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Simultaneous chiral separation and determination of carvedilol and 5'-hydroxyphenyl carvedilol enantiomers from human urine by high performance liquid chromatography coupled with fluorescent detection

Research Article

Sylwia Magiera*, Weronika Adolf, Irena Baranowska

*Department of Analytical Chemistry,
Silesian University of Technology,
44-100 Gliwice, Poland*

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Abstract: A sensitive and specific high performance liquid chromatography coupled with fluorescent detection (HPLC-FL) and tandem mass spectrometry detection (HPLC-MS/MS) methods for separation and determination of carvedilol (CAR) enantiomers and 5'-hydroxyphenyl carvedilol (5'-HCAR) enantiomers has been developed and validated. The analysed compounds were extracted from human urine by solid phase extraction. Good enantioseparation of the studied enantiomers was achieved on CHIRALCEL® OD-RH column using 0.05% trifluoroacetic acid and 0.05% diethylamine in water and acetonitrile in a gradient elution. The mass spectrometric data were acquired using the multiple reaction monitoring mode by positive electrospray ionisation. The method was validated over the concentration range from 25.0 ng mL^{-1} to 200 ng mL^{-1} for the analysed compounds. The limit of quantification varied from 14.2 ng mL^{-1} to 24.2 ng mL^{-1}. Both the repeatability and inter-day precisions were below 10.0%, and the accuracy varied from -13.2% to 3.77%. The extraction recoveries ranged from 79.2% to 108%. The present paper reports the method for the simultaneous determination of CAR enantiomers and their metabolite enantiomers (5'-HCAR) in human urine samples. This newly developed method was successfully used to analyse the aforementioned analytes in human urine samples obtained from patients suffering from cardiovascular disease.

Keywords: *Chiral separation • HPLC • Method validation • Carvedilol enantiomers • 5'-Hyroxyphenyl carvedilol enantiomers*

1. Introduction

Carvedilol ((±)-1-(carbazol-4-yloxy)-3-[[2-(*O*-methoxyphenoxy)ethyl]amino]-2-propanol (CAR), which has been prescribed as both an antihypertensive and antianginal agent, is considered an effective agent for treating congestive heart failure (CHF). CAR is known as an antagonist of adrenergic a and b-receptors. CAR exhibits minimal inverse agonist activity and, consequently, produces reduced negative chronotropic and inotropic effects [1].

CAR is administered clinically as a racemic mixture of the *R*(+)- and *S*(−)-enantiomers. The CAR enantiomers exhibit different pharmacological effects, the blockade of the β adrenergic receptor being primarily attributed to the *S*(−)-CAR (50–500 times higher than *R*(+)-CAR), whereas the two enantiomers are considered to be equipotent with respect to the blockade of the a adrenergic receptor. Additionally, each enantiomer displays a unique pharmacokinetic behaviour, where the *R(+)*-enantiomer attains higher plasma concentrations, bioavailability and protein binding [2]. Therefore, it is very important to measure the biological fluid concentrations of each CAR enantiomer to clarify the pharmacokinetic and pharmacodynamic characteristics of CAR upon clinical application [3,4].

Only CAR enantiomers (without metabolite 5'-HCAR) have been determined in plasma, urine and other biological fluids using techniques such as high performance liquid chromatography (HPLC) coupled to

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* *E-mail: sylwia.magiera@polsl.pl*

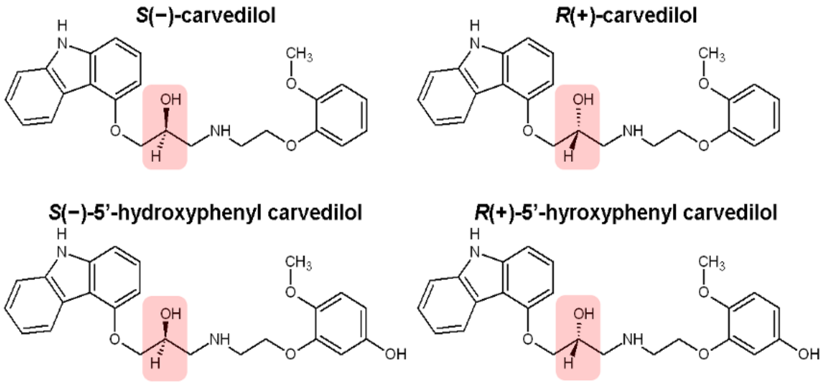

Figure 1. Chemical structure of CAR and 5'-HCAR stereoisomers.

fluorometric (FL) [5–12] and tandem mass spectrometry (MS/MS) [13] detection. Another method currently available for determining CAR focuses on the separation of the enantiomers using capillary electrophoresis (CE) coupled to UV-Vis detection [12]. Some papers have been published on CAR enantiomer separation using an enantioselective column [9] or indirectly after derivatising with an enantiomerically pure reagent [14–17]. Liquid–liquid extraction (LLE) [5,9–11], protein precipitation (PPE) [6] and solid phase extraction (SPE) [7] were used as the sample preparation techniques. However, these methods requiring time-consuming sample clean-up and the need to derivatise of CAR.

The methods that have been developed for the determination of CAR in biological fluids include HPLC [18–30], gas chromatography (GC) [31,32] and capillary electrophoresis (CE) [33]. Separation is typically performed by HPLC coupled with diode array (DAD) [18], electrochemical (ED) [19], FL [20–27], MS [28,29], or MS/MS [30] detection. The most widely used columns are packed with C18 sorbent and the chromatographic elution systems are typically binary with an acidified aqueous polar solvent, such as acetic acid, phosphoric acid, or formic acid and a less polar organic solvent, such as methanol or acetonitrile.

In this study, a method for simultaneous determination enantiomers of carvedilol and enantiomers of its metabolite – 5'-hydroxyphenyl carvedilol was developed. Some methods for determination of carvedilol enantiomers, without its metabolite enantiomers, are reported in the literature. This is a new method for simultaneous determination of mentioned above compounds.

