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Quantification of GR90291 in human blood by high resolution gas chromatography–mass selective detection (HRGC–MSD)

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Abstract: A specific and sensitive high resolution gas chromatography–mass spectrometry method for the determination of GR90291 in human blood is described. The extraction of GR90291 from blood required a polar organic solvent mixture. The crude extract was further purified by successive liquid–liquid partitioning prior to esterification with an HCl–n-butanol solution. This derivative was analysed using a deuterium-labelled internal standard by selected ion monitoring mass spectrometry. The calibration curve ranged from 1 to 100 ng ml^{-1}. The method is reliable for the determination of GR90291 pharmacokinetics in human subjects.

Keywords: *Gas chromatography/mass spectrometry; GC/MS; selected ion monitoring; SIM; GR90291; GI87084; remifentanil; human blood.*

Introduction

The compound GR90291 is a rapidly formed carboxylic acid metabolite of remifentanil or GI87084 (Fig. 1). Remifentanil is a propionyl-anilinopiperidine developed by Glaxo Inc. having analgesic properties [1–5]. In order to investigate the clinical pharmacokinetics of GR90291 following an infusion of remifentanil, a sensitive and specific method was required. It was important to investigate the clinical pharmacokinetics of GR90291 in blood to ascertain its accumulation and excretion. A limit of quantification of 1 ng ml^{-1} of blood was necessary due to the low administered dose of remifentanil.

Although remifentanil and GR90291 have structural similarities to the fentanyl compounds, analytical methods that have been developed for the fentanyls have limited applicability to GR90291. The development of this analytical method was complicated by (1) the rapid hydrolysis of parent drug in whole blood, (2) the zwitterionic nature of GR90291 at neutral pH, and (3) its affinity for cell debris and lipids. The first condition required extraction of the parent drug immediately after sample collection [6, 7]. The latter conditions required a polar solvent mixture for drug extraction. Further purification steps involving liquid–liquid extraction were necessary prior to derivatization.

Derivatization involved the conversion of GR90291 to an n-butyl ester of the *N*-propionic acid. This resulted in less chromatographic interference relative to other derivatization agents tested. This paper describes a gas chromatographic–mass spectrometric (GC–MS) method using a selected ion monitoring (SIM) technique for the quantification of GR90291 in human blood and its application to pharmacokinetic human studies.

Experimental

Chemicals and reagents

The drug, GR90291A (GR90291 trifluoroacetic acid salt), and internal standard, GR90291C ($^2H_4$-GR90291), were supplied by Glaxo Inc. Research Institute. Acetonitrile, 1-propanol, ethyl acetate, methylene chloride, 1-chlorobutane, 1-butanol and methanol, all glass distilled, were obtained from BDH (Montreal, Canada). Anhydrous sodium sulphate (certified), 0.1 N HCl solution (certified), and reagent grade hydrochloric acid (37%) were supplied by Fisher (Montreal, Canada). All aqueous solutions were prepared

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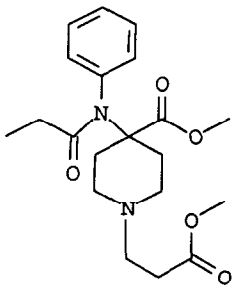
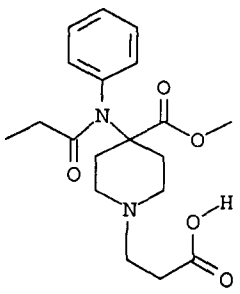


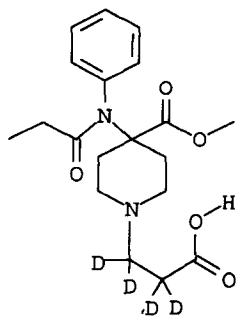
GI87084

methyl-3-(4-methoxycarbonyl-4-[{1-oxopropyl} phenylamino]-1-piperidine)-propionate
$C_{20}H_{28}N_2O_5$; Mol. wt.: 376.45



GR90291

4-methoxycarbonyl-4-[{1-oxopropyl} phenylamino]-1-piperidine-propionic acid
$C_{19}H_{26}N_2O_5$; Mol wt.: 362.43



GR90291C

4-methoxycarbonyl-4-[{1-oxopropyl} phenylamino]-1-piperidine-prop-(2,2',3,3'-tetradeutero)-ionic acid
$C_{19}H_{22}D_4N_2O_5$; Mol wt.: 366.43

Figure 1
Structures of GI87084, GR90291 and GR90291C.

with Type I Millipore water (Millipore; Toronto, Canada). Drug-free human blood was obtained from Biological Specialties (PA, USA). 0.3 N HCl in n-butanol was prepared by diluting 2.5 ml of concentrated HCl (37%, w/v) to 100 ml with n-butanol.

