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Journal of Chromatography B, 774 (2002) 157–164

JOURNAL OF CHROMATOGRAPHY B

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High-performance liquid chromatographic determination of 2-hydroxypropyl-γ-cyclodextrin in different biological fluids based on cyclodextrin enhanced fluorescence

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Received 18 September 2001; received in revised form 11 February 2002; accepted 27 March 2002

Abstract

A high-performance size exclusion chromatographic method with analyte enhanced fluorescence detection is described for the analysis of 2-hydroxypropyl-γ-cyclodextrin (HPGCD) in different biological fluids. The principle of detection was the in situ complexation of 8-anilinonaphthalene-1-sulfonic acid (ANS) by HPGCD. When HPGCD eluted from the column the increased fluorescence was measured at excitation and emission wavelengths of 270 and 512 nm, respectively. Solid-phase extraction cleanup and concentration of samples resulted in higher than 78% recovery of HPGCD for each of the studied biological fluids. Some important details of the method development as well as the validation of the method for rabbit plasma, rabbit aqueous humour, monkey plasma and monkey urine are given. The limits of quantification varied between 1 and 10 nmol/ml (correspond to 1.5–15 μg/ml) depending on the biological matrix used. The method was successfully adapted in another laboratory proving that HPGCD had not absorbed into aqueous humour and plasma after topical application of HPGCD containing eye drop in rabbits.

Keywords: 2-Hydroxypropyl-γ-cyclodextrin

1. Introduction

Hydroxypropyl-γ-cyclodextrin (HPGCD) consists of eight glucopyranose units, in which some hydroxy groups are statistically substituted with 2-hydroxypropyl groups at different positions of the glucose moieties. The average degree of substitution of the commercial product is 4.6/cyclodextrin (CD) ring. The 2-hydroxypropyl groups are mainly positioned on the secondary side of the ring.

HPGCD forms inclusion complex with various guest molecules modifying their physicochemical properties, e.g., their solubility, chemical reactivity,

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00182-4

fluorescence, etc. Therefore, HPGCD—as other CDs—can also be taken into consideration for using in drug formulations to improve their stability, dissolution, bioavailability and reduction of side effects [1].

The availability of a suitable analytical method is of crucial importance in pharmaceutical formulations, particularly, when a new excipient is used, like HPGCD. The analysis of CDs in biological media is extremely difficult, because, since these carbohydrates contain no chromophores, the customary detection technique (UV) is not applicable. Moreover, most of the CD derivatives consist of large number of structurally related isomers.

In the published HPLC methods refractive index [2], UV following derivatisation [2], indirect spectrophotometric [3,4], pulsed amperometric [5–7] and spectrofluorimetric [8–10] detection were used for determination of CDs in biological matrices. The spectrofluorimetric detection of CDs is based on the increase of fluorescence intensity of fluorophores upon formation of inclusion complex [11]. The fluorescence detection is very sensitive and provides some selectivity, because the cavity of the CD ring is detected.

The isomeric distribution of CD derivatives can be characterised by a fingerprint HPLC chromatogram (by reversed-phase or normal-phase chromatography). In biological samples, in which the CD derivatives should be quantitatively determined, size exclusion chromatography is used, where all isomers are—on purpose—compressed into one peak [4,8–10]

The only method known from the literature for determination of HPGCD is a gas chromatographic method with mass spectrometric detection. HPGCD was hydrolysed to the respective monosaccharides and the resulting 2-hydroxypropyl-glucose units were derivatized and quantified by gas chromatography–mass spectrometry [12].

Here we report on the development and validation of a relatively simple, rapid, specific and reproducible HPLC method for measuring HPGCD in various biological matrices. The method utilises solid-phase extraction for sample preparation prior to size exclusion chromatography with 8-anilinonaphthalene-1-sulfonic acid in the HPLC mobile phase for the fluorescence detection of HPGCD. There was no need to use an internal standard.

