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Fully automated assay for the determination of GR117289X in human plasma using mixed mode solid-phase extraction and high-performance liquid chromatography with ultraviolet detection

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Abstract: GR117289X, 3-{3-Bromo-2-[2-(1H-tetrazol-5-yl)-phenyl]-bezofuran-5-ylmethyl}-2-butyl-5-chloro-3H-imidazole-4-carboxylic acid, 1, is an angiotensin II receptor antagonist. A sensitive, accurate and precise assay for the determination of 1 in human plasma and validation data is described. The assay consists of a novel solid-phase extraction using Certify II® followed by a reversed-phase gradient HPLC separation with UV detection. The extraction procedure has been fully automated by a Zymate XP robot and linked on-line to the HPLC system. The analytical range for the assay is 5–250 ng ml^{-1} over which the assay is linear and specific for 1 with respect to endogenous plasma components and its tetrazole N_2 glucuronide, the major circulating metabolite 2. For the fully automated procedure the intra-assay data indicate a maximum bias and coefficient of variation across the calibration range of +8.0 and 9.4%, respectively. The inter-assay data indicate a maximum bias and coefficient of variation across the calibration range of +8.0 and 11.1%, respectively. The extraction efficiency of the assay was approximately 75%. Both a manual and fully automated assay were applied to the analysis of 1 in plasma of volunteers from a number of clinical studies. The assay has been shown to be robust in sustained use over several months.

Keywords: *Solid phase extraction; reversed-phase chromatography; automation.*

Introduction

GR117289X, 3-{3-Bromo-2-[2-(1H-tetrazol-5-yl)-phenyl]-bezofuran-5-ylmethyl}-2-butyl-5-chloro-3H-imidazole-4-carboxylic acid, 1, is a novel and potent angiotensin II receptor antagonist [1]. Previously, 1 has been analysed in plasma from pre-clinical species using liquid–liquid extraction and HPLC analysis. The assay was suitably sensitive, accurate and precise; however, sample throughput was low and the extraction manually intensive. The clinical development of 1 required the development of an accurate, precise, specific, linear, robust and sensitive assay to determine the pharmacokinetics of the drug. An assay strategy was required which would enable processing of large numbers of samples and also be easily transferable to other laboratories without robotic technology.

The paper describes both a manual and fully automated assay for the determination of 1 in human plasma samples from clinical studies and their validation according to published procedures [2]. Certify II® is a chemically modified silica gel material supporting hydrophobic, polar and ion exchange interactions. The use of it for the selective extraction of 1 from plasma is described and a comparison made of the validation data from the manual and automated assays.

Experimental

Chemicals, standards and materials

1 was supplied by the Pharmaceutical Development Department of Glaxo Group Research Ltd. A known plasma metabolite, GR175013X (2) was synthesized by the Process Research Department, Glaxo Group Research Ltd. Acetonitrile and methanol, both HPLC grade, were from Rathburn Chemicals Ltd (Walkerburn, UK). Concentrated formic acid, glacial acetic acid, ammonium acetate, sodium hydroxide and sodium carbonate (AnalaR®) were from BDH Chemicals Ltd (Poole, UK).

