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Determination of the chiral isomers of CGS 26214, a synthetic thyromimetic agent, in human plasma using microbore chiral chromatography–tandem mass spectrometry

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Abstract

CGS 26214 is a racemic compound having cholesterol-lowering activity in rats, dogs, and monkeys. This compound has two equipotent chiral components CGS 28934(−) and CGS 28935(+). An analytical challenge was to develop a sensitive liquid chromatography/tandem mass spectrometry (LC/MS/MS) method for the analysis of the chiral components in human plasma following clinical doses of 1 mg or less. Several issues had to be addressed in order to devise a LC/MS/MS assay for the above compounds. First, the compounds were esters and susceptible to hydrolysis under experimental conditions. Second, a lower limit of quantitation (LLOQ) of 0.4 ng/ml was needed. Third, positive electrospray ionization of CGS 26214 did not yield sufficient sensitivity needed for the studies in humans. Consequently, LC/MS/MS conditions were optimized for negative ion mode of detection. Fourth, sample preparation steps proved to be critical in order to reduce the possibility of microbore chiral-HPLC column (100 × 1.0 mm i.d.) obstruction, chromatographic deterioration, and matrix mediated electrospray ion suppression. Although the present method addressed the above challenges, its major drawback was limited sample throughput capability. Nonetheless, the method was successfully applied to generate plasma concentration–time profiles for human subjects after oral doses (0.9 mg) of the racemate as well as the optically pure isomers.

Keywords: CGS 26214; Cholesterol-lowering agent; Chiral; Electrospray; Tandem mass spectrometry

1. Introduction

Novartis compound CGS 26214 is a synthetic thyromimetic agent tested for its cholesterol lowering activity in rats, dogs and monkeys [1–3].

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This compound acts as an inhibitor of the enzyme HMG CoA reductase and lowers plasma low-density lipoprotein (LDL)-cholesterol and triglycerides [1]. It also increases high-density lipoprotein (HDL)-cholesterol in plasma and is virtually devoid of cardiac and thermogenic effects [1–3]. CGS 26214 rapidly hydrolyzes to the free acid in the biological system (Fig. 1), suggesting that it functions primarily as a prodrug. CGS 26214 is a racemic mixture of the chiral components CGS 28934(−) and CGS 28935(+) which have similar biological activities. Therefore, it was necessary to have a method to determine each of the chiral components CGS 28934 and CGS 28935 to support clinical studies of the drug.

The present report describes a chiral LC/MS/MS method for the quantitative determination of the stereoisomers CGS 28934 and CGS 28935 in human plasma. The method was successfully validated and applied to support several clinical studies.

2. Experimental

2.1. Chemicals

All solvents were of HPLC grade and all other chemicals were of analytical grade and used without further purification. Ethanol was purchased from Quantum Chemical Corporation (Tuscola, IL, USA). Methanol and n-propanol were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water, glacial acetic acid, ammonium acetate, and ammonium hydroxide were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Blank human plasma was purchased from Biological Speciality Corporation (Colmar, PA, USA). The following reference/standard compounds were supplied by Novartis Pharmaceuticals Corporation (East Hanover, NJ, USA): optically and chemically pure CGS 28934(−) and CGS 28935(+) ($C_{25}H_{24}O_6NF$), molecular weight (MW) = 453.47; the racemic internal standard [^{13}C,D_4]-CGS 26214 ($C_{24}^{13}CH_{20}^{2}H_4O_6NF$), MW =

