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Fluorescence determination of sulphobutylether-β-cyclodextrin in human plasma by size exclusion chromatography with inclusion complex formation

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Abstract

A selective method for the determination of sulphobutylether-β-cyclodextrin (SBECD) in human plasma has been developed and validated over the range 4–200 μg ml^{-1}. SBECD is extracted from plasma using end-capped cyclohexyl solid phase extraction cartridges. This is followed by high performance size exclusion chromatography with a mobile phase consisting of 1-naphthol (0.1 mM) in methanol-potassium nitrate (0.2 M) (1:9 v/v), 1 ml min^{-1}. The high aqueous content of the mobile phase quenches the fluorescence of 1-naphthol. However, the naphthol forms an inclusion complex with SBECD. The non-polar 'bucket' environment of the inclusion region restores the fluorescence, which is measured at excitation and emission wavelengths of 290 and 360 nm, respectively, when SBECD elutes from the column.

Keywords: Sulphobutylether-β-cyclodextrin; Analysis; Human plasma; Size exclusion chromatography; Inclusion complex; Fluorescence detection

1. Introduction

Cyclodextrins are cyclic oligosaccharides composed of α-1,4-linked glucopyranose units which form a truncated cone with a hydrophilic outer surface and a hydrophobic cavity. Although, larger rings have been produced, the three most common cyclodextrins contain 6, 7 or 8 glucopyranose units, denominated α-, β-, and γ-cyclodextrin, respectively. Each glucopyranose unit contributes one primary hydroxyl group to the narrow edge of the cavity and two secondary hydroxyl groups to the wide edge. These groups can be substituted with a wide variety of other groups to modify the cyclodextrin properties.

Because of their structure, cyclodextrins are able to form inclusion complexes with lipophilic

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guest molecules. This has lead to their use as additives in pharmaceutical, food and cosmetic products [1]. A number of reviews describe their use in the pharmaceutical industry where they have been used as excipients to improve the solubility, bioavailability, tolerability, and stability of drugs [2–5].

A novel excipient for use with drugs of limited aqueous solubility, sulphobutylether-β-cyclodextrin sodium (SBECD, Fig. 1) is a mixture of very polar, structurally related compounds in which butylsulphonic acid groups replace up to 10 of the hydroxyl hydrogens in β-cyclodextrin. SBECD has been used in a formulation designed to facilitate intramuscular administration of the anti-psychotic agent ziprasidone to patients whose symptoms preclude oral administration of this drug [6]. Pfizer is also currently investigating its use to enable an intravenous formulation of the novel antifungal agent voriconazole.

Although they have been extensively used as tools in the chromatographic separation of enantiomers [7,8], the cyclodextrins themselves are extremely difficult to analyse in biological media using standard techniques because, with only a few exceptions, they contain no chromophore and exist as a mixture of structurally related compounds.

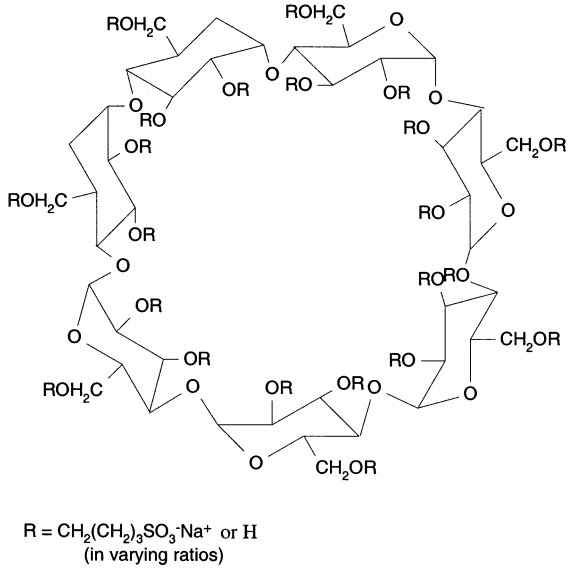

Fig. 1. Structure of SBECD.

The few methods that have been published over the last two decades use pulsed amperometric [9,10], refractive index [11], ultraviolet (following derivatisation) [11] and mass spectrometer [12] detectors. A particularly interesting property of the cyclodextrins is their ability to restore the quenched fluorescence of certain guest molecules in aqueous solutions [13]. This property has been used for the spectrophotometric determination of methyl-β-cyclodextrin in plasma and urine [14,15].

