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Quantitative determination of CGS 26214, a cholesterol lowering agent, in human plasma using negative electrospray ionization liquid chromatography–tandem mass spectrometry

Tapan K. Majumdar*, Shari Wu, Francis L.S. Tse

Department of Drug Metabolism & Pharmacokinetics, Novartis Institute for Biomedical Research, East Hanover, NJ 07936, USA

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Abstract

CGS 26214 is a synthetic cholesterol-lowering agent shown to be active in the rat, dog and monkey. The present work was conducted to develop a sensitive liquid chromatography–tandem mass spectrometry (LC–MS–MS) method for quantitative determination of the compound in human plasma following clinical doses of 10–100 μg per day. A number of analytical challenges were encountered during the development of the assay. The compound was an ester and susceptible to hydrolysis under experimental conditions. A lower limit of quantitation of 50 pg/ml was needed due to the low clinical doses. Positive electrospray ionization of CGS 26214 yielded insufficient sensitivity needed for the studies. Consequently, LC–MS–MS conditions were optimized for the negative ion mode of detection. The sample preparation steps proved to be critical in order to reduce the possibility of microbore column (50 mm×1.0 mm I.D.) obstruction, chromatographic deterioration, and matrix mediated electrospray ion suppression. The present method addressed the above issues. The method was accurate and reproducible and was successfully applied to generate plasma concentration–time profiles for human subjects after low oral doses of the compound.

Keywords: Cholesterol; CGS 26214

1. Introduction

Novartis compound CGS 26214 is a synthetic cholesterol-lowering agent shown to be active in the rat, dog and monkey [1–3]. 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 simulated gastric or intestinal fluid (Fig. 1), suggesting that it functions primarily as a prodrug. An analytical method was required for the trace level

*Corresponding author. Building 405, Room 230, 59 Route 10, Novartis, East Hanover, NJ 07936, USA. Tel.: +1-973-7813-582; fax: +1-973-7816-076.

E-mail address: tapan.majumdar@pharma.novartis.com (T.K. Majumdar).

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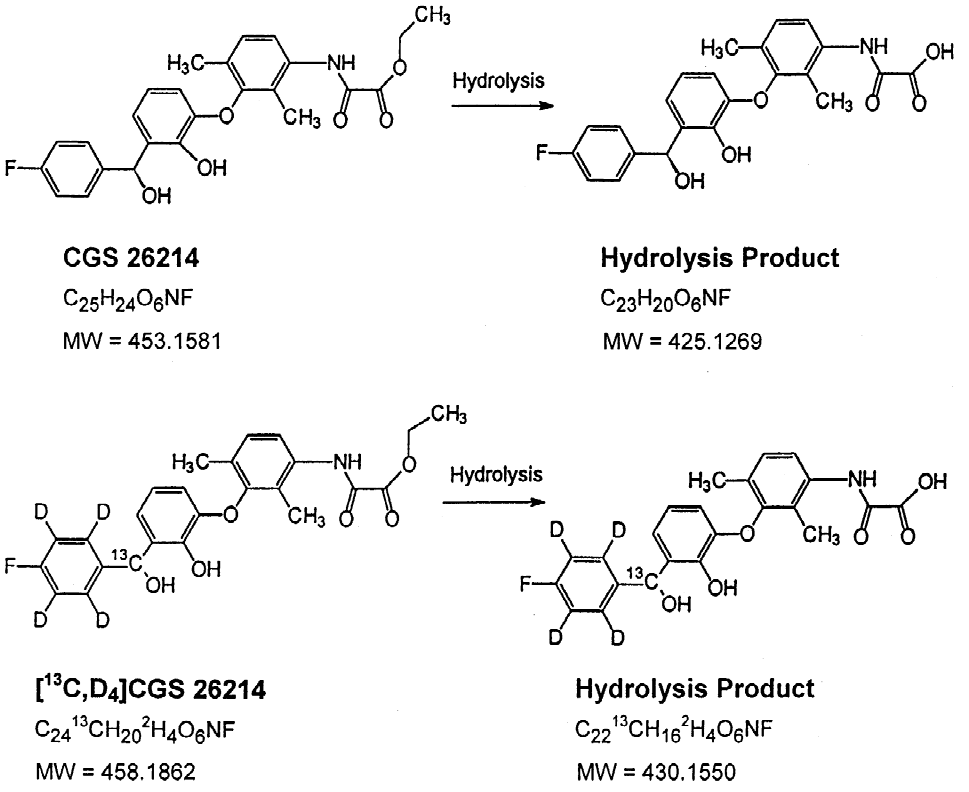

Fig. 1. Structures of CGS 26214, the internal standard, and their hydrolysis products.

quantitative analysis of this unstable compound in human plasma to support several low-dose studies.

