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Quantitative analytical methods for the determination of a new hypertension drug, CGS 25462, and its metabolites (CGS 25659 and CGS 24592) in human plasma by high-performance liquid chromatography

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Abstract

Two simple and sensitive reversed-phase high-performance liquid chromatography (HPLC) methods were developed and validated for the quantitative determination of a novel hypertension drug CGS 25462 and its major metabolites CGS 24592 and CGS 25659 in human plasma. CGS 25462 and CGS 25798 (internal standard) were purified by one-step liquid–liquid extraction with methylene chloride. The metabolites were analyzed on HPLC after plasma protein precipitation with 10% trichloroacetic acid (TCA). Separations were achieved on a Zorbax RX C_{18} column. All compounds were detected by using a fluorescence detector. The excitation wavelength was 254 nm, and emission was monitored at 325±12.5 nm. Assessment of recovery and reproducibility indicated good accuracy and precision. Over the validation concentration range of 10 to 1000 ng/ml for CGS 25462 and 25 to 5000 ng/ml for both metabolites, overall mean relative recoveries were 96% for CGS 25462, 101% for CGS 25659 and 107% for CGS 24592, and the coefficients of variation were 4.6 to 13% for CGS 25462, 9.5 to 13% for CGS 25659 and 7.7 to 15% for CGS 24592. The limits of quantification (LOQs) were 10 ng/ml for CGS 25462 and 25 ng/ml for CGS 24592 and CGS 25659, which were of sufficient sensitivity to measure the concentrations of CGS 25462, CGS 25659 and CGS 24592 in plasma samples from normal volunteers following a single 800 mg oral dose.

Keywords: CGS 25462; CGS 25659; CGS 24592

1. Introduction

(*S*)-*N*-[2-(Diphenylphosphonomethylamino)-3-(4-biphenyl)-propionyl]-3-aminopropionic acid (CGS 25462), the diphenyl phosphonate prodrug of is a novel selective neutral endopeptidase (NEP) inhibitor. The compound after oral administration to rats was metabolized to a pharmacologically active compound aminophosphonate (CGS 24592). CGS 24592 elevates plasma atrial natriuretic factor (ANF) levels by blocking the endopeptidase 24.11, which is an important mediator of degradation of ANF [1]. This compound showed antihypertensive activities in deoxycorticoste acetate (DOCA) salt hypertensive rats. After oral administration of 30 mg/kg of CGS 25462 mean arterial pressure was significantly re-

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duced up to −35±7 mmHg at 5 h postdosing [1,2] (1 mmHg=133.322 Pa). Since ANF has been shown to decrease blood pressure, and to stimulate natriuresis and diuresis in a variety of biological systems, the potentiation of ANF by CGS 24592, is expected to have therapeutic antihypertensive and diuretic efficacy for patients with hypertension or congestive heart failure.

This paper describes two simple high-performance liquid chromatography (HPLC) methods, one for the determination of CGS 25462 [prodrug, (**I**)] and other for the determination of two metabolites CGS 25659 [intermediate, (**II**)], the monophenyl phosphonate and CGS 24592 [active, (**III**)], the aminophosphonate in human plasma. Both methods were successfully used to analyze clinical samples from a pilot single-dose study in normal healthy volunteers.

2. Experimental

2.1. Materials

Compounds **I**, **II**, **III** and internal standard [CGS 25798, **IV**], Fig. 1, were supplied by Ciba-Geigy, Summit, NJ, USA. 4-Biphenylacetic acid (98%) (Fig. 1) was purchased from Aldrich (Milwaukee, WI, USA). Sodium perchlorate (analytical-reagent

