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Bioanalysis and preliminary pharmacokinetics of the acridonecarboxamide derivative GF120918 in plasma of mice and humans by ion-pairing reversed-phase high-performance liquid chromatography with fluorescence detection

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Abstract

We have developed and validated a sensitive and selective method for the determination of the P-glycoprotein modulator GF120918 in murine and human plasma. Chlorpromazine is used as internal standard. Sample pretreatment involves liquid–liquid extraction with *tert*-butyl methyl ether. Chromatographic separation is achieved by reversed-phase high-performance liquid chromatography using a Symmetry C_{18} column and detection was accomplished with a fluorescence detector set at excitation and emission wavelengths of 260 and 460 nm, respectively. The mobile phase consists of acetonitrile–50 m*M* ammonium acetate buffer, pH 4.2 (35:65, v/v). To achieve good separation from endogenous compounds and to improve the peak shape the counter-ion 1-octane sulfonic acid (final concentration 0.005 *M*) was added to the mobile phase. The lower limit of quantitation was 5.7 ng/ml using 200 μl of human plasma and 23 ng/ml using 50 μl of murine plasma. Within the dynamic range of the calibration curve (5.7–571 ng/ml) the accuracy was close to 100% and within-day and between-day precision were within the generally accepted 15% range. The stability of GF120918 was tested in plasma and blood from mice and humans incubated at 4°C, room temperature, and 37°C for up to 4 h. No losses were observed under these conditions. This method was applied to study the pharmacokinetics of orally administered GF120918 in humans and mice. The sensitivity of the assay was sufficient to determine the concentration in plasma samples obtained up to 24 h after drug administration.

Keywords: Acridonecarboxamide; GF120918

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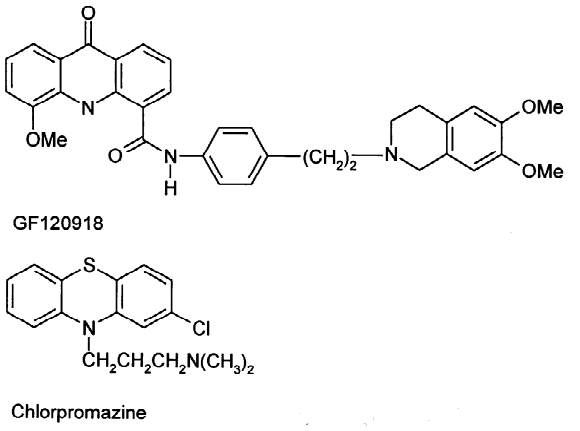

Fig. 1. Structures of GF120918 and the internal standard chlorpromazine.

1. Introduction

GF120918 (Fig. 1) is an acridonecarboxamide derivative which has been developed in the search for more potent and selective inhibitors of P-glycoprotein (P-gp). P-gp is a product of the MDR1 gene and is located in the cell membrane where it can extrude a wide variety of many different substances from the cell [1]. Its expression in drug-selected or transfected mammalian cancer cell lines is known to confer the multidrug resistance phenotype to these cells. The presence of P-gp in tumors of patients is believed to be associated with the clinical resistance of these tumors [2]. Besides its expression in tumor cells, P-gp is also expressed in a number of normal tissues, especially at barrier sites, such as the gut epithelium, brain endothelial cells and in the placenta where it plays a prominent role in the protection of the host [3–5].

Since the observation that non-cytotoxic dose levels of verapamil were able to inhibit P-gp functioning and restore the cytotoxicity of vinca alkaloids in a vincristine-resistant murine leukemia cell-line both in vitro and in vivo [6], numerous other so called reversal agents have been identified with higher potency and less toxicity, including GF120918 [7]. By using in vitro models it has been shown that GF120918 was able to block P-gp completely at concentrations of 0.05–0.1 μ*M*, rendering this compound about 100-fold more potent than the P-gp modulator cyclosporin A [7]. This level of GF120918 can be achieved in plasma of animals without significant systemic toxicity's, although the plasma concentration required for in vivo inhibition of P-gp may be higher due to protein binding [8,9].