2. Experimental

2.1. Chemicals

All solvents were HPLC grade and all other chemicals were of analytical grade and used without further purification. Ethanol was purchased from Quantum (Tuscola, IL, USA). Methanol was obtained from Fisher Scientific (Fair Lawn, NJ, USA). Water and ammonium hydroxide (4.96 *M*) were purchased from Aldrich (Milwaukee, WI, USA). Blank heparin treated human plasma was purchased from Biological Speciality (Colmar, PA, USA). The following reference/standard compounds were supplied by Novartis (East Hanover, NJ, USA): chemically pure CGS 26214 ($C_{25}H_{24}O_6NF$), molecular mass (M_r)=453.1581 and the internal standard [$^{13}C,D_4$]CGS 26214 ($C^{13}_{24}CH^2_{20}H_4O_6NF$), M_r= 458.1862. Chemical structures of the drug and the internal standard are shown in Fig. 1.

2.2. Standard and quality control (QC) solutions

A stock solution (1.0 mg/ml) of CGS 26214 was prepared in ethanol. Standard spiking solutions at concentrations ranging from 0.5 to 50 ng/ml were prepared by serial dilutions of the stock solution with methanol–water (1:1, v/v). The spiking solutions (0.1 ml) were added to 1 ml of blank (drug-free) human plasma to prepare calibration samples having concentrations of 50, 100, 250, 500, 1000, 2500 and 5000 pg/ml.

A separate mixed stock solution of CGS 26214 was used to prepare 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

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 50 ng/ml, 0.1 ml of which was used for spiking each sample. All solutions were prepared in glass volumetric flasks and stored at approximately −20°C until analysis.

2.3. Sample preparation

The plasma samples were thawed to room temperature. Plasma (1 ml) samples were pipetted in tubes and a 0.1-ml aliquot of the standard spiking solution or a blank solution of methanol–water (1:1, v/v) was added to each tube of standard/actual samples. Then 0.1 ml of the internal standard solution (50 ng/ml) was added and mixed by vortexing. The content of each tube was made alkaline by mixing with 2 ml of a freshly prepared aqueous solution of 0.5 *M* ammonium hydroxide and kept on the bench for 60 min at room temperature. This incubation under alkaline conditions allowed complete hydrolysis of the analytes to the corresponding acid (Fig. 1). The hydrolysate was treated with 0.4 ml of glacial acetic acid to make the content slightly acidic (pH 4–5). The samples were extracted on an automated ASPEC XL4 solid-phase extraction (SPE) system (Gilson, Middleton, WI, USA) using C_{18} SPE cartridges (Bond Elute C_{18}, 500 mg, 3 ml capacity, from Varian, Harbor City, CA, USA). The samples were loaded onto the SPE cartridges previously conditioned with 1 ml each of methanol and water. Each of the SPE cartridges was washed with 5% methanol in water (2.0 ml). The SPE cartridges were dried with air-suction for 1 min. The analytes were eluted from each SPE cartridge using 1.5 ml of 50% methanol in water. Each of the extracts was dried in a Savant evaporator at room temperature. The residue was reconstituted in 25 μl of 0.25 *M* ammonium hydroxide and a 5-μl aliquot was injected during liquid chromatography–tandem mass spectrometry (LC–MS–MS) analysis.