Gas chromatography–mass spectrometry

A Hewlett-Packard Model 5890 gas chromatograph equipped with an HP 5971A mass selective detector and an HP 5971A DOS workstation was utilized. Samples were injected using an HP 7673A autosampler using splitless injection and a purge off time of 2.0 min. The temperatures of the injection port and transfer line were set at 280°C. Electron impact ionization (EI) was performed at 70 eV at an estimated ion source temperature of 200°C.

A DB-5 fused silica capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness; J&W, CA) was utilized. The column was initially operated at 175°C for 2 min, after which the temperature was increased at a rate of 30°C min^{-1} to 310°C with a final hold time of 1.5 min. The carrier gas was helium with a column inlet pressure of 35 kPa resulting in a linear velocity of 40 cm s^{-1} at an oven temperature of 280°C.

The Hewlett-Packard Autotune program was used to tune the mass spectrometer. In order to obtain the desired sensitivity, the SIM mode was used for the determination of GR90291. The ions selected were *m/z* 210 and 269 for GR90291 and *m/z* 273 for the internal standard GR90291C. A 50 ms dwell time was used for each ion. The ion monitoring commenced at 6.1 min after injection with a

duration of 1.0 min. The retention time for GR90291 and the internal standard were approximately 6.52 and 6.51 min, respectively.

Preparation of calibration standards and quality control samples

Standards and quality control samples (QCs) were prepared by spiking blank human blood with acetonitrile solutions of differing GR90291 concentrations. The ratio of the volume of acetonitrile to blood was kept at 1% for all standards and QCs. After mixing and equilibration, 1.2 ml aliquots of standards and QCs were dispensed in polypropylene tubes and stored at −20°C until analysis. The calibration curve ranged from 1 to 100 ng ml^{-1}.

Sample, standard and QC pretreatment

Blood samples, standards and QCs (1 ml) were pipetted into 16 × 125 mm screw cap tubes containing 2 ml of acetonitrile, 4 ml of dichloromethane and 50 μl of an acetonitrile–internal standard solution (equivalent to 100 ng of GR90291C). The tubes were immediately capped and vortexed for 30 s. Following centrifugation at 1850 ***g*** for 10 min, the organic phase was removed and discarded. If necessary, the pretreated blood samples were stored at −20°C pending analysis.

Extraction procedure

After sample thawing, 2 ml of 1-propanol was added to each tube and vortexed for approximately 30 s. Four millilitres of methylene chloride were added to each tube and the tubes were capped and mixed for 10 min on a reciprocating shaker. Following centrifugation at 10°C (1850 ***g*** for 10 min), the lower organic layer was transferred into 13 × 100 mm screw cap culture tubes containing approximately 800 mg of anhydrous sodium sulphate. Following shaking and centrifugation, the dried organic phase was transferred into a clean 13 × 100 mm screw cap tube and was evaporated to dryness under a gentle stream of nitrogen at 60°C.

The residue was redissolved in 500 μl of 0.01 N HCl. This solution was washed three times with 5 ml of 1-chlorobutane on the reciprocating shaker (5 min), centrifuged at 10°C (1850 ***g*** for 5 min), and the organic supernatant was discarded. After the last wash, 1 ml of methanol was added to the remaining aqueous layer in each tube and vortexed. The mixture was evaporated to dryness under a gentle stream of nitrogen at 60°C.

Derivatization

To the residue was added 250 μl of a 0.3 N HCl–butanol solution. Each tube was capped and vortexed for approximately 15 s and placed in a dry bath at 70°C for 1 h. Following reaction, excess reagent was evaporated overnight to dryness under vacuum in the Speed Vac Evaporator (Savant; NY, USA). The residue was reconstituted in 50 μl of ethyl acetate.

Quantitation

Each calibration curve was determined by fitting a curve to the data pairs of peak area ratio and nominal standard concentrations by the method of least squares. The peak area ratio was calculated by dividing the peak area for GR90291 (m/z = 269) by the peak area for internal standard GR90291C (m/z = 273). A log polynomial equation known as the Wagner regression was utilized. The form of the equation is as follows:

$$\ln(y) = a[\ln(x)]^2 + b[\ln(x)] + c,$$

where x is the GR90291 concentration in ng ml^{-1} of blood, y is the peak area ratio of GR90291 over GR90291C, and a, b and c are the curve parameters. The concentrations of analytes in QC and study samples were calculated by substituting the peak area ratio in the equation for the determined regression curve and solving for x.