To our knowledge, there is no method for the determination of native SBECD in plasma or urine; the only method we are aware of for the determination of SBECD in plasma detects ^{14}C radiolabelled SBECD [16]. The present paper describes a relatively simple, rapid and reproducible method for measuring SBECD in human plasma with a calibration range 4–200 μg ml^{-1}. The method utilises solid phase extraction as the only sample preparation prior to size exclusion chromatography and fluorescence determination of an inclusion complex. No internal standard was required.

2. Experimental

2.1. Chemicals

Methanol (super purity solvent grade) was purchased from Romil Chemicals (Cambridge, UK). Phosphoric acid (85%), potassium nitrate and disodium hydrogen orthophosphate 12-hydrate (Analar grade) were purchased from Merck (BDH) (Lutterworth, UK). Aspirin, paracetamol, ampicillin, 3′-azido-3′-deoxythymidine (AZT), oxytetracycline, prednisolone, theophylline and 1-naphthol were purchased from Sigma (Poole, UK). Ziprasidone and voriconazole were obtained from Pfizer (Sandwich, UK). SBECD with an average degree of substitution of four was purchased from Higuchi Biosciences Centre (Kansas University, USA). All other batches of SBECD were supplied by Pfizer (Connecticut, USA). SBECD with an average degree of substitution of 6.5 was used for all experiments except where otherwise stated. Lithium-heparinised control human plasma was purchased from Charterhouse

Clinical Research Unit (Royal Masonic Hospital, UK). Ultra-pure water was provided by a Milli-Q-Plus water purification unit (Millipore, Watford, UK). IST end-capped cyclohexyl isolute solid phase extraction cartridges (100 mg, 3 ml) were purchased from Jones Chromatography (Mid-Glamorgan, UK).

2.2. Instrumentation

The high performance size exclusion system was constructed from the following components. Valco EHC6WP fast switching valve (Thames Chromatography, Maidenhead, UK), Shimadzu SIL-6B autoinjector, SCL-6B controller and two LC-6A pumps (Dyson Instruments, Hetton, UK), Merck-Hitachi L7480 fluorescence detector (Merck, Lutterworth, UK). Data collection and processing was carried out by an Ezchrom Elite V2.1 chromatography data system (Aston Scientific, Stoke Mandeville, UK).

The autoinjector was equipped with a 200 μl stainless steel loop and methanol–water (1:4 v/v) wash (1.25 ml per sample). The detector was equipped with a 12 μl flow cell, xenon lamp and 15 nm slit and was set at 290 nm excitation, 360 nm emission, 8 s time constant and low PMT voltage.

The analytical column was a 300×7.8 mm BioSep-SEC-S3000 (5 μm diol-silica, 290 Å) size exclusion column with a 35×7.8 mm guard packed with the same material (Phenomenex, Macclesfield, UK). The mobile phase was 1-naphthol (0.1 mM) in methanol-potassium nitrate buffer (0.2 M) (1:9 v/v), 1 ml min^{-1}. The mobile phase was degassed by filtration through a 0.2 μm nylon filter under vacuum before the naphthol was added. The vessel containing the mobile phase was wrapped with aluminium foil to protect the naphthol from light. All chromatography was performed at ambient temperature (~ 21°C).

To prevent 1-naphthol from building up on the detector flow cell, a post-column switching valve directs a methanol–water (7:3 v/v) wash to the detector for 5 min at 2 ml min^{-1} immediately following the injection of each sample.

2.3. Sample preparation

A stock solution of SBECD was prepared in water (~ 10 mg ml^{-1}). This was diluted with water to give a 100 μg ml^{-1} working solution. Calibration standards were prepared by adding the stock and working SBECD solutions (1–42 μl) to 2 ml aliquots of control human plasma to give concentrations of 4, 6, 10, 20, 30, 50, 100 and 200 μg ml^{-1} and total volumes within 1% of 2 ml (1.95 ml plasma was used for the 200 μg ml^{-1} standard).