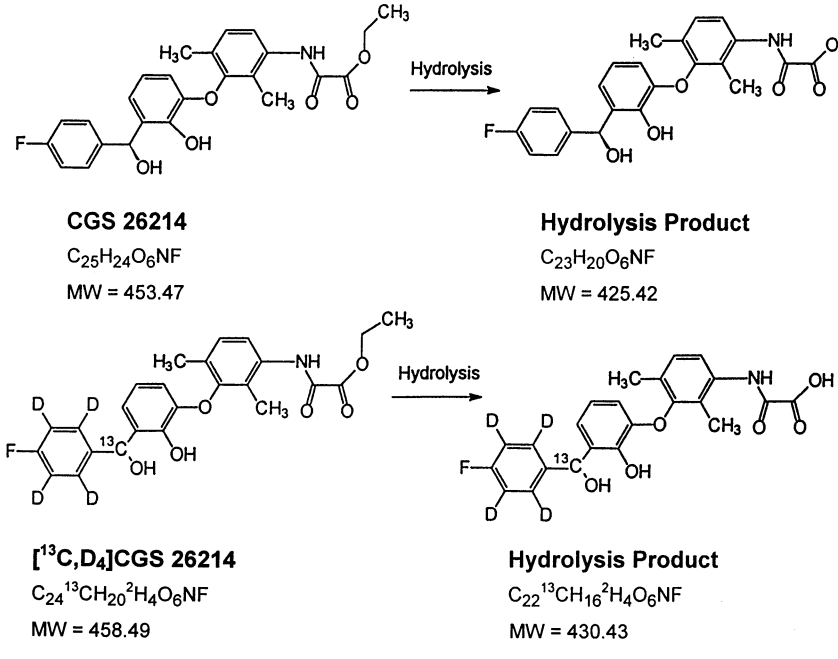

Fig. 1. Structures of CGS 26214 (racemic mixture of CGS 28934 and CGS 28935), the internal standard, and their hydrolysis products.

458.49. Chemical structures of the drug and the internal standard are shown in Fig. 1.

2.2. Standard and QC solutions

Stock solutions (1.0 mg/ml) of CGS 28934 and CGS 28935 were prepared in ethanol and combined (1:1, v/v) to yield a mixed stock solution. Standard spiking solutions at concentrations ranging from 4 to 250 ng/ml were prepared by appropriate dilution of the mixed stock solution with methanol:water (1:1, v/v). The spiking solutions (0.1 ml) were added to 0.9 ml of blank (drug-free) human plasma to prepare calibration samples having concentrations of 0.40, 1.0, 5.0, 10, and 25 ng/ml.

A separate mixed stock solution of the chiral compounds was used to prepare quality control (QC) samples. The QC samples for validation and stability experiments were prepared at concentrations corresponding to the low, middle and upper level of the calibration range according to the procedure described above. The racemic internal standard ([^{13}C,D$_4$]-CGS 26214) stock solution was prepared at a concentration of 1.0 mg/ml in ethanol. Further dilution of the stock solution with methanol:water (1:1, v/v) yielded a spiking solution having a concentration of 250 ng/ml, 0.1 ml (25 ng) of which was used for spiking each sample. All solutions were prepared in glass volumetric flasks and stored at about −20°C until further analysis.

2.3. Sample preparation

The plasma samples (1 ml) were thawed to room temperature, spiked with 0.1 ml of internal standard solution (25 ng/ml) and mixed by vortexing. The content of each of the sample tubes was made alkaline by mixing with 2 ml of freshly prepared aqueous solution of 0.5 M NH_4OH and kept on a bench for 60 min at room temperature. This incubation under alkaline condition allowed complete hydrolysis of the analytes to the corre-

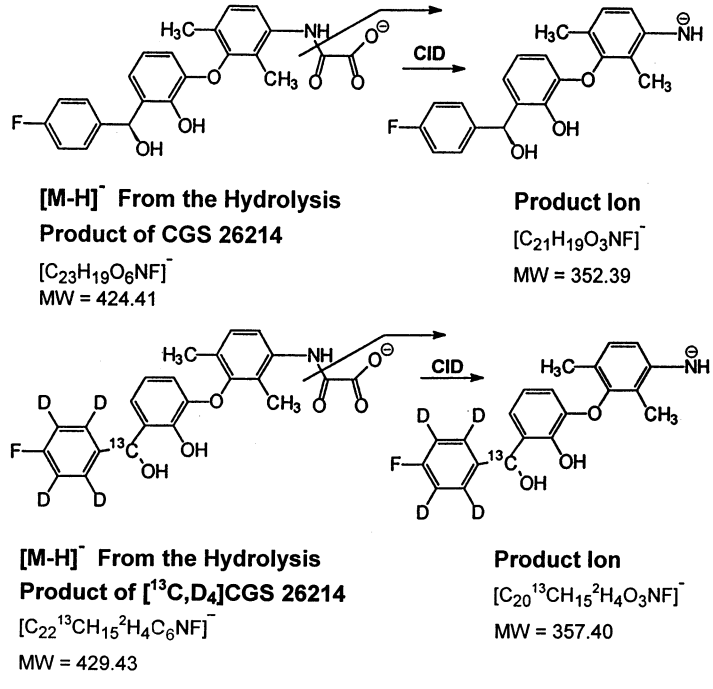

Fig. 2. Structures of the [M–H]$^-$ ions generated in the ESI source and the product ions produced during the MS/MS reactions from the free acids of CGS 26214 and the internal standard.

