ELSEVIER

Journal of Chromatography B, 772 (2002) 317–325

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Validation of a liquid chromatographic–tandem mass spectrometric method for the measurement of (*R*)-1-[2,3-dihydro-2-oxo-1-pivaloylmethyl-5-(2-pyridyl)-1H-1,4-benzodiazepin-3-yl]-3-(3-methylaminophenyl)urea (YF476) in human plasma

M.J. Redrup[a,*], F.C. Leaf[a], A. Miyashita[b], T. Watanabe[b], S. Higuchi[b], L.F. Chasseaud[a], K.N. Cheng[a]

[a]*Department of Mass Spectrometry, Huntingdon Life Sciences, P.O. Box 2, Huntingdon, Cambridgeshire, PE18 6ES, UK*
[b]*Drug Metabolism Laboratories, Yamanouchi Pharmaceutical Co. Ltd., 1-8 Azusawa 1-chome, Itabashi-ku, Tokyo 174, Japan*

Received 29 June 2001; received in revised form 15 February 2002; accepted 19 February 2002

Abstract

A sensitive and specific liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method has been validated for the measurement of YF476 in human plasma. The method involves a simple liquid–liquid extraction procedure, chromatography of the extracts on a C_{18} column, atmospheric pressure chemical ionisation and detection in the multiple reaction monitoring mode. The calibration line was linear over the concentration range 0.1 ng/ml (the limit of quantification) to 25.0 ng/ml. Intra- and inter-batch precision was <14% and intra- and inter-batch accuracy was <11% over the entire calibration range. The bioanalytical method is robust and has been used for the analysis of many samples from human subjects involved in early clinical studies (Phase I). © 2002 Published by Elsevier Science B.V.

Keywords: YF476; Benzodiazepines

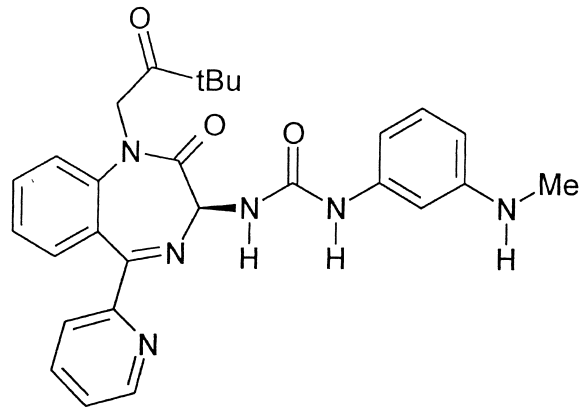
1. Introduction

Gastrin is a gastrointestinal tract hormone that exists in multiple forms in man [1–3]. Gastrin release is stimulated by protein ingestion and gastric distension. Its main physiological role is the stimulation of gastric acid secretion. (*R*)-1-[2,3-dihydro-2-oxo-1-pivaloylmethyl-5-(2-pyridyl)-1H-1,4-benzodiazepin-3-yl]-3-(3-methylaminophenyl)urea, YF476 (I, see Fig. 1), is a novel, potent and selective gastrin/cholecystokinin-β antagonist that inhibits basal and meal-stimulated gastric acid secretion [4].

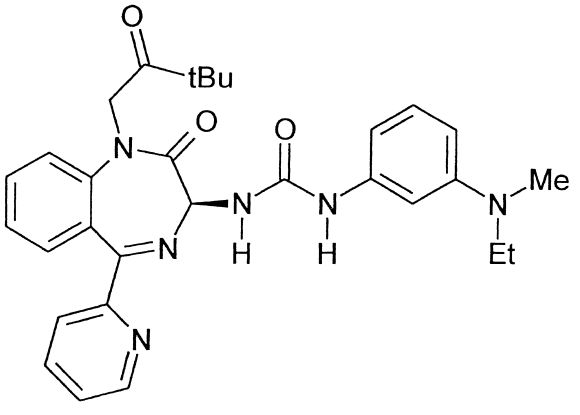
Due to the drug's potency, low plasma concentrations are anticipated for the dosage regimes proposed for its therapeutic use. Thus the bioanalysis of clinical samples required a sensitive and specific method with a low limit of quantification (100 pg/ml), so as to maximise the amount of pharmacokinetic data obtained during clinical studies. A high-performance liquid chromatographic (HPLC–UV) method has previously been developed and validated for the bioanalysis of YF476 in toxicity study samples [5] but its sensitivity is inadequate for

*Corresponding author. Tel.: +44-1480-892-000; fax: +44-1480-890-693.

