm, Agilent Technologies, USA).

The composition of acetonitrile–water mobile phase (0.65 ml/min) was 4:96 (v/v) for α-CD analyses and 7:93 (v/v) for β-CD and γ-CD analyses, respectively. The injection volume was 20 μl and the column temperature was 30 °C. The post-column addition of 0.5 M NaOH solution (0.65 ml/min) was used to adjust the pH of mobile phase (pH > 12) for the detection of the carbohydrate analytes. The detector setup was as follows: acquisition delay (AD) = 300 ms, data acquisition potential ($E_1$) = 100 mV, data acquisition time ($t_1$) = 600 ms, oxidation potential ($E_2$) = 1000 mV, oxidation time ($t_2$) = 150 ms, reduction potential ($E_3$) = −600 mV, reduction time ($t_3$) = 250 ms, and range ($R$) = 10 μC.

### 2.4. Standard solutions and method calibration

The standard stock solutions (27.5 mg/ml α-CD, 10.1 mg/ml β-CD and 28.3 mg/ml γ-CD) were prepared by dissolving a precisely weighed amount of a CD in ultrapure water. The calibration standard solutions (8–9 concentrations at the range of 0.33–110 μg/ml α-CD, 0.606–121 μg/ml β-CD or 0.566–141 μg/ml γ-CD) were obtained by dilution of the stock solution with the mobile phase. The calibration standard solutions were analysed by HPLC without pretreatment.

The quality control (QC) stock solutions (19.5 mg/ml α-CD, 11.5 mg/ml β-CD and 43.5 mg/ml γ-CD) were prepared by dissolving a precisely weighed amount of a CD in ultrapure water. The QC stock solution was diluted with HBSS-Hepes solution to obtain the QC samples, with water to obtain the reference samples, and with the mobile phase to obtain the reference standard solutions. The QC samples and reference samples were prepared at three concentration levels (3.9, 78 and 975 μg/ml for α-CD; 3.7, 92 and 1150 μg/ml for β-CD; and 4, 104 and 1300 μg/ml for γ-CD). The reference standard solutions were prepared at three concentration levels which were proportional to the concentrations of the QC samples after the pretreatment (8.8, 35.1 and 87.8 μg/ml for α-CD; 8.3, 41.4 and 103.5 μg/ml for β-CD; 9.4, 47 and 117 μg/ml for γ-CD), respectively.

### 2.5. Sample preparation

The samples were obtained from the permeation studies with Calu-3 bronchial epithelial cells [26]. In brief, the Calu-3 cells were grown at air-interface on Transwell® polycarbonate filters (1.12 $cm^2$, 0.4 μm pore size, Corning Costar, NY, USA). The permeation of natural CDs was studied across the confluent Calu-3 cell layers (37 °C, 150 rpm). The cells were exposed to CD solutions for 4 h with initial apical concentration of 1 mM α-CD, β-CD or γ-CD in HBSS-Hepes solution (2.1). The samples were withdrawn from the apical and basolateral compartments and the CD concentration of the samples was determined using the present analysis method.

Since the HBSS-Hepes solution (2.1) was used as the reservoir medium in the cell permeation studies, the QC samples with a known amount of CD were prepared in HBSS-Hepes solution (2.4) and used for the evaluation of the method validity. Solid-phase extraction (Discovery DSC-18, 1 ml, 100 mg, Supelco, USA) was used to remove the interfering compounds from the sample matrix. The SPE-cartridges were pre-conditioned with 1.0 ml of methanol following 1.0 ml of ultrapure water. After that, 450 μl of sample was loaded into the cartridges and washed with 1.0 ml of ultrapure water to remove the interfering compounds. The analytes were eluted by 2.0 ml of methanol–water (75:25, v/v). The eluent was evaporated to dryness under a nitrogen flow (40 °C) and the residue was dissolved in 200 μl or 1000 μl of acetonitrile–water mobile phase, after which the pretreated samples were analysed with HPLC. If necessary, the samples were further diluted with the mobile phase. The concentrations of the pretreated samples were calculated against the calibration curve (2.4). The original sample concentrations were back-calculated based on the known dilution factors from the sample pretreatment and the correction factor from the daily recovery % values of the QC samples (see Section 2.6.2).

