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Liquid chromatographic determination of β-cyclodextrin derivatives based on fluorescence enhancement after inclusion complexation

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ABSTRACT

A liquid chromatographic method using fluorescence detection for the determination of β-cyclodextrin (βCD) and its derivatives is presented. The chromatographic system is based on size-exclusion chromatography with the addition of the fluorophoric compound 1-naphthol to the mobile phase. Detection is based on fluorescence enhancement caused by the formation of inclusion complexes. By incorporating 10^{-4} *M* 1-naphthol in the mobile phase, detection limits of 90, 27, 370 and 37 pmol were obtained for βCD, hydroxypropyl-βCD, trimethyl-βCD and dimethyl-βCD, respectively. The method was applied to the determination of dimethyl-βCD in urine: the minimum detectable concentration was 0.2 μg/ml after preconcentration of 10 ml of urine.

INTRODUCTION

Methylated β-cyclodextrins (βCDs, Fig. 1) consist of seven glucopyranose units, in which two of the three OH groups of every unit, at the 2- and 6-positions, are methylated to an OCH_3 group. The formation of inclusion complexes with βCDs changes a variety of physicochemical properties of the guest molecule, *e.g.* its solubility, chemical reactivity or dissociation constant. βCDs and their derivatives are therefore used in pharmaceutical research to improve drug stability, dissolution rate and bioavailability [1]. Since unmodified βCDs are only rarely soluble in water, cyclodextrins modified by methylation, for example, are used for pharmaceutical applications [2].

Some βCDs and derivatives, such as dimethyl-βCD (DMβCD), have been applied as solubilizers and enhancers of drug absorption in nasal

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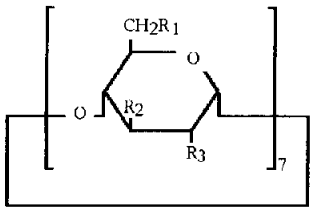

COMPOUND	R_1	R_2	R_3
DMβCD	-OCH_3	-OH	-OCH_3
TMβCD	-OCH_3	-OCH_3	-OCH_3
βCD	-OH	-OH	-OH
HPβCD	R_1, R_2 or R_3: -O-[CH_2-CHOH-CH_3]$_n$		

Fig. 1. Structures of β-cyclodextrin and its derivatives.

drug delivery [3,4]. From animal experiments it is known that DMβCD after oral administration is not metabolized and is absorbed only in traces [5]. After intraveneous administration DMβCD is cleared by glomerular filtration [6]. In order to perform pharmacokinetic studies, sensitive bioanalytical methods are required for the determination of βCD derivatives in, for example, plasma or urine. A major obstacle to the development of such methods is the total absence of chromophoric, electroactive or chemically reactive functional groups. Although unmodified cyclodextrins can be detected rather sensitively by pulsed-amperometric detection [7,8], modification of the hydroxyl groups by methylation leads to a drastic decrease in sensitivity using this detection principle. Analytical methods for methylated cyclodextrins described so far are based either on indirect detection [9,10] or on inclusion complex formation [11–13]. In the latter case the decrease of colour intensity of alkaline phenolphthalein upon inclusion complexation with βCDs has been exploited in the post-column reaction mode.

The most elegant feature of this detection principle is its inherent selectivity, considering the limited number of compounds capable of reducing the colour intensity of alkaline phenolphthalein. Since detection is based on the reduction of the detector signal against a high background, the detection limits obtained are still rather high. This paper describes the use of fluorescence enhancement as a detection method for modified cyclodextrins. Detection is based on the increase of fluorescence intensity of fluorophores upon formation of inclusion complexes. A thorough discussion of the factors that influence this increase has been presented by Frankewich *et al.* [14]. This technique is widely used for the determination of fluorophoric compounds where fluorescence is quenched by water molecules or solvent-borne quenchers [15,16]. Since the formation of fluorescent inclusion complexes is an equilibrium reaction, the use of fluorophores for the determination of cyclodextrins should equally be possible.