In order to support the development of GF120918, an HPLC assay has been developed. Some of the technical details of this method are briefly described by Hyafil et al. [7]. Following alkaline extraction with diethyl ether, GF120918 was separated on a non-silica based PRPI column using a mobile phase with a pH of 11.5. These alkaline conditions were chosen because of an improvement in fluorescence signal at higher pH allowing quantification as low as 10 ng/ml.

In a later paper, Witherspoon et al. [10] described the use of liquid–liquid extraction with *tert*-butyl methyl ether. The separation of GF120918 and the internal standard (a structural analog) from endogenous substances was achieved using a Hypersil ODS column and a mobile phase at a pH of 4.0. In addition the pH of the column effluent was increased by adding 0.1 *M* NaOH as a post-column reagent.

For the support of our pharmacokinetic studies with GF120918 in wild-type and P-gp deficient mice and cancer patients we developed and validated a simple and sensitive assay requiring only 50 μl of mouse plasma or 200 μl of human plasma. To avoid the use of post-column reagents or alkaline mobile phases we decided to explore the usefulness of fluorescence detection at pH values compatible with a standard silica based column. Since structural analogs of GF120918 are not readily available we successfully tried to use chlorpromazine as internal standard. We also tested the stability of GF120918 in biological matrices (plasma, whole blood) to establish handling and storage conditions during pharmacokinetic studies.

2. Experimental

2.1. Chemicals

GF120918·HCl (Batch CD-0030, purity 97.5%) was kindly provided as a powder by Glaxo Wellcome (Research Triangle Park, USA). Chlorpromazine·HCl originated from BUFA (Uitgeest, The Nether-

lands). PIC B-8 (1-octane sulfonic acid) was purchased from Waters (Milford, MA, USA). 1-Chlorobutane (HPLC-grade) originated from Sigma–Aldrich, Steinheim, Germany. All other chemicals were of analytical or Lichrosolv gradient grade and were purchased from E. Merck (Darmstadt, Germany). Water was purified by the Milli-Q Plus system (Millipore, Milford, MA, USA). Blank human plasma was obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

2.2. Stock solutions

Stock solutions of GF120918 (≅ 110 μg/ml) were prepared by accurately weighing 5.5 mg of pure substance and dissolving in 50 ml of ice-cold (0–4°C) *N*,*N*-dimethylacetamide. Aliquots of these solutions were stored at room temperature, 4 or −20°C.

Chlorpromazine was used as internal standard. A 1 mg/ml stock solution was prepared by dissolving 25 mg of accurately weighed chlorpromazine in 25 ml of purified water. This stock solution was stored at −20°C. An internal standard working solution of 1 μg/ml of chlorpromazine was prepared by a 1000-fold dilution of the stock solution in acetonitrile–50 m*M* ammonium acetate buffer, pH 4.2 (35:65, v/v) and stored at −20°C.

2.3. HPLC instrumentation and conditions

The HPLC system consisted of a SpectroFlow 400 solvent delivery system (Kratos, Ramsey, USA), a Model 360 autosampler (Kontron, Basel, Switzerland) provided with a 100-μl sample loop and a Model FP920 fluorescence detector (Jasco, Hachioju City, Japan). Chromatographic separations were performed using a stainless steel column (150×4.6 mm I.D.) packed with 3.5 μm Symmetry C_{18} material (Waters, Milford, MA, USA). The mobile phase was prepared by mixing 350 ml of acetonitrile with 650 ml of 50 m*M* ammonium acetate buffer, pH 4.2, containing 1 bottle of Pic B-8 (resulting in a final concentration of a 0.005 *M* 1-octane sulfonic acid). The mobile phase was delivered with a flow-rate of 1 ml/min, and fluorescence detection was used with excitation wavelength and emission wavelength set at 260 and 460 nm, respectively. The bandwidth of emission monochromator was set at 40 nm and the gain at 100.