Stability

The stability of a GR90291 stock solution prepared in acetonitrile was evaluated, as well as in human blood at 4.0 and 80.2 ng ml^{-1}, stored under various conditions.

Collection of clinical samples

Subjects were administered GI87084 by intravenous infusion. Blood samples were collected prior to administration of the drug, during, and after infusion at the sampling times indicated in the clinical protocols. A typical protocol called for sampling times at 1, 2, 3, 5, 7, 10, 15, 20, 25, 30, 45, 60, 90, 120, 150 and 170 min during infusion, and post-infusion times of 1, 3, 5, 7, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min. All blood samples were immediately treated as described

in the *Sample, standard and QC pretreatment* section and stored at −20°C until analysis.

Results

Linear fit

For the method's quantitation range of 1–100 ng ml^{-1}, a correlation coefficient (r) >0.9991 was achieved in 22 consecutive analytical runs.

Within-batch GR90291 accuracy and precision

Replicate human blood blanks were spiked, washed and frozen on a single day with GR90291 at concentrations of 1.0, 4.0, 40.1 and 80.2 ng ml^{-1}. These were quantitated using a freshly prepared calibration standard set analysed on a single day. The results are summarized in Table 1. The relative standard deviations (RSDs) for the within-batch quality control and limit of quantitation precision results ranged from 4.0 to 10.7%. Comparison of actual and calculated quality control concentrations of GR90291 ranged from 94.3 to 110.9% of nominal.

Between-batch GR90291 accuracy and precision

Replicate human blood blanks were spiked, washed and frozen on a single day with GR90291 at quality control concentrations of 4.0, 40.1 and 80.2 ng ml^{-1}. The quality control standards were analysed on four separate days using calibration standard curves prepared on each of the analytical days. The results obtained are summarized in Table 2. The RSDs for the between-batch quality control precision results ranged from 3.5 to 7.4%. Comparison of actual and calculated quality control concentrations of GR90291 ranged from 98.2 to 106.1% of nominal.

Extraction recovery

Recovery was determined by comparing the peak area ratio of extracted QCs at 4.0, 39.9 and 79.9 ng ml^{-1}, to an unextracted standard curve of GR90291. The recovery of GR90291 from 1 ml of human blood ranged from 76.8% at 4 ng ml^{-1} to 59.3% at 80 ng ml^{-1}.

Stability

Immediately after collection, GR90291 is paired in blood samples with the stable isotope internal standard. The concentration stability of GR90291 in clinical samples is compared relative to a species with nearly identical physico-chemical properties. No significant deviations in the $^{1}H_4$-GR90291/$^{2}H_4$-GR90291 ratio are seen in clinical samples, calibration standards, or quality controls.

The stability data for GR90291 in blood at room temperature for 9 h, frozen at −20°C for 6 months, following three freeze–thaw cycles, and left as dry post-derivatized residue at room temperature for 24 h are presented in Table 3. No statistically significant degradation of

Table 1
Within batch precision and accuracy for the quantitation of GR90291 in human blood

Concentration (ng ml^{-1})					
Theoretical	Found	SD	RSD (%)	Accuracy (%)*	n
1.0	1.11	0.118	10.7	110.9	8
4.0	3.78	0.245	6.5	94.3	10
40.1	42.87	1.338	3.1	106.8	10
80.2	83.08	3.34	4.0	103.5	10

*Defines as $\frac{[\text{Found}] \times 1.0}{[\text{Theoretical}]}$.

Table 2
Between batch precision and accuracy for the quantitation of GR90291 in human blood

Concentration (ng ml^{-1})						
Theoretical	Found	SD	RSD (%)	Accuracy (%)	n	Number of analytical runs
4.0	3.94	0.20	7.4	98.2	36	18
40.1	42.57	2.12	5.0	106.1	35	18
80.2	79.36	2.76	3.5	98.9	35	18

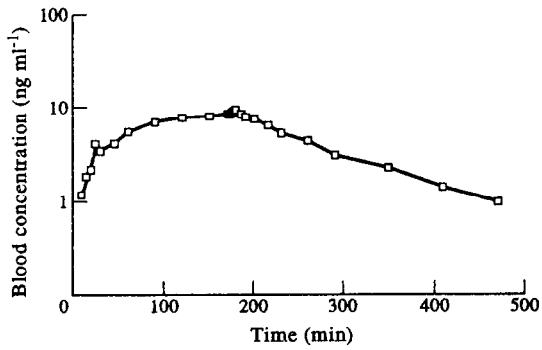


Figure 2
Representative concentration vs time profile for GR90291 in human blood.