**Table 1**
Summary of the CD analysis methods

| | α-CD | β-CD | γ-CD |
|---|---|---|---|
| Column | Zorbax SB-Aq 5 μm, 4.6 mm × 150 mm (30 °C) | Zorbax SB-Phenyl 5 μm, 4.6 mm × 150 mm (30 °C) | Zorbax SB-Phenyl 5 μm, 4.6 mm × 150 mm (30 °C) |
| Mobile phase | ACN:$H_2O$ 4:96 0.65 ml/min | ACN:$H_2O$ 7:93 0.65 ml/min | ACN:$H_2O$ 7:93 0.65 ml/min |
| pH adjustment | 0.5 M NaOH 0.65 ml/min | 0.5 M NaOH 0.65 ml/min | 0.5 M NaOH 0.65 ml/min |
| Resolution to glucose[a] | 2.9 | 3.2 | 2.7 |
| LOQ μg/ml (RSD)[b] | 0.78 (10.6) | 0.46 (8.1) | 0.53 (16.3) |

[a] Calculated by the USP method, $n = 3$, representative chromatograms are shown in Fig. 2.
[b] Original sample concentration before pretreatment ($n = 4$–5); relative standard deviation in parentheses.

### 2.6. Validity of methods

#### 2.6.1. Specificity

The chromatograms of seven different injections were compared in order to evaluate the specificity of the method: (a) ultrapure water, (b) mobile phase, (c) CD standard solution (55, 50 or 71 μg/ml α-CD, β-CD or γ-CD in mobile phase, respectively), (d) glucose standard solution (5 μg/ml in mobile phase), (e) CD and glucose standard solution (12, 23 or 26 μg/ml α-CD, β-CD or γ-CD, respectively, together with 5 μg/ml glucose in mobile phase), (f) blank sample without CD and (g) artificial sample containing CD and glucose. The blank sample consisted of plain HBSS-Hepes solution (2.1). The artificial sample consisted of α-CD, β-CD or γ-CD (12, 23 or 26 μg/ml, respectively) together with glucose (1.3 μg/ml) in HBSS-Hepes solution. The blank and artificial samples were analysed after the SPE pretreatment (2.5).

The effect of sample matrix was evaluated by analysing QC samples in HBSS-Hepes solution ($n = 5$) and the reference samples in water ($n = 5$) at three concentration levels (2.4) after the SPE pretreatment. The selectivity was evaluated by comparing the obtained mean concentration values of the QC samples and the reference samples.

#### 2.6.2. Recovery

The QC samples ($n = 3$) and reference standard solutions ($n = 3$) at three concentration levels (2.4) were prepared. The QC samples were analysed with HPLC after the SPE pretreatment while the reference standard solutions were analysed without pretreatment. The recovery was determined by comparing the mean peak areas of the QC samples to those of the reference standard solutions at proportional concentration levels (2.4).

As the calibration standards were analysed without any pretreatment (2.4) while the samples were analysed after the SPE pretreatment, it was necessary to take the daily recovery into account when the results were calculated. Therefore, a correction factor which was based on the daily recovery of the QC samples (mean value of three concentration levels, $n = 3$ at each level, total $n = 9$), was determined. Thus, a single correction factor was obtained for the whole concentration range of samples. The correction factor was determined as the reciprocal of the mean daily recovery % value of the QC samples and used for the quantitation of all the samples in a sequence, including the QC samples.

#### 2.6.3. Precision and repeatability

The QC samples ($n = 3$) at three concentration levels (2.4) were prepared and analysed with HPLC after SPE pretreatment. The precision (1 day) was evaluated by calculating the relative standard deviation (RSD %) of the results for each concentration level ($n = 3$). The repeatability (3 days) was evaluated by calculating the RSD % of the results of 3 days for each concentration level ($n = 9$).

#### 2.6.4. Accuracy

The QC samples ($n = 3$) at three concentration levels (2.4) were prepared by the SPE pretreatment and analysed with HPLC. The sample concentration was determined against the calibration curve and multiplied with the appropriate dilution and correction factors (2.6.3). Method accuracy was calculated as the percentage ratio of the determined sample concentration (mean, $n = 3$) to the nominal CD concentration.

#### 2.6.5. Limit of quantification

The limit of quantification (LOQ) was evaluated by analysing various LOQ samples at concentrations of 0.78–1.6 μg/ml α-CD, 0.46–0.93 μg/ml β-CD and 0.53–1.05 μg/ml γ-CD in HBSS-Hepes ($n = 5$), respectively. The LOQ was defined as the original sample concentration which gave an acceptable RSD value (RSD < 20%).

#### 2.6.6. Stability

The stability of the pretreated samples was evaluated during an 1-day storage in HPLC vials in the autosampler. The stability was expressed as the percentage ratio of the results from the second analysis (day 2) and the first analysis (day 1) of the solutions. During both days, freshly prepared standard solutions were used for the calibration.