Peak recording and integration were performed on an SP4600 Datajet integrator connected to a WINner/8 system provided with WINner for windows software (ThermoSeparations Products, Fremont, CA, USA).

Emission and excitation spectra were recorded using the FP920 fluorescence detector with the detector cell filled with 10 μg/ml of GF120918 in buffer solutions of pH ranging from 3.6 to 11.2 with 5 μg/ml of GF120918 in 0.1 *M* NaOH.

2.4. Calibration standards and quality control samples

Different stock solutions with separate weighing of the analyte were used for preparing the calibration standards and the quality control samples. For each run, calibration samples were prepared containing GF120918 (free base) at concentrations of 5.3, 10.7, 21.3, 53.3, 106, 213, and 533 ng/ml by dilution of the 100 μg/ml stock solution in drug-free human plasma.

Quality control samples in human plasma were prepared by dilution of the 100 μg/ml stock solution in drug-free human plasma to final concentrations of 5.7, 11.4, 57.1, and 228 ng/ml. Unknown specimens with concentrations higher than the upper limit of quantitation (ULQ) will be diluted in drug-free human plasma. Given the similarities between the matrix of the standards and the quality controls in human plasma we did not include a QC-sample with a concentration above the ULQ.

Quality control samples in mouse plasma were prepared by diluting the 100 μg/ml stock solution by a factor of ten with drug-free human plasma. Subsequently, this solution was further diluted with drug-free mouse plasma to achieve final concentrations of 22.8, 114 and 571 ng/ml.

2.5. Sample pretreatment

A volume of 200 μl of human plasma or 50 μl of mouse plasma supplemented with 150 μl of blank human plasma was pipetted into glass tubes provided with teflon lined screw caps. Samples with concentrations of GF120918 above the dynamic range of

the calibration curve were diluted in blank human plasma prior to sample pretreatment. Volumes of 50 μl of the internal standard solution and 3 ml *tert*-butyl methyl ether were added and the vials were mixed vigorously for 5 min. After centrifugation for 5 min at 2000 *g*, the aqueous layer was frozen by dipping the vials in a bath of ethanol–solid carbon dioxide. The upper organic layer was decanted into a glass tube and evaporated at 37°C under a gentle stream of nitrogen. The residue was reconstituted by sonication for 5 min in 150 μl of acetonitrile–50 m*M* ammonium acetate buffer, pH 4.2 (30:70, v/v). The solution was transferred into an Eppendorf vial and placed in the HPLC autosampler.

2.6. Assay validation

The assay was validated according to generally accepted principles [11]. Statistical analyses were done with the computer program SPSS (version 9.0; SPSS Inc., Chicago, IL, USA).

2.7. Linearity

Linear regression of the peak ratios of GF120918 and internal standard versus concentration was weighed by $1/x^2$ (the reciprocal of the square of the concentration). The *F*-test for lack of fit ($\alpha = 0.05$) was used to evaluate the linearity of the calibration curves.

2.8. Accuracy and precision

Quality control samples in human plasma in five-fold and in mouse-plasma in six-fold were processed and analyzed in three different analytical runs. The accuracy was calculated by dividing the observed concentration and the nominal concentration and multiplied by 100%. An estimate of the between-day precision was obtained by one-way analysis of variance (ANOVA) for each control sample using the run day as the classification variable. The day mean square (DayMS), error mean square (ErrMS) and the grand mean (GM) of the observed concentrations across run days were obtained. The following formulas were used to calculate the within-day precision (WDP) and between-day precision (BDP) for each quality control sample:

$$\mathrm{WDP\%} = (\mathrm{ErrMS})^{0.5}/\mathrm{GM} \cdot 100\%$$

$$\mathrm{BDP\%} = [(\mathrm{DayMS} - \mathrm{ErrMS})/n]^{0.5}/\mathrm{GM} \cdot 100\%$$

where n is the number of replicates in each run.

2.9. Selectivity

Blank human plasma from six different healthy donors and blank plasma from four FVB-mice were processed and analyzed to determine whether endogenous plasma peaks co-eluted with GF120918 or the internal standard.