Therefore, the aim of this study was to establish and validate a chiral high performance liquid chromatography coupled with fluorescence detection (HPLC-FL) method with high sensitivity and specificity for simultaneously determining CAR and 5'-HCAR enantiomers (Fig. 1) in human urine. This HPLC-FL method was then applied to urine samples collected from patients with cardiovascular disease who were treated using CAR. Furthermore, a HPLC-ESI-MS/MS method was used to confirm the presence of the selected enantiomers in the human urine.

2. Experimental procedure

2.1. Chemicals and reagents

Both *S*(−)- and *R*(+)-carvedilol enantiomers (*S*(−)-CAR and *R*(+)-CAR) (≥99% purity) and *S*(−)- and *R*(+)-5'-hyroxyphenyl carvedilol enantiomers (*S*(−)-5'-HCAR and *R*(+)-5'-HCAR) (≥99% purity) were purchased from Toronto Research Chemicals Inc. (North York, Canada). HPLC-grade acetonitrile (ACN), water, ammonium hydroxide (AH), diethylamine (DEA), trifluoroacetic acid (TFA), formic acid (FA) and acetic acid (AA) were purchased from Merck (Darmstadt, Germany). Analytical-grade methanol and acetone were purchased from POCH S.A. (Gliwice, Poland).

2.2. Preparation of stock solutions, calibration standards and quality control (QC) solutions

The primary stock solutions (1.00 mg mL^{-1}) of the CAR enantiomers and 5'-HCAR enantiomers were prepared in methanol. The series of working solutions were prepared fresh from these stock solutions by serial dilutions with methanol.

Calibration standards with concentrations of 25.0, 40.0, 50.0, 75.0, 100 and 200 ng mL^{-1} were prepared for each enantiomer by the serial dilution of the working standard solutions with drug-free human urine (after SPE procedure). The urine used for this spiking was examined to ensure that it was free of drug interferences before preparing the standard calibration curve.

The quality control (QC) samples were prepared by spiking an appropriate amount of the working standard solutions into drug-free human urine (after SPE procedure) to achieve three concentration levels: the low concentration of quality control (LQC), 25.0 ng mL^{-1}; medium concentration quality control (MQC), 40.0 ng mL^{-1}; and high concentration quality control (HQC), 100 ng mL^{-1}. All solutions were stored at 4°C and brought to room temperature before use.

2.3. Instrumentation and analytical conditions

The reversed-phase HPLC system consisted of a Model L-6200A Intelligent Pump (Merck Hitachi) with a dynamic mixing chamber, Model L-4500A (Merck Hitachi), diode array(DAD) (Model L-7480), fluorescence detector (FL) (Model L-7360) and thermostat (Lachrom® Merck). The analytes were separated on a CHIRALCEL® OD-RH (150×4.6 mm; 5 μm) column (Daicel/Chiral Technologies, Illkirch, France) equipped with a CHIRALCEL® OD-RH analytical guard column. The samples were injected using a Rheodyne valve with a 20 μL loop. Data acquisition was performed using a HPLC System Manager HSM D-7000, Version 2.1 (Merck-Hitachi).

A gradient mobile phase system consisting of (A) 0.05% trifluoroacetic acid (TFA) and 0.05% diethylamine (DEA) in water and (B) acetonitrile was employed for the sample analysis. The gradient elution method was as follows: 0–5 min at 65% B (1.0 mL min^{-1}), 5–20 min at 60% B (0.3 mL min^{-1}) and 20–60 min at 65% B (1.0 mL min^{-1}). Fluorescence detection was conducted using an excitation wavelength of 254 nm and an emission wavelength of 356 nm. The column was maintained at 25°C.

Additionally, an HPLC-MS/MS system was used to confirm the presence of the selected enantiomers in the samples. These HPLC-MS/MS analyses of the urine samples were performed using a Dionex HPLC (Dionex Corporation, Sunnyvale, CA, USA) system consisting of an UltiMate 3000 RS (Rapid Separation) pump, an UltiMate 3000 autosampler, an UltiMate 3000 column compartment with a thermostable column and an UltiMate 3000 variable wavelength detector operated using Dionex Chromeleon™ 6.8 software. The chromatographic separation was performed using the column and gradient elution program described above. DEA and TFA additives negatively affected the analyte response under positive ion mode electrospray (ESI$^+$) detection even at very low concentrations. Therefore, formic acid (FA) and ammonium hydroxide (AH) in water were used as a component of the mobile phase if an MS/MS detector was employed during sample analysis.

The HPLC system was coupled to an API 4000 Q TRAP tandem mass spectrometer equipped with an electrospray ionisation (ESI) source (Applied Biosystems/MDS SCIEX, Foster City, CA, USA). The mass spectrometer operated in the positive ion mode. The crucial detector parameters, such as ionisation voltage (IS), collision assisted dissociation gas (CAD), source temperature (TEM), nebuliser gas (GS1), heater gas (GS2), and curtain gas (CUR), were optimised *via* flow injection analysis (FIA) to obtain better ionisation. The compound-dependent parameters were tuned and optimised for the analytes and IS *via* the direct infusion of a 1.0 μg mL^{-1} standard solution into the ion source using a Harvard syringe pump with a flow rate of 10 μL min^{-1}. Continuous mass spectra were obtained by scanning from *m/z* 50 to 800. The dwell time was maintained at 500 ms for each analyte. The data were acquired using Analyst software, Version 1.4.

2.4. Sample preparation

Urine samples were obtained from patients being treated with CAR (3.5 mg). Drug-free urine samples were collected before the patients took the drugs. Control drug free urine samples were obtained from ten subjects and used as matrix to prepare calibration standards and quality control samples. Urine samples were stored in a freezer at –20°C. The collection and use of human urine samples from patients was approved by the Institutional Ethics Committee.

