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A sensitive assay for ZD1839 (Iressa [1]) in human plasma by liquid–liquid extraction and high performance liquid chromatography with mass spectrometric detection: validation and use in Phase I clinical trials

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Abstract

A specific and sensitive high performance liquid chromatography method for the quantitative determination of ZD1839 ('Iressa') concentrations in treated healthy volunteers and patients with cancer has been developed and validated. Plasma samples (0.5 ml) were extracted, at basic pH, with methyl-t-butyl ether using deuterated ZD1839 as an internal standard. The extracts were chromatographed on an Inertsil ODS3 column eluted with acetonitrile/ammonium acetate and ZD1839 and the internal standard quantified by mass spectrometric detection. The method was validated with respect to linearity, selectivity, precision, accuracy, limit of quantification (LOQ), recovery and stability. The precision and accuracy of the assay were good and the LOQ was 0.5 ng/ml. The assay has been successfully applied to a number of clinical and pharmacokinetic studies and been shown to be robust and reliable during routine use.

Keywords: ZD1839; Quantification; Epidermal growth factor receptor inhibitor; Liquid–liquid extraction; High performance liquid chromatography; Phase I trials

1. Introduction

Inhibition of the epidermal growth factor receptor (EGFR) has been achieved by various methods, including inhibition of the EGFR tyrosine kinase (EGFR-TK) by small molecule inhibitors [1–8]. ZD1839, 4-(3-chloro-4-fluoroanilino)-7-methoxy-6-(3-morpholinopropoxy) quinazoline, ('Iressa') is an orally active, selective EGFR-TK inhibitor (EGFR-TKI). A low molecular weight anilinoquinazoline, ZD1839 (Fig. 1), potently inhibits EGFR-TK in vitro and exhibits significant antitumour activity in a broad range of solid human tumour xenografts in vivo [7–9]. Preclini-

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cal toxicology studies have demonstrated that ZD1839 has good oral bioavailability and that chronic oral dosing with ZD1839 is generally well tolerated (data on file, AstraZeneca). The favourable tolerability profile of ZD1839 seen in volunteers [10] has also been borne out in cancer patients [11,12].

This paper describes the validation of a specific and sensitive high performance liquid chromatography (HPLC) method developed for the quantitative determination of plasma concentrations of ZD1839. This has been used to generate pharmacokinetic data in both healthy volunteers and cancer patients during the clinical development of ZD1839 [13].

2. Experimental

2.1. Reagents and materials

ZD1839 was supplied by AstraZeneca Pharmaceuticals (Macclesfield, UK) with a purity of 99%. Deuterated (d8)-ZD1839 was prepared by the Drug Metabolism and Pharmacokinetics Department, AstraZeneca, UK. Acetonitrile (far UV grade), methyl-t-butyl ether (MTBE) (HPLC grade) and methanol (HPLC grade) were supplied by Fisher Scientific UK Limited (Loughborough, UK); ammonium acetate and sodium hydroxide (AnalaR grade) were supplied by BDH Chemicals (Lutterworth, UK). Soda glass extraction tubes were supplied by Samco Limited (Surrey, UK); borosilicate glass drying tubes by VWR Scientific Inc (Germany) and autosampler vials by Chromacol Ltd (Welwyn Garden City, UK).

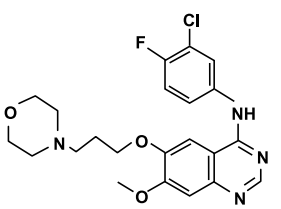

Fig. 1. Chemical structure of ZD1839.

2.2. Stock solution preparation

Stock solutions of ZD1839 and deuterated (d8)-ZD1839 (internal standard) were prepared by dissolving appropriate amounts, typically 5–10 mg, of solid compound in methanol to give 1 mg/ml solutions. Further methanolic solutions were prepared by serial dilution of the 1 mg/ml solutions. The ZD1839 solutions were used to prepare calibration standards and a 1 μg/ml solution of deuterated ZD1839 was used as internal standard.