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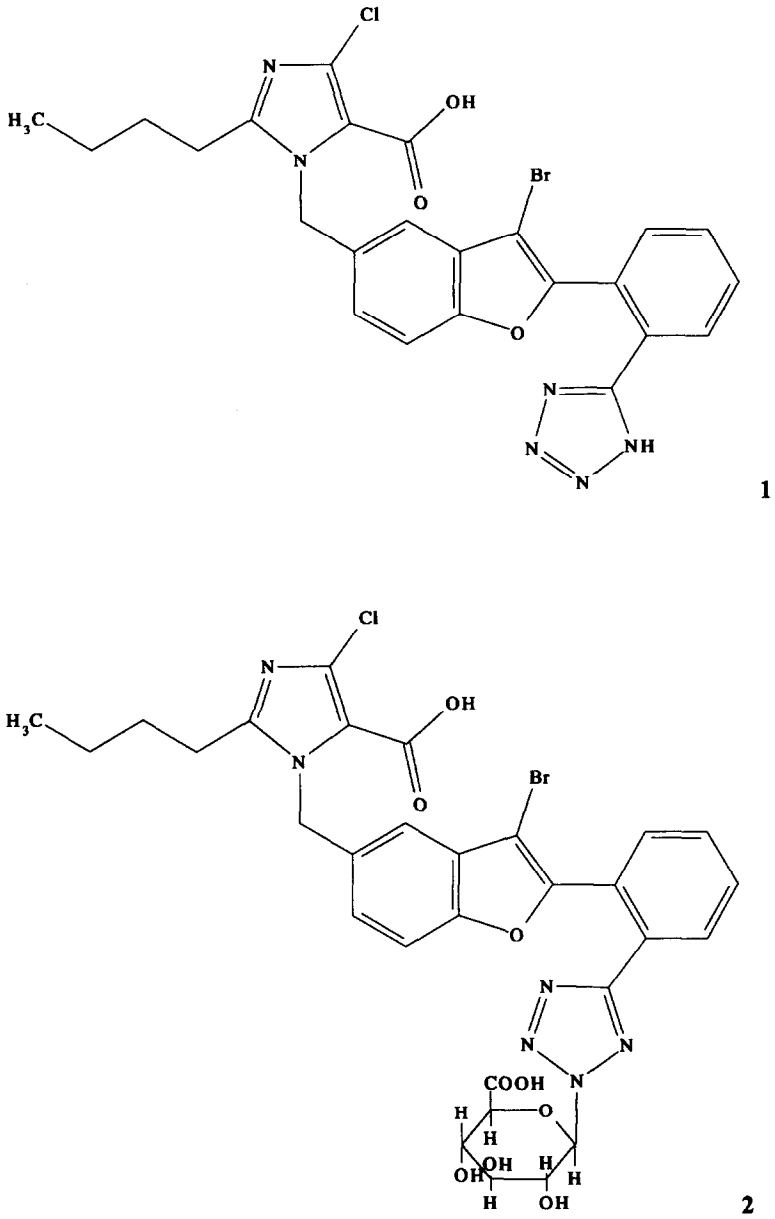

Figure 1
(1) GR117289X; (2) GR175013X.

Water was double distilled from glass and stored in glass. Bond Elut Certify II® sample preparation cartridges (200 mg) were supplied by Jones Chromatography (Hengoed, UK).

Preparation of standards

Calibration standards (5, 10, 50, 100, 200 and 250 ng ml^{-1}) and quality control (QC) samples (12, 125, 220 ng ml^{-1}) were prepared by mixing control plasma with aqueous solutions of 1. The calibration standards in plasma could be prepared either prior to each assay run or in a batch to be stored frozen (−20°C) until required. The QC samples were prepared in a batch and stored frozen (−20°C) until required. 1 is stable in control plasma for at least 11 months when stored at −20°C.

Sample preparation

Samples and standards were prepared for analysis by using solid-phase extraction. The extraction procedure detailed below describes the manual procedure based on a vacuum extraction box [3].

Bond Elut Certify II® extraction cartridges (200 mg) were washed sequentially with meth-

anol (3 ml) and distilled water (3 ml). The liquids were drawn through by reduced pressure, but the cartridges were not allowed to dry. Plasma (1 ml) was acidified with concentrated formic acid (25 μl), methanol (100 μl) was added to aid solubility, samples were diluted with distilled water (1 ml), vortex mixed, then drawn through the extraction cartridges. The eluate was discarded. When the sample had passed through, the cartridges were allowed to dry. The cartridges were then washed sequentially with methanol (3 ml), distilled water (3 ml) and 0.1% v/v formic acid in methanol (3 ml), and were allowed to dry after each washing step. 1 was eluted with 2% v/v formic acid in methanol (2.5 ml) into Eppendorf tubes (Sarstedt, Leicester, UK) and the solvent was removed at elevated temperature and reduced pressure; 45°C and $<8 \times 10^{-1}$ Torr on a Savant Speed Vac Concentrator (Stratech Scientific Limited, London, UK). Pressures of approximately 130 and 380 mmHg were used to draw solutions through and to dry the cartridges, respectively. The dried extracts were reconstituted with 0.03 M sodium carbonate by vortex mixing and a portion injected onto the HPLC system. The use of basic pH to reconstitute the sample was necessary to achieve complete re-dissolution. Practical experience has shown that the extracts remain stable at ambient temperatures for at least 24 h.