Human urine (3 mL) (CS, QC and patient urine samples) was transferred to a 10 mL Eppendorf cup, and protein precipitation was performed using 1.5 mL acetonitrile and 1.5 mL methanol. The samples were centrifuged using a Universal Centrifuge Z 323 K at 6500 rpm for 15 minutes at room temperature (ca. 22°C). Extraction was performed using a BAKERBOND spe-12G system (J.T. Baker Inc., Deventer, Netherlands). The sample was loaded onto an extraction Oasis HLB cartridge (6 mL, 500 mg, Waters) that had been pre-conditioned with 6 mL of methanol followed by 6 mL 0.1% formic acid in water. The loaded cartridge was left for at least 2 min at room temperature. Subsequently, the elution was carried out with 5 mL of methanol:acetone:formic acid (4.5:4.5:1; v/v/v) mixture, and the eluate was evaporated to dryness under a nitrogen stream [34]. Finally, the residue was dissolved in 0.5 mL of methanol and filtered through a 0.20 μm membrane before injecting a 20 μL / 5 μL aliquot into the HPLC-FL / HPLC-MS/MS system for analysis.

2.5. Method validation

A chromatographic comparison of the drug-free human urine extracts from six different sources, drug-free urine extract spiked with the analytes and urine sample extracts from patients treated with CAR was conducted to evaluate the selectivity of the method. Each individual sample was prepared according to the sample preparation procedure described above. The response of co-eluting interferences upon comparison of the chromatograms for the drug-free urine sample extract and corresponding spiked urine samples extract should be less than 20% of the response for the analytes.

The calibration curves (analyte peak on the Y-axis and analyte concentration on the X-axis) were obtained based on the least square linear regression fit (y=ax+b). The correlation coefficient (r^2) was used to estimate linearity. The data obtained were subjected to a regression analysis, and correlation coefficients were calculated for each compound using Excel. The accuracy of the method was required to be within ±15% of the nominal concentration, except at the LOQ, which was required to be within ±20%.

The limit of quantification ($LOQ=10\times SD_{xy}/b$, where SD_{xy} is the standard deviation and b is the slope) were calculated according to precision and accuracy standards as described by Konieczka [35]. In detail, drug-free samples (after extraction) were spiked with decreasing amounts of the target compounds. Based on these measurements, calibration curves for each analyte were established, which were then utilized to calculate the LODs and LOQs.

The repeatability and inter-day precisions were determined by assessing the measured results for the QC samples at the LQC, MQC and HQC levels. The precision was estimated as the relative standard deviation (RSD) for each QC sample, which was calculated by dividing the standard deviation (SD) of the measured concentrations by the mean concentration: $RSD(\%)= (SD/C_{mean,mes})\times100$. The accuracy was defined as the difference between the measured (C_{mes}) and nominal (C_{nom}) concentrations of the low QC, medium QC samples and high QC samples and was expressed as the relative error: $RE(\%)=[(C_{mes}-C_{nom})/C_{nom}]\times100$. To evaluate the repeatability and accuracy, six aliquots of each sample were analysed on the same day. For inter-day precision and accuracy, six aliquots of each sample were analysed on three consecutive days (samples were prepared independently every day). The repeatability and inter-day precisions were below 15%, and the accuracies were within ±15%.

The extraction recoveries (ER) for *S*(−)-CAR, *R*(+)-CAR, *S*(−)-5’-HCAR and *R*(+)-5’-HCAR were calculated by comparing the experimental results of two sets of solutions at three concentrations. In the first set, drug-free urine samples were spiked with all analytes at LQC, MQC and HQC concentration levels before the extraction step (set A), while in the second set the standard solution spikings (at the same concentrations) were made on the drug-free urine extracts (set B). The ratio (A/B×100) is defined as the ER. The reproducibility of the extraction procedure was determined using the RSD (%).

3. Results and discussion

3.1. Method development

Because CAR and 5’-HCAR enantiomers share the same ion transition channel, the baseline resolution of the two isomers on the column was necessary. The liquid chromatographic conditions that could influence the separation, including the stationary phase, both the composition and pH value of the mobile phase, the column temperature and the flow rate, were investigated. The peak shape, response intensity and retention time were the main aspects studied during the HPLC optimisation. No chiral separations for *S*(−)-CAR, *R*(+)-CAR, *S*(−)-5’-HCAR and *R*(+)-5’-HCAR were obtained using the following chiral columns: Lux Amylose-2 (amylose tris(5-chloro-2-methylphenylcarbamate)) and Lux Cellulose-2 (cellulose tris(4-methylbenzoate)). The CHIRALCEL® OD-RH column with cellulose tris-(3,5 dimethylphenyl-carbamate) as the packing material showed satisfactory chiral selectivity; therefore, this column was used for the following experiments.

The chromatographic conditions, especially the mobile composition, were optimised through several trials to achieve good resolution and a symmetrical peak shape. Methanol and acetonitrile were used to investigate the influence of the organic modifier on the enantioseparation and retention. A better separation was achieved with acetonitrile than with methanol. The elution order of the enantiomers remained constant. The nature of the additives can significantly influence the peak shape. To optimise the peak shape, the effects of adding trifluoroacetic acid (TFA), formic acid (FA), either acetic acid (AA) or ammonium hydroxide (AH), diethylamine (DEA) and triethylamine (TEA) to the mobile phase were examined. The results indicated that no enantioseparation was obtained in the presence of aqueous AA. When aqueous FA and TFA were used, enantioseparation was observed for both *R*(+)-5’-HCAR and *S*(−)-5’-HCAR and *S*(−)-CAR and *R*(+)-CAR. TEA, one of the most commonly used amine additives, had very little effect on the retention and selectivity compared to DEA. Using TEA created experimental problems relating to the stability of the baseline and negative peaks in the chromatogram. However, the addition of AH to the mobile phase did not reduce the selectivity and efficiency. An acceptable enantioseparation for *S*(−)-