2.3. Sample preparation

Plasma (0.5 ml) from a standard or sample was dispensed into a glass tube, the internal standard added (25 μl of a 1 μg/ml methanolic solution) and the tubes loaded onto a Zymate robot (Zymark Ltd, Warrington, UK). Sodium hydroxide (0.5 ml of a 1 M aqueous solution) and methyl-t-butyl ether (MTBE; 6 ml) were added and the tube contents mixed thoroughly by vortex mixing for 2 min. Each tube was centrifuged for 30 s to separate the organic and aqueous layers then 5.5 ml of the organic layer transferred to a clean glass tube and evaporated to dryness at 20 °C under a stream of oxygen-free nitrogen. The dry residues were reconstituted with 250 μl HPLC eluant, vortex mixed, transferred to autosampler vials and 15–100 μl (dependent upon instrument response) injected onto the reversed phase HPLC system.

2.4. Chromatography

The extracts were chromatographed on a 15 cm × 4.6 mm id Inertsil ODS3 column (HiChrom Ltd, Reading, UK), protected by an Inertsil ODS3 cartridge guard column eluted at a flow rate of 1 ml/min with 80% acetonitrile: 20% aqueous ammonium acetate (1% w/v). Detection was by Perkin Elmer Sciex API-300 tandem mass spectrometer with a heated nebuliser source for atmospheric pressure chemical ionisation. Detection was based on precursor transitions to the strongest intensity product ions. These were $447.2 \rightarrow 128$ for ZD1839, and $455.4 \rightarrow 136$ for the internal standard. Multiple reaction monitoring scans were employed with a dwell time of 50 ms

Table 1
Recovery of ZD1839 and deuterated (d8)-ZD1839 over the working range of the assay

Compound	Test concentration (ng/ml)	% recovery (± S.D.)
ZD1839	1.5	122.4 ± 7.3
	80	111.4 ± 4.3
Deuterated ZD1839	50	79.1 ± 9.4

and a 2 ms pause. The scans were acquired for 5 min.

2.5. Calibration and quality control

Calibration standards were prepared in a total of 0.5 ml of control (i.e. drug free) plasma. Known volumes of ZD1839 spiking solutions were added to the plasma such that the volume of spiking solution did not exceed 10% of the total volume. Eight calibration standards were prepared to produce a calibration series, which covered the range 0.5–100 ng/ml ZD1839.

The calibration curve, a plot of peak height ratio of ZD1839/internal standard against concentration of ZD1839, was fitted by forcing the line through the origin. In each analytical run quality control (QC) samples spiked at three independent concentrations (1.5 or 1.72, 40 and 90 ng/ml) were included and used to accept/reject the run. Each of the QC samples was analysed once at the beginning and once at the end of each run. Each QC result was considered acceptable if the result obtained from the analysis lay within ± 20% of the nominal concentration. For the run to be accepted at least four of the six QC results had to be acceptable, with one acceptable QC at each concentration. During analytical runs where a large number of sample dilutions were required, the middle QC was replaced by a QC sample spiked at 400 ng/ml. This QC was diluted before analysis and the acceptance/rejection criteria described above were used.

3. Results and discussion

3.1. Method development

Liquid–liquid extraction of ZD1839 was our first choice of sample preparation and this gave clean extracts free from endogenous interference. For the chromatography, a Hichrom RPB column (Hichrom Ltd) was initially employed with a mobile phase containing trifluoroacetic acid (TFA). These chromatographic conditions did not prove to be robust and non-linear calibration curves were sometimes achieved. The Hichrom RPB column was replaced by an Inertsil ODS 3 column, which improved the linearity of the calibration curve, and the TFA in the mobile phase was replaced by ammonium acetate, bringing about an improvement in the peak shape. The large organic component of the eluent allowed retention times of approximately 2.5 min to be achieved giving a high sample throughput.

3.2. Linearity

The linear range of the assay extended from 0.5 to 100 ng/ml. The slopes of 13 separate calibration curves used in analysis of samples from one volunteer trial ranged from 0.013 to 0.023, with a mean value of 0.019 ± 0.002 S.D.

3.3. Recovery

The recovery from plasma of ZD1839 at 1.5 and 80 ng/ml and deuterated (d8)-ZD1839 at 50 ng/ml was investigated. Recovery was determined by expressing the mean peak height from five extracted samples spiked at known concentrations of ZD1839 and (d8)-ZD1839 as a percentage of the mean peak height obtained from five solutions of HPLC eluent spiked at the same concentrations (taking into account the concentration step involved in the extraction procedure). The recoveries of ZD1839 and internal standard are presented in Table 1. The recovery of both compounds was good with the mean recovery of ZD1839 at both concentrations being 116.9% (± 7.1 S.D.) and the recovery of (d8)-ZD1839 being 79.1% (± 9.4 S.D.). The use of the theoretical concentration

may have introduced inaccuracies, which could account for the greater than 100% recovery observed for ZD1839.