The present method has been applied to the determination of DMβCD in urine. In a volunteer pilot study, DMβCD levels were measured in six urine samples from a female taking Estranasal (estradiol–DMβCD 2% nasal spray) and four urine samples from a male taking a DMβCD 5% nasal spray.

EXPERIMENTAL

Chemicals

βCD and DMβCD were obtained from Avebe (Veendam, Netherlands), hydroxypropyl-βCD (HPβCD) from Janssen (Tilburg, Netherlands) and trimethyl-βCD (TMβCD) from Janssen Biotech (Geel, Belgium). 1-Naphthol and 2-naphthol (both from Merck, Darmstadt, Germany), Rhodamine 6G and 1-anilino-8-naphthalenesulphonate (ANS) (both from Sigma, St. Louis, MO, USA) were used as obtained.

The buffers used were prepared from sodium hydroxide and phosphoric acid 85%, both of analytical grade (Merck). Sodium chloride was of high purity and obtained from Merck. Ethanol and methanol were obtained from Baker (Deventer, Netherlands). Acetonitrile was purchased from Rathburn (Walkerburn, UK). Demineralized water was filtered over a 0.2-μm filter.

Apparatus

The liquid chromatographic system consisted of a Jasco Model Familic-300S isocratic pump

(Japan Spectroscopic, Tokyo, Japan) and a Promis autosampler (Spark Holland, Emmen, Netherlands) equipped with a 20-μl loop. Fluorescence detection was performed with a Shimadzu (Kyoto, Japan) fluorescence spectromonitor, Model RF 530, operated at an excitation wavelength of 290 nm and an emission wavelength of 360 nm. The detector signal was monitored by an Axxi-Chrom 727 chromatography data station (Axxiom Chromatography, Calabasas, CA, USA). All experiments with regard to the choice of fluorophore and the influence of organic modifier were carried out in batch on a Perkin-Elmer (Beaconsfield, UK) fluorescence spectrophotometer, Model MPF 4.

Chromatographic system

Chromatography was performed on different stationary phases: a CarboPac PA1 column (250 mm × 4.6 mm I.D.) (Dionex, Sunnyvale, CA, USA), a laboratory-packed LiChrosorb RP-2, 5-μm column (100 mm × 3 mm I.D.) (Merck), a TSK gel 3000 SW column, 10 μm (300 mm × 7.5 mm O.D.) (Hewlett Packard, Amstelveen, Netherlands) and a TSK guard column SWXL (40 mm × 6 mm I.D.). The eluent on the TSK guard column was 10^{-4} *M* 1-naphthol in methanol–water (1:99, v/v). Chromatography was performed at ambient temperature at a flow-rate of 0.25 ml/min.

Sample pretreatment

The extraction cartridges consisted of glass Pasteur pipettes (100 mm × 5.0 mm I.D.) containing a plug of cotton-wool. The pipettes were dry-packed with 100 mg of Polygosil C_{18} 40–63 μm (Macherey & Nagel, Düren, Germany), conditioned before analysis with 5 ml of methanol and 7 ml of water.

The urine samples (10 ml) were loaded onto the C_{18} extraction cartridges, which were washed afterwards with 2 ml of water and 8 ml of methanol–water (50:50, v/v). The residual on-column liquid phase was discarded by vacuum, and the column was eluted with 2 ml of methanol. This residue was evaporated in a vortex evaporator (Haake Büchler Instruments, Lenexa, KS, USA) at 35°C. The residue was dissolved in 0.25 ml of mobile phase, and 20 μl were injected into the chromatographic system. Calibration curves were obtained by adding known amounts of the analyte to blank urine of a healthy volunteer.