## 3. Results

### 3.1. Calibration

The concentration–response relationship was observed to be non-linear over the concentration range studied. The curve fitting showed that the unweighed quadratic equation (general form $y = ax^2 + bx + c$) gave a better description of the calibration data compared to the linear equation (general form $y = ax + b$) (evaluated with the data of α-CD with SPSS 14.0 for Windows, data not shown). In the present study, the reduction of the concentration range to achieve the linear relationship was not practicable because the application to the cell permeation study required the whole concentration range to be used. It has been suggested that in this kind of case, a non-linear model for the calibration curve should be selected [27]. In the present study, all of the methods showed a good correlation ($r \geq 0.999$) between the concentration and the response with the concentration ranges studied (Fig. 1).

### 3.2. Specificity

The representative chromatograms showing the specificity of CD analysis methods are shown in Fig. 2. All of the CD peaks gave an excellent symmetry, and sufficient separation between a CD and glucose was achieved (Table 1). The sample pretreatment did not cause any interference with the quantification of CDs and did not alter the retention times of CDs. Furthermore, the sample matrix did not affect the detector response as the mean concentration values of the QC samples (prepared in HBSS-Hepes) and the reference samples (prepared in water) were comparable (Table 2). Therefore, the analysis methods for α-CD, β-CD and γ-CD were considered as specific.

**Table 2**
Determined concentrations of QC samples (HBSS-Hepes solution as sample matrix) and reference samples (water as sample matrix) after the SPE pretreatment

| CD | Nominal concentration (μg/ml) | Determined concentration (μg/ml) (mean ± SD, $n = 5$) | |
|---|---|---|---|
| | | QC sample | Reference sample |
| α-CD | 3.9 | 4.5 ± 0.4[a] | 4.0 ± 0.4 |
| | 78 | 71.0 ± 4.0 | 82.1 ± 6.9 |
| | 975 | 977.5 ± 51.6 | 1035.7 ± 50.9 |
| β-CD | 3.7 | 3.2 ± 0.0 | 3.5 ± 0.1 |
| | 92 | 85.4 ± 3.1 | 86.3 ± 1.5 |
| | 1150 | 1145.3 ± 18.7 | 1012.1 ± 71.2 |
| γ-CD | 4.2 | 3.8 ± 0.1 | 3.5 ± 0.1 |
| | 104 | n.a. | n.a. |
| | 1300 | 1048.2 ± 32.5 | 891.6 ± 12.9 |

n.a. = not available.
[a] $n = 4$.

### 3.3. Recovery

The mean recovery of all the CDs was >90% (Table 3) while the amount of glucose in the pretreated samples was <0.5% of the original concentration. The correction factor, which was expressed as the reciprocal of the daily mean recovery % value (Section 2.6.2), improved the accuracy of the results. For example, if the mean daily recovery of a CD in the QC samples was 97% ($n = 9$), the corresponding correction factor was 1.03. By using this correction factor, the accuracy of the results was improved from the value of 94% (obtained without the correction factor) to the value of 97%.

### 3.4. Precision, repeatability and accuracy

All of the methods showed acceptable precision (RSD ≤ 5.1%) and repeatability (RSD ≤ 13.7%) (Table 3). By using the correction factor (Section 2.6.2) it was possible to eliminate the effect of daily variation in the solid-phase extraction and obtain an acceptable accuracy as shown in Table 3.

### 3.5. Limit of quantification

The limits of quantitation for α-CD, β-CD and γ-CD were 0.78, 0.46 and 0.52 μg/ml, respectively (Table 1). These sample concentrations correspond to the injected amount of 34, 33 and 25 pmol/injection, respectively. As the obtained RSD values were markedly below 20%, it can be suggested that the method offers a possibility to further decrease the sample concentrations in the described conditions.