CAR, *R*(+)-CAR, *S*(−)-5'-HCAR and *R*(+)-5'-HCAR was obtained using acetonitrile/TFA (or FA) and DEA (or AH) in a water solvent system. After optimising the mobile phase components, a gradient solvent system was finally chosen as the mobile phase. Higher concentrations of the additives can also positively influence the enantioselectivity and efficiency. The influence of TFA (and FA) and DEA (and AH) concentrations ranging from 0.02% to 0.2% was investigated. The effect of the TFA (and FA) and DEA (and AH) content on the enantioseparation was inconspicuous. Both the retention and resolution decreased with increasing DEA content. The optimal concentration for both TFA/FA and DEA/AH was found to be 0.05%.

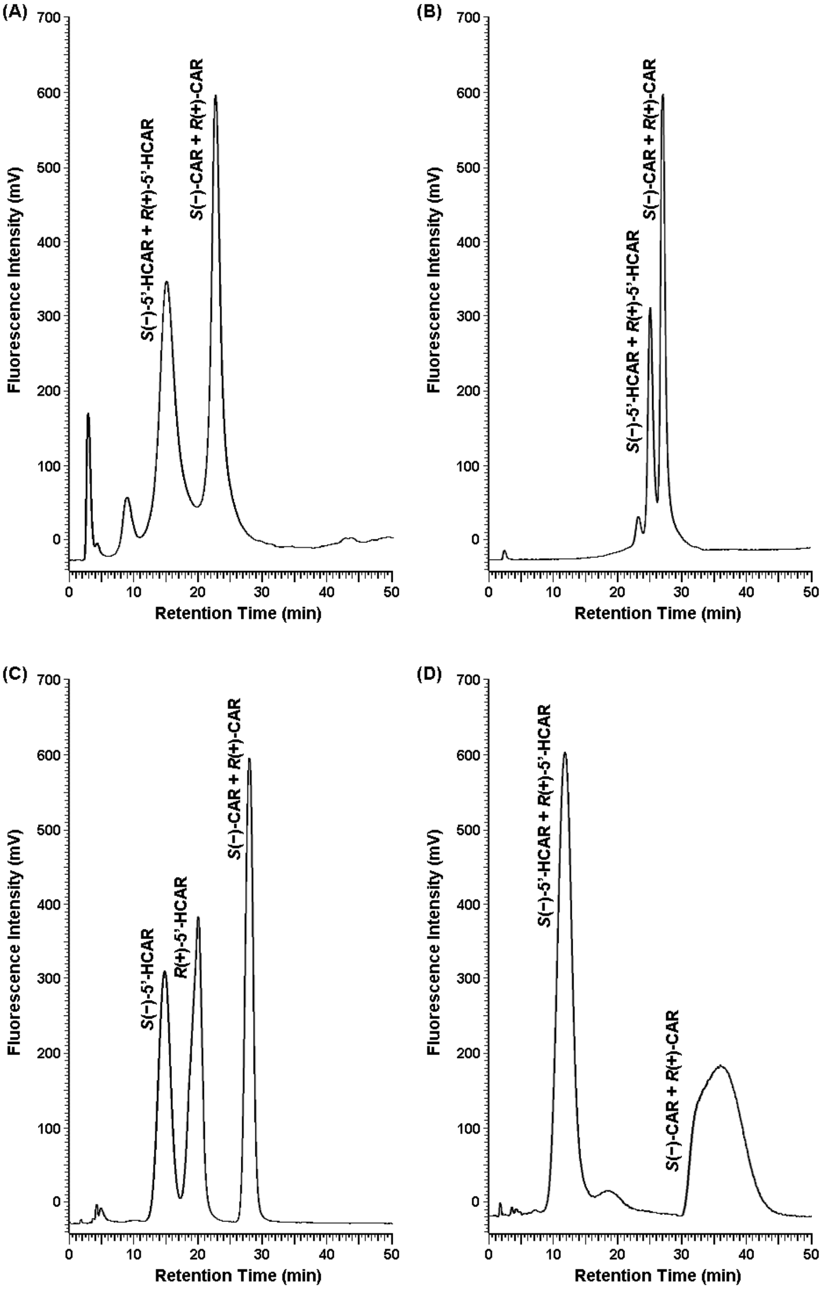

Figure 2. HPLC-FL chromatograms obtained from a mixed standard solution containing both CAR and 5'-HCAR enantiomers at concentration of 100 ng mL^{-1} obtained using different chromatographic conditions: (A) Lux Amylose-2 column, acetonitrile/0.05% TFA and 0.05% DEA in water as a mobile phase; (B) Lux Cellulose-2 column, acetonitrile/0.05% TFA and 0.05% DEA in water as a mobile phase; (C) CHIRALCEL® OD-RH column, acetonitrile/0.05% AA and 0.05% DEA in water as a mobile phase; (D) CHIRALCEL® OD-RH column, methanol/0.05% TFA and 0.05% DEA in water as a mobile phase.