RESULTS AND DISCUSSION

Optimization of the detection system

Choice of fluorophore. The detection of βCDs by means of fluorescence enhancement requires either the incorporation of the fluorophore into the eluent or its post-column addition. In both cases a suitable fluorophore should provide high enhancement factors but produce only low background fluorescence. Table I shows the enhancement factors obtained in batch experiments for 1-naphthol, 2-naphthol, Rhodamine 6G and ANS in the presence of different cyclodextrins. Obviously, only βCD derivatives can form inclusion complexes strong enough to yield an enhan-

TABLE I

FLUORESCENCE ENHANCEMENT FACTORS OF DIFFERENT CYCLODEXTRINS

Label	$\lambda_{ex}/\lambda_{em}$ (nm)	Concentration (*M*)	Enhancement factor					
			αCD	βCD	γCD	DMβCD	TMβCD	HPβCD
1-Naphthol	290/360	10^{-4}	1.5	21.1	1.9	31.1	13.0	25.1
2-Naphthol	310/360	10^{-4}	1.1	1.5	1.1	2.0	1.3	1.8
ANS	390/510	10^{-3}	1.3	3.1	4.3	9.8	3.2	21.1
Rhodamine 6G	525/550	10^{-7}	1.0	1.1	1.0	2.0	2.1	1.6

cement effect, whereas α- and γCDs form only weak complexes with the fluorophore investigated. The enhancement effect is most pronounced for 1-naphthol, which is in agreement with the results of Frankewich *et al.* [14]. 1-Naphthol is the most suitable candidate with respect to background fluorescence. 1-Naphthol, 2-naphthol and ANS are weakly fluorescent, producing only low background fluorescence, but Rhodamine 6G is a strong fluorophore and could be used only at very low concentrations (10^{-7} *M*). All further experiments were carried out with 1-naphthol.

The stability constants for different βCDs with 1-naphthol were determined by measuring the fluorescence at fixed βCD concentrations and varying 1-naphthol concentrations. Data analysis was performed using non-linear least-squares fitting. The stability constants for the 1-naphthol complexes of βCD, DMβCD, TMβCD and HPβCD (assuming 1:1 stoichiometry) are 2310, 1820, 3530 and 1780, respectively.

Influence of organic modifier content on fluorescence enhancement. Since the formation of inclusion complexes is disturbed by organic solvents, *e.g.* mobile phase modifiers such as methanol or acetonitrile [12,14], the influence of common chromatographic solvents on fluorescence enhancement was investigated. Fig. 2 shows the dependence of the fluorescence enhancement factor of DMβCD on the contents of methanol, acetonitrile and ethanol. It demonstrates that at a methanol content higher than 10% a considerable decrease in fluorescence enhancement occurs. The effect is even more pronounced for acetonitrile and ethanol. A maximum content of 10% methanol in the eluent, however, strongly limits the choice of stationary phase unless a post-column dilution step is intended. The influence of these results on the design of the chromatographic system is discussed below.

Chromatographic system

Fluorescent inclusion complexes can be formed by the addition of the fluorophore to the mobile phase, thereby avoiding a post-column reaction detection system, which would require an additional pump and result in extra-column bandbroadening. Using *in situ* complex formation, the choice of the chromatographic system is strongly determined by the physicochemical conditions at which the inclusion complex is stable.