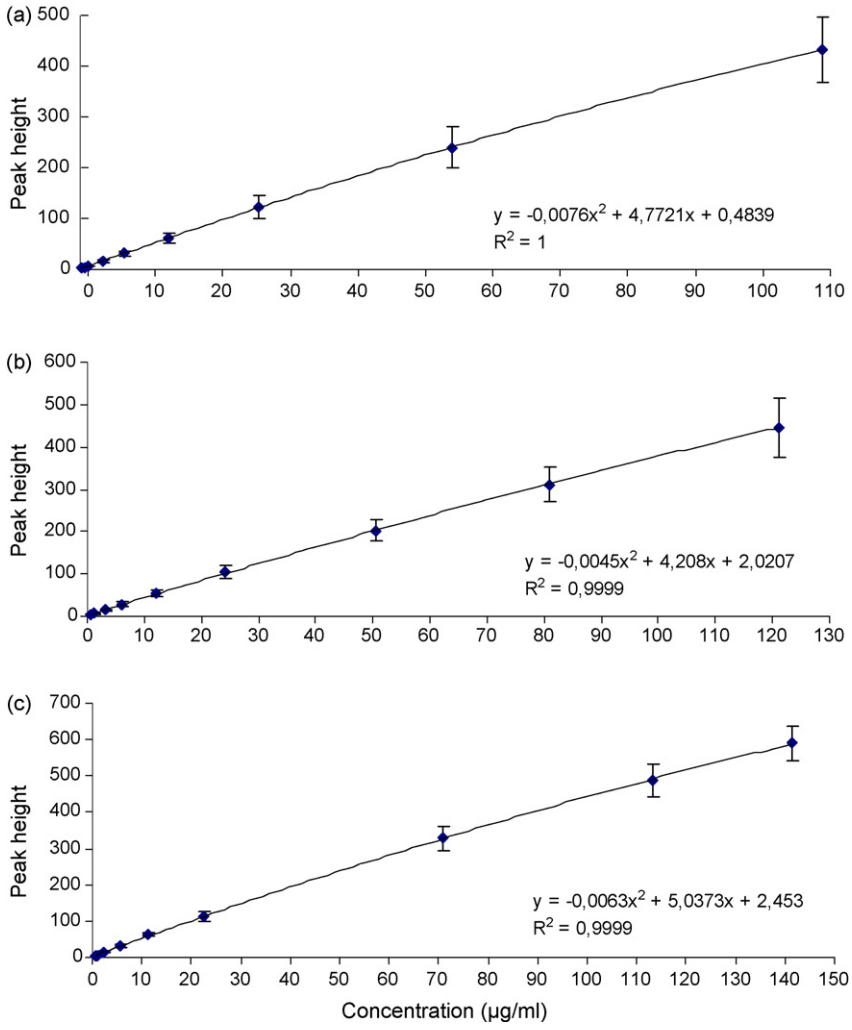


**Fig. 1.** Calibration of the CD analysis methods. Calibration range covers the concentrations of (a) 0.330–110 μg/ml α-CD, (b) 0.606–121 μg/ml β-CD and (c) 0.566–141 μg/ml γ-CD (mean ± SD, 8–9 data points).

**Table 3**
Recovery, repeatability and accuracy (%)

| CD | Nominal concentration (μg/ml) | Precision[a] RSD (%) | Repeatability[b] RSD (%) | Recovery (%)[b] Mean ± SD | Accuracy (%)[b] Mean ± SD |
|---|---|---|---|---|---|
| α-CD | 3.9 | 2.7 | 10.9 | 97 ± 16 | 107 ± 12 |
| | 78 | 4.5 | 3.4 | 91 ± 19 | 96 ± 3 |
| | 975 | 0.7 | 7.7 | 91 ± 18[c] | 94 ± 7 |
| β-CD | 3.7 | 5.1 | 13.7 | 102 ± 8 | 104 ± 14 |
| | 92 | 0.7 | 8.0 | 104 ± 4 | 100 ± 8 |
| | 1150 | 4.5 | 7.6 | 104 ± 10[c] | 99 ± 8 |
| γ-CD | 4.2 | 3.1 | 5.9 | 96 ± 8 | 94 ± 5 |
| | 104 | 2.6 | n.a. | 104 ± 2[a] | 103 ± 3[a] |
| | 1300 | 1.4 | 5.3 | 110 ± 7[c] | 87 ± 5 |

n.a. not available.
[a] 1 Day, $n = 3$.
[b] 3 Days, $n = 9$ ($n = 3$/day).
[c] 3 Days, $n = 8$ ($n = 2$–3/day).

### 3.6. Stability

All of the pretreated samples were stabile during the 1-day storage in the HPLC vials in the autosampler. The concentration of the analytes remained unchanged, as the CD concentration was 97 ± 3% (α-CD), 104 ± 4% (β-CD) and 91 ± 4% (γ-CD) of the initial concentration after the 1-day storage in the autosampler, respectively. No interfering peaks were detected after the storage.