An aliquot of each sample (0.16 ml) was pipetted into a 12 ml tapered polypropylene tube (Sarstedt, Leicester, UK) and sodium phosphate buffer (1.5 ml) (pH 7.0; 0.2 M) added. The mixture was vortex mixed briefly. End-capped cyclohexyl cartridges (100 mg, 3 ml) were conditioned with methanol (1 ml) followed by sodium phosphate buffer (1 ml) (pH 7.0; 0.2 M). 1 ml of the diluted sample was loaded onto the cartridge and allowed to elute under gravity. The cartridge was washed with sodium phosphate buffer (1 ml) (pH 7.0; 0.2 M) and excess buffer was removed by brief suction, taking care not to dry the cartridge. The cartridge was transferred to a 12 ml tapered polypropylene tube and methanol–water (0.5 ml) (15:85 v/v) added. Two drops were gently pushed through with a teat and the rest allowed to elute under gravity. Excess solvent was pushed through and the sample blown to dryness under a stream of nitrogen in a TurboVap LV evaporator (Zymark, Warrington, UK) set at 40°C. The dried sample extract was reconstituted in methanol–water (0.15 ml) (10:90 v/v), transferred to a 0.3 ml polypropylene autosampler vial (Phase Separations, Queensferry, UK) and 0.12 ml injected into the chromatography system.

2.4. Data processing

The difference in the buffer content of the reconstituted sample and the mobile phase results in a baseline drop at the tail of the SBECD peak which complicates automatic baseline fitting (Fig. 2). We tried washing the Isolute cartridge with water and 1% solutions of trifluoroacetic acid, ammonia and triethylamine to remove the sodium