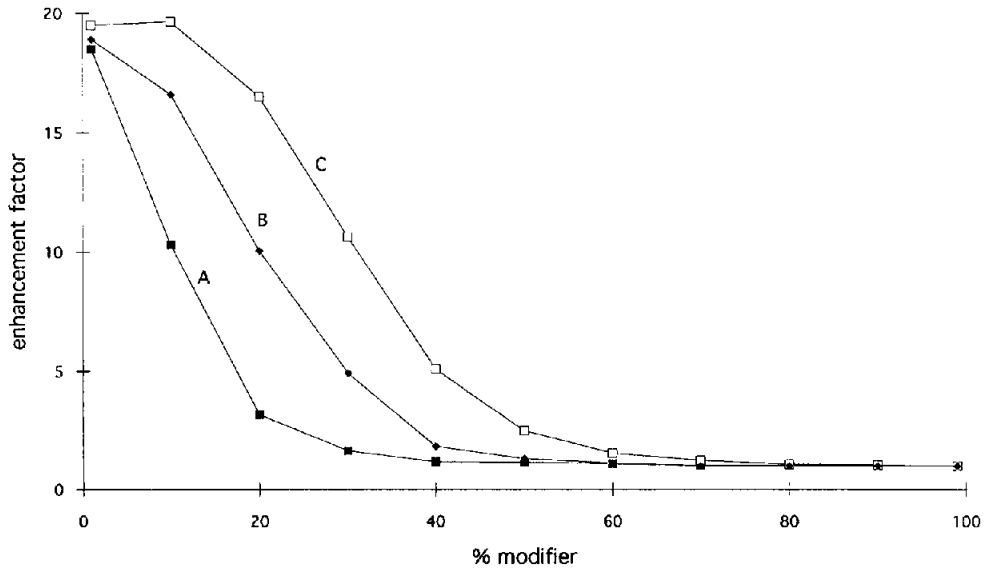

Fig. 2. Dependence of the fluorescence enhancement factor of DMβCD on the organic modifier content. A, acetonitrile; B, ethanol; C, methanol.

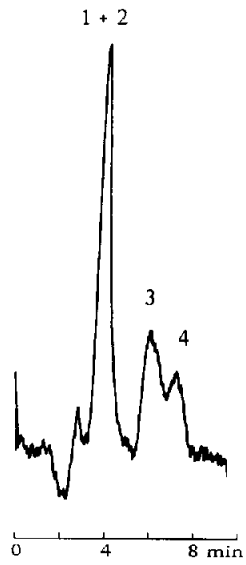

Fig. 3. Chromatogram of (1) HPβCD, (2) βCD, (3) DMβCD and (4) TMβCD on a TSK guard column using the fluorescence enhancement detection system. Conditions as in Experimental.

Since organic modifier concentrations of higher than 10% result in a drastic decrease of fluorescence enhancement (see Fig. 2), reversed-phase stationary phases (C_{18}, C_8 and C_2 bonded silica) would provide retention times that were too long to be useful. On ion-exchange stationary phases, such as CarboPac PA1, on the other hand, DMβCD was not retained at all. Size-exclusion supports, such as TSK gel 3000 SW, were the most suitable support for fast analysis of DMβCD. Both the analytical column (300 mm × 7.5 mm I.D., 10-μm particles) and the guard column (40 mm × 6 mm I.D., 10-μm particles) were packed with TSK 3000 SW. Owing to the high selectivity of the detection principle, the guard column provided sufficient separation efficiency to allow the determination of DMβCD in urine.

Addition of 1-naphthol to the mobile phase did not change the chromatographic behaviour of DMβCD. Fig. 3 shows a chromatogram of HPβCD, βCD, DMβCD and TMβCD on the TSK guard column; HPβCD has the same retention as βCD. The rather broad peak obtained for DMβCD can be explained by the heterogeneous character of the analyte. The DMβCD formulation used in these experiments consisted of a mixture of DMβCDs containing methyl groups at positions 14, 15 and 16 [17]. With the present chromatographic system and using *in situ* inclusion complexation (10^{-4} *M* 1-naphthol), detection limits (signal-to-noise ratio 3:1) of 90, 27, 370 and 37 pmol were obtained for βCD, HPβCD, TMβCD and DMβCD, respectively. The detector response was linear over two orders of magnitude.

Determination of DMβCD in urine

Solid-phase extraction on C_{18} bonded silica was used to perform off-line preconcentration. Because of the high retention of DMβCD it was