sponding acids (Fig. 1). Then 0.4 ml of glacial acetic acid was added to neutralize the content in each tube. The samples were loaded onto C_{18} solid phase extraction (SPE) cartridges (Bond Elute C_{18}, 500 mg, 3-ml capacity, from Varian, Harbor City, CA, USA) previously conditioned with methanol and water. Each of the SPE cartridges was washed sequentially with water (2.0 ml) followed by 40% methanol in water (1.0 ml). The SPE cartridges were dried with air-suction for 2 min. The analytes were eluted from the SPE cartridge using 1.5 ml of 80% methanol in water. Each of the extracts was dried in a Savant evaporator at room temperature. The residue was reconstituted in 0.5 ml of 20% methanol in water. The extract was further purified by injecting through a micro-preparative Asahipak ODP (Keystone Scientific, Bellefonte, PA, USA)

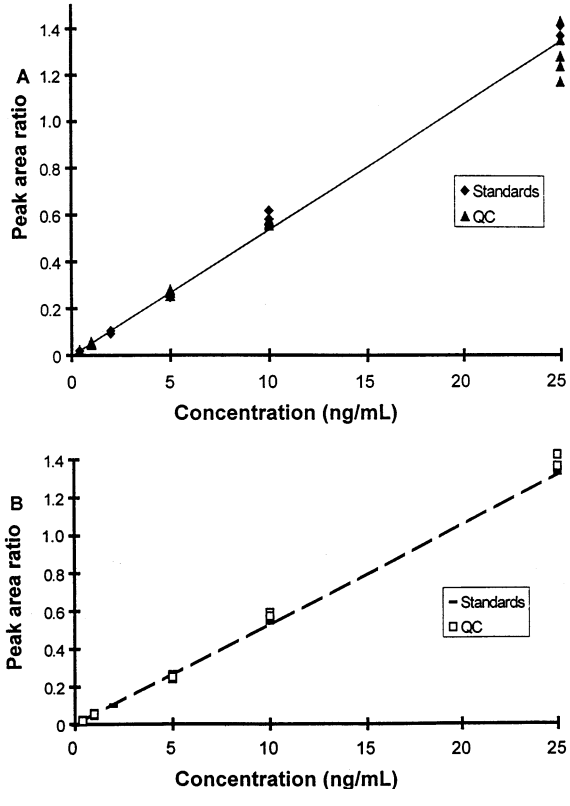

Fig. 3. Panel A, representative calibration curve for CGS 28934 in human plasma, $y = 0.0543x - 0.00411$, $r = 0.9952$; panel B, representative calibration curve for CGS 28935 in human plasma, $y = 0.0552x - 0.00618$, $r = 0.9975$.

column (150 × 2 mm, 5 μm) at 15°C. Separation was performed using water:methanol:5 N NH_4OH (60:40:5, v/v) at a flow rate of 0.2 ml/min. The fraction containing the compounds of interest and internal standards was collected between 7 and 9.5-min time window into a 1-ml autosampler vial. The solvent was evaporated and reconstituted in 10 μl of 2.8% ethanol in 0.03% ammonium acetate (pH 7.0), and a 2-μl aliquot was injected during LC/MS/MS analysis.

2.4. LC/MS/MS instrumentation

The liquid chromatographic separation was performed on a Chiral AGP Micro column (100 × 1 mm, 5 μm; Regis Technology, Morton Grove, IL, USA) using an isocratic flow of 4% n-propanol in 0.03% ammonium acetate in water (pH 7.0) as the mobile phase at a rate of 40 μl/min. The injection volume was 2 μl. The analytical column was guarded with a 0.5-μm prefilter (Upchurch Scientific, Oak Harbor, WA, USA) and maintained at ambient temperature. The LC system consisted of a LC-10AD Shimadzu pump (Columbia, MD, USA) and an SCL-10A controller. An on-line solvent degasser (Metachem, Torrance, CA, USA) was utilized. The autosampler was a CTC A200S unit obtained from Leap Technologies, Chapel Hill, NC, USA.