2. Experimental

2.1. Materials

Hydroxypropyl-γ-cyclodextrin (HPGCD, degree of substitution 4.6, average molecular mass 1500) is the product of Wacker Chemie (Munich, Germany). Methanol (gradient grade) was purchased from Merck (Darmstadt, Germany), 8-anilinonaphthalene-1-sulfonic acid (ANS) and 1-naphthol were obtained from Sigma–Aldrich Chemie (Steinheim, Germany). Water (gradient HPLC grade) was purchased from Scharlau Chemie (Barcelona, Spain). Rabbit (Fauve de Bourgogne) and monkey (Cynomolgus) plasma, rabbit aqueous humour and monkey urine were obtained from LPT Laboratory (Hamburg, Germany).

2.2. Instrumentation

The chromatographic apparatus consisted of Agilent (Hewlett-Packard) 1050 pumping system, 1100 Series thermostatted autosampler, fluorescence detector and thermostatted column compartment. For data processing and analysis version A 06.03. of ChemStation software was used (Waldbronn, Germany).

Two type of analytical columns were tested: Nucleogel aqua-OH 40-8 (300×7.7 mm with 75×7.7 mm guard column, Macherey-Nagel, Düren, Germany) and TSKgel G 3000 SW (300×7.5 mm with 75×7.5 mm guard column, TosoHaas, Stuttgart, Germany).

2.3. Chromatographic conditions

The mobile phase was prepared by mixing water, methanol and 5 m*M* ASN methanolic stock solution (920:76:4, v/v), filtered and degassed ultrasonically prior to use. The mobile phase flow-rate was 1 ml/min, the column temperature was 40 °C, 100 μl sample was injected from the vials which were thermostatted to 25 °C in the autosampler. After injection a number of samples (20–25) a column washing procedure was carried out to eliminate the interference caused by the adsorption of plasma components on the column (see Section 3.2). The optimised excitation and emission wavelengths were 270 and 512 nm, respectively. Other detector parameters: PMT-Gain 10, peakwidth >0.2 min.

2.4. Sample preparation

The stock solution of HPGCD was prepared in water (~1.5 mg/ml, corresponds to 1 mmol/dm^3). Rabbit and monkey plasma, rabbit aqueous humour and monkey urine samples were diluted with saline (0.9% sodium chloride) solution before solid-phase extraction. Calibration standards were prepared by spiking the biological matrix with various amount of HPGCD.

Solid phase extraction (SPE) was carried out using a Vac Elut SPS 24 vacuum manifold on Supelco-Clean LC-PH solid-phase extraction columns (3 ml, 500 mg, Supelco, Sigma–Aldrich, Steinheim, Germany). The concentration and cleanup of the samples was based on inclusion complex formation of HPGCD with the phenyl groups of the used sorbent in the SPE columns. After conditioning the columns with 1 ml methanol and 2 ml saline the prepared samples containing HPGCD were aspirated slowly through the columns. The columns were washed with 2 ml 0.01 *M* hydrochloric acid and 2 ml of 10% aqueous methanol. HPGCD was eluted with 2 ml 60% aqueous methanol. The solvent was carefully evaporated under a stream of nitrogen at ~80 °C. The residue was dissolved in 200 μl of mobile phase used for chromatography.

2.5. Specificity, linearity and range

Specificity was checked by comparing the retention times of endogenous compounds in biological matrices with those of HPGCD.

For determination of linearity the peak areas of HPGCD were plotted against the theoretical concentration. Calibration curves were obtained from least-squares linear regression analysis of the data. The linearity of the method was confirmed by regression statistics. The studied concentration ranges were 1–10 nmol/ml (1.5–15 μg/ml) for rabbit plasma, 10–100 nmol/ml for rabbit aqueous humour, 25–1500 nmol/ml for monkey plasma and 5–1500 nmol/ml for monkey urine, respectively.

2.6. Recovery, precision and accuracy

The extraction yield of HPGCD from plasma was determined at three different concentration levels. Plasma samples were spiked with a known amount of HPGCD and then extracted with the SPE procedure as described. For calculation of the recovery control calibration samples were prepared, and HPGCD stock solution was added to the samples obtained from SPE procedure of blank plasma. The chromatograms of spiked plasma samples were evaluated by the control calibration curve.