1570-0232/02/$ – see front matter
PII: S1570-0232(02)00118-6



YF476 (I)



CH-3058A (II)

Fig. 1. Chemical structures of YF476 and the internal standard, CH-3058A.

the much lower doses likely to be used clinically. LC–MS–MS has become established as the technique of choice for the analysis of biological samples arising from clinical studies because of its higher throughput and greater sensitivity, typically in the pg/ml range [6–8]. A range of benzodiazepines are bioanalysed by LC–MS–MS [9–12].

This paper describes the validation of a high-performance liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method, that has been developed in our laboratories, for the measurement of YF476 in human plasma (1 ml) using a close chemical analogue, CH-3058A (II, see Fig. 1), as an internal standard chosen because it would be expected to have very similar physicochemical properties to YF476.

2. Experimental

2.1. Materials and reagents

YF476 and an analogue CH-3058A suitable for use as an internal standard, were provided by Yamanouchi Pharmaceutical Co. Ltd., Tokyo, Japan. Heparinised blank human plasma was obtained from an accredited commercial source. Super purity grade water was purchased from Romil Ltd, Cambridge, UK, and HPLC grade methanol, acetonitrile, methyl-*tert*.-butyl ether, and analytical grade ammonium acetate and sodium hydrogen carbonate from Fisher Scientific, Loughborough, UK. Argon (research grade) and nitrogen (research and zero grades) were supplied by BOC Ltd, London, UK.

2.2. Instrumentation and LC–MS–MS conditions

The LC–MS–MS system consisted of a HP1090 auto-injector and pump (Hewlett-Packard Ltd, Stockport, UK), with a C_{18} Prodigy 5 μm, 30×4.6 mm I.D. column (Phenomenex, Macclesfield, UK), connected to the heated nebuliser assisted atmospheric pressure chemical ionisation (APCI) interface of an API III$^+$ triple quadrupole mass spectrometer (Sciex, Toronto, Canada).

The respective retention times of YF476 and the internal standard were about 0.9 and 1.4 min, respectively (Fig. 3).

Chromatography was performed isocratically using aqueous ammonium acetate (0.05 *M*, ca. pH 7)–acetonitrile (2:3 v/v) as the mobile phase at a flow-rate of 1 ml/min. The mass spectrometer was operated in the positive ion detection mode, which is appropriate for a basic compound such as YF476 [7]. The nebuliser temperature was set at 500 °C, the nebuliser gas (N_2) at 0.8 l/min and the auxiliary gas (N_2) at 2 l/min. The curtain gas was set at 1.2 l/min and the orifice voltage at 40 V. Argon was used as the collision gas at a thickness of ca. 3×10^{15} atoms/cm^2 and a collision energy of 15 eV. The ion

transitions of m/z 499→351 and m/z 527→351 were monitored for YF476 and its internal standard, respectively. The dwell time was set at 290 ms per channel with a pause time of 10 ms.

2.3. Preparation of stock solutions, calibration standards, quality control samples, stability samples and system suitability test solution

Primary stock solutions of YF476 were prepared from separate weighings for calibration standards and quality control (QC) samples. The primary stock solutions were prepared in acetonitrile and the subsequent working stock solutions were prepared by dilution with 0.05 *M* ammonium acetate–acetonitrile (3:1 v/v). CH-3058A internal standard solution was prepared in 0.05 *M* ammonium acetate–acetonitrile (3:1 v/v) at a concentration of 50 ng/ml. All solutions were stored at ca. 4 °C when not in use.