A TSQ-700 triple quadrupole mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionization (ESI) interface [4–10] was used for the detection of the compounds. Experiments were performed in the negative ionization mode of detection [11]. In place of the most commonly utilized fused silica capillary, a hypodermic stainless steel capillary (0.008 in. o.d. × 0.004 in. i.d.) was used in the ESI assembly, which resulted in a relatively low chemical noise and produced electrospray at lower voltages than typically observed [11]. Nitrogen gas at 50 psi was used as the sheath gas to assist liquid nebulization and desolvation. A potential of 3.0 kV was applied to the electrospray tip to provide an ion current of 0.5–1.0 μA during the analysis. The heated capillary was set at 200°C. The precursor ions produced in the ESI source were mass-selected by the first quadrupole and

Table 1
Summary of calibration curve obtained for the analysis of CGS 28934 in human plasma

Analysis day	Nominal concentration (ng/ml)					Slope	y-Intercept	Correlation coefficient (r)
	0.4	2	5	10	25			
	Back-calculated concentration (ng/ml)							
Day 1	0.440	2.26	4.74	11.1	25.2	0.0540	−0.00520	0.9942
	0.370	1.79	4.71	11.1	24.1			
Day 2	0.400	1.99	4.98	11.1	26.1	0.0551	−0.00386	0.9955
	0.420	1.70	4.82	11.1	25.4			
Day 3	0.410	2.00	4.65	10.9	25.5	0.0538	−0.00327	0.9959
	0.410	1.76	4.83	11.5	26.3			
Mean ($n=6$)	0.408	1.92	4.79	11.1	25.4	0.0543	−0.00411	0.9952
S.D.	0.023	0.208	0.116	0.197	0.779	–	–	–
CV (%)	5.64	10.8	2.42	1.77	3.07	–	–	–
Recovery (%)	102	96.0	95.8	111	102	–	–	–
RE (%)	2.00	−4.00	−4.20	11.0	1.60	–	–	–

Table 2
Summary of calibration curve obtained for the analysis of CGS 28935 in human plasma

Analysis day	Nominal concentration (ng/ml)					Slope	y-Intercept	Correlation coefficient (r)
	0.4	2	5	10	25			
	Back-calculated concentration (ng/ml)							
Day 1	0.400	2.11	4.72	10.4	27.2	0.0568	−0.00706	0.9962
	0.400	1.72	4.89	10.7	26.3			
Day 2	0.410	1.96	4.56	10.7	24.3	0.0557	−0.00822	0.9983
	0.390	1.98	5.19	10.8	25.2			
Day 3	0.380	2.04	4.91	10.6	25.2	0.0531	−0.00326	0.9980
	0.430	1.90	4.63	10.7	25.3			
Mean ($n=6$)	0.402	1.95	4.82	10.7	25.6	0.0552	−0.00618	0.9975
S.D.	0.017	0.134	0.230	0.138	1.01	–	–	–
CV (%)	4.23	6.87	4.77	1.29	3.95	–	–	–
Recovery (%)	101	97.5	96.4	107	102	–	–	–
RE (%)	0.500	−2.50	−3.60	7.00	2.40	–	–	–

allowed to undergo collisionally induced dissociation (CID) in the second quadrupole. Argon was used as the collision gas at 2.5 mTorr of pressure and the collision energy was set at 16.4 eV. Experiments were performed in the multiple reaction monitoring (MRM) mode to detect precursor to product ion transitions of m/z 424 $[M–H]^- \rightarrow 352$ (scan time, 3 s) for CGS 28934 and CGS 28935 and m/z 429 $[M–H]^- \rightarrow 357$ (scan time, 3 s) for the (−) and (+) isomers of the racemic internal standard. The reactions monitored during the MRM scans are shown in Fig. 2. The first quadrupole was set at 6 Da for precursor ions while the third quadrupole was set at 4 Da for product ions.

2.5. Calibration curve and quantification of samples

All calibrators were processed in duplicate at five different concentrations 0.4, 2.0, 5.0, 10, and 25 ng/ml. Calibration curves ($y = mx + b$),

represented by the plots of the peak area ratios (y) of the analyte peaks to the respective internal standard peaks versus the concentrations (x) of the calibration samples, were generated using weighted ($1/x^2$) linear least-square regression model. Representative standard calibration curves for CGS 28934 and CGS 28935 are shown in Fig. 3A and B, respectively. Concentrations in the QC and stability samples were calculated from the resulting peak area ratios and the regression equation of the calibration curve.