Instrumentation

The HPLC system comprised a Perkin–Elmer Series 410 solvent delivery system, (Beaconsfield, UK), a Gilson 231 sample injector (Anachem, Luton, UK), a Spherisorb ODS-2 HPLC column (5 μm, 100 × 4.6 mm i.d.; Phase Sep, Deeside, UK) protected by a Upchurch in-line filter, a Kratos SF783 programmable absorbance detector (Anachem), and a Magnus Data Sigma Filter (Anachrom, Uxbridge, UK). Data handling was carried out using a Multichrom Chromatography Data Handling System (VG Data Systems Ltd, Altringham, UK).

Robotics

For the fully automated procedure the sample preparation was performed as above for the manual procedure with the following exceptions.

The instrumentation used was a Zymark System V XP robot (Zymark Ltd, Warrington, UK) with the following modules: balance pysection with Sartorious 1712 balance, dilute and dissolve pysection, liquid/solid extraction pysection customized with six solvent lines, evaporation pysection, HPLC sipping injection pysection (200 μl rheodyne loop) and personal computer.

Solvents were forced through the extraction columns by positive air pressure of approximately 5 psi. The elution solvent was removed under nitrogen using a heating block set at 40°C.

The assay procedure was serialized with the various modules working in concurrent mode. The robot thus processed up to eight samples at any one time, preparing a sample for HPLC analysis every 10 min.

Chromatographic analysis

Chromatographic analysis was carried out at ambient temperature, using a Spherisorb reversed-phase column as above. The mobile phase consisted of a binary mixture of acetonitrile and ammonium acetate buffer (0.1 M, adjusted to approximately pH 4.1 with glacial acetic acid) gradient. The solvent programme was from 25 (organic): 75 (aqueous) to 50:50 v/v in a linear gradient over 10 min, held for 3 min, then returned to 25:75 v/v and allowed to re-equilibrate for 7 min. The flow rate was 1 ml min^{-1} and detection by UV absorbance (300 nm, 0.001 AU). A Magnus Data Sigma Filter (M.H. Scientific, Aylesbury, UK) was placed between the detector analogue output and the chromatography data system; this provided filtering (0.02 Hz) to remove electrical noise, and a 10-fold gain on the analogue signal.

Quantification

Calibration standards (six concentrations between 5.0 and 250 ng ml^{-1}) and control plasma were analysed in duplicate. All calibration standards were extracted and analysed as described. Peak areas were recorded, and calibration lines were constructed using weighted linear regression ($1/X$). Concentrations of 1 in QC and study samples were quantified by comparison with these standard lines.

The calibration range of 5–250 ng ml^{-1} was chosen based on the expected concentrations of 1 in plasma following oral administration at the anticipated therapeutic dose. Samples containing 1 at concentrations in excess of 250 ng

ml^{-1} were analysed after dilution with control plasma to within the calibration range of the assay.

Validation

Validation of the assay procedure was carried out to establish intra- and inter-assay variability, specificity, linearity and extraction efficiency over the calibration range. The inter- and intra-assay variability were determined to assess accuracy and precision. The accuracy was expressed by the bias in sample values (difference from the theoretical) and the precision was expressed by the relative standard deviation (RSD). Both bias and RSD are presented as percentages.