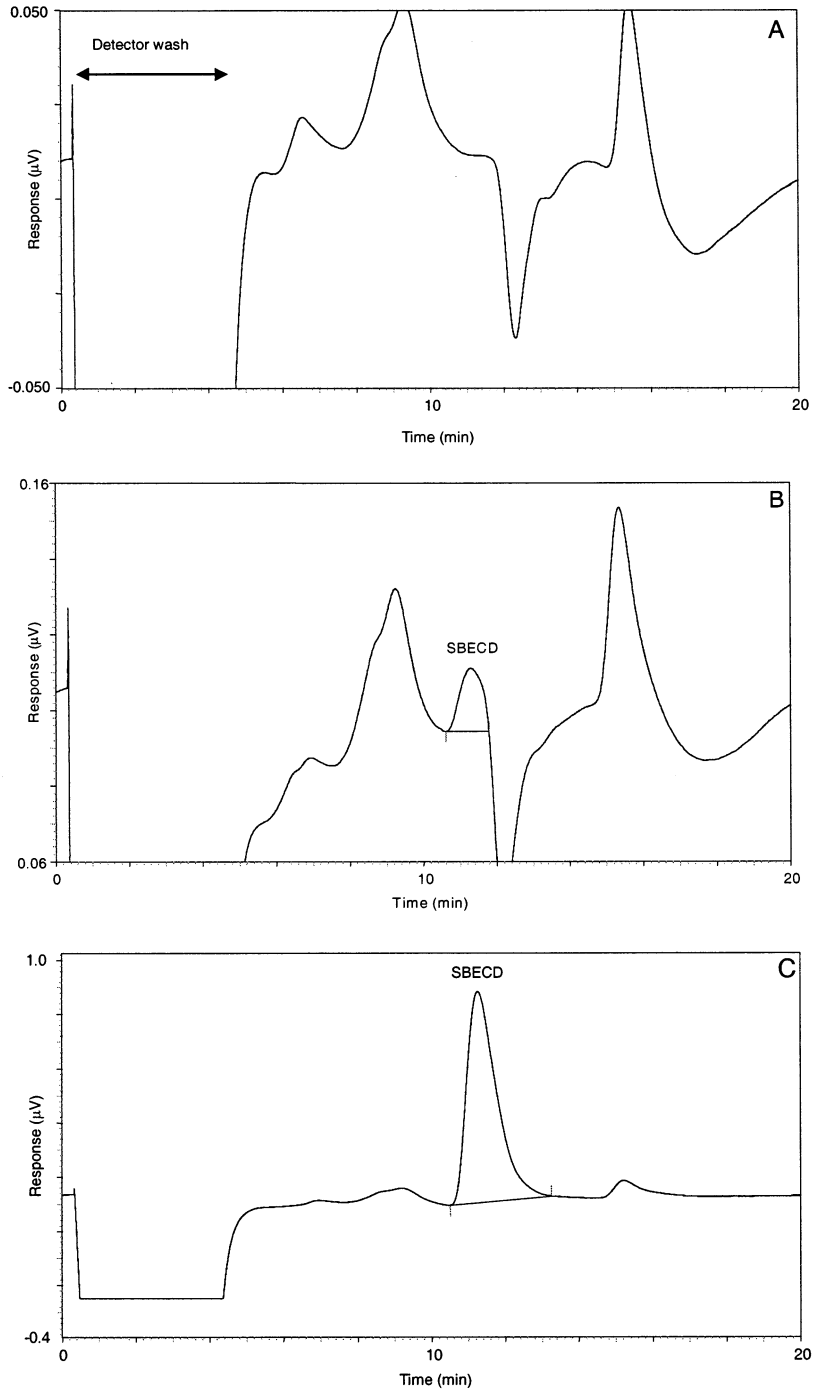

Fig. 2. Chromatograms of extracts of human plasma containing (A) no SBECD, (B) SBECD, 4.00 μg ml^{-1} and (C) SBECD, 200 μg ml^{-1}.

phosphate. While these washes reduced the baseline disturbance, they also resulted in significant loss of SBECD and hence could not be incorporated into the extraction procedure.

The baseline problem was therefore resolved by using Ezchrom integration parameters which force a horizontal baseline across the base of the SBECD peak (Fig. 2B). At high concentrations this horizontal fitting was considered inappropriate and the baseline was manually fitted (Fig. 2C).

Calibration curves were constructed by plotting the peak height of SBECD against concentration using a weighted (1/Y) least squares regression in the Ezchrom data system.

2.5. Accuracy and precision

Accuracy and precision were assessed on three separate occasions by performing six replicate analyses of control human plasma fortified with SBECD to 4, 50 and 200 μg ml^{-1}. Accuracy was calculated as the percentage difference between the expected and measured concentrations. Precision was calculated as the relative standard deviation (RSD).

2.6. Selectivity and recovery

Six different batches of control human plasma were analysed to check for chromatographic interference from co-eluting endogenous compounds. One of these batches was visibly lipaemic and another contained haemolysed erythrocytes, prepared by mixing control human plasma (9.5 ml) with human blood (0.5 ml) which had been frozen and thawed three times. The same six batches of plasma were fortified with SBECD to 4 and 200 μg ml^{-1} and analysed in triplicate to check for interference by endogenous plasma components on the extraction procedure.

Ziprasidone, voriconazole and seven other commonly used drugs were added to separate aliquots of plasma containing 20 μg ml^{-1} SBECD to give 5 μg ml^{-1} of each compound. The plasma samples were then analysed in triplicate for SBECD. The potential co-administered drugs tested were aspirin, paracetamol, ampicillin, AZT, oxytetracycline, prednisolone and theophylline.

The extraction recovery of SBECD was determined by fortifying control human plasma with 100 μg ml^{-1} SBECD, extracting six replicates, and comparing the mean peak height of SBECD with that obtained by directly injecting, in triplicate, a solution of 100 μg ml^{-1} SBECD in water.

It has been reported [17] that the degree of substitution of SBECD (and other cyclodextrins) can affect the host-guest binding constant and thereby change the amount of inclusion complex formed per mole of cyclodextrin. Therefore, to check the affect of the degree of substitution on response, solutions of seven different batches of SBECD (0.1 mg ml^{-1}) with average degrees of substitution ranging from 4 to 6.9 were prepared in mobile phase and 90 μl injected, in duplicate, to the chromatography system. For the SBECD batch with an average degree of substitution of 6.9, the number of injections was increased to six in order to determine the variation.

2.7. Stability

Control human plasma was fortified with SBECD to 100 μg ml^{-1} and analysed in triplicate fresh and after three freeze–thaw cycles. Plasma was also fortified with SBECD to 50 μg ml^{-1} and analysed in triplicate fresh and after storage at either fridge temperature ($\sim$4°C) or room temperature ($\sim$20°C) for 24 h.