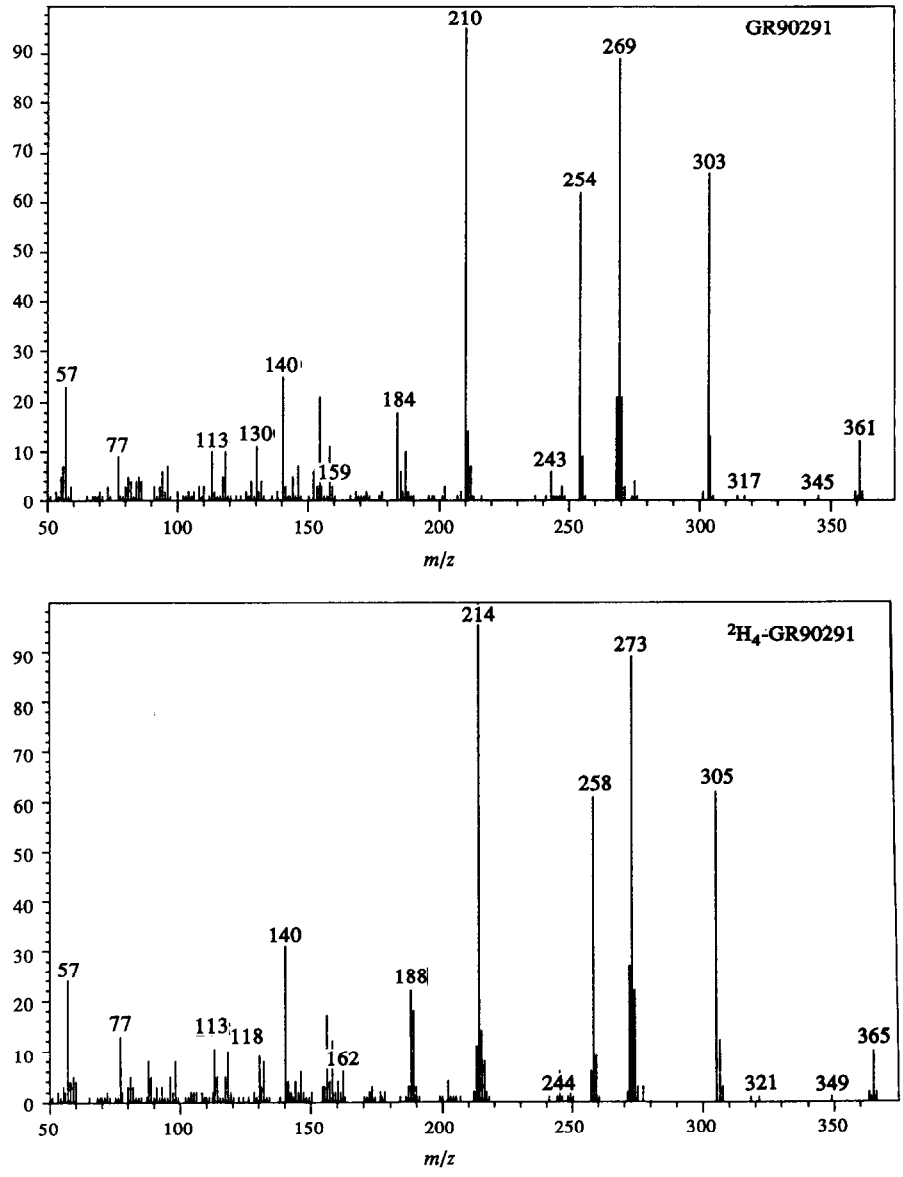


Figure 3
Mass spectra of GR90291 and $^2H_4$-GR90291 n-butylester derivatives.