2.10. Lower limit of quantitation (LLQ)

The LLQ was determined by spiking the blank plasma samples from the six healthy volunteers and was established when both the accuracy was within the 100±20% range and the WDP% was smaller than 20%.

2.11. Recovery

To determine the extraction recovery, the slopes of the processed calibration curves were compared to standard curves prepared by dilution of the GF120918 stock solution in acetonitrile–50 m*M* ammonium acetate buffer, pH 4.2 (35:65, v/v). The recovery of the internal standard was calculated from the mean peak area of processed samples versus an aliquot of internal standard diluted three-fold in acetonitrile–50 m*M* ammonium acetate buffer, pH 4.2 (35:65, v/v).

2.12. Stability

The stability of GF120918 was tested at concentrations of 200 ng/ml in human plasma and blood and 500 ng/ml in mouse plasma and blood. Aliquots were stored on ice (0–4°C), at room temperature (22–26°C) and at 37°C for up to 4 h.

2.13. Glaxo Wellcome analytical method

Serum concentrations of GF120918 were determined by a validated HPLC method developed by Glaxo Wellcome (unpublished) consisting of liquid–

liquid extraction followed by reverse phase HPLC using fluorescence detection. Alkalinized serum samples were extracted with methyl tertiary butyl ether and reconstituted in mobile phase. The mobile phase consisted of acetonitrile–50 m*M* ammonium acetate buffer, pH 4.0 (45:55, v/v) at a flow-rate of 1 ml/min. The separation was performed on a BDS Hypersil C_{18} column (25 cm×4.6 mm). Sodium hydroxide (0.25 *M*) was added post-column at a flow-rate of 1 ml/min through a mixing tee, and the combined effluent was monitored by fluorescence detection (excitation 270 nm, emission 524 nm). Sample processing was fully automated using a Zymark robotics system. Quantitation was based on a calibration curve of GF120918 concentration versus the ratio of analyte peak height/internal standard peak height. The range of the calibration included concentrations from 2.5 to 1000 ng/ml. The within-day and between-day accuracy of the mean for the quality control samples ranged from 96 to 101% and 97 to 103%, respectively of the nominal concentrations. The within-day and between-day precision for the quality control samples was 4.1 and 3.6%, respectively. The method was linear over the concentration range investigated.

2.14. Cross-validation

A batch of 62 samples from a clinical study in which patients received orally a dose of 1000 mg of GF120918 were analyzed in different analytical runs. This clinical study was aimed to enhance the oral bioavailability of paclitaxel, an anti-tumor agent, which is efficiently transported by P-gp in the gut wall, when given in combination with GF120918. Plasma samples for GF120918 were taken at several time points up to 49 h after intake and placed immediately on ice until shipment on dry-ice to Glaxo Wellcome where the analysis of GF120918 was performed. A second set of samples was collected in parallel for the bioanalysis of paclitaxel at our Institute. These samples were thawed and frozen at least once for the determination of paclitaxel, before they were used for quantification of GF120918. The data obtained from Glaxo Wellcome were compared with our data by using weighted Deming regression analyses. These regression analyses were done with the computer program Method Validator (version 1.16; by P. Marquis, http://perso.easynet.fr/~philimar).

3. Results and discussion

3.1. Detection

We investigated the fluorescence properties of GF120918 by recording emission and excitation spectra using the FP920 detector at pH values ranging from 3.6 to 13 because we wanted to eliminate the need for using an alkaline mobile phase or make-up flow (Fig. 2). When diluted in 0.1 *M* NaOH the emission maximum was found at 560 nm with excitation at 280 nm. When the pH of the solvent was decreased to values compatible with regular HPLC conditions the emission maximum shifted to a lower wavelength. The intensity at the emission maximum was about two-fold lower at this