Calibration standards were prepared on an individual and daily basis by adding an appropriate volume of YF476 working stock solution to aliquots of blank pooled human plasma at concentrations of 0.1, 0.2, 1.0, 5.0, 10.0, 20.0 and 25.0 ng/ml. Bulk quality control samples were prepared at concentrations of 0.1, 0.3, 10.0 and 20.0 ng/ml. QC samples were stored as individual aliquots (1 ml) at ca. −20 °C until required for analysis. Stability samples were prepared in bulk and divided into aliquots (1 ml) at concentrations of 0.3 and 20.0 ng/ml. These stability samples were either analysed immediately to provide time zero data, left at room temperature (ca. 22 °C) for 2 h, subjected to three freeze–thaw cycles or stored at ca. −20 °C for up to 3 months prior to analysis.

The system suitability test solution was prepared by adding YF476 and CH-3058A to aqueous 0.05 *M* ammonium acetate–acetonitrile (3:1 v/v) at a concentration of ca. 20 ng/ml for both compounds.

2.4. Sample preparation and extraction procedure

Calibration standards and QC samples in plasma, and study samples (1 ml), were spiked with internal standard CH-3058A solution (50 ng/ml, 100 μl), and saturated sodium hydrogen carbonate (1 ml) was added to each sample followed by methyl-*tert*.-butyl ether (5 ml). The samples were rotary shaken for ca. 10 min followed by centrifugation, also for ca. 10 min, at ca. 2700 *g*. The organic supernatant was removed and evaporated to dryness under oxygen-free nitrogen at 35 °C. The resultant residue was reconstituted in 0.05 *M* ammonium acetate–acetonitrile (3:2 v/v, 250 μl), which was vortex mixed, sonicated (ca. 5 min) and then centrifuged at ca. 2500 *g* for ca. 10 min. A 70 μl portion of the reconstituted sample was injected onto the LC column for analysis.

2.5. Data acquisition and processing

Chromatographic data were acquired and processed using the data system (Apple Macintosh Power PC 8100) supplied with the Sciex mass spectrometer and the associated MacQuan V1.4 software (PE-Sciex, Toronto, Canada).

2.6. Assay validation

2.6.1. System suitability test

A system suitability test sample (70 μl) was analysed prior to each batch of samples so as to ensure that the instrument response, retention time and chromatographic peak shape for both YF476 and CH-3058A were as expected.

2.6.2. Linearity

Calibration standards at seven concentrations of YF476 in human plasma over the range 0.1–25.0 ng/ml were analysed singly. The linearity of the calibration line was assessed by fitting peak area ratios of YF476 to CH-3058A versus plasma YF476 concentrations using sum of least squares regression analysis with $1/x^2$ ($1/\text{concentration}^2$) weighting.

2.6.3. Precision and accuracy

QC samples ($n=6$) at each of four concentrations (0.1, 0.3, 10.0 and 20.0 ng/ml) were analysed to determine intra-day precision and accuracy, by comparing measured concentrations against theoretical values. Inter-day precision and accuracy were assessed on three separate occasions.

2.6.4. Extraction recovery

The recovery (extraction efficiency) of YF476 through the extraction procedure was determined at low (0.3 ng/ml), medium (10.0 ng/ml) and high (20.0 ng/ml) concentrations, by adding known volumes of YF476 working stock solution to the control human plasma prior to extraction. The responses of these samples were compared to those in which YF476 working solution was added to blank plasma extract to provide the same target concentrations ($n=6$). The extraction recovery of CH-3058A was assessed similarly at a concentration of ca. 5 ng/ml.

2.6.5. Specificity

The initial specificity of the method was assessed by extracting and analysing six different batches of blank and fortified human plasma, and comparing these with control plasma fortified with YF476 and CH-3058A. The ion chromatograms were examined for chromatographic peaks from endogenous substances that might interfere with the method. There were no such peaks from blank human plasma and a consistent response was obtained from spiked plasma. The method is achiral. However, conversion of YF476 to its opposite antipode would not be expected as metabolism or instability at the chiral centre is unlikely.

2.6.6. Storage stability

The stability of YF476 added to control human plasma at low (0.3 ng/ml) and high (20.0 ng/ml) concentrations was assessed after storage at ambient temperature (ca. 22 °C for 2 h), at ca. −20 °C for 1 week, 1 month, 3 months or after three freeze–thaw cycles. The concentrations of YF476 measured in the stored samples were compared with concentrations measured at time zero.