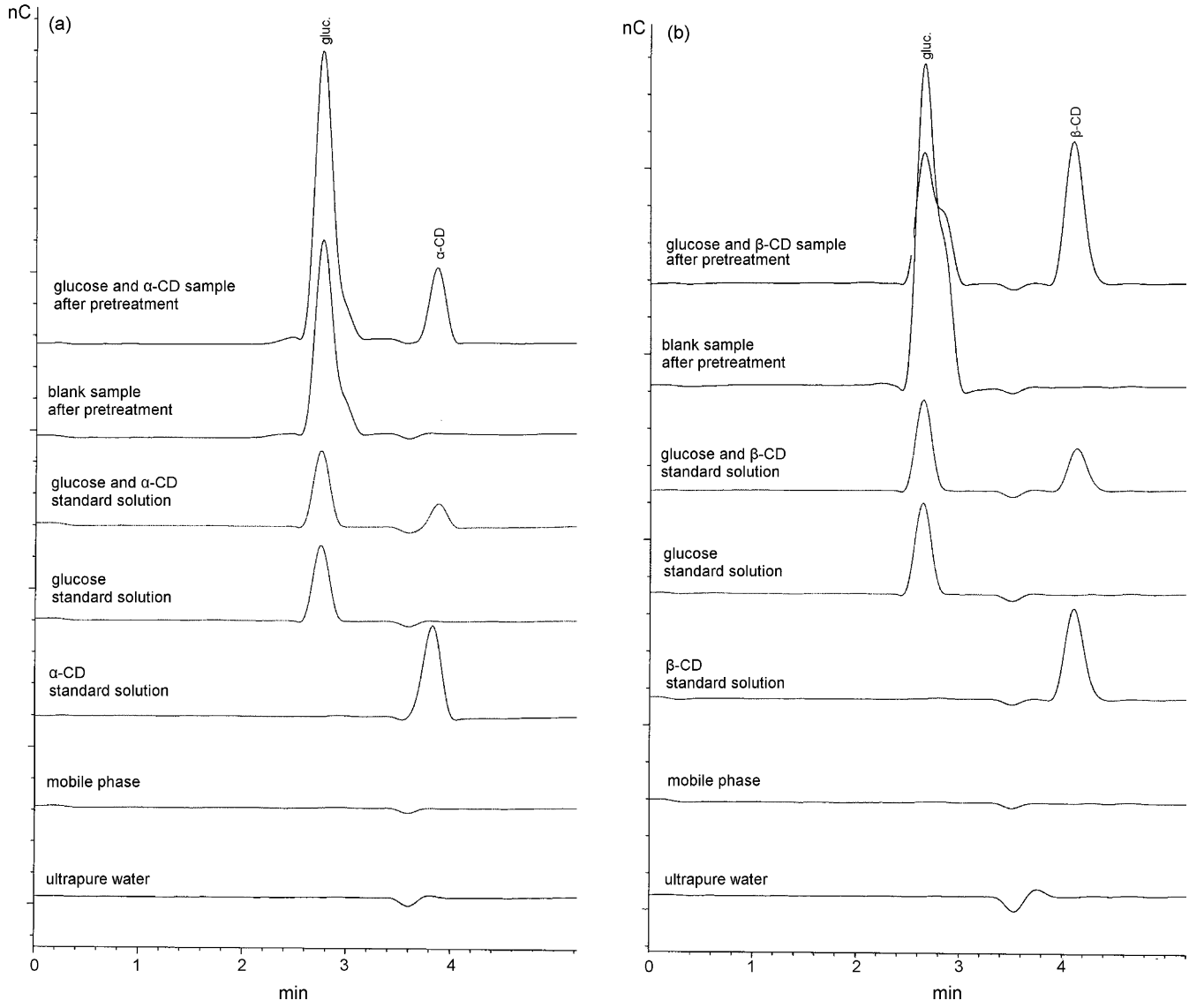


**Fig. 2.** HPLC chromatograms representing the specificity of (a) α-CD, (b) β-CD and (c) γ-CD analysis methods. See the sample concentrations in Section 2.6.1. and chromatographic conditions in Table 1.