3.4. Limit of quantification

Five calibration series each composed of 10 standards covering a concentration range of 0.3–100 ng/ml were analysed. The coefficient of variation (CV) in peak height of ZD1839 was calculated for each calibration level and used to produce a response error relationship over the range 0.3–100 ng/ml. The limit of quantification (LOQ) of the assay without addition of internal standard was 0.5 ng/ml, the concentration at which the assay CV fell below 10%. The total assay CV was 6.1%.

3.5. Specificity

The assay was investigated to ensure that it was specific to ZD1839 alone and that the calculated concentrations would not be influenced by any underlying endogenous interference with either ZD1839 or internal standard. This was determined by using control human plasma from ten individuals. Three aliquots of each individual matrix were extracted. One extract was subjected to the chromatographic procedure as described, to the second extract ZD1839 at a concentration equivalent to the LOQ was added and to the third ZD1839 and internal standard at a concentration equivalent to that present in the final extracted sample was added. Reference solutions of ZD1839 and internal standard were chromatographed alongside the extracts. The peak heights of deuterated (d8)-ZD1839 and ZD1839 from the two reference solutions were compared by paired t-test with the respective human plasma samples in order to assess whether there was any significant endogenous interference.

There was no evidence of interference with the deuterated (d8)-ZD1839. There was some evidence of endogenous interference with ZD1839 from plasma but this interference was equivalent to less than 0.1 ng/ml and, since the LOQ of the assay was set at 0.5 ng/ml, was not considered to be analytically significant.

3.6. Precision/accuracy

The assay precision and accuracy were investigated. Aliquots of control human plasma were bulk spiked with nominal concentrations of ZD1839 0.5, 8, 40 and 90 ng/ml. Each of these samples was split into subaliquots and stored at -20 °C until analysed in quadruplicate on four separate occasions. The concentration data were investigated by analysis of variance to determine the within and between batch precision. Assay accuracy was assessed by expressing the determined concentration as a percentage of the nominal concentration. The results are presented in Table 2. Overall, the precision was good with a mean CV across the range of the assay of $4.4 \pm 0.89\%$ (S.D.). The accuracy was good across the range 8–90 ng/ml, with values of $< 110\%$ being achieved. At 0.5 ng/ml the accuracy was 125%. This value exceeds the suggested limit of $\pm 20\%$ given in the Guidance for Industry Bioanalytical Method Validation [14], but was considered acceptable. The mean overall accuracy was $114 \pm 15.0\%$ (S.D.).

Table 2
Precision and accuracy data generated across the calibration range 0.5–100 ng/ml

Nominal concentrations (ng/ml)	Mean result (ng/ml)	Intra-assay variation (%)	Inter-assay variation (%)	Overall assay variation (%)	Overall accuracy (%)	S.D.	n
0.5	0.682	5.8	NC	5.1	125	19.5	12
8.0	8.29	4.1	NC	3.9	104	4.02	16
40	42.2	3.3	1	3.4	106	3.61	16
90	98.0	3.1	4.3	5.2	109	5.63	16

NC: not calculated.

Table 3
Summary of assay performance obtained across 7 clinical trials

Target concentration (ng/ml)	Mean concentration (ng/ml)	Intra-batch CV (%)	Inter-batch CV (%)	Total CV (%)	Accuracy (%)
1.72	1.81	8.4	NC	7.3	105
1.5	1.78	8.5	4.5	9.6	119
40	39.2	4.8	6.0	7.6	97.9
90	87.3	4.5	4.8	6.6	97.1
400	399	4.2	NC	4	99.6

NC: not calculated.