2.6.7. Effect of dilution

The effect of dilution was assessed by preparing control plasma samples fortified with YF476 at concentrations of 125 ng/ml and 1250 ng/ml which were then diluted 1 in 10 and 1 in 100, respectively with blank plasma. The concentrations of YF476 found in the replicate diluted samples ($n=6$) were compared with theoretical concentrations.

2.6.8. Injection carry-over

The LC–MS–MS system was checked for injection carry-over by injecting solvent blanks after the injection of a high concentration matrix standard.

3. Results and discussion

3.1. Mass spectrometry and chromatography

Heated nebuliser assisted atmospheric pressure chemical ionisation was preferred to ionspray (pneumatically assisted electrospray) for the analysis of YF476 because it provided a better response and because it would be less susceptible to matrix effects.

Under the mass spectrometric conditions used, YF476 and CH-3058A gave intense protonated molecular ions (precursor ions) at m/z 499 and 527, respectively (Fig. 2a, b). These ions were fragmented by collision activated dissociation (CAD) with argon at 15 eV in the Q2 region of the mass spectrometer to generate the product ions. The product ion spectra of YF476 and CH-3058A are presented in Fig. 2c, d. The most abundant product ion for both YF476 and CH-3058A (m/z 351) was monitored by multiple reaction monitoring (MRM) from the corresponding precursor ion. Q2 settling software was employed to eliminate MRM channel cross-talk. This product ion arose from loss of the alkylaminophenyl carbamoyl sidechain from both YF476 and the internal standard.

Under the liquid chromatographic conditions used, YF476 and CH-3058A were eluted with retention times of ca 0.9 and 1.4 min, respectively with a chromatographic cycle time of 3 min. Representative chromatograms of both MRM channels (YF476; m/z 499→351 and CH-3058A; m/z 527→351) for extracts of blank human plasma, and plasma containing YF476 at concentrations of 0, 0.1 ng/ml (LLOQ) and 25.0 ng/ml are presented as Fig. 3a–d, respectively. There was no detectable response from extracts of blank human plasma obtained from six different individuals as illustrated by Fig. 3a.

Examination of the MRM chromatograms obtained during the analysis of plasma from many different subjects showed that the within-day response for the internal standard was constant thus indicating the absence of matrix effects. It was not