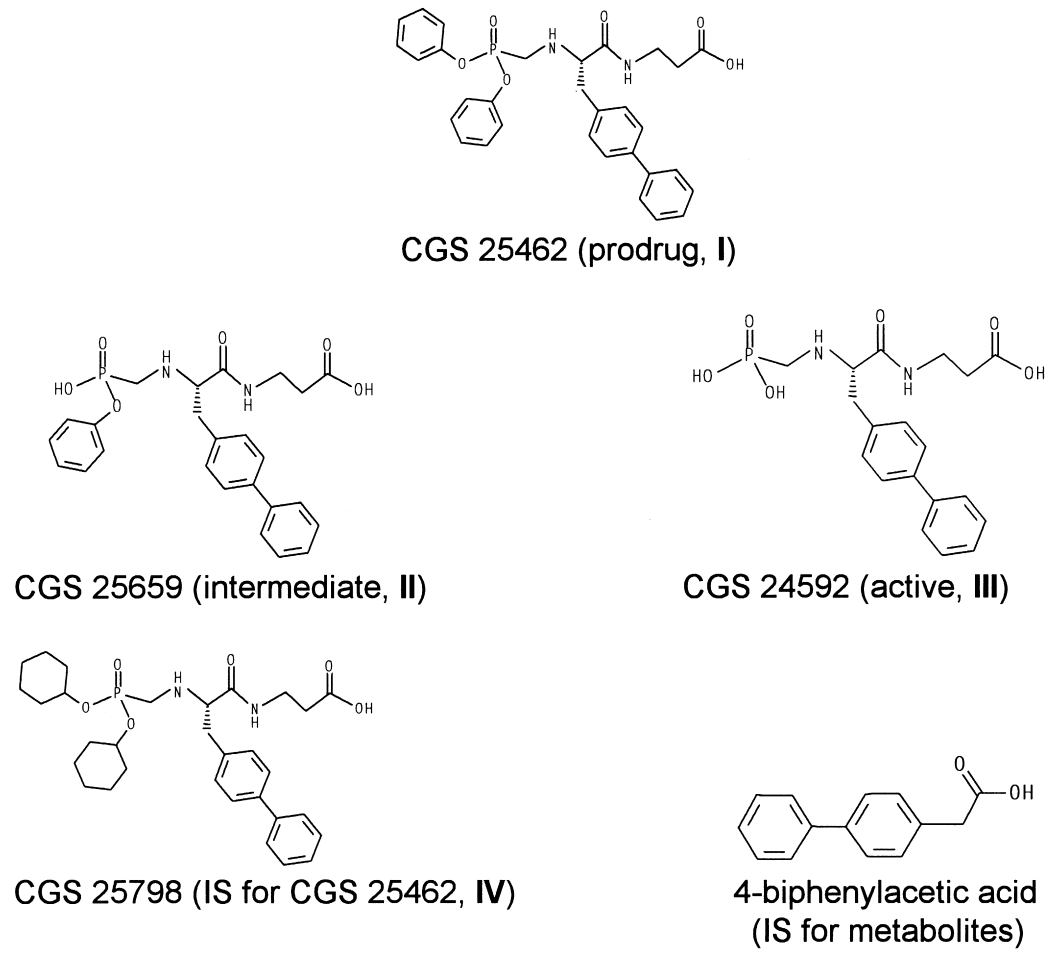

Fig. 1. Structures of CGS 25462, CGS 25659, CGS 24592, CGS 25798 and 4-biphenylacetic acid.

grade) and *o*-phosphoric acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Acetonitrile, methanol, water and methylene chloride (HPLC grade) were purchased from Burdick and Jackson (Muskegon, MI, USA). Sodium bicarbonate (reagent grade) and trichloroacetic acid (TCA, reagent grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA). Human plasma from heparinized blood was purchased from Biological Specialty Corporation (Lansdale, PA, USA).

2.2. Preparation of standard solutions

Stock solutions (2.0 mg/ml) were prepared in acetonitrile for **I**, in 0.25 *M* sodium bicarbonate–acetonitrile (1:1, v/v/) for **II** and in 0.25 *M* sodium bicarbonate for **III**.

Plasma spiking solutions (1.0, 2.0, 5.0, 10, 25, 50 and 100 μg/ml) of **I** and both metabolites (0.5, 1.0, 2.0, 10, 20, 50 and 100 μg/ml, each) were prepared by serial dilutions of the 2.0 mg/ml stock solution in acetonitrile for **I** and in sodium bicarbonate–acetonitrile (1:1, v/v) for both metabolites.