3. Results and discussion

3.1. Calibration curves

The calibration curve parameters of CGS 28934 and CGS 28935 from 3 days of validation are summarized in Table 1 (plot in Fig. 3A) and Table 2 (plot in Fig. 3B), respectively. The correlation coefficients were greater than or equal to 0.9942 and 0.9962 for CGS 28934 and CGS 28935, respectively. The fit of the calibration standards to the curves (Tables 1 and 2) was assessed from the relative error (RE, %): $100 \times$ [(back-calculated concentration from the regression line equation) − (nominal concentration)]/(nominal concentration). For CGS 28934 the coefficient of variation (%CV) did not exceed 10.8% and the differences for mean back-calculated concentrations did not exceed 11.0% from the theoretical value. For CGS 28935 the coefficient of variation (%CV) was within 6.87% and the differences for mean back-calculated concentrations did not exceed 7.00% from the theoretical value. The low relative errors for both the compounds indicated good fits of the regression model over the concentration range of the calibration curves.

Table 3
Intraday and interday accuracy and precision of the QC samples for CGS 28934

Nominal concentration (ng/ml)	Recovery (%)			
	Day 1	Day 2	Day 3	Interday ($n = 15$)
0.4	97.5	97.5	110	
	95.0	100	118	
	100	97.5	110	
	95.0	98.0	103	
	92.5	95.0	100	
Mean	96.0	97.6	108	101
S.D.	2.85	1.78	7.01	6.98
CV (%)	2.97	1.82	6.49	6.91
5.0	96.0	98.0	96.0	
	94.0	94.0	102	
	90.0	90.0	96.0	
	96.0	92.0	100	
	94.0	98.0	106	
Mean	94.0	94.4	100	96.1
S.D.	2.45	3.58	4.24	4.31
CV (%)	2.61	3.79	4.24	4.48
25	107	108	106	
	96.8	99.2	92.0	
	109	96.8	87.2	
	103	93.5	95.2	
	98.6	107	104	
Mean	103	101	96.9	100
S.D.	5.24	6.37	7.97	6.65
CV (%)	5.09	6.31	8.22	6.65

Table 4
Intraday and interday accuracy and precision of the QC samples for CGS 28935

Nominal concentration (ng/ml)	Recovery (%)			
	Day 1	Day 2	Day 3	Interday ($n = 15$)
0.4	107	108	115	
	108	108	93.2	
	97.2	113	98.5	
	99.7	120	87.0	
	103	115	102	
Mean	103	113	99.1	105
S.D.	4.62	5.07	10.5	8.97
CV (%)	4.49	4.49	10.6	8.54
5.0	89.0	96.0	98.8	
	91.8	102	92.7	
	95.7	88.0	100	
	97.4	86.0	94.5	
	89.9	96.0	95.8	
Mean	92.8	93.6	96.4	94.2
S.D.	3.65	6.54	3.02	4.60
CV (%)	3.93	6.99	3.13	4.88
25	102	100	103	
	92.0	93.2	108	
	94.8	96.8	108	
	102	107	115	
	95.2	108	112	
Mean	97.2	101	109	102
S.D.	4.55	6.41	4.55	7.10
CV (%)	4.68	6.35	4.17	6.96

3.2. Accuracy and precision of the QC samples

Both intraday and interday accuracy and precision of the method were evaluated from five replicates of QC samples of known concentrations. The experiments were repeated on 3 different days and the data are displayed in Tables 3 and 4 for CGS 28934 and CGS 28935, respectively. Accuracy was determined by calculating the mean recovery for the observed concentrations in percent of the nominal concentrations in QC samples. Precision was assessed from the %CV of the mean recoveries. As shown in Table 3, the intraday mean recoveries varied from 94 to 108% over 0.4–25 ng/ml concentration range of CGS 28934, and the corresponding %CV values ($n = 5$) varied from 1.82 to 8.22. The interday mean recoveries for CGS 28934 varied from 96.1 to 101% and the corresponding %CV values ($n = 15$) varied from 4.48 to 6.91 over the 0.4–25 ng/ml concentration range. For CGS 28935 (Table 4) the intraday mean recoveries varied from 92.8 to 113% over the concentration range 0.4–25 ng/ml, and the corresponding %CV varied from 3.13 to 10.6%. The interday mean recoveries varied from 94.2 to 105% and the %CV ($n = 15$) varied from 4.88 to 8.54% over the concentration range 0.4–25 ng/ml of CGS 28935.