2.4. LC–MS–MS instrumentation

The liquid chromatographic separation was performed on an Asahipak ODP microbore column (50 mm×1 mm, 5 μm) supplied by Keystone Scientific (Bellefonte, PA, USA). An isocratic flow of methanol–water–5 *M* ammonium hydroxide (60:55:5, v/v) was used as a mobile phase at a rate of 10 μl/min. The column temperature was maintained at 45°C and the injection volume was 5 μl. The analytical column was guarded with a 0.5-μm pre-filter (Upchurch Scientific, Oak Harbor, WA, USA). 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]. Nitrogen gas at 344.7 kPa (50 p.s.i.) was used as the sheath gas to assist liquid nebulization and desolvation. A potential of 3.6 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 allowed to undergo collisionally induced dissociation (CID) in the second quadrupole. Argon was used as the collision gas at 333.3 kPa (2.5 mTorr) of pressure and the collision energy was set at 16.4 eV. Experiments were performed in the selected reaction monitoring (SRM) mode to detect precursor to product ion transitions of *m/z* 424 $[M-H]^- \rightarrow 352$ (scan time=3 s) for CGS 26214 and *m/z* 429 $[M-H]^- \rightarrow 357$ (scan time=3 s) for the internal standard. The reactions monitored during the SRM scans are shown in Fig. 2. The first quadrupole (peak width at 50% of height) was set at 3 u for precursor ions while the third quadrupole was set at 2 u for product ions.

2.5. Calibration curve and quantification of samples

All calibrators were processed in duplicate at seven different concentrations 50, 100, 250, 500, 1000, 2500 and 5000 pg/ml. Calibration curves ($y=mx+b$), represented by the plots of the peak area

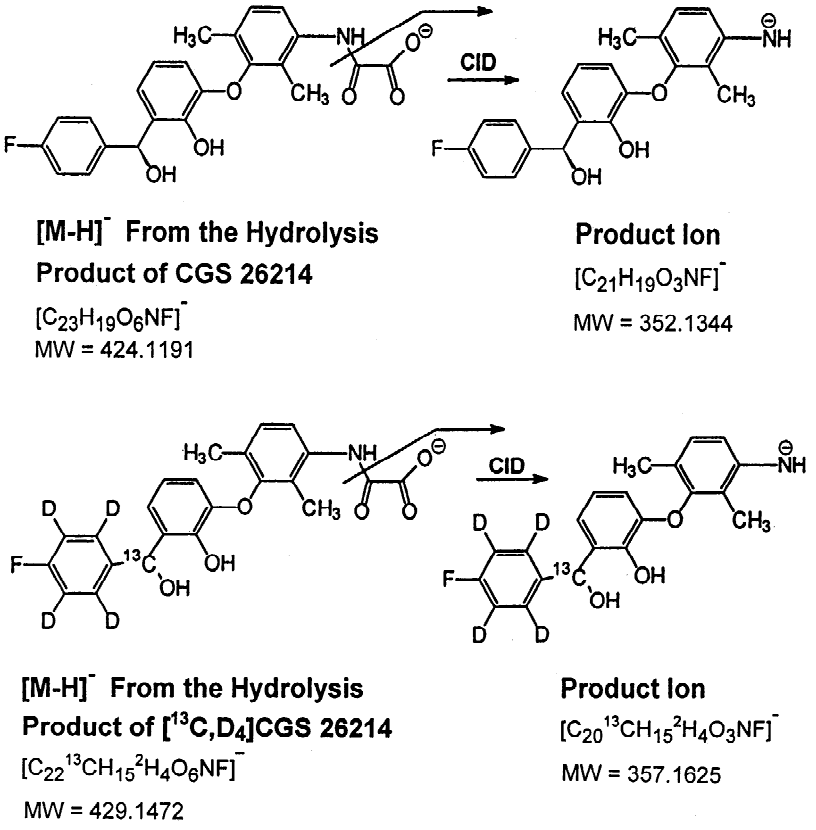

Fig. 2. Structures of the $[M-H]^-$ ions generated in the ESI source and the product ions produced during the MS–MS reactions from the free acids of CGS 26214 and the internal standard.

ratios (y) of the analyte peaks to the respective internal standard peaks versus the concentrations (x) of the calibration samples, were generated using a weighted ($1/x^2$) linear least-squares regression model. Calibration curve parameters are shown in Table 1. The calibration curves obtained during 3 days of validation were linear with an average correlation coefficient of 0.998. 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 26214 from 3 days of validation are summarized in Table 1. The correlation coefficients were greater than or equal to 0.9970. The fit of the calibration standards to the curve (Table 1) was assessed from the relative error (RE, %): 100×[(back-calculated concentration from the regression line equation)−(nominal concentration)]/(nominal concentration). For CGS 26214 the differences for mean back-calculated concentrations were within 2.66% of the theoretical value in the 50 to 5000 pg/ml concentration range, and the relative standard deviation (RSD) did not exceed 7.83%. The predefined acceptable limits of RSD were 20% for the lower limit of quantitation (LLOQ) and 15% for other concentrations.