Internal standard (**IV** or 4-biphenylacetic acid) stock solution was prepared at a concentration of 2.0 mg/ml in acetonitrile, and was diluted to give the spiking solution final concentration of 25 μg/ml of **IV** and 100 μg/ml of 4-biphenylacetic acid.

2.3. Preparation of calibration standards and quality control samples

Calibration standards were prepared in duplicate, on a daily basis by adding 10-μl aliquots of the original spiking solutions to 1.0 ml of control human plasma for **I** and to 200 μl of plasma for both metabolites, to give final concentrations of 10, 20, 50, 100, 250, 500 and 1000 ng/ml for **I**, and 25, 50, 100, 500, 1000, 2500 and 5000 ng/ml for **II** and **III**.

Quality control and stability samples were prepared from the same spiking solutions as calibration standards at four different concentrations (10, 20, 500 and 1000 ng/ml) for **I** and (25, 50, 1000 and 5000 ng/ml) for **II** and **III** in control human plasma. The quality control samples were stored at −80°C until analyzed.

*2.4. Extraction procedure for **I***

A 10-μl aliquot of the 25 μg/ml internal standard (**IV**) spiking solution (final concentration 250 ng/ml) was added to each 1.0-ml plasma sample and vortexed at high speed for 5 s. One ml of 0.1 *M* sodium perchlorate (pH 1.95, adjusted with 85% *o*-phosphoric acid) was added and the compounds were extracted with 5 ml of methylene chloride. Polypropylene tubes were used for sample extraction due to adherence to glass tubes. The organic layer was transferred to a 15-ml polypropylene disposable tube and solvent was removed by evaporation at 37°C under nitrogen. The residue was reconstituted in 100 μl of acetonitrile. A 25-μl aliquot was used for HPLC analysis.

*2.5. Protein precipitation procedure for **II** and **III***

Plasma samples (200 μl) were transferred to a microcentrifuge tube. A 10-μl aliquot of the 100 μg/ml (5000 ng/ml plasma) internal standard (4-biphenylacetic acid) spiking solution was added and the tube was vortexed at high speed for 5 s. After the addition of 10% TCA (100 μl) the sample was vortexed for 5 s and centrifuged at 8600 *g* for 5 min using a microcentrifuge (Biofuge A, Baxter Scientific Products). A 25-μl aliquot of supernatant was used for HPLC analysis.

2.6. Instrumentation

Analysis of all compounds was performed using an HP 1090 system (Hewlett-Packard, Paramus, NJ, USA) equipped with a temperature-controlled autosampler. Detection was performed using a Spectraflow (Kratos Analytical, Model 783, Ramsay, NJ, USA) fluorescence detector. The excitation wavelength was 254 nm, and the emission was monitored at 325±12.5 nm by using a bandpass filter. Chromatographic separations were performed at ambient temperature on a Zorbax RX C_{18} column (5 μm, 250×4.6 mm; MAC-MOD Analytical, Chadds Ford, PA, USA). A two-solvent gradient system was applied for separation of both metabolites. Solvent A was 0.1 *M* sodium perchlorate at pH 2.3 (adjusted with 85% *o*-phosphoric acid), while solvent B was 26% of solvent A in acetonitrile. The mobile phase

was delivered at a flow-rate of 1.0 ml/min under linear gradient condition from 33% B to 70% B in 6 min.

Chromatographic separation of **I** was performed at ambient temperature on the column as described above. The mobile phase consisted of 0.1 *M* sodium perchlorate (pH 2.3)–10% 0.1 *M* sodium perchlorate (pH 2.3) in acetonitrile (33:67, v/v) and was delivered at a flow-rate of 1.0 ml/min under isocratic conditions. Analysis run time was 15 min.