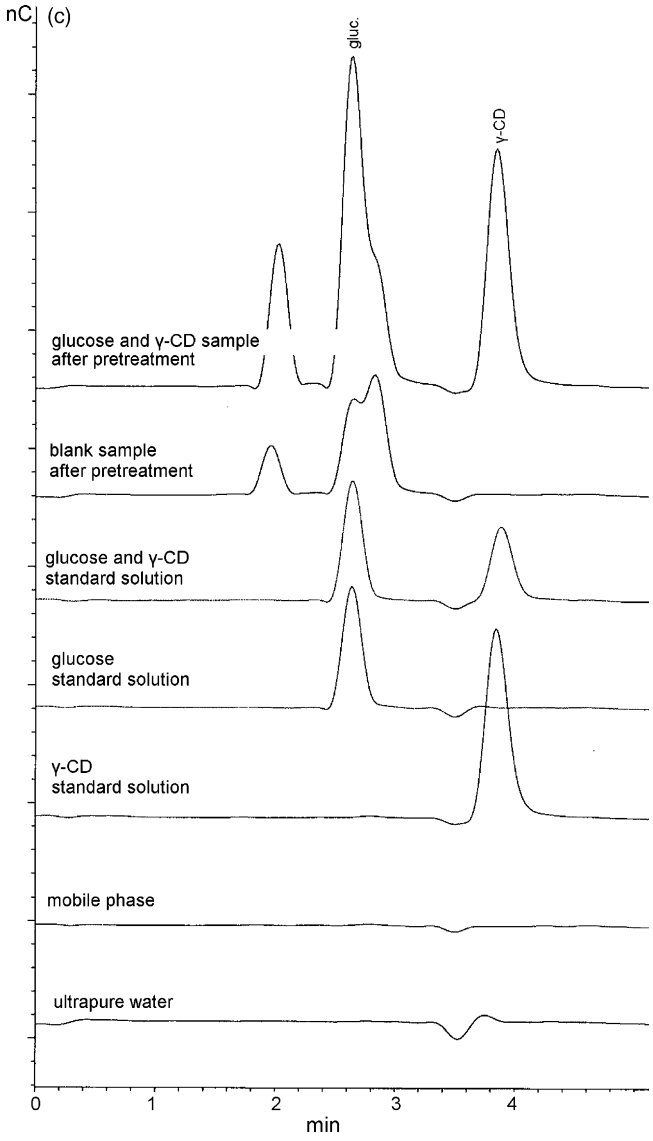


**Fig. 2.** (*Continued*).

### 3.7. Application to the cell permeation study

The present method has been successfully applied to the cell permeation studies of natural CDs, which are described in more detail in a separate manuscript [26]. Shortly, the CD permeation across the Calu-3 bronchial cell line was studied with 1 mM α-CD, β-CD and γ-CD solutions prepared in HBSS-Hepes, which was used as the QC sample matrix in the present study. It is commonly known that CDs are able to extract cellular components, such as cholesterol, from the biological membranes [28]. However, the visual inspection of the HPLC chromatograms revealed that the authentic sample matrices did not interfere with the analysis of the samples even at the lowest sample concentrations (Fig. 3). The results from the cell permeation study are reported elsewhere [26], while the present study is the first one to report the applied CD analysis methods in detail.

## 4. Discussion

In most cases, the separation of natural CDs has been carried out by HPLC with reversed-phase columns [4,8–10,12–15], while few applications with normal phase [2,25] and anion exchange [11] chromatography have been described. Furthermore, solid-phase extraction [5,8,10], ultrafiltration [9,12] and centrifugation [13,14] have been used as the pretreatment methods of biological samples containing natural CDs. In the present study, the reversed-phase columns (Zorbax SB-Aq and Zorbax SB-Phenyl) provided good chromatographic performance for the aqueous α-CD, β-CD and γ-CD samples. In general, depending on the mobile phase composition, both phase materials were able to give acceptable resolution between a CD and glucose. However, during the method development, marked peak tailing for β-CD and γ-CD was observed after a few weeks when the Zorbax SB-Aq column was used, though the peak shape of α-CD remained excellent. Therefore, this phase material was changed to Zorbax SB-Phenyl which was found to give an acceptable resolution for a CD and glucose as well. However, this column was not suitable for the α-CD analysis, as α-CD peak splitting was observed in the conditions needed to achieve sufficient resolution to glucose. Therefore, Zorbax SB-Aq was chosen for the α-CD analyses and Zorbax SB-Phenyl was used for the β-CD and γ-CD analyses in order to retain the sufficient resolution and excellent peak shape for the analytes. However, the sample pretreatment was done with C-18 solid-phase extraction which was shown to be suitable phase material for all these CDs.

In the present study, an on-column sensitivity at picomole level was obtained. The QC sample concentrations at the lowest level were equivalent to the molar concentrations of 3.9 μM α-CD (148 pmol/inj.), 3.7 μM β-CD (131 pmol/inj.), and 4.2 μM γ-CD (130 pmol/inj.) which correspond to the reported sensitivities of previously published PAD methods for the analysis of natural CDs. For example, Fang et al. [7] reported the linear ranges of 5.0–600 μM (α-CD), 4.0–600 μM (β-CD) and 3.0–600 μM (γ-CD) with a CE-PAD method with a Cu disk electrode. In addition, HPLC-PAD methods were reported with linear ranges of 20–2500 pmol/inj. of β-CD (extracted from urine) [8], 6.25–200 pmol/inj. of β-CD and γ-CD (extracted from serum) [10], 20–1500 pmol/inj. of β-CD (extracted from plasma) [12], and 10–1000 pmol of α-CD in water [11]. In addition, a linear range of 25–500 pmol of β-CD and γ-CD was reported in the same study, though the authors admitted that the method gave poor peak shapes for these CDs.