2.8. Determination of SBECD in plasma samples from human volunteers

SBECD was assayed in plasma samples collected from two healthy male human volunteers following administration of 1600 mg SBECD as a single 15 min iv infusion. The batch of SBECD used to prepare the calibration standards (and quality controls) was the same as that administered to the volunteers.

3. Results and discussion

3.1. Accuracy and precision

Calibration curves were linear over the range

Table 1
Accuracy and precision ($n = 6$) on three separate occasions for SBECD added to control human plasma

Occasion	SBECD concentration (μg ml^{-1})		Accuracy (%)	Precision (RSD %)
	Prepared	Found (mean)		
1	4.00	3.91	97.7	4.67
	50.0	50.9	102	4.44
	200	197	98.5	7.45
2	4.00	4.36	109	16.3
	50.0	52.8	106	9.18
	200	196	98.0	3.04
3	4.00	4.10	103	4.52
	50.0	51.0	102	5.56
	200	192	96.2	5.83

4–200 μg ml^{-1} with mean values ($n = 5$) for slope of 4102 (S.D. 702), intercept −1096.68 (S.D. 3354) and correlation coefficient, 0.9985 (S.D. 0.0012). On three separate occasions, the accuracy and precision were within 9 and 17%, respectively, at the lowest concentration tested (4 μg ml^{-1}) and within 6 and 10%, respectively, at the higher concentrations (Table 1).

The limit of quantification (LOQ) in bioanalytical methods is generally accepted [18–20] as a concentration at which accuracy is within ±20% of nominal value with an RSD ≤ 20%. Since the accuracy and precision were within these limits at the lowest SBECD concentration examined, that concentration (4 μg ml^{-1}) was taken as the LOQ.

3.2. Chromatography, selectivity and recovery

Typical chromatograms obtained for blank human plasma and for plasma fortified with 4 and 200 μg ml^{-1} SBECD are shown in Fig. 2A–C, respectively. The retention time of SBECD was 11.3 min. The assay was selective for SBECD as no significant peaks due to endogenous compounds were observed which would compromise the determination of SBECD at 4 and 200 μg ml^{-1} when six different batches of human plasma were analysed (Table 2). The lipaemic plasma gave the lowest recovery at both of these concentrations although the accuracy is still within the generally accepted limits of ±20% at the LOQ and ±15% at higher concentrations. In our experience of Phase I clinical trials, plasma samples of such a lipaemic nature usually form only a very small proportion of those collected in a study. This observation was therefore considered to be a minor issue to be considered on a per study basis. The drugs, which may be co-administered with SBECD did not affect the chromatography or recovery of SBECD.

Table 2
SBECD extracted in triplicate from six different batches of human plasma[a,b]

Plasma	SBECD concentration (μg ml^{-1})		Accuracy (%)
	Prepared	Found (mean ± S.D.)	
A	4.00	4.25 ± 0.170	106
B	4.00	4.25 ± 0.289	106
C	4.00	4.37 ± 0.725	109
D	4.00	4.27 ± 0.506	107
E	4.00	3.30 ± 0.280	82.5
F	4.00	3.77 ± 0.285	94.1
A	200	189 ± 15.1	94.3
B	200	183 ± 13.7	91.6
C	200	180 ± 14.4	90.0
D	200	198 ± 1.32	98.8
E	200	172 ± 5.11	86.0
F	200	185 ± 13.8	92.6

[a] Plasma E was visibly lipaemic.
[b] Plasma F contained haemolysed erythrocytes.