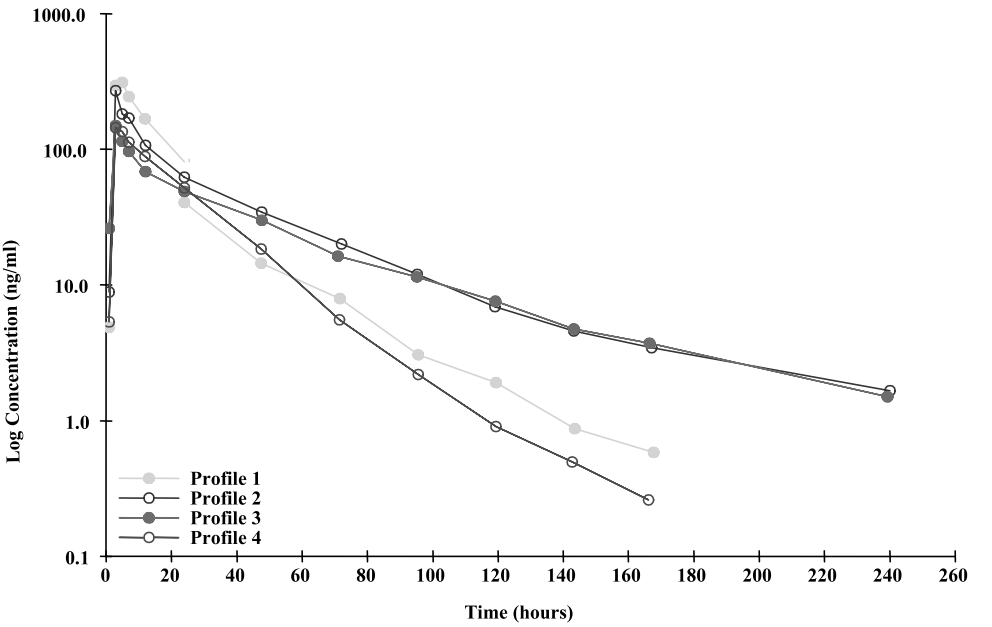

Fig. 2. Example ZD1839 plasma concentration–time profiles.

The mean accuracy at all four concentrations was over 100% indicating a possible positive bias, however the individual data used to generate the mean values showed positive and negative deviations from the nominal values. In addition, when the assay was used to analyse clinical samples, the QC samples showed positive and negative deviations from the nominal values (Table 3).

3.7. Stability

The stability of ZD1839 has been assessed at various stages of the assay, as methanolic stock solutions at 4 °C, in human plasma at room temperature and at −20 °C, as a dry residue after extraction and after reconstitution in HPLC eluent. For investigation of each assay stage, five samples stored under the conditions listed were assayed alongside five freshly prepared samples and the peak height data compared using the method of Timm et al. [15] to assess any instability.

Stock solutions of ZD1839 at concentrations of 1 mg/ml and 0.01 μg/ml have been shown to be stable for 29 and 7 days respectively, while stock solutions of (d8)-ZD1839 have been shown to be

stable for up to 146 days. In human plasma, ZD1839 was stable for 24 h if stored at room temperature and for up to 12 months when stored at −20 °C. In HPLC eluent, ZD1839 was stable when stored at room temperature for 24 h followed by 48 h at 4 °C. The internal standard was also stable to these conditions. ZD1839 samples were reconstituted in HPLC eluent as soon as possible after the MTBE layer had been evaporated to dryness.

3.8. Quality control data from routine use

The assay described in this paper has been used routinely to derive plasma concentrations for several clinical trials. A typical plasma concentration–time profile for ZD1839 is presented in Fig. 2.

The QC data generated from five patient trials and two volunteer trials has been assessed to determine the within and between batch variabil-