3.3. Extraction efficiency and the limit of quantitation

The extraction efficiencies of CGS 28934 and CGS 28935 from plasma were assessed by comparison of the peak areas from extracted samples to those from non-extracted spiked standards ($n = 6$). The mean $\pm$ S.D. values of the extraction efficiencies for CGS 28934 in human plasma were 50.3 ± 4.8, 30.4 ± 3.6 and 37.1 ± 1.0% at concentrations of 0.4, 10 and 25 ng/ml, respectively. The

extraction efficiencies for CGS 28935 were 41.8 ± 6.0, 29.2 ± 3.0 and 39.4 ± 0.4% at concentrations of 0.4, 10 and 25 ng/ml, respectively. The extraction efficiencies for the (−) and (+) isomers of the internal standard were 39.3 ± 2.8 and 39.1 ± 3.0%, respectively, at 25 ng/ml. The lower limit of quantitation (LLOQ), defined as the lowest concentration on the standard curve that could be measured with acceptable accuracy and precision, was 0.4 ng/ml using 1 ml of human plasma for both CGS 28934 (Table 3) and CGS 28935 (Table 4).

3.4. Specificity

The instrument was operated in the MRM mode to achieve optimum specificity and sensitivity. The fragmentation reactions of m/z 424 → 352 and m/z 429 → 357 were monitored for the analytes and the internal standards, respectively (Fig. 2). A representative chromatogram of extracts of blank human plasma spiked with the internal standard is shown in Fig. 4. Absence of any peak in the blank human plasma (upper trace of Fig. 4) indicated the high specificity of the method for CGS 28934 and CGS 28935. Peak assignment was established from samples spiked individually with each compound and treated as described in the sample preparation procedure. CGS 28934, CGS 28935, and the internal standard peaks had similar retention times (Fig. 5). Absence of 'cross-talk' in the presence of the internal standard was shown by the absence of any peak in the MRM channel of the analytes CGS 28934 and CGS 28935 (upper trace of Fig. 4) at appropriate retention times (~6.8 and 8.7 min). Specificity of the method was investigated further by analysis of six different lots of blank human plasma samples. Ion chromatograms of CGS 28934 and CGS 28935 spiked at different and equal amounts in plasma samples in the presence of 25 ng/ml internal standards are shown in Fig. 5A and B.

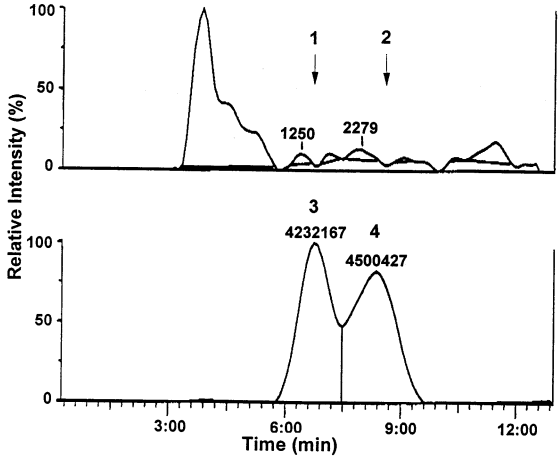

Fig. 4. HPLC/ESI/MS/MS ion-chromatogram of blank human plasma spiked with 25 ng/ml of internal standard. Arrows 1 and 2 refer to the approximate retention times of CGS 28934 and CGS 28935, respectively. Peaks 3 and 4 are the (−) and (+) isomers of the racemic internal standard.