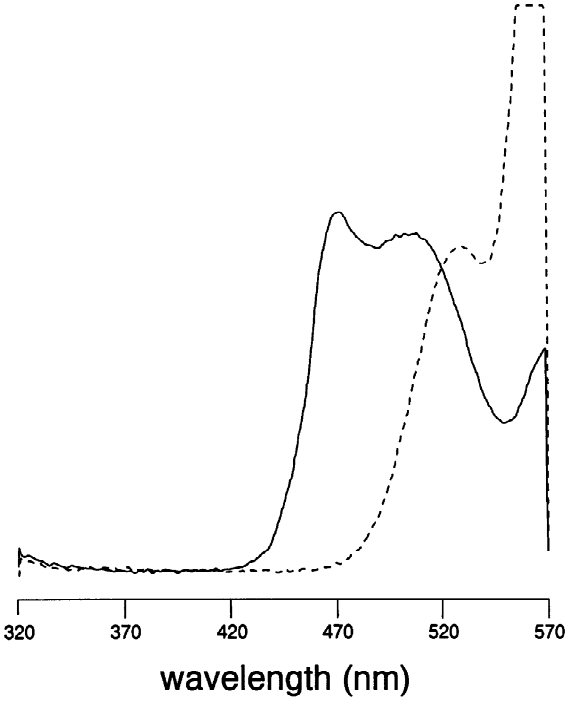

Fig. 2. Emission spectrum of GF120918 (10 μg/ml) with pH 3.6 (———) and GF120918 (5 μg/ml) with pH 13 (-------) at wavelengths of 320–570 nm and at excitation wavelength of 280 nm.

lower pH, however the total amount of energy emitted remained about similar, albeit distributed over a broader wavelength range (440–540 nm). The detector signal can be maximized by setting the bandwidth of the emission monochromator to the maximum value (40 nm).

This lower wavelength for emission has the additional advantage that it is more distant from 560 nm, the wavelength which fits twice the excitation wavelength of 280 nm. Grating-monochromators are also transparent for wavelengths which are *n*-fold (*n* being 1, 2, 3, ...) shorter than the preset wavelength and consequently, light coming from the excitation source which is scattered by particles in the detector cell may reach the photomultiplier tube resulting in increased noise. This scatter was more present in the alkaline solution, even when the detector cell was filled with NaOH solution without GF120918 in, which may be due to the fact that this solution was not filtered before use.

Later, when appropriate HPLC conditions had been established (see below), we established the optimum signal: noise ratio by repeated injections of solutions of GF120918 and stepwise increments of the emission (440 to 520 nm) and excitation (240 to 280 nm) wavelengths. We found an optimum at 260 and 460 nm for excitation and emission, respectively.

3.2. Chromatography

Chlorpromazine was tested as internal standard because it shares a number of structural similarities with GF120918. Moreover, an assay for chlorpromazine had been reported [12], describing the use of chromatographic conditions that showed similarities with the method described for GF120918 [10]. Different packing materials have been tested for the separation of chlorpromazine showing marked differences in chromatographic behavior. In line with these observations we also experienced poor chromatographic behavior of GF120918 and chlorpromazine using Spherisorb ODS-2 packing material (data not shown). On the other hand, chromatography using Symmetry C_{18} stationary phase resulted in very acceptable peak shapes for GF120918 as well as chlorpromazine using a mobile phase of acetonitrile–ammonium acetate buffer, pH 4.2 (35:65, v/v).

Peaks from endogenous substances that eluted close to the compounds of interest were, however, present in most plasma samples analyzed (Fig. 3). We tried to optimize the separation between these peaks and those of GF120918 and chlorpromazine by changing the pH of the mobile phase buffer. Shifting the pH to more acidic conditions selectively decreased the retention times of GF120918 and chlorpromazine, without changing those of the interferences. However, the analytes shifted towards the solvent front, where other interferences were also present, resulting in chromatographic conditions that were considered insufficiently robust. Shifting the peaks of the analytes away from the solvent front was possible by increasing the pH of the mobile phase, however, at the expense of serious deterioration of peak shapes. In order to further optimize the separation between these peaks and the peaks of chlorpromazine and GF120918 a negatively charged counter ion 1-octane sulfonic acid (PIC B-8, Waters) was added to the mobile phase. The retention time of the positively charged GF120918 and chlorpromazine was increased, yielding a good separation from the endogenous peaks (Fig. 4). The final composition of the mobile phase for good selectivity was obtained with acetonitrile–50 m*M* ammonium