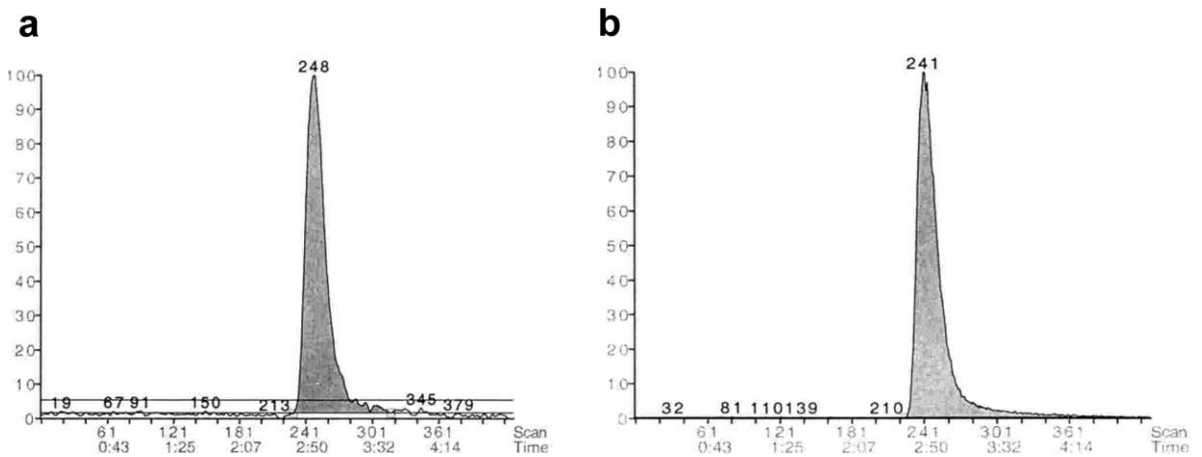

Fig. 3. Chromatogram of ZD1839 QC sample (nominal concentration 1.5 ng/ml). (a) ZD1839; (b) internal standard.

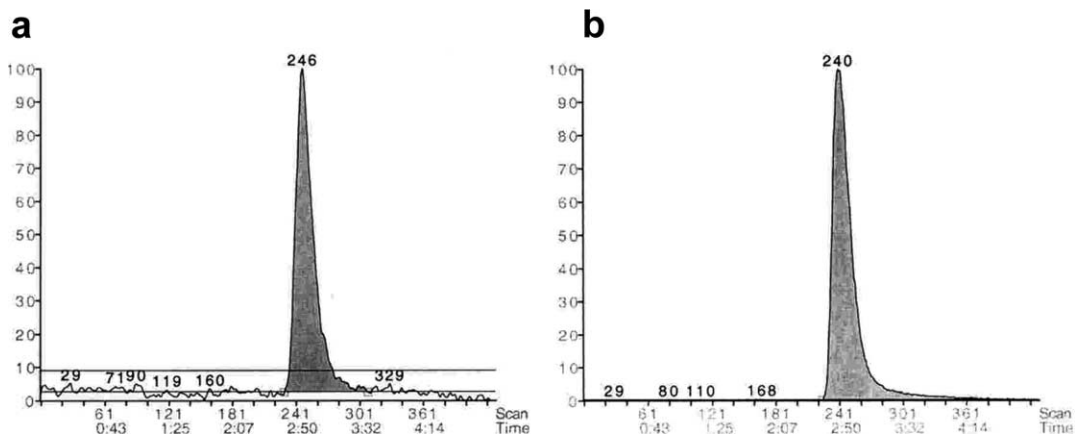

Fig. 4. Chromatogram of ZD1839 sample taken 120 h after administration of 100 mg ZD1839 (concentration = 0.856 ng/ml). (a) ZD1839; (b) internal standard.