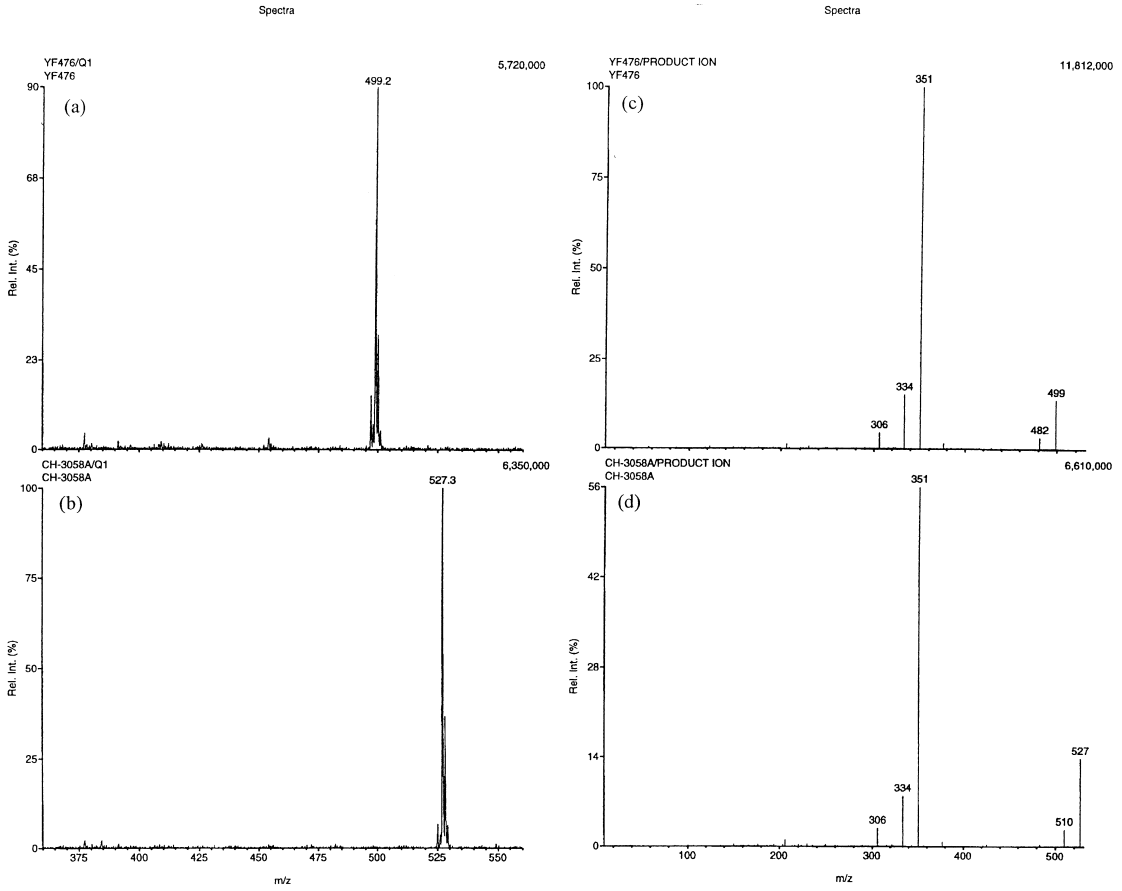


Fig. 2. Precursor ion and product ion mass spectra of (a, c) YF476 and (b,d) CH-3058A, respectively.

necessary to modify the method during the analysis of any clinical samples from Phase I studies in healthy volunteers.

3.2. Linearity and lower limit of quantification

The relationship between peak area ratios of YF476 to CH-3058A and concentrations of YF476 in human plasma was linear ($r \geq 0.9979$) over the calibration range 0.1–25.0 ng/ml. The lower limit of quantification (LLOQ) of the assay was 0.1 ng/ml (the lowest calibration point) using 1 ml of plasma. At the LLOQ, the inter-day precision on three occasions was 11% with a relative error of the mean of −7% (Tables 1 and 2). The sensitivity of the method for the measurement of YF476 was limited by the presence of an impurity (probably YF476 present in the internal standard) in the internal standard (see Fig. 3b), and the limit of quantification was set at four times that of the impurity. In the event, the lowest concentrations measured in clinical samples exceeded that of the impurity by some eighty times, and no ion corresponding to YF476 was detectable in the internal standard Q1 spectrum (Fig. 2b).

3.3. Precision and accuracy

Intra-batch precision of the method, as indicated by the coefficients of variation of the measured concentrations of replicate quality control samples ($n = 6$) on three occasions was 7–14% at 0.1 ng/ml, 3–8% at 0.3 ng/ml, 1–2% at 10.0 ng/ml and 2–6% at 20.0 ng/ml (Tables 1 and 2).