3.2. Accuracy and precision of the QC samples

Intra- and inter-day accuracy and precision of the method were evaluated from six replicates of QC samples of known concentrations. These QC samples

Table 1
Summary of calibration curves obtained for the analysis of CGS 26214 in human plasma

Analysis day	Nominal concentration (pg/ml)							Slope	*y*-Intercept	Correlation coefficient (*r*)
	50	100	250	500	1000	2500	5000			
	Back-calculated concentration (pg/ml)									
Day 1	49	96	243	479	1029	2369	4979	0.00042	0.0041	0.9988
	53	99	259	501	1098	2496	5087			
Day 2	48	103	233	484	985	2524	5132	0.00042	0.0024	0.9990
	51	107	239	495	1016	2572	5203			
Day 3	51	89	242	468	912	2484	5042	0.00042	0.0076	0.9970
	51	111	272	533	1013	2562	5353			
Mean (n=6)	51	101	248	493	1009	2501	5133	0.00042	0.0047	0.9983
SD	1.76	7.91	14.6	22.7	60.6	73.5	132	0	0.0027	0.0011
RSD (%)	3.45	7.83	5.89	4.60	6.01	2.94	2.57	0	56.4	0.11
Recovery (%)	102	101	99	99	101	100	103	–	–	–
RE (%)	2.00	1.00	−0.80	−1.40	0.90	0.04	2.66	–	–	–

were prepared at three different concentrations corresponding to the lowest (50 pg/ml), the middle (1000 pg/ml), and the highest level (5000 pg/ml) of the calibration curve. The experiments were repeated on 3 different days and the data are displayed in Table 2. Accuracy was determined by calculating the mean recovery for the observed concentrations in percent of the nominal concentrations in standard samples. Precision was assessed from the RSD of the mean recoveries. The acceptable limits of recovery for the method were 80% at the lowest QC and 85% for other QC samples. The respective limit of RSD was 20% for the lowest QC and 15% for other QC concentrations. As shown in Table 2, the intra-day mean recoveries (n=6) varied from 94.6 to 105% over the 50–5000 pg/ml concentration range of CGS 26214, and the corresponding RSD values varied from 1.29 to 15.0%. The inter-day mean recoveries (n=18) varied from 97.8 to 103% and the corresponding RSD values varied from 2.76 to 9.71% over the concentration range. Therefore, the precisions and recoveries were within the tolerance limits.

3.3. Lower limit of quantitation

The LLOQ, defined as the lowest concentration on the standard curve that could be measured with acceptable accuracy and precision, was 50 pg/ml using a 1-ml volume of human plasma (Table 2). The mean (n=18) inter-day accuracy at LLOQ was 97.8% and the precision was 8.18%. These parameters were well within the acceptance criteria of the intra-day accuracy of 80% and the precision of 20% for LLOQ.

3.4. Specificity

The fragmentation reactions of m/z 424→352 and m/z 429→357 occurred during the MS–MS process were monitored for the analytes and the internal standards, respectively (Fig. 2). The instrument was operated in the SRM mode to achieve optimum specificity and sensitivity. A representative chromatogram of extracts of blank human plasma spiked with the internal standard (15 ng/ml) is shown in Fig. 3. Peak assignment was established from samples spiked individually with each compound and treated as described in the sample preparation procedure. The mass resolved CGS 26214 and the internal standard peaks had similar retention times (Figs. 4 and 5). Fig. 3 shows the presence of a very low intensity chemical noise peak in the blank plasma at the retention time of CGS 26214. This negligible noise peak was due to trace level cross-contamination present in the internal standard. The upper trace in Fig. 3 displayed the chemical noise which was enhanced by spiking internal standard at concentration three times higher than that actually used in the validation. The intensity of the noise peak was approximately 10 times less than the