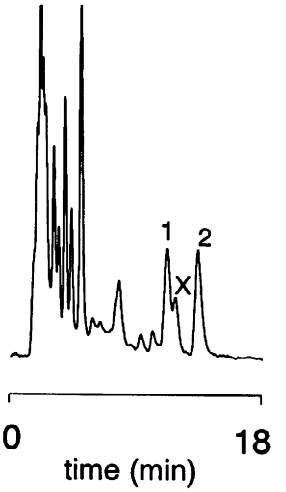

Fig. 3. Chromatogram of a mouse plasma using a mobile phase of acetonitrile–50 m*M* ammonium acetate buffer, pH 4.2 (35:65, v/v) without the counter ion PIC B-8. Note the poor separation between GF120918 (1) and an endogenous peak (X). Chlorpromazine is designated 2.

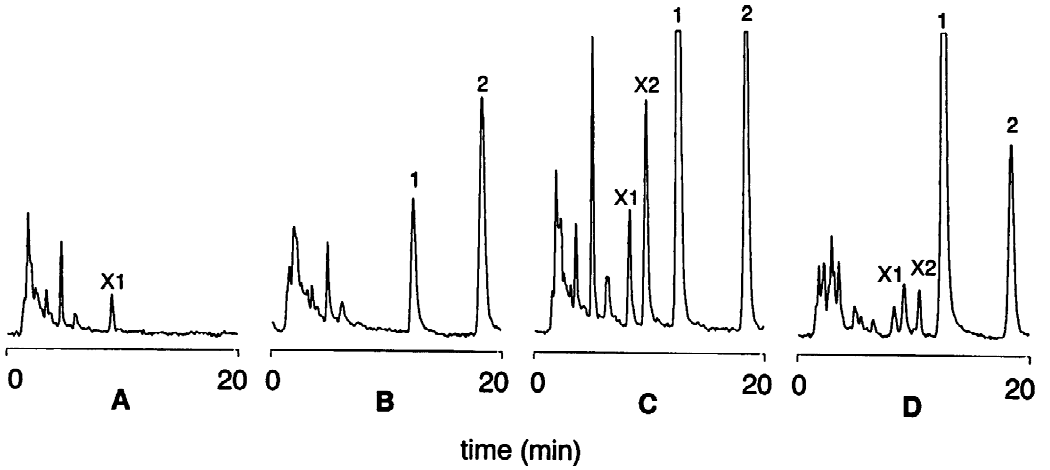

Fig. 4. Typical chromatograms of blank human plasma (A), human plasma spiked with 50 ng/ml of GF120918 (B), plasma sample from a mouse obtained at 4 h following 100 mg/kg oral GF120918, resulting in a concentration of 676.5 ng/ml (C), and a patient plasma obtained at 2.5 h following 1000 mg oral GF120918, resulting in a concentration of 235.1 ng/ml (D). Peaks: 1=GF120918, 2=chlorpromazine (internal standard), X1 and X2=peaks from endogenous substances. (attenuation=50 mV full scale).

acetate buffer, pH 4.2, PIC B-8 (35:65, v/v). No late eluting peaks were observed, allowing a total run time of 25 min per sample.