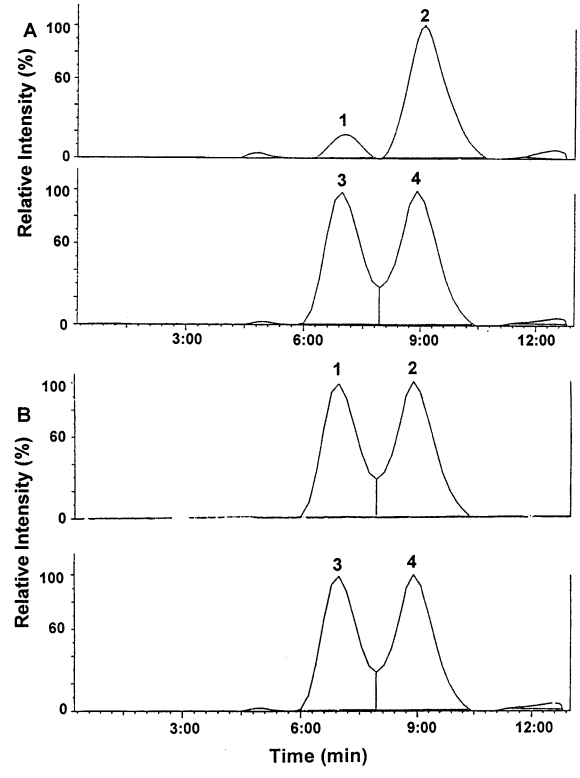

Fig. 5. HPLC/ESI/MS/MS ion-chromatograms. Panel A, peaks 1 and 2 are 0.4 ng/ml of CGS 28934 and 10 ng/ml of CGS 28935, respectively. Peaks 3 and 4 are the (−) and (+) isomers of the internal standard (25 ng/ml). Panel B, peaks 1 and 2 are 25 ng/ml each of CGS 28934 and CGS 28935, respectively. Peaks 3 and 4 are the (−) and (+) isomers of the internal standard (25 ng/ml).

Table 5
Stability data for CGS 28934

Storage period and storage conditions	Nominal concentration (ng/ml)	Recovery (%)		Mean recovery (%)
		First replicate	Second replicate	
0 h, 25°C	0.4	97.5	100	98.8
	5	98.0	94.0	96.0
	25	109	99.2	104
6 h, 25°C	0.4	115	110	113
	5	88.0	94.0	91.0
	25	90.0	98.8	94.4
24 h in autosampler at 25°C (extract only)	0.4	110	90	100
	5	92.0	94.0	93.0
	25	98.6	103.0	101
48 h in refrigerator, 6°C (extract only)	0.4	100	102	101
	5	92.0	98.0	95.0
	25	102	103	103
Three freeze–thaw cycles	0.4	103	120	112
	5	90.0	92.0	91.0
	25	102	103	103

Table 6
Stability data for CGS 28935

Storage period and storage conditions	Nominal concentration (ng/ml)	Recovery (%)		Mean recovery (%)
		First replicate	Second replicate	
0 h, 25°C	0.4	108	108	108
	5	96.0	102	99.0
	25	104	93.2	98.6
6 h, 25°C	0.4	115	120	118
	5	98.0	90.0	94.0
	25	96.0	91.0	93.5
24 h in autosampler at 25°C (extract only)	0.4	105	100	103
	5	102	106	104
	25	103	91.0	97.0
48 h in refrigerator, 6°C (extract only)	0.4	105	113	109
	5	108	104	106
	25	109	102	106
Three freeze–thaw cycles	0.4	118	100	109
	5	99.4	94.1	96.8
	25	96.4	103	99.7