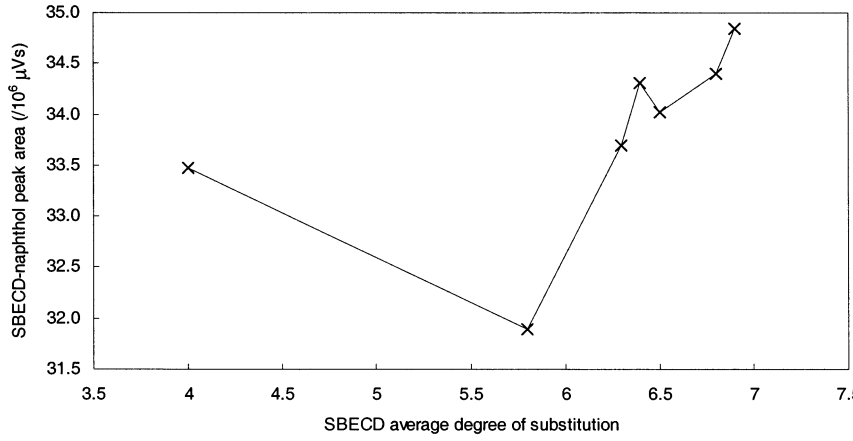

Fig. 3. Variation of the SBECD-naphthol peak area with the SBECD average degree of substitution. For the SBECD batch with an average degree of substitution of 6.9, the precision (RSD, $n = 6$) of the peak area was 1.27%.

The recovery of SBECD from plasma was 69% for the solid phase extraction procedure. The overall recovery is less than this due to the experimental design, which allows for the sacrifice of 52% of the sample to maximise the accuracy and precision of its handling in the absence of an internal standard.

One would expect that, all other factors being equal, as the average degree of substitution is increased, the detector response per unit weight of SBECD would decrease due to the reduced number of 'buckets' available to form an inclusion complex. However, it was observed that as the substitution increased from 4 to 6.9, the peak area of SBECD generally increased (Fig. 3). This effect was even greater for the peak height due to a sharpening of the SBECD peak as the degree of substitution increased. Unfortunately, there is an insufficiently reliable correlation to allow for between-substitution correction. Therefore, the batch of SBECD used to prepare the calibration standards should have the same average degree of substitution as that used to produce the samples in which SBECD is to be quantified.

3.3. Stability

SBECD in human plasma was stable to three freeze–thaw cycles with changes of -3.7, -6.5 and -2.8% from the initial measured concentration of 108 µg ml^{-1} after 1, 2 and 3 cycles, respectively. SBECD in human plasma was also stable to storage for 24 h at 4 and 20°C with changes of -2.2 and -1.8%, respectively, from the initial measured concentration of 51.2 µg ml^{-1}.

3.4. SBECD in plasma samples from human volunteers

Fig. 4 shows the SBECD plasma concentration–time profiles in two healthy male human volunteers who each received 1600 mg SBECD as a single 15 min iv infusion. The concentration of SBECD in plasma samples collected from 8 h onwards were below the limit of quantification (4 µg ml^{-1}).

4. Conclusion

A selective method for the assay of SBECD in human plasma has been developed and validated over the range 4–200 µg ml^{-1}. It is simple, requiring solid phase extraction as the only sample preparation prior to high performance size exclusion chromatography and fluorescence determination of an inclusion complex.

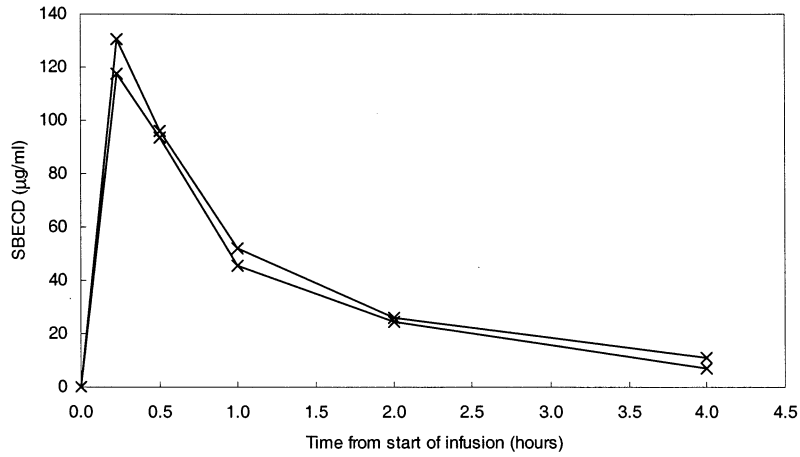

Fig. 4. Plasma concentrations of SBECD in two healthy male human volunteers following administration of 1600 mg SBECD as a single 15 min iv infusion.

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