2.7. Calibration and sample quantification

The calibration standards were prepared in duplicate daily, then extracted and analyzed as described above. Calibration curves were generated using weighted ($1/y$, where $y=c+bx$) linear least squares regression as the mathematical model, and were represented by plots of the peak area ratios of each compound to internal standard versus concentration of the calibrator. Quantification of quality control, stability and clinical samples were obtained by interpolation from the regression equations for the calibration curves.

Peak areas for the prodrug, metabolites and internal standard were measured using a Model 970 dual channel interface and a TurboChrom chromatography workstation (PE Nelson Systems, Cupertino, CA, USA). The chromatographic data were processed for peak area ratios of the compound to internal standard using TurboChrom II 2700 (Version 3.1).

3. Results and discussion

Compound **I** is a prodrug which is metabolized in animals to **I** and in humans to **II** (an intermediate) and **III** (an active NEP inhibitor). Initially, we attempted to develop a sensitive bioanalytical method for the simultaneous determination of all three compounds in human plasma. Due to chemical structure differences it was not possible to find a common reconstitution solution for all three compounds in which the diester (**I**), monoester (**II**) and acid (**III**) would be stable, have acceptable peak shape and good sensitivity. Compound **I** was stable in acetonitrile; however, the best peak shape for **II** and **III** was in acidic pH. In order to achieve best sensitivity and to avoid stability problem two separate assays were ultimately developed. One analytical method was developed for the determination of **I** in human plasma using liquid–liquid extraction with methylene chloride and another using TCA protein precipitation (**II** and **III**). After liquid–liquid extraction the organic solvent was evaporated to dryness and the residue was redissolved in acetonitrile. The absolute recoveries were 75% for **I** and 78% for **IV** (internal standard). Both metabolites **II** and **III** were analyzed directly after protein precipitation with 10% TCA. The absolute recoveries were 25% for **II**, 60% for **III** and 9.1% for 4-biphenylacetic acid (internal standard). All three compounds were stable at ambient temperature during HPLC analysis.

The plasma stability data are shown in Table 3. Compound **I** showed approximately 30% loss of the compound in plasma over the concentration range of 10 to 1000 ng/ml during 6 h storage at room temperature, but no loss was observed after 7, 14 and 29 days storage at −80°C. Approximately 40% of **I** was lost during the freeze–thaw experiment. Mean percent recoveries for **I** were 66.8% at 10 ng/ml, 59.3% at 500 ng/ml and 57.1% at 1000 ng/ml.

The metabolites showed no loss of compounds in plasma over the concentration range of 25 to 5000 ng/ml during 6 h storage at room temperature, after 8, 13 days, 1 month and 7 months storage at −80°C and during the freeze–thaw experiment.

Chromatograms of extracts from control human plasma and plasma spiked with 1000 ng/ml of **I** and internal standard are shown in Fig. 2A and B, respectively. The prodrug was eluted with a retention time of 5.2 min and internal standard 7.2 min. The chromatograms of extracts from control human plasma and plasma spiked with 5000 ng/ml each of **II**, **III** and internal standard are shown in Fig. 3A and B, respectively. Metabolites were eluted with retention times of 6.7 min for **III**, 9.8 min for **II** and internal standard 14.7 min. The specificity of the method was demonstrated by the lack of interfering peaks at the retention times of the parent compound and its metabolites upon analysis of control plasma from heparinized blood of adult volunteers receiving no medication.

The calibration curve data are shown in Table 1.

Table 1
Plasma stability of CGS 25462, CGS 25659 and CGS 24592 in human plasma

Storage conditions	Added concentration (ng/ml)	Mean % relative recovery ($n=2$)		
		CGS 25462	CGS 25659	CGS 24592
Day 1, 0 h, 25°C	10	96		
	25		104	83
	1000	100		
	5000		105	101
Day 1, 6 h, 25°C	10	74		
	25		84	92
	1000	71		
	5000		94	94
1 week, −80°C	10	119		
	25		90	116
	1000	92		
	5000		106	99
2 weeks, −80°C	10	114		
	25		130	82
	1000	96		
	5000		106	110
1 month, −80°C	10	100		
	25		92	100
	1000	85		
	5000		81	98
7 months	25		100	83
	5000		98	91
3 Freeze–thaw cycles	10	67		
	25		88	102
	1000	59		
	5000		105	92