Table 2
Intra- and inter-day accuracy and precision of the QC samples for CGS 26214

Nominal concentration (pg/ml)	Recovery (%)			Inter-day (n=18)
	Day 1	Day 2	Day 3	
50	86.8	99.9	85.2	
	99.2	107	89.7	
	99.8	100	98.3	
	99.1	110	97.6	
	100	109	86.7	
	93.9	88.9	110	
Mean	96.5	102	94.6	97.8
SD	5.25	7.96	9.27	8.00
RSD (%)	5.44	7.80	9.80	8.18
1000	106	105	92.4	
	98.7	106	98.9	
	110	97.8	110	
	100	93.6	102	
	100	112	92.5	
	90.9	104	134	
Mean	101	103	105	103
SD	6.56	6.49	15.7	10.0
RSD (%)	6.50	6.30	15.0	9.71
5000	104	103	105	
	103	98.5	100	
	101	99.4	93.2	
	105	99.8	101	
	103	100	102	
	103	102	103	
Mean	103	100	101	101
SD	1.33	1.70	4.06	2.79
RSD (%)	1.29	1.70	4.02	2.76

LLOQ and had no impact on the analysis. Specificity of the method was further investigated by acquiring analogous peak areas for the drug and the internal standard after spiking into six different lots of blank human plasma samples. Observation of interference to the drug peak in plasma samples from dosed patients is a common phenomenon during bioassay. Such interference can distort the plasma concentration profile during actual study. No such interference was observed during the analysis of plasma from post dosed patients during our regular studies. Ion chromatograms of CGS 26214 spiked at 25 and 5000 pg/ml in plasma samples in the presence of 5000 pg/ml internal standard are shown in Figs. 4 and 5, respectively.

3.5. Stability

QC samples containing blank human plasma spiked with CGS 26214 were prepared at 50, 100, 1000 and 5000 pg/ml concentrations and analyzed immediately. Stability of CGS 26214 in human plasma was studied under different conditions at these four concentrations and compared with data obtained from freshly prepared samples. CGS 26214 was stable in plasma for at least 72 h at room temperature (~25°C), the respective mean recoveries at 50, 100, 1000 and 5000 pg/ml being 96.7, 96.0, 113 and 94.7%. The compound was also stable in human plasma when stored at −20°C for 210 days. The respective mean recoveries at 50, 100, 1000, and