Intra-assay variability was measured from six-fold replicates of the sample matrix spiked at each calibrant concentration, which were then assayed against a duplicate calibration line prepared in the same way.

Inter-assay variability was measured by analysis of duplicate-spiked plasma samples, corresponding to each calibration point, on four separate occasions. An independent calibration line was prepared in duplicate each time.

Specificity was studied by analysing control human plasma by the described procedures and the chromatograms examined for visible evidence of interfering endogenous compounds. The single major metabolite 2 was run

Table 1
Intra-assay accuracy and precision

Theoretical concentration (ng ml^{-1})	Mean observed concentration (ng ml^{-1})	Standard deviation (ng ml^{-1})	Accuracy (Bias) (%)	Precision (RSD) (%)
Manual				
4.9	5.2	0.4	+6.1	8.5
9.8	9.0	0.7	−8.2	8.3
49.1	44.9	4.2	−8.6	9.4
98.2	94.8	4.7	−3.5	5.0
196	199	6.0	−1.5	3.5
246	241	8.4	−2.0	3.5
Automated				
5.0	5.3	0.5	+6.0	9.4
10.0	10.8	0.5	+8.0	4.5
50.0	53.0	1.6	+6.0	3.0
100	107	6.7	+7.0	6.3
200	206	9.3	+3.0	4.5
250	250	6.5	0.0	2.6

Number of replicates (n) = 6.
The manual and automated assays were validated independently. As a result the theoretical concentrations differ slightly.

Table 2
Inter-assay accuracy and precision

Theoretical concentration (ng ml^{-1})	Mean observed concentration (ng ml^{-1})	Standard deviation (ng ml^{-1})	Accuracy (Bias) (%)	Precision (RSD) (%)
Manual				
4.9	5.6	0.5	+14.3	8.1
9.8	10.1	1.5	+3.1	14.9
49.1	50.5	3.7	+2.9	7.4
98.2	103	8.9	+4.9	8.6
196	212	13.6	+8.2	6.4
246	274	23.0	+11.4	8.4
Automated				
5.0	5.2	0.6	+4.0	11.1
10.0	10.8	0.5	+8.0	4.5
50.0	53.0	1.6	+6.0	3.0
100	107	6.7	+7.0	6.3
200	207	19.0	+3.5	9.2
250	248	21.6	−1.0	8.7

Number of replicates (n) = 4 × 2.
The manual and automated assays were validated independently. As a result the theoretical concentrations differ slightly.

in the HPLC system to ascertain its retention time.

The linearity of the assays was determined by comparing the mean values of each spiked plasma sample concentration obtained during intra-assay validation with the theoretical concentration.

The extraction efficiency of 1 was measured on two separate occasions by comparing the peak area of duplicate quality control (QC)