Average correlation coefficients (r) of 0.9981 for **I**, 0.9986 for **II** and 0.9979 for **III**, indicated good fits to the weighted linear regression model. The mean slope data associated with the coefficients of variation (C.V.s) of 5.6% for **I**, 10% for **II** and 16% for **III** indicated good inter-day reproducibility. The data presented in Table 2 show calculated results for the inter- and intra-day accuracy and precision for **I**, **II** and **III** quality control samples. The results are expressed as a mean percent found and percent of variation (C.V.). The inter-day values were calculated using all the determinations ($n=16$ for **I** and $n=12$ for both metabolites) at the indicated concentrations. Slightly higher mean relative recoveries for the individual concentrations were allowed based on acceptable overall mean percent relative recovery (107%).

The limit of quantification (LOQ) is defined as the lowest compound concentration with acceptable accuracy (mean relative recovery 100±15%) and precision (C.V.≤20%). For these methods the LOQ was 10 ng/ml for **I** and 25 ng/ml for both metabolites.

Overall mean relative recoveries (accuracy) were 96% for **I**, 101% for **II** and 107% for **III**. The data showed good intra-day precision with C.V. values of 6.5 to 20% for **I**, 9.6 to 12% for **II** and 8.7 to 11% for **III** at the respective LOQs. Furthermore, C.V.s did not exceed 12% at the other concentrations for **I** or its metabolites. The inter-day precision was also good, with C.V. values of 13% for **I**, 9.5% for **II** and

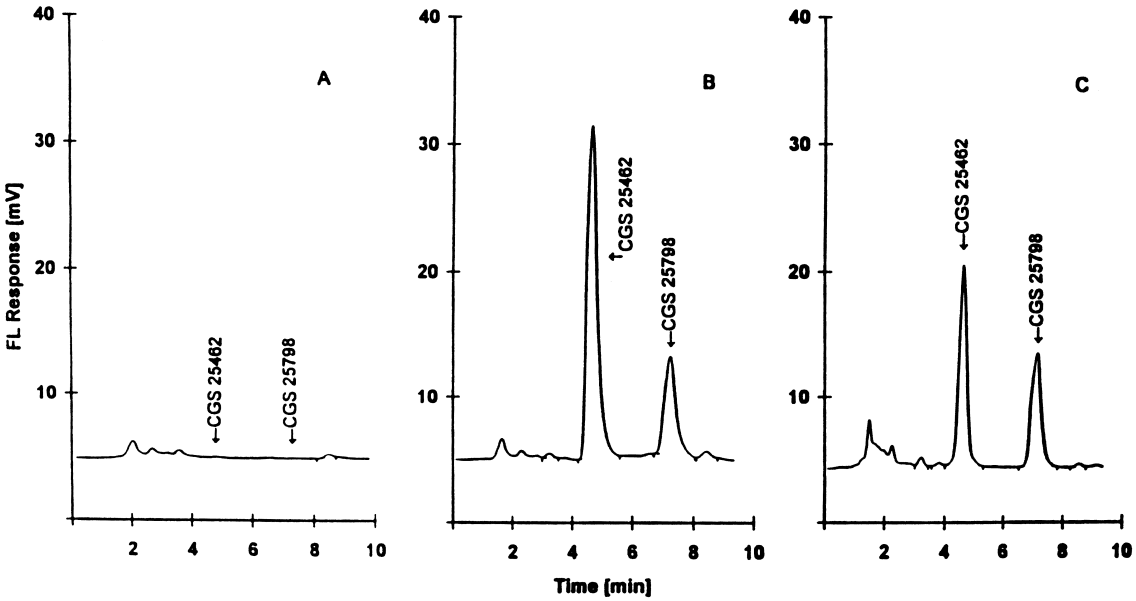

Fig. 2. Typical chromatograms of human plasma extracts of CGS 25462. (A) Control human plasma; (B) control human plasma spiked with 1000 ng/ml CGS 25462 and 250 ng/ml of CGS 25798 (internal standard); (C) plasma sample from a normal male volunteer 3 h after oral administration of an 800 mg dose of CGS 25462.