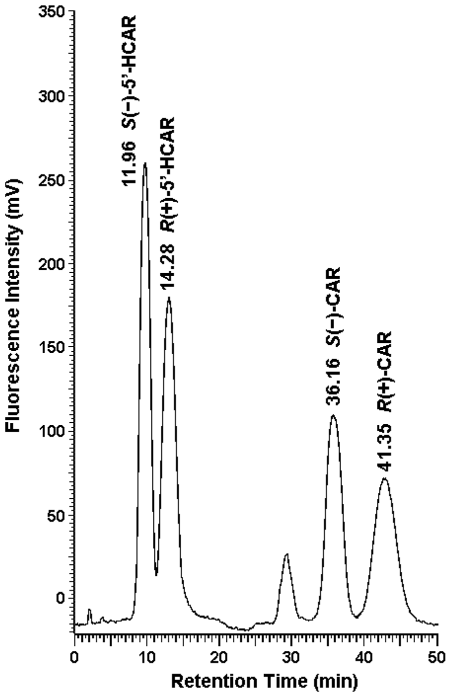

Figure 3. HPLC-FL chromatograms obtained from a standard solution containing both CAR enantiomers and 5'-HCAR enantiomers at concentration of 100 ng mL^{-1} (CHIRALCEL® OD-RH column, acetonitrile/0.05% TFA and 0.05% DEA in water as a mobile phase).

Acetonitrile/TFA and DEA in water were used as the mobile phase, and detection was performed using an FL detector and acetonitrile/FA and AH in water with a tandem ESI-MS/MS detector. It was also found that the best separation was obtained using a gradient elution with a constant column temperature of 25°C and a flow rate ranging from 0.3 mL min^{-1} to 1.0 mL min^{-1}. According to the fluorescence spectra, the analytes were monitored at an excitation wavelength of 254 nm and an emission wavelength of 356 nm. The analytical run time under these optimised chromatographic conditions was 50.0 min (Fig. 3) with retention times for *S*(−)-5'-HCAR, *R*(+)-5'-HCAR, *S*(−)-CAR and *R*(+)-CAR of 11.96 min, 14.28 min, 36.16 min and 41.35 min, respectively, and a relative standard deviation below 2.34%.

During the development of the MS/MS detection, two main ionisation sources, APCI and ESI, were compared, and ESI was chosen for its superior ionisation efficiency. To optimise the ESI conditions for CAR and 5'-HCAR enantiomers, quadrupole full scans were performed in the positive ion detection mode. The analysis was performed in the multiple reaction monitoring (MRM) mode using both precursor ions and their corresponding product ions. Following detailed optimisation of the mass spectrometry conditions (provided in the instrumentation and chromatographic conditions section), the transition from an m/z 407.1 precursor ion to m/z 99.9 was used to quantify the CAR enantiomers. Similarly, the transition from an m/z 423.0 precursor ion to m/z 99.9 was used to quantify the 5'-HCAR enantiomers. Fig. 4 shows both the product ion mass spectra of the analytes.

The source-dependent parameters for all analysed compounds were optimised by observing the maximum product ion responses. The MS/MS operating conditions were systematically evaluated using the standard solutions to optimise the analyte ionisation: CUR (10–50 psi, selected 10 psi), GS1 (40–90 psi, selected 90 psi), heater gas (GS2) (30–80 psi, selected 80 psi), heated TEM (300–750°C, selected 500°C), IS (1500–4500 V, selected 4000 V) and CAD (low, medium, high, selected medium). Furthermore, the additional compound-dependent parameters, such as the declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP), were also optimised. The optimum conditions were as follows: DP: 76 V for CAR and 86 V for 5'-HCAR; EP: 5 V for CAR and 5 V for 5'-HCAR; CE: 43 V for CAR and 43 V for 5'-HCAR; CXP: 6 V for CAR and 6 V for 5'-HCAR.

3.2. Method validation

Fig. 5 shows typical chromatograms for (A) a drug-free urine sample extract and (B) a drug-free urine sample extract spiked with *S*(−)-CAR, *R*(+)-CAR, *S*(−)-5'-HCAR, *R*(+)-5'-HCAR (at a concentration of 40.0 ng mL^{-1}). No significant interference from the endogenous urine components was observed at the retention times corresponding to the analysed compounds, which demonstrates that the method is selective for the tested compounds and is free of interference from the urine components.

The calibration curves for the CAR enantiomers and 5'-HCAR enantiomers were constructed using five calibration standards (25.0–200 ng mL^{-1}). These calibration curves had a reliable reproducibility for the standard concentrations across the calibration range. All of the mean correlation coefficients (r^2) were above 0.9984 (Table 1). The RSD at each level for *S*(−)-CAR, *R*(+)-CAR, *S*(−)-5'-HCAR and *R*(+)-5'-HCAR was less than 6.5%. Thus, the calibration curves exhibited good linearity within the chosen range (Fig. 6).

The limit of quantification (LOQ) was between 14.2 ng mL^{-1} and 24.2 ng mL^{-1} for the analysed compounds (Table 1).

The results of the repeatability and inter-day precision and accuracy studies are presented in Table 2. The REs were between -7.49% and 3.77% (intra-day) and between -13.2% and -7.70% (inter-day); the RSDs ranged from 2.04% to 5.80% (repeatability)