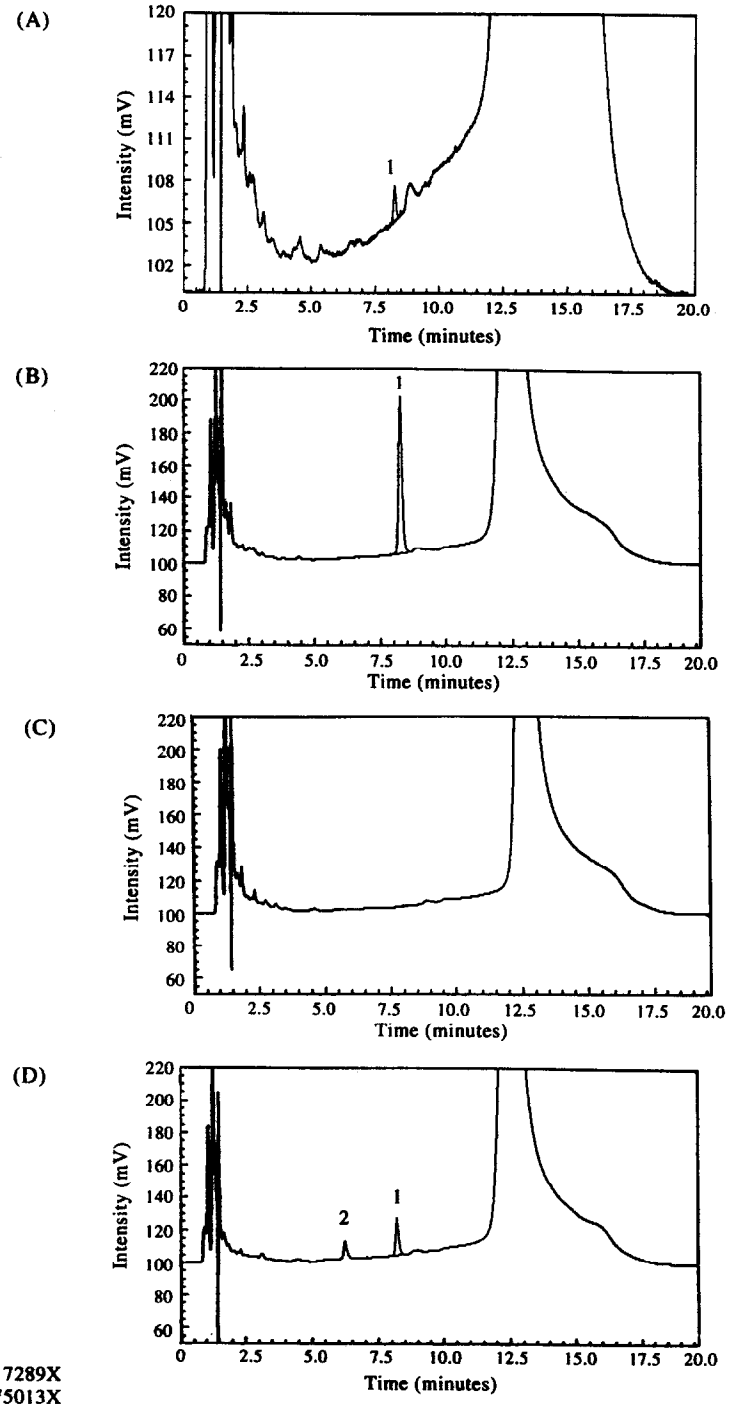

Figure 2
Chromatograms of plasma extracts: (A) and (B) standards containing 1 at 5 and 250 ng ml^{-1}, respectively; (C) pre-dose; (D) 6-h post-dose sample from a volunteer administered a 250 mg oral dose of 1.

samples at three concentrations using the assay described, with direct injection of aqueous solutions of 1.

Results

The validation data presented in Tables 1 and 2 show that both a manual and fully automated assay are suitable for the determination of 1 in human plasma.

Examples of chromatograms obtained from a standard curve, and pre- and post-dose samples are shown in Fig. 2.

Intra-assay accuracy and precision across the calibration range, derived from meaned data, in the manual assay indicated maximum values of −8.6 and 9.4%, respectively (Table 1). Corresponding values for the automated assay were +8.0 and 9.4%, respectively. Inter-assay accuracy and precision across the calibration range of the manual assay was +14.3 and 14.9%, respectively (Table 2). Similarly derived values for the automated assay were +8.0 and 11.1%, respectively. High accuracy and precision were thus achieved by this external standard method of calibration, so the inclusion of an internal standard was not thought likely to make a significant improvement to the quality of the data obtained. The law of propagation of error [4, 5] suggests the use of an internal standard in this assay could have impaired the assay precision. Observations concerning the use of internal standards in the analysis of drugs in biological fluids, and some comparative data, have been reported elsewhere [6–8]. Chromatograms from control plasma were free from material at the retention time of 1 (approximately 8 min) and the known tetrazole N_2 glucuronide metabolite 2 was well separated. Thus, the assays have been shown to be specific for 1 with respect to endogenous plasma components and this metabolite. The linearity of the calibration lines were good. Using linear weighted ($1/X$) regression due to the 50-fold range of calibration, typically the coefficient of determination was greater than 0.99. Linearity of the assays was also good as demonstrated by the intra-assay accuracy. Mean extraction efficiencies of the manual and automated assays was essentially the same being 75.1 and 76.2%, respectively.