Table 3
Stability of GR90291 during sample preparation (expressed as a percentage of the nominal concentration)

Stability conc. GR90291 (ng ml^{-1})	Room temp stability (blood) (9 h)	Freeze–thaw stability (blood) (3 cycles)	Dried extract stability (24 h)
40	101.6%	105.0%	98.0%
80.2	101.1%	98.5%	99.3%

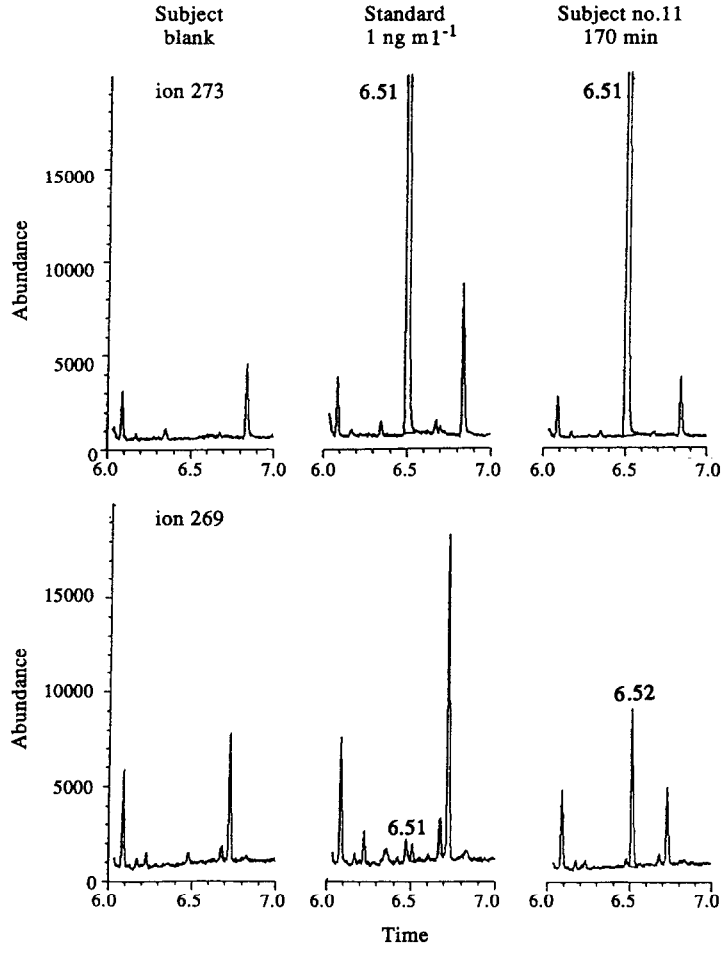


Figure 4
Ion chromatograms obtained from extracted samples of subject blank, extracted standard at 1 ng ml^{-1} and subject 11 at 170 min of infusion.

GR90291 at 4 and 80 ng ml^{-1} was observed. For the dry post-derivatized residue, although the ratio of the metabolite to internal standard remains constant for these storage conditions, analyte response diminishes. It is recommended that samples are analysed within 24 h of preparation.

The stability of GR90291 in acetonitrile was also evaluated for a 50 μg ml^{-1} solution. No evidence of degradation was found after a period of 217 days at a storage temperature of 20°C.

Clinical pharmacokinetics

The described analytical method was used to determine the subject blood concentrations of GR90291 obtained during infusion of GI87084. A representative blood concentration vs time profile obtained from a subject with a total dose of 10.5 μg kg^{-1} administered via

computer-assisted continuous infusion (CACI) over 180 min is presented in Fig. 2.

Discussion

The mass spectra of GI87084 and the GR90291 n-butyl ester derivative are shown in Fig. 3. SIM mode was chosen for its specificity and sensitivity. The *m/z* 269 ion for GR90291 and *m/z* 273 ion for GR90291C were preferred to the more abundant base peaks. These ions were generally less interfered than the base peak ions. The *m/z* 210 ion for GR90291 was also monitored to provide confirmation of method specificity. Use of the stable isotope internal standard corrected for imprecisions associated with numerous sample preparation steps and fluctuations in the electron multiplier response.

A minimum of 5:1 *S/N* response at the lower limit of quantitation was observed for the quantitation ions. The ion chromatograms from extracted blood samples of a 1 ng ml^{-1} standard, subject blank and subject sample (collected at 170 min) are shown in Fig. 4.

During development of this method several esterification reagents were tested. The use of anhydrous or more concentrated HCl in n-butanol resulted in partial transesterification of the methyl ester at the 3-acetate function. It was also determined that the derivatization reagent required evaporation overnight in a vacuum centrifuge. When conventional evaporative techniques were attempted, chromatograms often showed no analyte or internal standard peaks. It is possible that catalytic amounts of the derivatization reagent cause the derivative to hydrolyse.

It may be that the techniques described herein for the isolation of a polar zwitterionic metabolite may be useful in the analysis of other compounds of this type.

Conclusion

The method described herein is a sensitive, accurate and reliable assay for the determination of GR90291 in human blood and has been successfully used for pharmacokinetic studies.

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