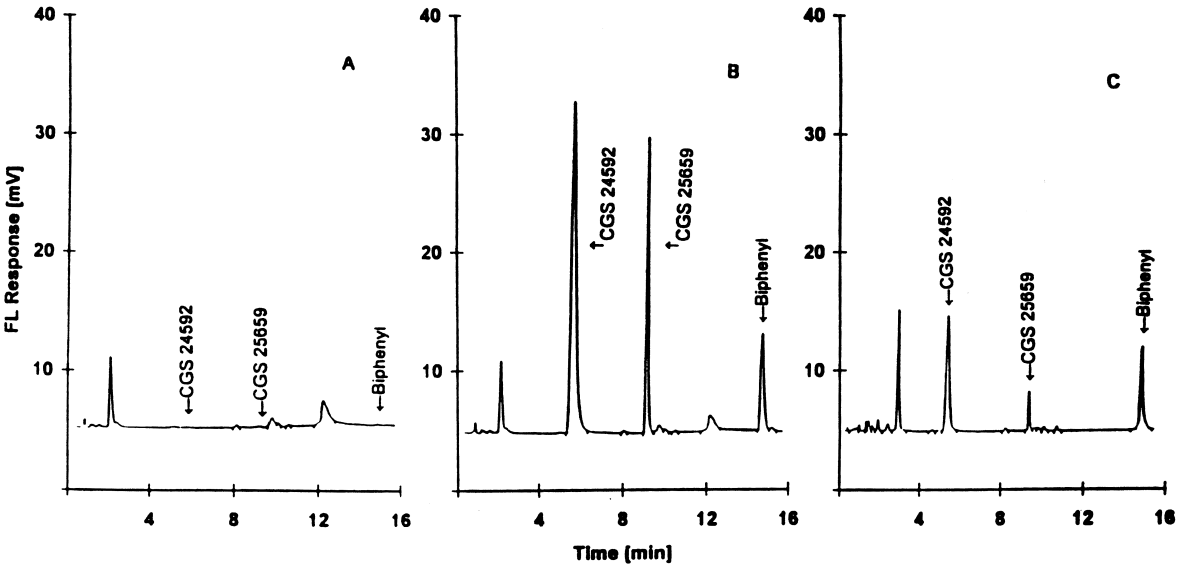

Fig. 3. Typical chromatograms of human plasma extracts of CGS 25659 and CGS 24592. (A) Control human plasma; (B) control human plasma spiked with 5000 ng/ml of CGS 25659 and CGS 24592 and 5000 ng/ml of 4-biphenylacetic acid (internal standard); (C) plasma sample from a normal male volunteer 3 h after oral administration of an 800 mg dose of CGS 25462.

Table 2
Reproducibility of CGS 25462, CGS 25659 and CGS 24592 daily calibration curves

Analysis day	Slope	Intercept	Correlation coefficient
CGS 25462			
1	0.0030	−0.0042	0.9998
2	0.0032	−0.0079	0.9975
3	0.0034	−0.0032	0.9954
4	0.0031	−0.0029	0.9995
Mean	0.0032		0.9981
S.D.	0.0002		0.0020
C.V. (%)	5.6		0.2
CGS 25659			
1	0.00028	0.0048	0.9981
2	0.00034	0.0022	0.9985
3	0.00029	0.0038	0.9992
Mean	0.00030		0.9986
S.D.	0.00003		0.0006
C.V. (%)	10		0.1
CGS 24592			
1	0.00074	−0.0020	0.9975
2	0.00102	−0.0083	0.9975
3	0.00089	−0.0082	0.9987
Mean	0.00088		0.9979
S.D.	0.00014		0.0007
C.V. (%)	16		0.1