Repeatability and intermediate precision (by different persons on three different days) and accuracy of the method were calculated after analysis of six replicates of spiked samples (at three different concentration levels) evaluated with a calibration curve. Precision was calculated as the relative standard deviation (RSD), while accuracy was evaluated as the percentage difference between the expected and measured concentration.

2.7. Determination of the limit of detection and limit of quantification

The limit of detection (LOD) was defined as the HPGCD concentration resulting from a signal/noise ratio of 3. The limit of quantification (LOQ) was defined as the lowest HPGCD concentration, which can be determined with an acceptable accuracy and precision of $<$20%.

3. Results and discussion

3.1. Selection of fluorophore for detection of HPGCD

The detection of CDs by fluorescence enhancement requires a suitable fluorophore, which should provide a high enhancement factor, while producing a low background fluorescence. The observed fluorescence enhancement of fluorophore molecules in the presence of CDs is affected by numerous factors. The first factor that influences the magnitude of fluorescence enhancement observed in CD containing aqueous solution of the fluorophore is the type of CD. Emission enhancement depends upon the size and geometry of the fluorophore molecule in relation to the dimensions of the cavity of the specific CD employed. Therefore, the first step of the work was the selection of the most suitable fluorophore molecule for detection of HPGCD.

A screening test was carried out involving 29

fluorophore substances. The effect of HPGCD (5 μmol/dm^3) on the fluorescence intensity of fluorophores (20 μmol/dm^3) in aqueous solution was measured. The fluorescence enhancement was in acceptable range (5–10%) with five molecules, while almost 20% enhancement of emission was measured in ANS solution in the presence of HPGCD. 1-Naphthol, which was successfully used for detection of βCD derivatives [8–10] was found to be unsuitable for HPGCD possessing a larger CD ring, only 1% emission enhancement was obtained.

3.2. Development of HPLC method

First the detector parameters were optimised. By controlling all spectra on the chromatogram slight wavelength shifts (2–3 nm) of the excitation and emission maxima was observed in the elution range of the HPGCD (Fig. 1). The signal/noise ratio was better at the lower excitation wavelength, 270 nm, than that at 370 nm. A medium photo-multiplier gain value, 10 proved to be adequate.

Two types of gel filtration HPLC column were tested. The characteristic data of the HPGCD peak were much better using the polymer based Nucleogel aqua-OH column, but the baseline was unstable. The peak shape and signal-to-noise ratio were acceptable and the baseline was stable on the other, TSKgel G 3000 SW column, therefore, this type of column was selected for the quantitative determination of HPGCD.

The ANS concentration of mobile phase is a crucial factor of HPGCD detection. The characteristic of HPGCD peak and the signal/noise ratio (S/N) were the best at low ANS concentration (10 μmol/dm^3), however, the baseline was not stable. Twenty μmol/dm^3 ANS concentration in the mobile phase resulted in stable baseline, good peak shape and acceptable S/N ratio. Further increase of ANS concentration caused further decrease of S/N ratio as well as bad peak shape (Table 1).

Taking the principle of inclusion complex formation into consideration the solvent content of the mobile phase should be kept at minimum; therefore, only 2% methanol was applied. The use of a lower column temperature (30 °C) is also advantageous in the viewpoint of inclusion complex formation.

During the work, however, we had to modify the methanol content as well as the column temperature. Depending on the composition of biological matrices fast change of column performance was observed after 15–30 injections. The system and matrix peaks moved toward to the HPGCD peak resulting in poorer evaluation of the peak area, while the retention of HPGCD peak remained unchanged (Fig.