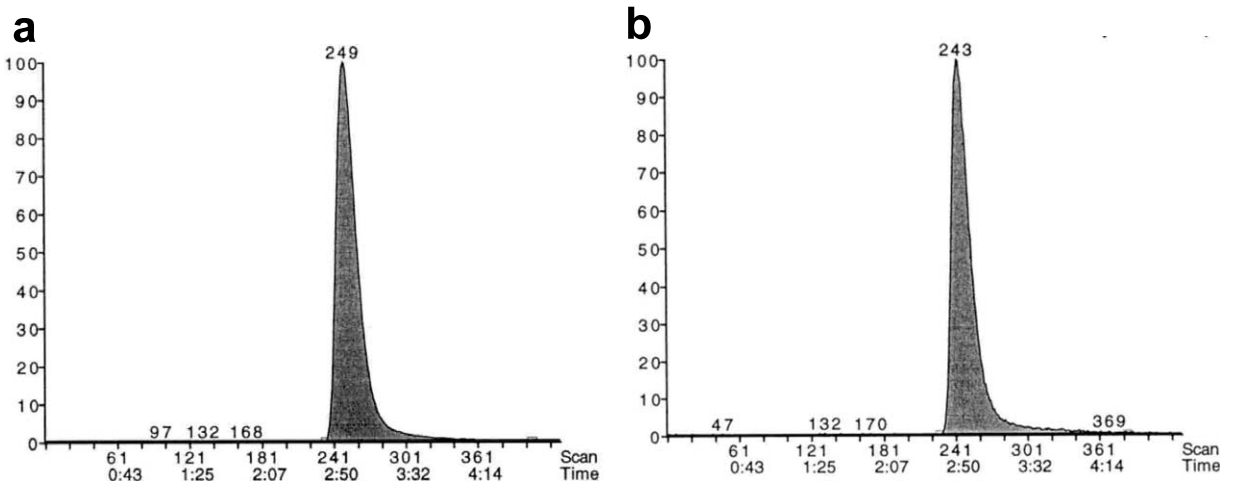

Fig. 5. Chromatogram of ZD1839 sample taken 72 h after administration of 500 mg ZD1839 (concentration = 83.1 ng/ml). (a) ZD1839; (b) internal standard.

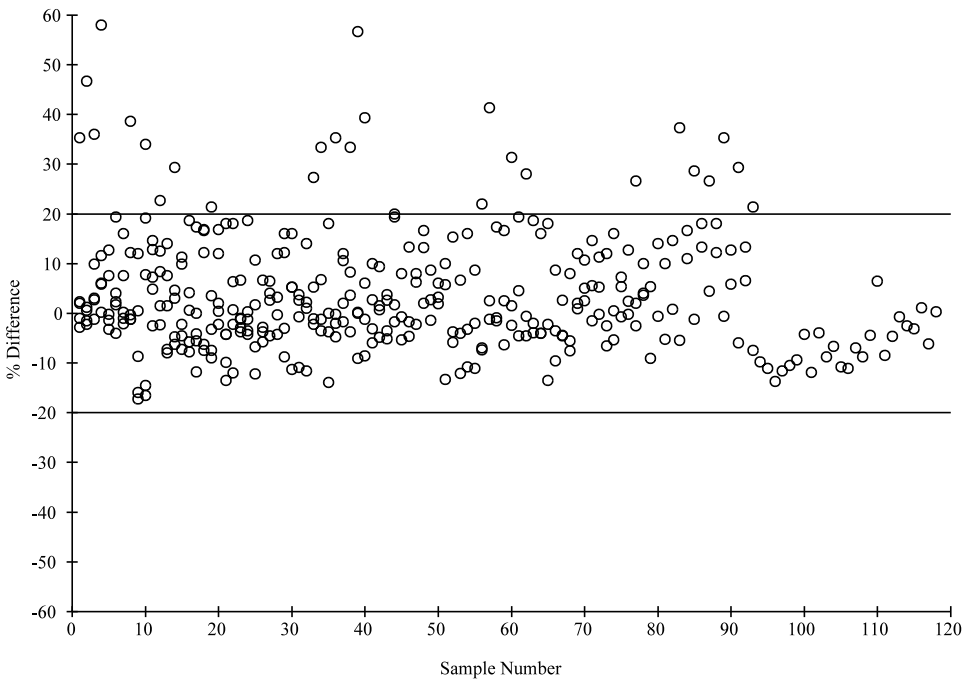

Fig. 6. Graph showing results obtained for QC samples at 1.5, 40, 90 and 400 ng/ml as percentage difference from nominal concentrations.

ity. These data are presented in Table 3 as an indication of the performance of the assay during routine use. Example chromatograms obtained during the analysis of QC samples spiked at 1.5 and 90 ng/ml, and ex vivo clinical trial samples are presented in Figs. 3–5.

The data show that the precision and accuracy were good with a mean overall CV of 7.02 ±

2.02% (S.D.) and a mean overall accuracy of $104 \pm 9.08\%$ (S.D.). The same QC data are presented in Fig. 6, where each QC concentration is expressed as a percentage difference from the nominal concentration. Overlaid on the data are the 20% limits that were used to accept or reject individual QC samples. Across the seven studies, a total of 58 analytical runs were performed with only four of these runs being rejected on the basis of the QC sample results.

4. Conclusion

A method has been developed and validated for determining ZD1839 concentrations in human plasma. The assay has been shown to have adequate specificity and sensitivity for use in clinical trials. It has a LOQ of 0.5 ng/ml that allows sufficient sensitivity for reliable estimation of the pharmacokinetics of ZD1839. The assay has been used to analyse over 2764 samples in over 58 analytical runs with a less than 7% typical rate of run rejection. The assay has been applied successfully to more than seven volunteer/patient trials and has proved to be both robust and reliable.

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