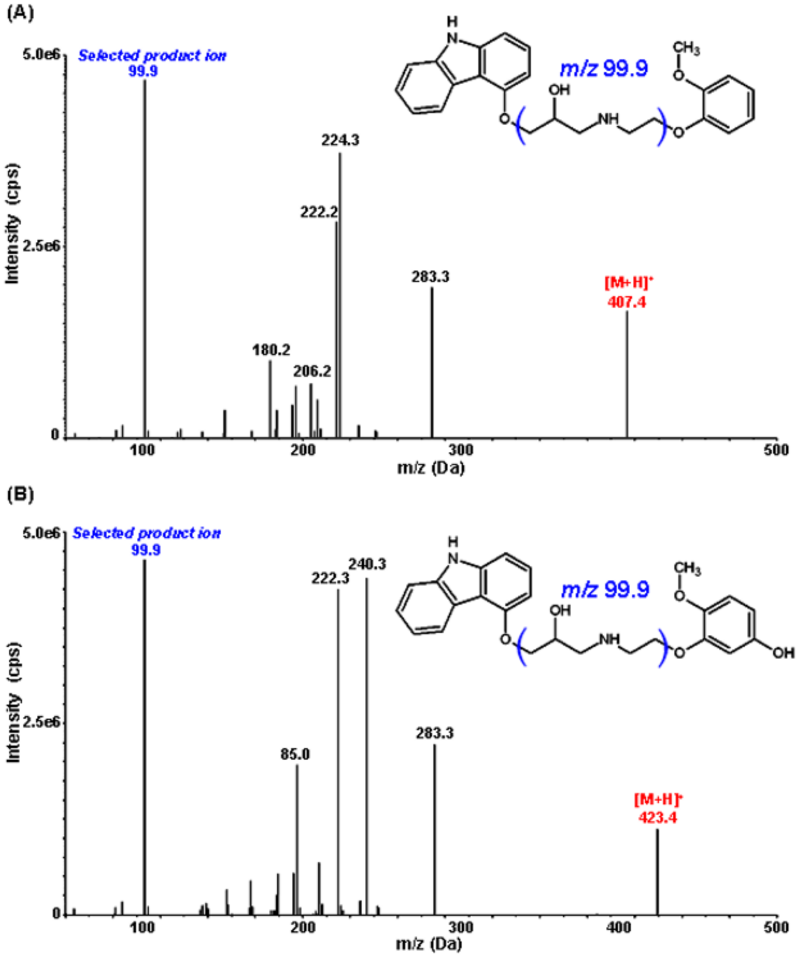

Figure 4. Mass spectra product ions for (A) CAR (*m/z* 407.1→99.9) and (B) 5'-HCAR (*m/z* 423.0→99.9) in positive electrospray ionisation mode.

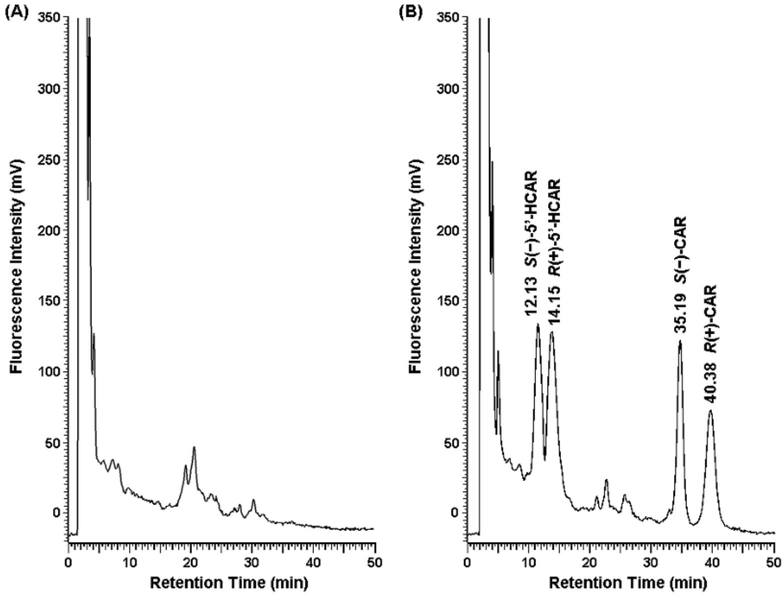

Figure 5. Representative chromatograms of the (A) drug-free urine sample extract and (B) drug-free urine sample extract spiked with the analytes.

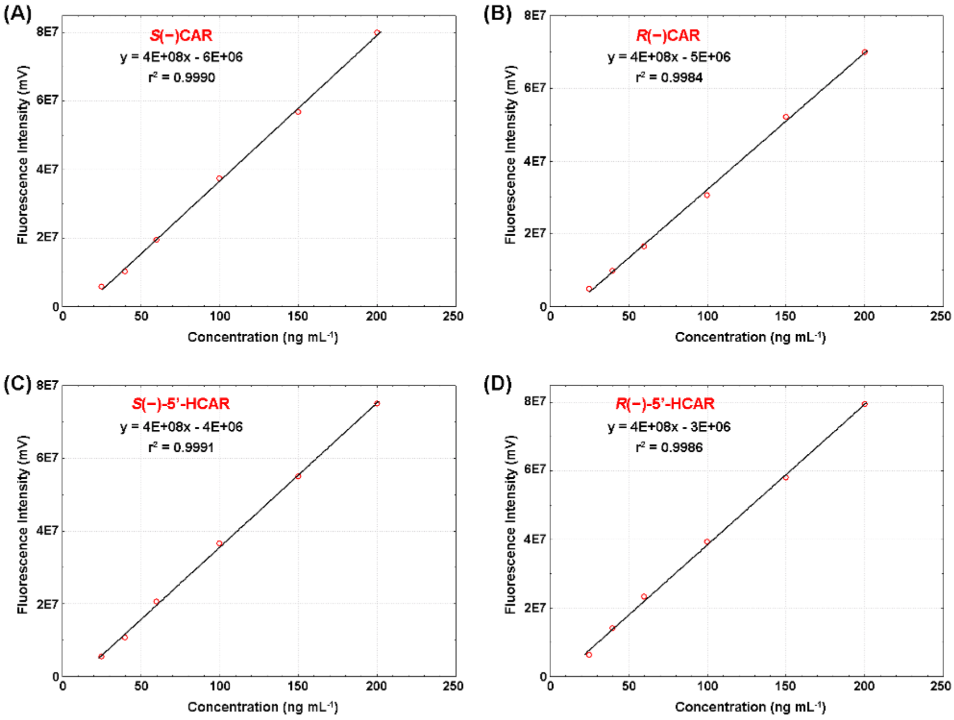

Figure 6. Calibration curves for enantiomers of CAR (A and B) and enantiomers of 5-HCAR (C and D).

Table 1. Analytical parameters for the calibration curves of the CAR enantiomers and 5'-HCAR enantiomers (n=6).