Discussion

The aim of this project was to develop a sensitive, accurate and precise assay that could be used in laboratories with or without robotic technology for the determination of 1 in human plasma in support of clinical studies.

The benefits of automation in routine bioanalysis are well documented, however, this technology is not yet widely available. As such this assay was designed so that it could be performed manually or in a fully automated manner dependant on the technology available to the particular laboratory.

Batches of up to 60 samples including QC samples and calibration standards have been successfully analysed over several months. QC data generated during study sample analysis indicated similar accuracy and precision to that generated during validation, demonstrating

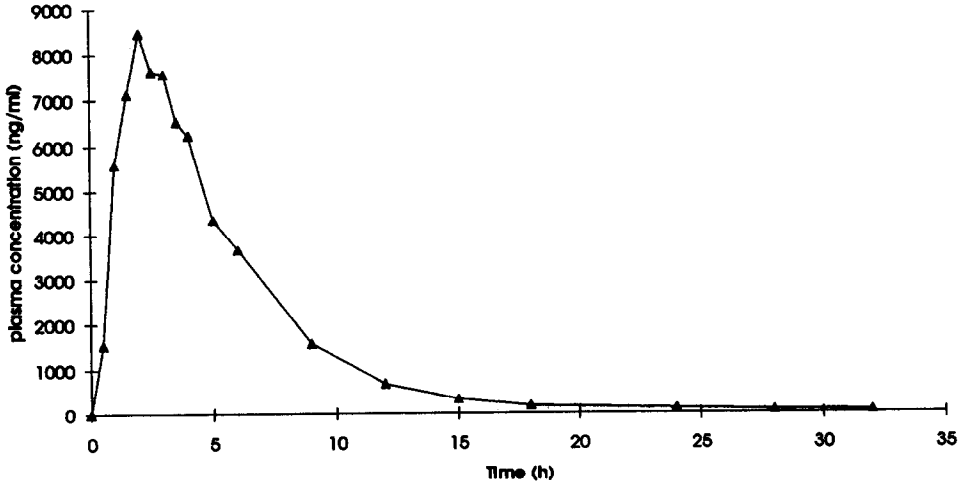

Figure 3
Concentration of GR117289X in plasma versus time profile in a volunteer receiving a 250 mg oral dose of 1.

that the assays are robust. In volunteers, the assays were of sufficient sensitivity to provide good pharmacokinetic data in man. A typical profile of plasma concentrations vs time after administration of an oral dose of 250 mg of 1 is shown in Fig. 3.

Apart from the solid-phase material, the strategy used for the assay was conventional. Bond Elut Certify II® is a chemically modified silica gel material supporting three different types of interactions: hydrophobic, polar and ion exchange (anion). Unlike conventional solid-phase extraction columns, this phase utilizes multiple interaction properties for selectively eliminating interfering materials and isolating the compound of interest. The silica gel is specially designed for the extraction of acidic and neutral molecules from biological fluids and a number of urine assays have been developed using this material [9–11]. However, this present study is the first documented plasma assay. In the extraction procedure used, the different types of interaction on the Certify II® cartridges have been exploited to retain 1 reproducibly. The material allowed the use of a greater variety of solvents than conventional materials to remove endogenous materials prior to the elution of the compound of interest.

Conclusions

An analytical procedure is described for the determination of 1 in human plasma which is accurate, specific, and linear over the calibration range 5–250 ng ml^{-1}.

The use of a solid-phase extraction column (Certify II® Bond Elut) has enabled the selective extraction and concentration of 1 from the plasma matrix.

The use of automation has resulted in a modest improvement in the accuracy and precision of the assay procedure, significantly reduced human involvement and allowed 24-h unattended operation.

The analytical procedure has been used to successfully support a number of clinical studies.

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