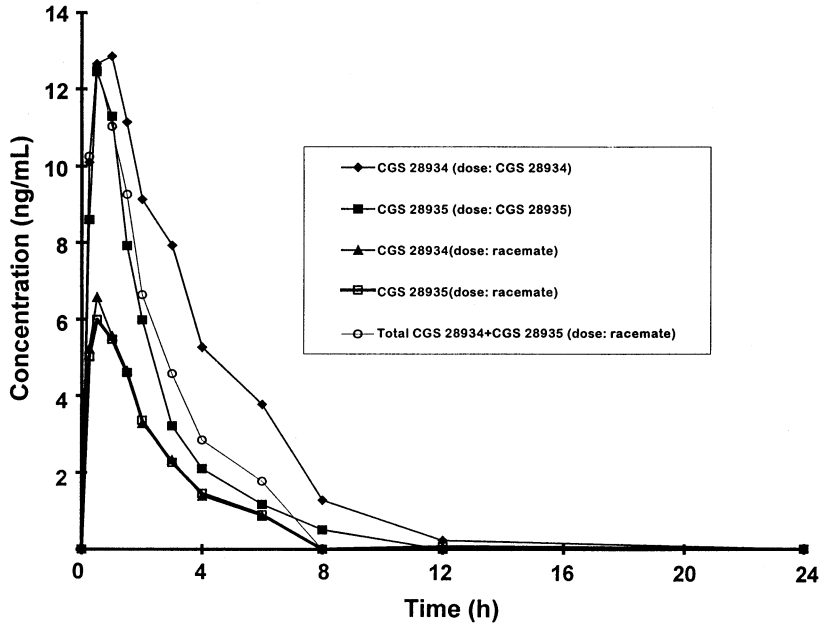

Fig. 6. Plasma concentration–time profiles for a patient dosed with the racemate CGS 26214 and the optically pure isomers, 900 μg each separately at 2-week intervals.

3.5. Stability

QC samples containing blank human plasma spiked with CGS 28934 and CGS 28935 were prepared at 0.4, 5.0, and 25 ng/ml concentrations and analyzed immediately. Furthermore, the stabilities of CGS 28934 and CGS 28935 in these samples under different conditions were studied and compared with data obtained from freshly prepared samples. Analyses of the plasma samples were performed in duplicate and the results are displayed in Tables 5 and 6 for CGS 28934 and CGS 28935, respectively. Both the isomers were stable in plasma for at least 6 h at room temperature (~ 25°C). The respective mean recoveries for CGS 28934 at 0.4, 5.0, and 25 ng/ml after 6 h storage at room temperature were 113, 91.0, and 94.4% (Table 5). The recoveries of CGS 28935 after 6 h storage at room temperature were 118, 94.0 and 93.0% at 0.4, 5.0, and 25 ng/nl (Table 6). Both the compounds were stable in the extracts after 24 h of storage of the extracts in the autosampler at room temperature. The mean recoveries were 100, 93, and 101% for CGS 28934 (Table 5) and 103, 104, and 97.0% for CGS 28935 (Table 6) at 0.4, 5.0, and 25 ng/ml, respectively. The compounds were also stable at all three concentrations in the extracts stored at ~ 6°C for at least 48 h. Effect of freeze (-20°C)–thaw cycles on the stability of the compounds was studied on freshly prepared QC samples. No discernible loss of either compound was detected after three freeze–thaw cycles, the respective mean recoveries of CGS 28934 being 112, 91.0 and 103% and those of CGS 28935 being 109, 96.8, and 99.7% of the nominal values of 0.4, 5.0, and 25 ng/ml.

The objective of the method was to establish a chiral quantitative method with a LLOQ of 0.4 ng/ml. The method was needed to support several clinical studies where oral doses of CGS26214 and the optical isomers were separately administered at ≤ 1 mg per subject. LC/MS/MS of the compounds displayed a superior sensitivity in the negative ionization mode using an ESI interface. In order to achieve the required LLOQ, two experimental measures were taken into consider-

ation. First, decreased LC flow rate compatible with ESI, resulting in an increased concentration (i.e. reduced extra-column dispersion effects) of eluting peaks from smaller column diameters was considered. Consequently, stable ion current and presumably higher ionization efficiency were realized in concert with a reduced flow rate (i.e. 40 μl/min). Second, suppression of ion intensities of the analytes was observed during the method development. It was difficult to separate the ion suppressants in this microbore chiral column. Addition of a HPLC preparatory step during sample preparation improved the ion intensities by minimizing matrix mediated electrospray ion suppression [12–14]. The major drawback of this approach, however, was its limited sample throughput. Nonetheless, the sensitivity of the method proved to be sufficient to meet the requirement of several clinical studies. Fig. 6 depicts an example of a plasma concentration–time profile for a patient. The subject was treated with oral doses of 900 μg each of CGS 28934, CGS 28935 and CGS 26214 sequentially with a 2-week washout period between treatments. As expected the method was adequate for depicting plasma concentration profiles of the compounds during the 24-h sampling period.

4. Conclusions

A chiral LC/MS/MS method has been developed and validated for the quantification of the chiral isomers CGS 28934 and CGS 28935 in human plasma. Excellent linearity was observed over the concentration range 0.4–25 ng/ml. The method exhibited ruggedness and was successfully utilized in the analysis of samples from several clinical studies.

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