Analyte	Linear range (ng mL^{-1})	Correlation coefficient r^2	LOD (ng mL^{-1})	LLOQ (ng mL^{-1})	LLOQ[a] (pmol/20 μL)
S(−)-CAR	25.0 – 200	0.9990	4.73	14.2	0.701
R(+)-CAR	25.0 – 200	0.9984	5.13	15.4	0.762
S(−)-5'-HCAR	25.0 – 200	0.9991	7.07	21.2	1.00
R(+)-5'-HCAR	25.0 – 200	0.9986	8.07	24.2	1.14

a. LLOQ re-calculated to account for the sample injection (20 μL volume)

and from 2.55% to 10.0% (inter-day). All the values of precision and accuracy including LOQ were within the specified ranges and therefore acceptable. The acceptable range of accuracy and precision are be low 15% bias or RSD. Based on the accuracy and precision of the presented data, it was concluded that the proposed method was sufficiently precise and accurate for determining CAR enantiomers and 5'-HCAR enantiomers in human urine.

Extraction recovery (ER) studies were performed for each analyte at the LQC, MQC and HQC concentrations by comparing the peak areas of the extracted urine samples to the spiked samples. The mean recoveries of all analytes extracted from human urine at these three QC concentrations ranged from 79.2% to 108%. The ER for the CAR enantiomers and 5'-HCAR enantiomers are shown in Table 3.

3.3. Method application

The proposed chiral HPLC-FL method was applied to patient urine samples for the study of *S*(−)-CAR, *R*(+)-CAR, *S*(−)-5'-HCAR and *R*(+)-5'-HCAR. The drug quantification was performed using the calibration curves and standard addition methods. Additionally, an HPLC-MS/MS method was used to confirm the presence of the selected enantiomers in the urine samples.

SPE was chosen for the extraction and purification of the analytes due to its high selectivity, simple efficiency, extraction speed, automation potential and the reduced volumes of organic solvents required relative to liquid-liquid extraction. The tested SPE sorbent proved that appropriate absolute recoveries can be obtained using Oasis HLB (Waters) after optimising the conditions, such as the solvent volume using for elution and the amount of sorbent.

Table 2. Repeatability and inter-day precision and accuracy for the CAR enantiomers and 5'-HCAR enantiomers in human urine samples (n=6).

Analyte	Added (ng mL^{-1})	Repeatability			Inter-day		
		Found (ng mL^{-1})	RSD [a] (%)	RE [b] (%)	Found (ng mL^{-1})	RSD [a] (%)	RE [b] (%)
S(−)-CAR	100	102	3.62	2.26	92.3	2.55	-7.70
	40.0	38.7	2.59	-3.21	36.6	5.88	-8.39
	25.0	23.4	5.54	-6.50	23.1	8.88	-7.72
R(+)-CAR	100	103	2.04	3.45	91.6	7.74	-8.38
	40.0	37.7	4.90	-5.60	36.3	7.96	-9.27
	25.0	23.1	4.37	-7.46	22.0	7.72	-11.9
S(−)-5'-HCAR	100	96.5	4.08	-3.49	90.1	5.65	-9.91
	40.0	37.0	3.51	-7.49	35.5	6.41	-11.3
	25.0	23.1	5.80	-7.43	21.7	10.0	-13.2
R(+)-5'-HCAR	100	96.1	3.17	-3.93	89.7	4.45	-10.2
	40.0	41.5	3.95	3.77	35.3	4.72	-11.8
	25.0	23.3	4.70	-6.66	21.9	7.73	-12.3

a. *Relative standard deviation*
b. *Relative error*

Table 3. Extraction recovery of CAR enantiomers and 5'-HCAR enantiomers from human urine (n=6).

Analyte	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD [a] (%)	ER [b] (%)
S(−)-CAR	100	93.9	4.52	93.9
	40.0	38.2	5.95	95.6
	25.0	24.2	3.20	97.0
R(+)-CAR	100	94.2	3.12	94.2
	40.0	33.7	2.63	84.3
	25.0	24.0	8.16	96.2
S(−)-5'-HCAR	100	95.3	3.45	95.3
	40.0	38.7	2.87	96.8
	25.0	26.9	3.49	108
R(+)-5'-HCAR	100	88.4	4.54	88.4
	40.0	33.7	6.12	84.2
	25.0	20.9	1.08	83.7

a. *Relative standard deviation*
b. *Extraction recovery*

For the analysis of CAR enantiomers and 5'-HCAR enantiomers in urine by HPLC-FL, these compounds exhibited well-separated peaks using a CHIRALCEL® OD-RH column. As an example of the application of this method, the concentrations of the drug and metabolite enantiomers in urine samples taken from patients treated with CAR were analysed. Table 4 shows the levels of *S*(−)-CAR, *R*(+)-CAR, *S*(−)-5'-HCAR and *R*(+)-5'-HCAR found in these urine samples. The representative FL chromatogram and MRM chromatograms of the extracts obtained from urine sample (Sample 1) (after SPE procedure) after the oral administration of CAR to patients are shown in Figs. 7 and 8, respectively.

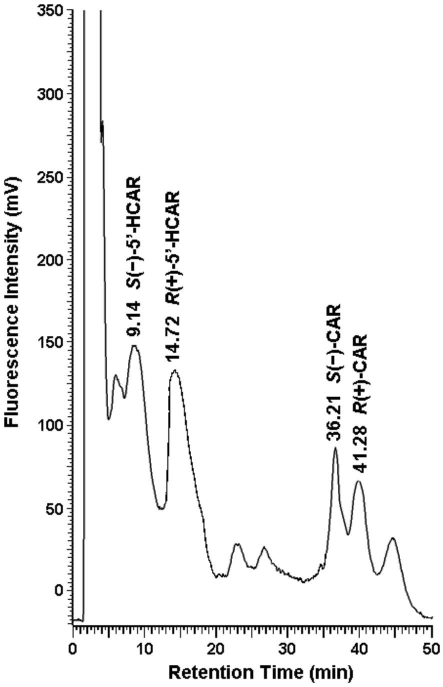

Figure 7. FL chromatogram corresponding to the extracts (after SPE procedure) of urine sample from a patient after the oral administration of CAR.