All of the above mentioned, previously published PAD methods (except the one for α-CD in water [11]) required reasonably long sample analysis times (approx. 20–30 min in HPLC) because of the simultaneous analysis of two or more CDs in one sample. In the present study, the samples contained only one of the CDs in the sample matrix at a time and therefore, sufficient resolution between the CD and glucose was the main requirement for the chromatographic separation. Compared to the previously published methods, the overall analysis time of CDs could be markedly reduced with the present HPLC-PAD analysis method. This is because the HPLC analysis time for a single sample was reduced (to 5.5–6 min) and the preparation steps for the calibration standards were minimised (as the time-consuming pretreatment step could be omitted). However, despite the relatively short analysis time, the present method provided sufficient resolution between a CD and glucose which

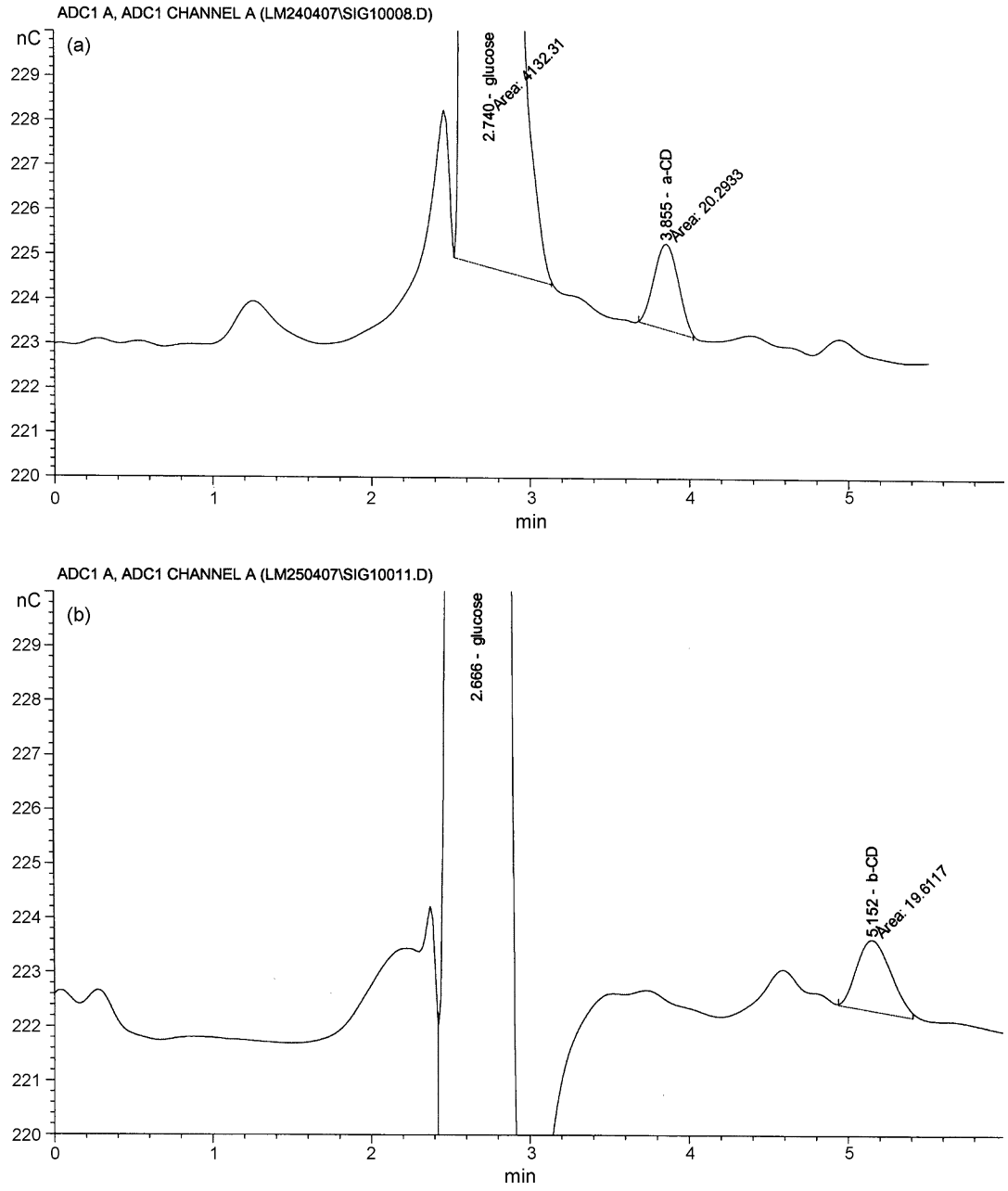


**Fig. 3.** Representative chromatograms of the pretreated samples obtained from the cell permeation studies with Calu-3 cells [26]. The CD concentrations in the injected solutions were (a) 3.72 μg/ml α-CD, (b) 5.23 μg/ml β-CD and (c) 5.85 μg/ml γ-CD.