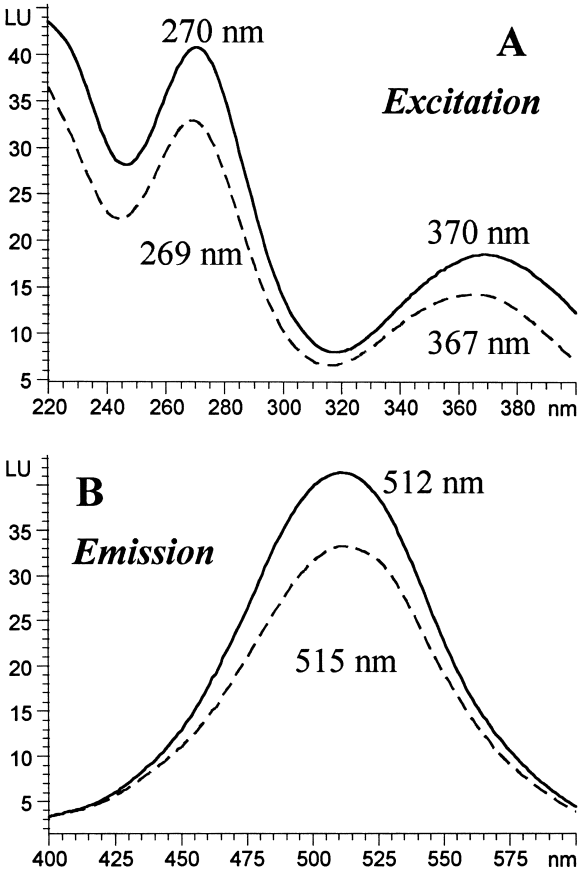

Fig. 1. Excitation (A) and emission (B) spectra of ANS in the baseline region (- - -) and at the chromatographic peak maximum of HPGCD (—).

Table 1
Characteristic data of HPGCD peak (10 nmol/ml) at different ANS concentration

ANS conc. (μmol)	Peak area	S/N ratio	n	Peak symmetry
10	122	156	3572	1.40
20	171	102	3070	1.45
50	313	53	1702	2.18

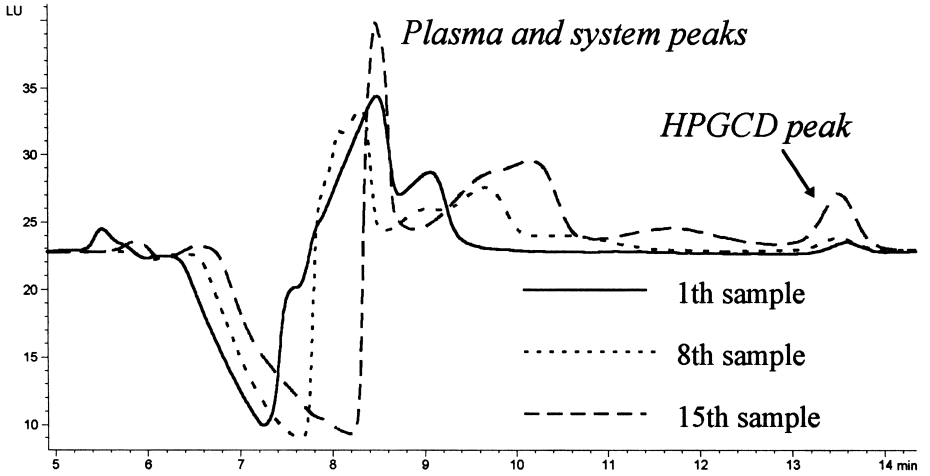

Fig. 2. HPLC chromatograms of some samples representing the change of column performance.

2). The possible reason of this phenomenon is the adsorption of matrix components of biological samples on the HPLC column. Increasing the methanol content of the mobile phase to 8% and the column temperature to 40 °C resulted in longer life time of the column.

More effective purification of the sample from the matrix components with SPE could not be carried out without drastic decrease of HPGCD recovery. Therefore, besides the increase of methanol content and column temperature development of a column-regeneration method was inevitable to eliminate the adsorbed interfering substances from the HPLC column. One of the developed column regenerating procedure was a simple washing of the column with methanol–water 90:10 mixture. This method was used every day at the end of the measurements. After measuring the last sample a gradient step was applied changing the mobile phase to the washing mixture (30 min). After washing the column with 100–120 ml of washing mixture the mobile phase was changed back (gradient step 30 min).