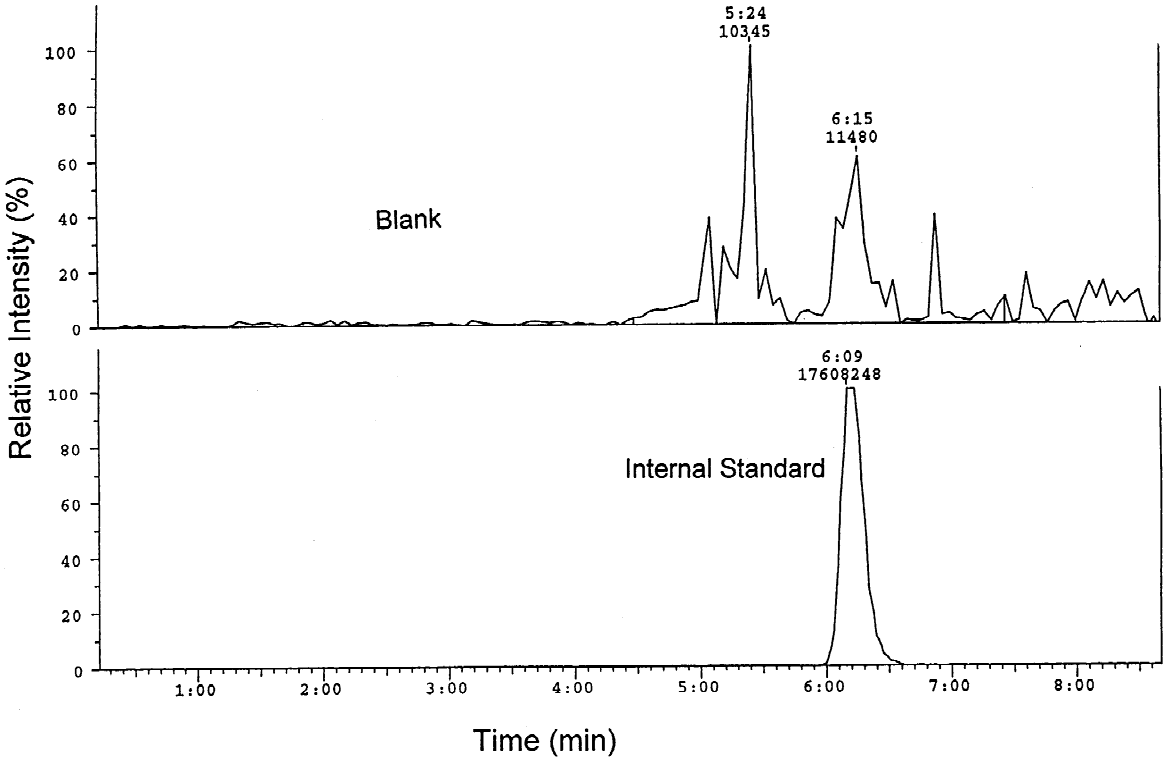

Fig. 3. HPLC–ESI-MS–MS ion chromatograms of blank human plasma spiked with 15 ng/ml of internal standard.

5000 pg/ml were 98.3, 98.7, 111, and 97.0%. Freeze–thaw stability was studied by measuring the recovery of freshly prepared QC samples after three freeze (−20°C)–thaw (~25°C) cycles. The respective mean recoveries after three freeze–thaw cycles were 97.0, 100, 111, and 97.7% of the nominal values of 50, 100, 1000, and 5000 pg/ml.

Stability of the hydrolysis product of CGS 26214 (Fig. 1) in human plasma was also investigated separately. The hydrolysis product was stable in plasma for at least 72 h at room temperature (~25°C), the respective mean recoveries at 50, 1000, and 5000 pg/ml being 97, 115 and 103%. The product was stable after three freeze–thaw cycles; the respective mean recoveries after three freeze–thaw cycles were 81 106 and 98% of the nominal values of 50, 1000 and 5000 pg/ml.

Several analytical problems were resolved during development of the method. CGS 26214 is an ester that was unstable under conditions used in the method. The stability problem of ester prodrugs is well known and has been documented in the literature [12–14]. The method described here does not measure CGS 26214 as an ester. It measures the hydrolysis product which is an acid (Fig. 1). The stable isotope-labeled CGS 26214 was used as internal standard to cancel any variation in the extraction procedure. This internal standard was also chosen to cancel any variation of CGS 26214 during ionization and the gas phase MS–MS reactions. Another problem was to achieve an LLOQ of 50 pg/ml. In order to minimize the LLOQ, three experimental measures were considered. First, the selection of appropriate ionization method. Positive electrospray ionization of CGS 26214 failed to provide the required sensitivity but a superior sensitivity was observed in the negative ionization mode using an ESI interface. Second, adding a hydrolysis step followed by an automated SPE during sample preparation was critical for cleanliness of the extract. This sample preparation procedure minimized the matrix mediated electrospray ion suppression [15–