3.3. Sample pretreatment

Sample pretreatment was performed by using *tert*-butyl methyl ether. Chloroform, diethyl ether and 1-chlorobutane were also tested, but endogenous peaks, interfering with GF120918 and the internal standard, were co-extracted and the recovery of the solvents were less than the recovery of *tert*-butyl methyl ether. The absolute recovery of the sample pretreatment procedure was less than 30% using chloroform or 1-chlorobutane, and 60% in case of diethyl ether. With *tert*-butyl methyl ether the recovery was 86% for GF120918 and 91% for chlorpromazine. It was not necessary to increase the pH of the sample to alkaline conditions as described previously [7,10]. Because of these high recoveries a second extraction was not executed. The recovery in 200 μl plasma of mice was less, resulting in unacceptable accuracy's when calculated on calibration curves prepared in human plasma. However, when using 50 μl of mouse plasma (supplemented with 150 μl blank human plasma) the recovery increased

Table 1
Validation parameters of GF120918 in human and mouse plasma

	Nominal concentration (ng/ml)	Interpolated concentration (ng/ml)	Accuracy (%)	Within-day precision (%)	Between-day precision (%)
Human	5.71	5.97	105	3.6	4.3
	11.4	11.7	103	3.0	2.4
	57.1	59.5	104	2.6	3.4
	228	245	107	2.9	1.9
Mouse	22.8	23.8	104	4.5	4.2
	114	118	103	6.3	1.3
	571	601	105	3.2	3.8

to the range observed for human plasma, while maintaining sufficient sensitivity.

3.4. Validation

The LLQ was established by spiking blank human plasma of six different individuals. At the 5.7 ng/ml concentration level the accuracy and precision met the requirements. The LLQ in mouse plasma was established at 23 ng/ml using 50 μl sample instead of 200 μl as for human plasma.

Calibration curves in human plasma were linear over a concentration range of 5.3 to 533 ng/ml. This range was considered acceptable for our pharmacokinetic studies. The optimum weight factor for fitting of the calibration curve was $1/x^2$ (reciprocal of the square of the concentration). Control specimens were prepared in blank human plasma and mouse plasma. The lowest concentration was equal to the concentration established as the LLQ.

Within the linear dynamic range of the assay the accuracy of quality control samples of human and mice were close to 100% (Table 1). The within-day precision and between-day precision were within the generally accepted 15% range.

When using the assay for routine analysis over 7 months (12 runs), the reproducibility of the quality control samples assayed in duplicate in each series was better than 10.0%.

3.5. Stability

GF120918 was stable in human plasma and blood and in mouse blood when stored at −4°C, at room temperature and at 37°C. In mouse plasma GF120918 was stable at −4°C and at room tempera-