When the column could not be regenerated with the above-described washing procedure, another washing method was used with reversed flow direction. First the column was washed with 0.1% aqueous phosphoric acid solution (pH 2.7) containing 10% methanol for 30 min, with a flow-rate of 1 ml/min. The next step was washing the column with methanol–water (90:10) mixture similarly to the above described procedure. After finishing the washing period the flow direction was reversed back and the washing solution changed back to the mobile phase (gradient step 30 min). The effect of washing methods is demonstrated in Fig. 3.

3.3. Adaptation of the method to different biological matrices

The described solid-phase extraction and HPLC methods (Section 2) were developed using rabbit plasma (sample size 1 ml). The methods could be applied with minor modification for determination of HPGCD in rabbit aqueous humour (sample size 100 μl) and monkey plasma (sample size 0.5 ml). In case of monkey urine (sample size 1 ml), the first washing step in the SPE procedure had to be modified: 6 ml water was used instead of 2 ml 0.01 *M* hydrochloric acid.

3.4. Validation of the method

The validation process is illustrated by the relevant data for determination of HPGCD in rabbit plasma. The method was successfully validated for other biological matrices, too (rabbit aqueous humour, monkey plasma and urine) but not described here more detailed.

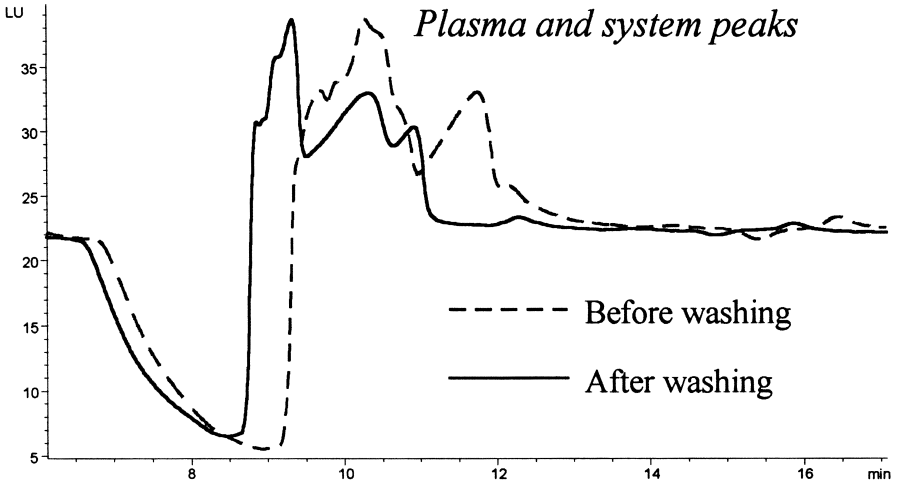

Fig. 3. Chromatograms representing the effect of washing method.

3.4.1. Specificity

The identical retention times of HPGCD in the control and in the biological samples prove the identity of HPGCD in the corresponding test samples. No peaks interfered at the retention times of HPGCD (Fig. 4A–D) in any of different biological matrices.

3.4.2. Linearity and range

The peak area of HPGCD varied linearly with concentration over the range used which was 1–10 nmol/ml (1.5–15 μg/ml) for rabbit plasma, 10–100 nmol/ml for rabbit aqueous humour, 25–1500 nmol/ml for monkey plasma and 5–1500 nmol/ml for monkey urine, respectively. The correlation coefficients were better than 0.997 for all four biological fluids. The confidence interval ($P=95\%$) of intercept contained zero and the scattering of residuals was accidental for each linearity curve.

3.4.3. Precision, accuracy and recovery

The relative standard deviation values assessed by analysing spiked samples were below 12% for each of the four different biological samples both for repeatability and intermediate precision. The accuracy was varied between 84 and 106%. The extraction yields of HPGCD were higher than 78% on the different concentration levels for all studied biological fluids. The obtained values of experiments with rabbit plasma are presented in Table 2.

3.4.4. Limit of quantification, limit of detection

The limit of quantification (LOQ) was 1 nmol/ml for rabbit plasma (precision $<12\%$, accuracy 84%). Ten nmol/ml HPGCD were measured with good precision (12%) and accuracy (91%) in rabbit aqueous humour, while LOQ were 10 and 5 nmol/ml for monkey plasma and urine, respectively.