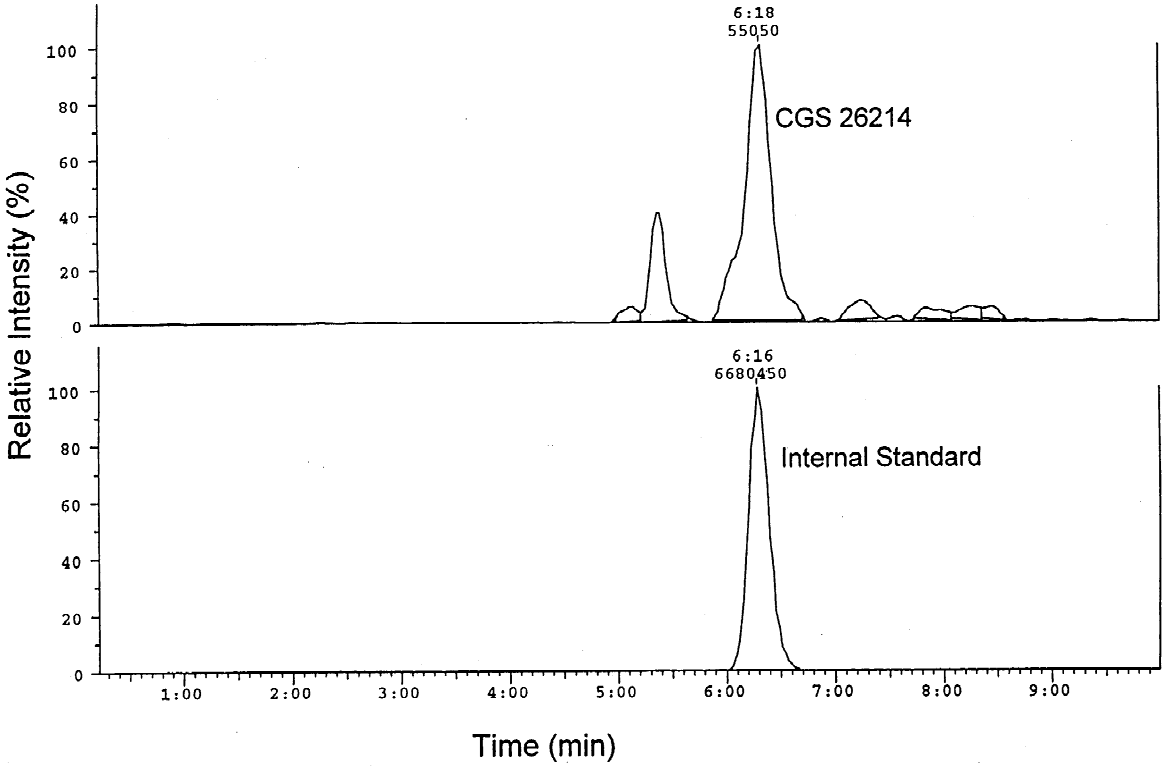

Fig. 4. HPLC–ESI-MS–MS ion chromatograms of blank human plasma spiked with 25 pg/ml of CGS 26214 and 5000 pg/ml of internal standard.

17]. Third, a narrow bore column was used to increase the peak concentration (i.e., reduced extra-column dispersion effects) of the analyte. Consequently, stable ion current and presumably higher ionization efficiency were realized in concert with a reduced flow-rate (i.e., 10 μl/min) used in this method. Clean extract obtained in this method was also critical to avoid plugging of the narrow bore column. The sensitivity of the method proved to be sufficient to meet the requirement of several clinical studies.

Fig. 6 depicts the plasma concentration–time profiles for three male subjects treated with CGS 26214 at doses of 10, 50 and 100 μg once daily for 7 days. In this study samples were collected in heparinized tubes at 0, 0.5, 1, 2, 3, 4, 6, 8 and 12 h post-dose on days 1 and 7. The concentration–time profiles clearly showed that all the subjects were exposed to the drug. The respective CGS 26214 exposure as measured by the $AUC_{0-24\ h}$ (area under curve) values were 4611 and 3347 pg h/ml for day 1 and day 7 of the 100 μg dose group. The $AUC_{0-24\ h}$ values for the lowest dose group (10 μg) were 242 and 180 pg h/ml on day 1 and day 7, respectively. The exposure increased in a dose over-proportional manner and no accumulation of CGS 26214 was observed after 7 days of treatment. The time to reach the highest concentration (T_{max}) varied from 0.5 to 3 h. The method clearly was adequate for monitoring plasma concentration profiles of the compound during the 12 h sampling period.