Table 4. Analyte concentrations in urine samples obtained from patients (n=6).

Sample	S(−)-CAR			R(+)-CAR			S(−)-5’-HCAR			R(+)-5’-HCAR		
	Concent.[a] (ng mL^{-1})	Concent.[b] (ng mL^{-1})	SD[c] (ng mL^{-1})	Concent.[a] (ng mL^{-1})	Concent.[b] (ng mL^{-1})	SD[c] (ng mL^{-1})	Concent.[a] (ng mL^{-1})	Concent.[b] (ng mL^{-1})	SD[c] (ng mL^{-1})	Concent.[a] (ng mL^{-1})	Concent.[b] (ng mL^{-1})	SD[c] (ng mL^{-1})
Sample 1	50.4	8.40	0.181	27.3	4.55	0.322	29.7	4.95	0.0201	43.4	7.24	0.0202
Sample 2	49.9	8.31	0.140	29.9	4.32	0.223	25.4	4.24	0.512	nd [e]	nd [e]	–
Sample 3	104	17.3	0.542	89.8	15.0	0.760	nq [d]	nq [d]	–	167	27.8	2.66
Sample 4	108	18.0	0.161	65.3	10.9	0.461	48.0	8.00	0.0105	nq [d]	nq [d]	–

a Concentration of analytes in 0.5 mL of eluate, after SPE procedure, calculated from calibration curve (after preconcentration of urine samples 6 times).
b Concentration of analytes in 1 mL of urine samples.
c. Standard deviation
d. Not quantified
e. Not detected
f. Highlighting row: it relates to the chromatogram, which is placed in the manuscript as a Fig. 7

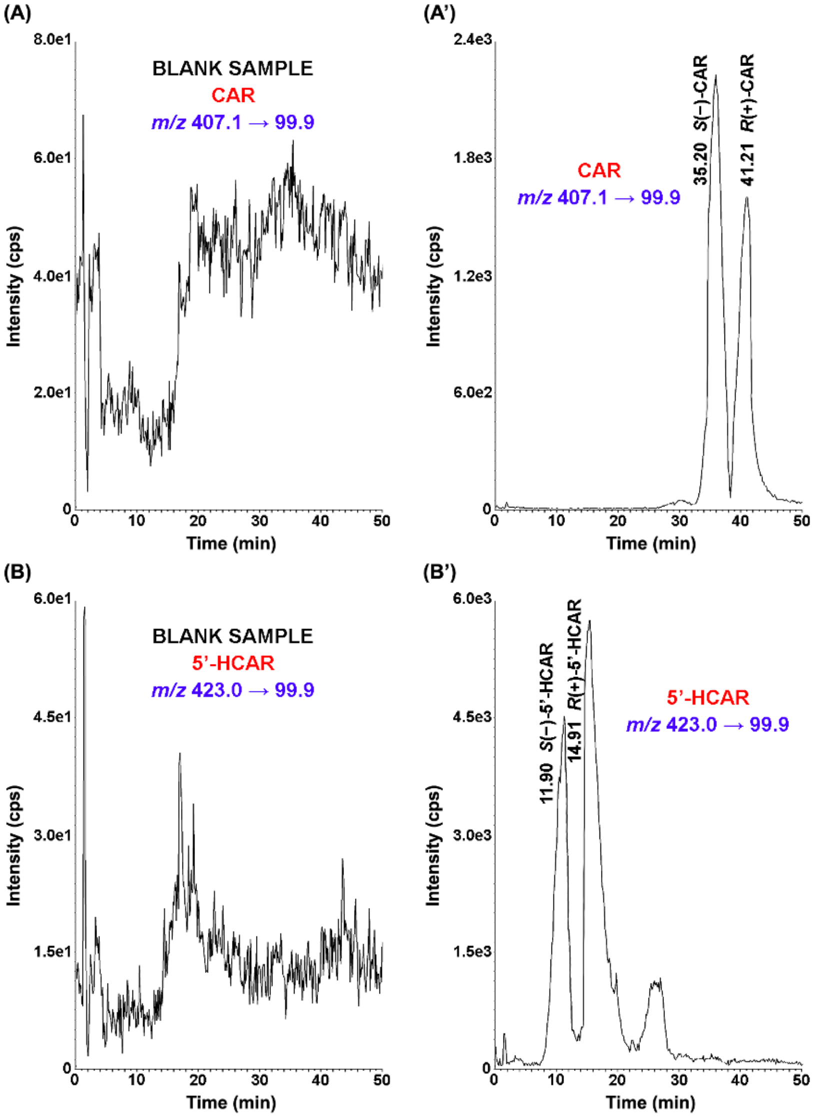

Figure 8. MRM chromatograms corresponding to the extracts (after SPE procedure) of (A and B) drug-free urine sample and (A’ and B’) urine sample analysed after CAR intake.

4. Conclusions

In conclusion, a new HPLC assay using fluorescence detection to simultaneously determination and quantification of CAR enantiomers and 5'-HCAR enantiomers from human urine was developed and validated. The established HPLC-FL method demonstrated good performance in terms of its linearity, quantification limits, precision and accuracy. An efficient SPE method was used for the extraction and the clean-up processes.

The high sensitivity makes this method a valuable tool for both clinical and basic research on the metabolism of CAR. Moreover, a high recovery indicates that the method can be used successfully. To the best of our knowledge, the HPLC method described herein is the new procedure that allows for the simultaneous determination of *S*(−)-CAR, *R*(+)-CAR, *S*(−)-5'-HCAR and *R*(+)-5'-HCAR.

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