The good detectability of HPGCD in rabbit plasma is shown in Fig. 5. The detection limits were 0.5 nmol/ml in rabbit plasma, 7 nmol/ml in rabbit aqueous humour, 5 and 3 nmol/ml HPGCD in monkey plasma and urine, respectively.

3.4.5. Robustness

The developed method is more sensitive for the changing of method parameters than the common methods, because the base of detection is the complex equilibrium between the HPGCD and the fluorophore additive, ANS. The most critical points are the presence of possible impurities in the mobile phase and the temperature in the SPE procedure. The accurate execution of the method is strictly advised in order to ensure good precision, accuracy and recovery mainly at low HPGCD concentration. To check the system suitability particular evaluation of a test sample is necessary before beginning of sample measurements. The shifting of the retention times of system and matrix peaks toward higher region (see Section 3.2) and decrease of peak area of HPGCD peak indicate the change of column performance.

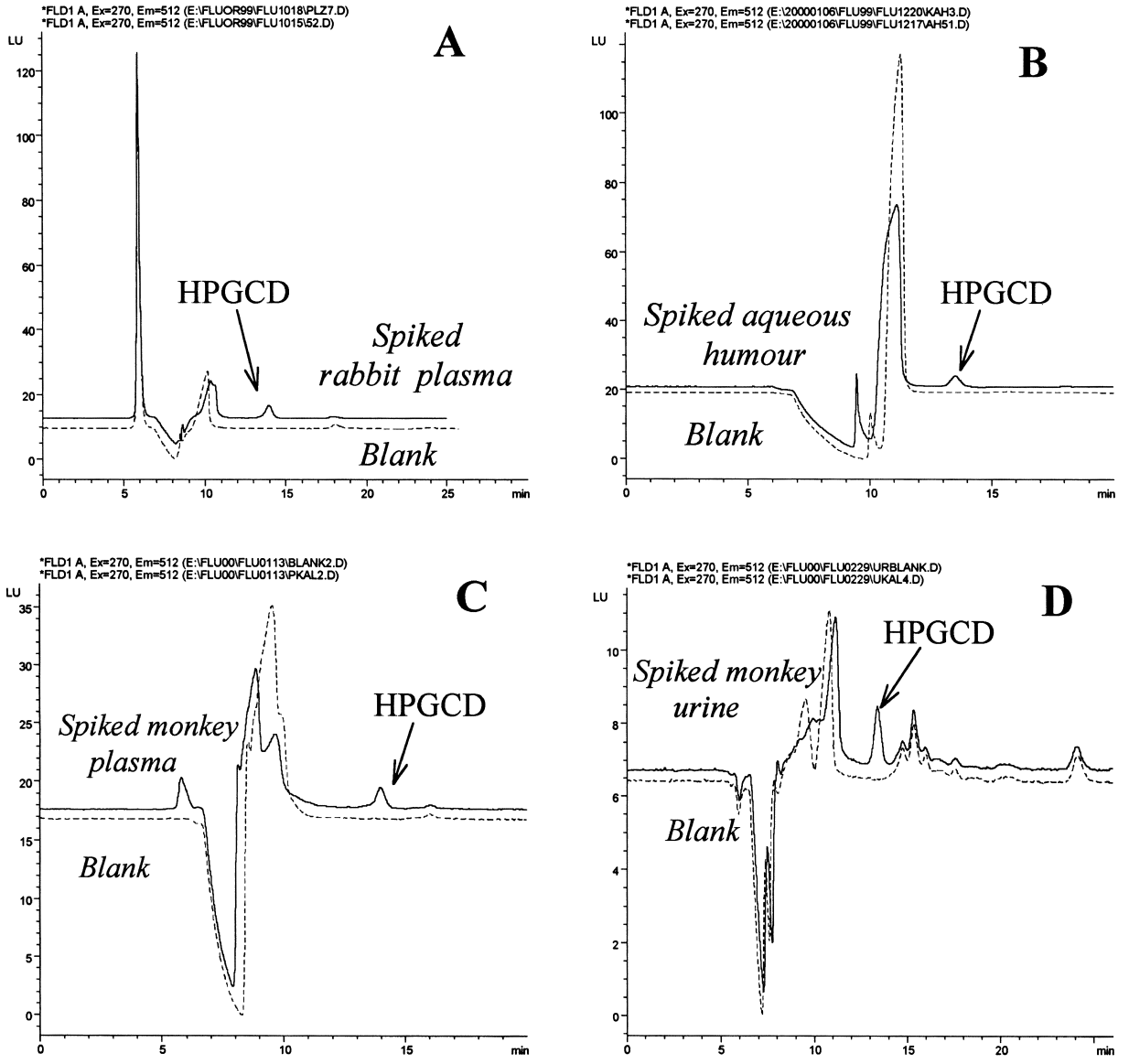

Fig. 4. Chromatograms of spiked (—) and blank (- - -) biological samples. (A) Rabbit plasma (10 nmol/ml), (B) rabbit aqueous humour (100 nmol/ml), (C) monkey plasma (50 nmol/ml), and (D) monkey urine (25 nmol/ml).

Table 2
Repeatability, accuracy and recovery of the HPGCD determination in rabbit plasma

Concentration level (nmol/ml)	Repeatability RSD (%)	Accuracy (%)	Recovery (%) (mean±SD)
1	11.73	84.0	93.2±11.9
4	7.47	96.5	87.2±7.8
10	2.63	94.4	96.2±3.5