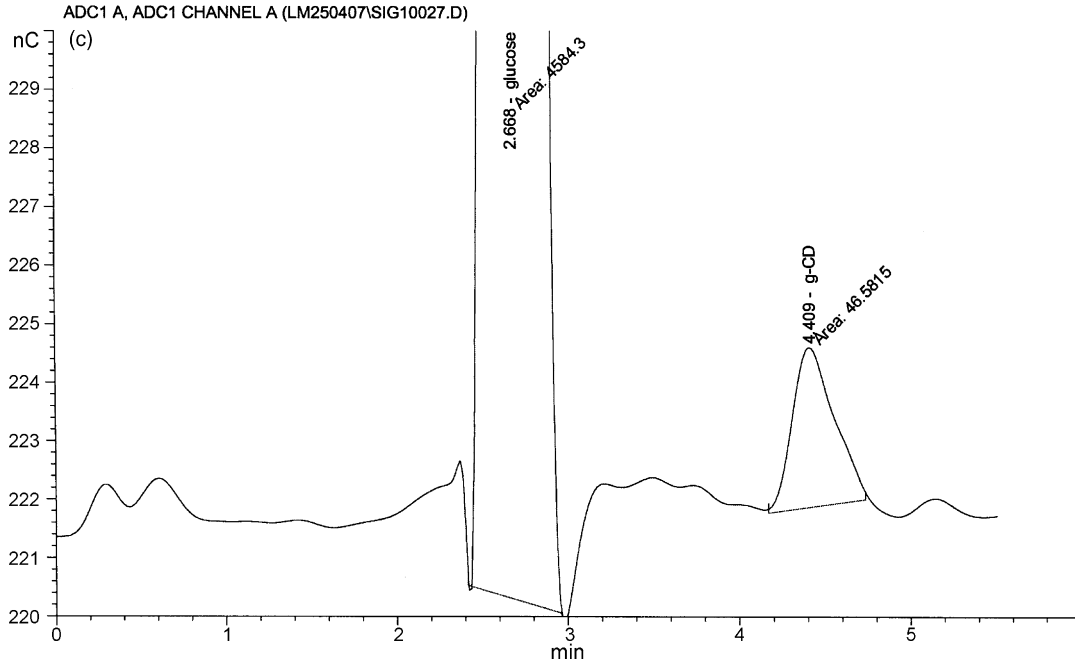


**Fig. 3.** (*Continued*).

was essential for the application to the related cell permeation study.

The present HPLC-PAD methods show a superior sensitivity when compared to other published detection methods used for the analysis of natural CDs in aqueous matrices. With CE-fluorescence methods, linear ranges of 0.21–63 mM α-CD, 5.2–1600 μM β-CD and 18–6100 μM γ-CD have been obtained by using 2,6-ANS as the fluorescent complexing reagent [18] and linear ranges of 0.12–14 mM α-CD, 50–1800 μM β-CD and 15–10,000 μM γ-CD by using 8,1-ANS as the fluorescent complexing reagent [29]. With HPLC-spectroscopic methods, linear ranges of 3–500 μM α-CD, 100–2500 μM β-CD and 200–5000 μM γ-CD have been obtained by using electrochemically created iodine as the colorimetric complexing agent [15].

By using the present HPLC-PAD methods, a similar sensitivity level was obtained for all the natural CDs, respectively. This has not been the case with the above-mentioned indirect detection methods where the sensitivity for each CD has been dependent on their complexing properties. However, by using PAD detector, it must be noted that the regular polishing of the gold-plated surface electrode is essential to prevent decrease in the response which is related to the overall method sensitivity. In the present study, two separate amperometric cells were used for the determination of CDs while three different persons were performing the laboratory work. Though a difference in the relative detector responses between the two-amperometric cells was observed, this did not affect the accuracy of the method. In addition, the present analysis method was also found to be insensitive to the changes in the laboratory personnel.

In summary, the present method development consisted of two phases: (a) the development of an HPLC analysis method for determination of CDs in aqueous solutions and (b) the development of a sample pretreatment method to remove the interfering compounds from the sample matrix prior to the HPLC analysis. As the present method is based on the use of pure calibration standard solutions, it can be applied both for the analysis of pure samples without pretreatment, or for the samples requiring sample pretreatment before the HPLC analysis. As an evidence to this, the present HPLC-PAD method has been successfully applied to the analysis of samples from dissolution studies (currently unpublished data) and cell permeation studies of CDs [26], respectively.

## 5. Conclusions

Simple, rapid and sensitive HPLC-PAD analysis methods were developed for the quantitative analysis of α-CD, β-CD and γ-CD in an aqueous medium. An accurate and repeatable sample pretreatment method was developed to remove the interfering compounds from the sample matrix and to enable the pre-concentration of the analytes prior to the HPLC analysis. In addition to the cell permeation studies (with the need for sample pretreatment), these analysis methods have been used for the quantitation of CDs in aqueous dissolution medium (without the need for sample pretreatment). Thus, the authors suggest that the present analysis method is utilisable for various pharmaceutical applications involving the analysis of natural CDs in aqueous matrices.

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