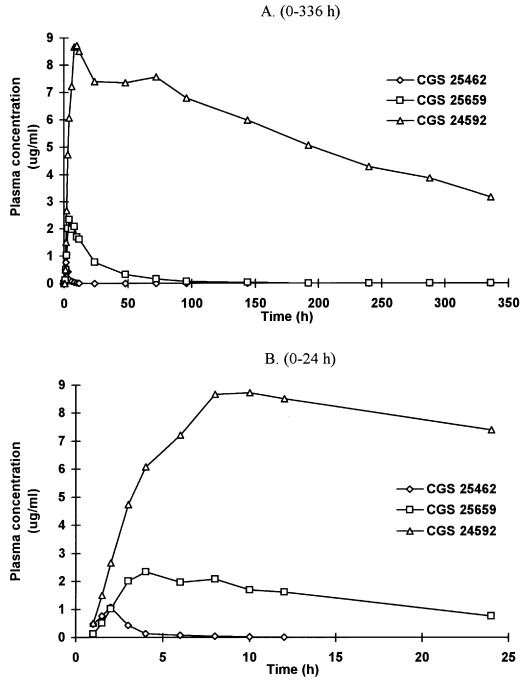

Fig. 4. Plasma concentration–time profiles of CGS 25462, CGS 25659 and CGS 24592 from a normal volunteer following oral administration of an 800 mg dose of CGS 25462. (A) Plot (0 to 336 h); (B) expanded plot (0–24 h).

Table 3
Intra- and inter-day accuracy and precision data for CGS 25462, CGS 25659 and CGS 24592 quality control samples

Added concentration (ng/ml)	Mean percent relative recovery (%C.V.)				
	Day 1	Day 2	Day 3	Day 4	Inter-day
CGS 24562					
10	109 (20)	112 (7.3)	106 (9.2)	97 (6.5)	106 (13)
20	106 (4.3)	97 (4.9)	100 (4.4)	92 (8.6)	99 (6.0)
500	96 (3.2)	88 (6.1)	88 (9.9)	88 (5.9)	91 (6.6)
1000	94 (1.0)	88 (2.1)	86 (3.8)	89 (5.8)	89 (4.6)
Overall mean recovery=96%					
CGS 25659					
25	101 (11)	109 (12)	104 (9.6)		105 (9.5)
50	114 (11)	111 (5.0)	101 (11)		109 (13)
1000	109 (2.0)	86 (1.6)	103 (1.6)		99 (11)
5000	107 (5.5)	80 (12)	100 (2.0)		96 (13)
Overall mean relative recovery=101%					
CGS 24592					
25	85 (8.7)	107 (11)	111 (11)		101 (15)
50	112 (8.4)	112 (8.7)	106 (5.8)		110 (7.8)
1000	127 (2.2)	112 (5.2)	107 (1.4)		115 (8.2)
5000	119 (7.6)	109 (4.7)	105 (3.1)		111 (7.7)
Overall mean relative recovery=107%					

Note: $n=4$ for intra-day determinations: $n=16$ (CGS 25462) and $n=12$ (CGS 25659 and CGS 24592) for inter-day determinations.

15% for **III** at the respective LOQs, and inter-day C.V. values that did not exceed 15% at the other concentrations.

These methods were used in support of a Phase I clinical trial. Fig. 2C and Fig. 3C show representative chromatograms (3 h postdose) from extracted human plasma of **I**, **II** and **III**, respectively. These plasma samples were collected from one healthy male volunteer who received a single 800 mg oral dose of **I**. Fig. 4 shows the **I**, **II** and **III** plasma concentration–time profiles from the same volunteer. The plasma concentrations for **I** indicated rapid compound absorption from the gastrointestinal (GI) tract and quick conversion to intermediate and to active metabolite. The prodrug disappeared from the plasma quickly (1.5 h half-life), while the intermediate (44 h half-life) and active metabolite declined slowly (383 h half life). Both metabolites were also detected in the urine.

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