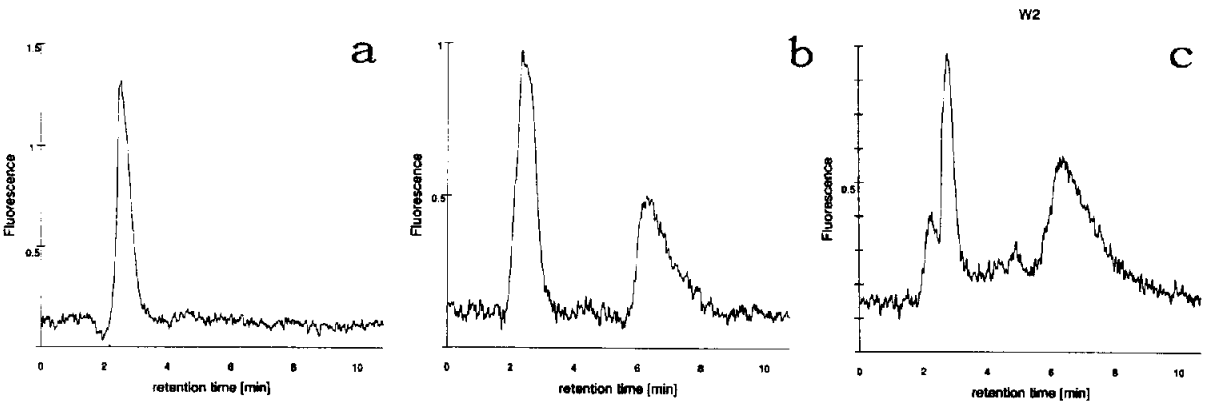

Fig. 4. Chromatograms of (a) blank urine, (b) urine spiked with 0.5 μg/ml DMβCD and (c) urine from a female volunteer receiving a nasal dose of 4 mg of DMβCD. Conditions as in Experimental.

TABLE II

VALIDATION OF THE METHOD

Concentration (μg/ml)	Precision (%)		Accuracy (%)
	Day-to-day (n = 3)	Within-day (n = 3)	
0.5	2.7	5.8	97.2
1.0	7.2	8.7	94.4
2.0	6.1	2.5	98.4
3.0	5.2	9.4	92.5
5.0	6.2	7.6	93.9

possible to wash the column with 10 ml of methanol–water (50:50, v/v) without breakthrough of the analyte. In this way an enrichment factor of 40 was obtained for DMβCD while the majority of low-molecular-mass urine components were removed. A chromatogram representing the determination of 0.5 μg/ml DMβCD in urine is shown in Fig. 4b.

The method was validated by a three-fold assay on three consecutive days of blank urine samples to which different amounts of the compound had been added. Six urine samples of 10 ml were spiked with concentrations ranging from 0.2 to 5 μg/ml, covering the relevant concentration range. A typical regression line with the standard deviation of slope and intercept is $y = (23\,948 \pm 408)x - (6283 \pm 1143)$, where y represents the peak area of DMβCD and x the concentration in μg/ml; the correlation coefficient is 0.999. The results of the validation are summarized in Table II. In the relevant concentration range the method is linear, and the mean recovery over this range is 93%. The determination limit, based on a signal-to-noise ratio of 10, was 150 ng; the corresponding minimum detectable concentration in urine was 0.2 μg/ml.

In a volunteer study, DMβCD levels were measured in six urine samples from a female taking Estranasal (estradiol–DMβCD 2% nasal spray) and four urine samples from a male taking a DMβCD 5% nasal spray. The levels of DMβCD that were found were 4.2 ± 2.1 and 2.5 ± 1.7%, respectively, of the concentration administered. This corresponds to a concentration of 0.2–2.3 μg/ml DMβCD in urine, indicating that DMβCD is hardly adsorbed in men after nasal application. An example for the determination of DMβCD in female urine (W2) is shown in Fig. 4c.

CONCLUSION

β-Cyclodextrin and its derivatives can be determined by the formation of fluorescent inclusion complexes with a suitable fluorophore. When size-exclusion chromatography is used, free and complexed βCDs show no significant difference in retention. Addition of the fluorophore to the mobile phase results in *in situ* complexation, making the use of a post-column reaction detection system unnecessary. The high selectivity of this detection principle is demonstrated by the determination of DMβCD in urine. After preconcentration of 10 ml of urine, virtually no interfering matrix components were detected.

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