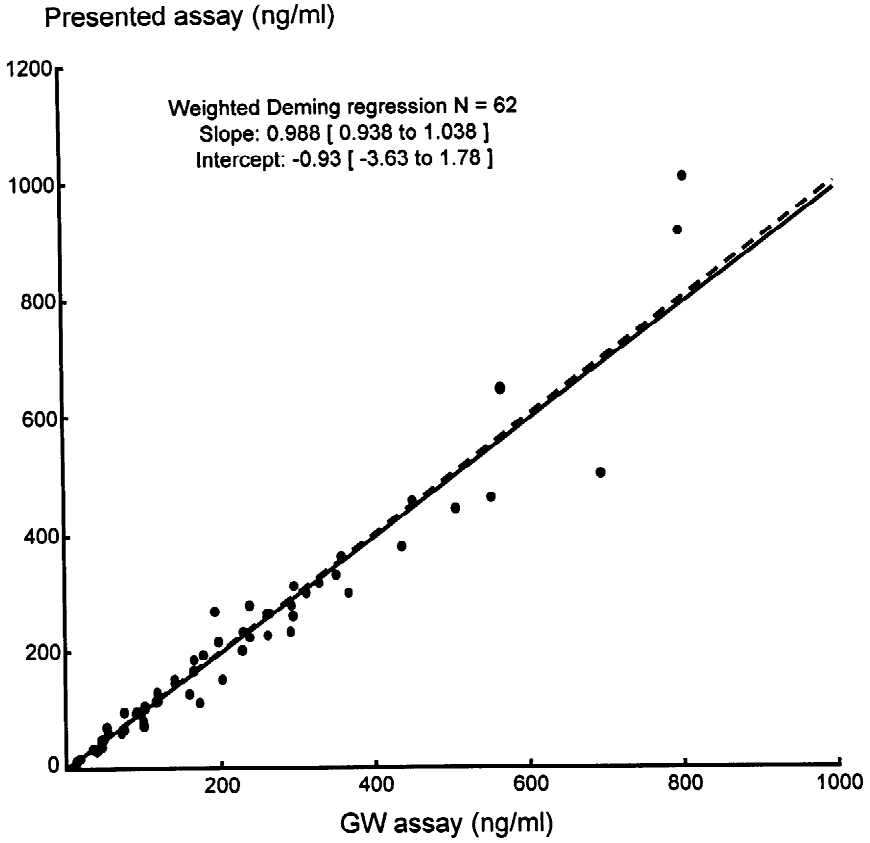

Fig. 5. Cross-validation results of concentrations of human plasma samples obtained with the developed method (*y*-axis) vs. the concentrations obtained from Glaxo Wellcome (*x*-axis). The solid line represents the line calculated and the dotted line the line of identity.

ture for up to at least 4 h. The concentrations of GF120918 did not deviate more than 5% of the nominal concentration.

The stability of GF120918 in mouse plasma incubated at 37°C was less (deviations up to 18.3%). We therefore recommend thawing of frozen plasma specimens at room temperature.

3.6. Cross validation

The results of the cross-validation are given in Fig. 5. The concentrations of GF120918 in human plasma samples obtained with the present method (*y*-axis) are plotted against the concentrations obtained from Glaxo Wellcome (*x*-axis). Weighted Deming regression was performed. The intercept and slope were not significantly different from 0 and 1, respectively, within the 95% level of significance.

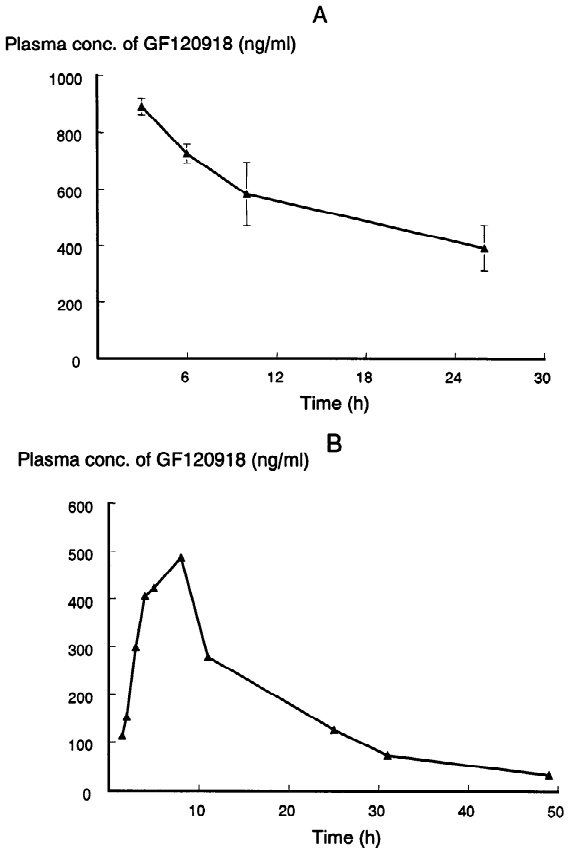

Fig. 6. (A) Plasma concentration–time curve of GF120918 in mice after administration of 100 mg/kg oral GF120918. Data are shown as the mean of four mice±the standard error. (B) Plasma concentration–time curve from a patient after receiving 1000 mg oral GF120918.

3.7. Application of the assay

The applicability of the assay was demonstrated in samples of mice receiving 100 mg/kg of GF120918 by oral administration (Fig. 4C) and from patients receiving 1000 mg oral GF120918 (Fig. 4D). As can be seen from the plasma concentration–time curves in mice (Fig. 6A) and a patient (Fig. 6B) the LLQ of this assay was by far sufficient for pharmacokinetic purposes in subjects given therapeutically relevant doses.

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