Therefore, two types of system suitability (SST) samples were used. First a 10 nmol/ml HPGCD solution was measured to control the suitability of detection. The other SST sample was a spiked biological fluid, the retention time of the last eluted matrix peak should be less than 12 min.

In spite of the speciality of the developed analytical method it was transferred to another labora-

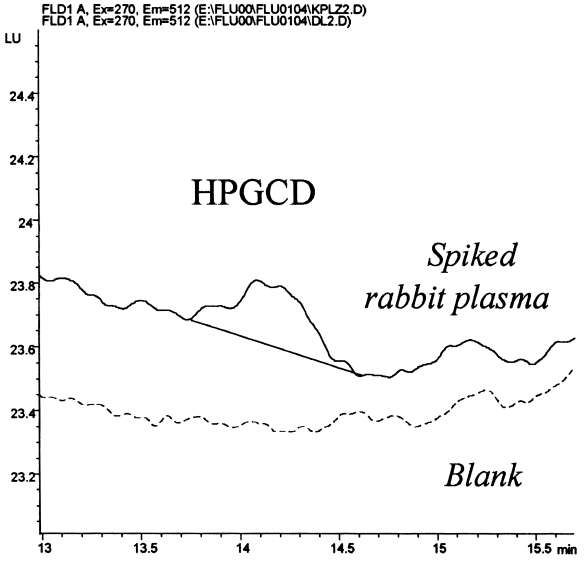

Fig. 5. Detectability of HPGCD in rabbit plasma (0.5 nmol/ml).

tory, where it was successfully used to prove that HPGCD got not absorbed into aqueous humour and plasma after topical application in rabbits.

4. Conclusion

HPGCD can be sensitively determined by the formation of a fluorescent inclusion complex with 8-anilinonaphthalene-1-sulfonic acid. The high sensitivity of this detection principle was demonstrated by the determination of HPGCD in different biological matrices. Good recovery values (higher than 78%) were obtained using solid-phase extraction procedure for sample concentration and cleanup prior to high-performance size exclusion chromatography. The developed column regenerating procedures increase the lifetime of the HPLC column enabling the analysis of a number of samples on the same HPLC column. The practical usefulness of the method was proved by its successful transfer to another laboratory.

Acknowledgements

This work—being a part of the development of a Diclofenac sodium/HPGCD eye drop formulation—has been performed in a co-operation between Novartis Ophtha (Basel, Switzerland, Duluth, GA, USA) and Cyclolab Ltd. (Budapest, Hungary). The authors are grateful to Ms. Zs. Zachar for her excellent technical assistance.

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