4. Conclusions

An LC–MS–MS method has been developed and validated for the quantification of CGS 26214 in human plasma. Several analytical problems such as stability of the analyte, cleanliness of extract, choice of column diameter, and ionization method to meet

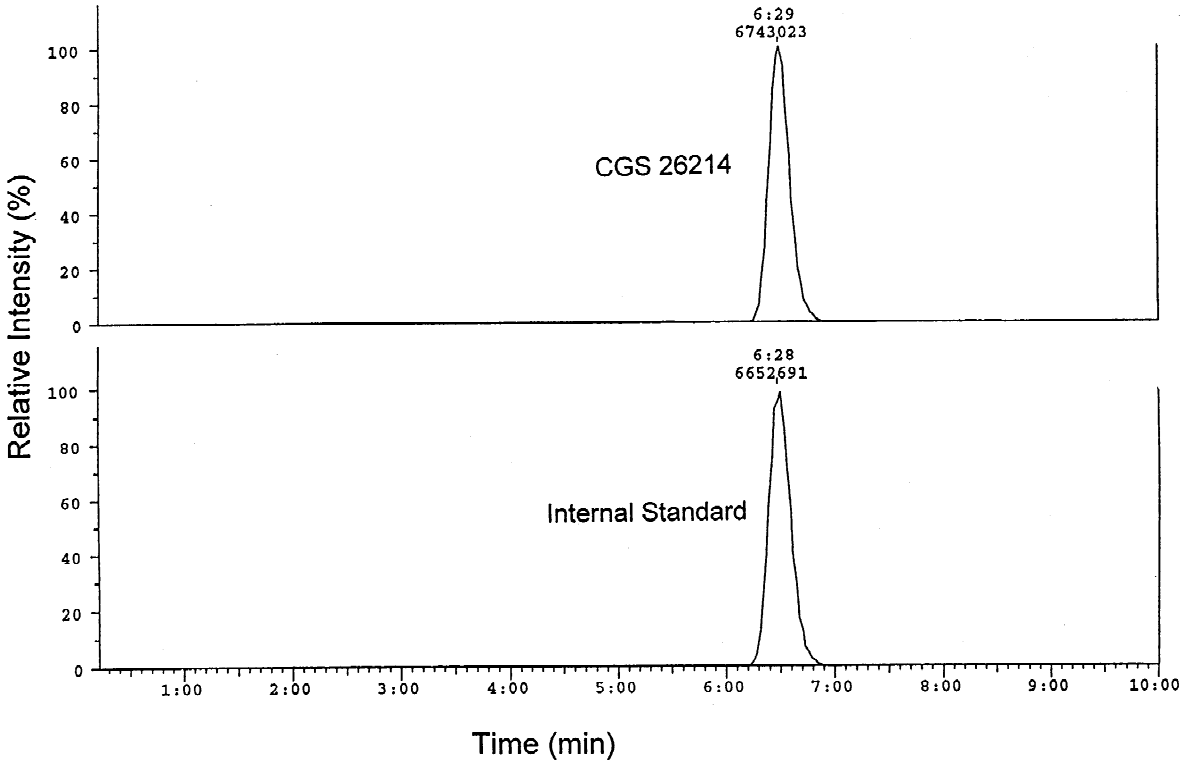

Fig. 5. HPLC–ESI-MS–MS ion chromatograms of blank human plasma spiked with 5000 pg/ml each of CGS 26214 and internal standard.

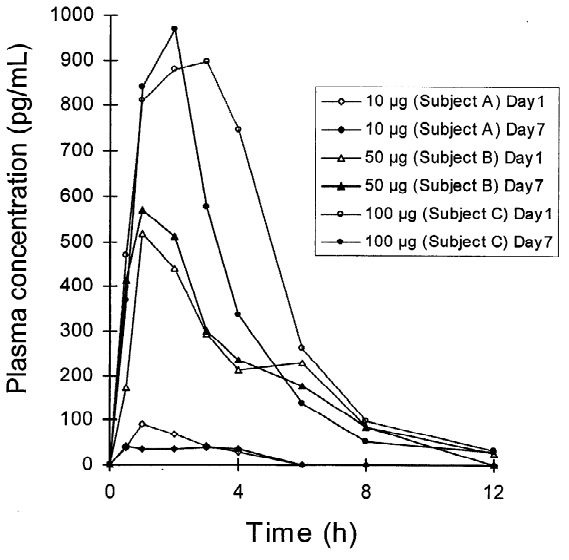

Fig. 6. Plasma concentration–time profiles for patients dosed with CGS 26214.

the LLOQ requirement were addressed. Excellent linearity was observed over the concentration range of 50 to 5000 pg/ml. The method exhibited ruggedness and was successfully utilized in the analysis of plasma samples from several clinical studies.

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