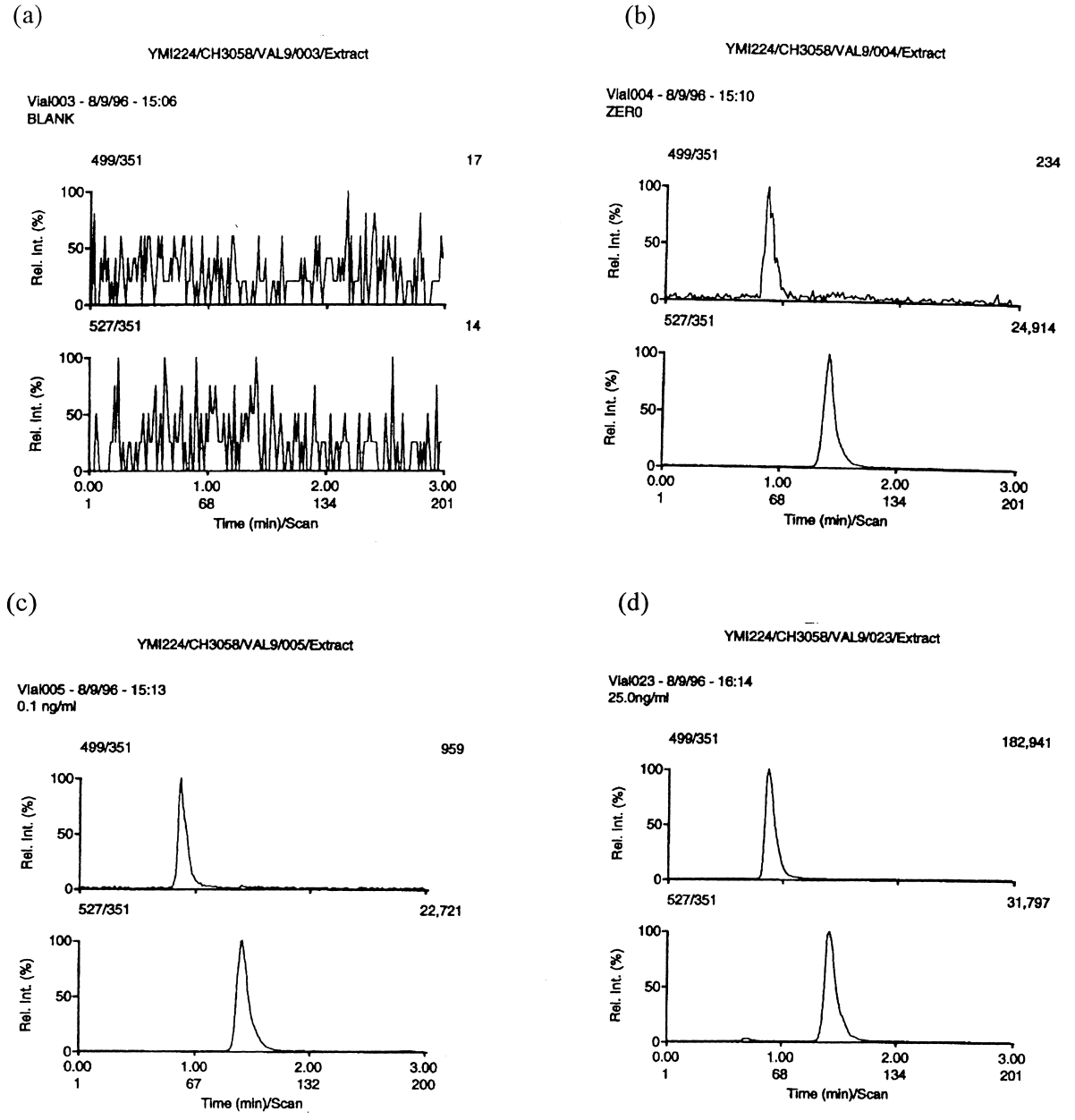


Fig. 3. Representative MRM chromatograms of YF476 (top trace) and internal standard (lower trace) for (a) blank human plasma, (b) 0.0 ng/ml, (c) 0.1 ng/ml (LLOQ) and (d) 25.0 ng/ml plasma calibration samples.

Intra-batch accuracy of the method, as indicated by the relative error of mean measurement of replicate quality control samples ($n=6$) on three occasions, was ±9% or less at 0.1 ng/ml, ±11% or less at 0.3 ng/ml, ±11% or less at 10.0 ng/ml and ±8% or less at 20.0 ng/ml (Tables 1 and 2).

Inter-batch precision of the method for three separate batches, calculated using analysis of vari-

Table 1
Analysis of replicate quality control samples on three occasions: intra-batch precision and accuracy

Batch	Conc (ng/ml)	Mean $n=6$[a]	SD	C.V. (%)	RE (%)
1	0.1	0.09	0.01	7	−8
2		0.09	0.01	10	−9
3		0.10	0.01	14	−2
1	0.3	0.30	0.01	4	1
2		0.29	0.02	8	−4
3		0.33	0.01	3	11
1	10.0	9.91	0.11	1	−1
2		8.91	0.11	1	−11
3		10.68	0.22	2	7
1	20.0	18.40	0.34	2	−8
2		20.69	1.21	6	3
3		20.22	1.18	6	1

SD, standard deviation; C.V., coefficient of variation; RE, relative error of mean. Mean, SD, C.V. and RE are all calculated using non-rounded data.

[a] For Batch 1 at 0.3 ng/ml, $n=5$.

ance, was 11% at 0.1 ng/ml ($n=18$), 9% at 0.3 ng/ml ($n=17$), 9% at 10.0 ng/ml ($n=18$) and 8% at 20.0 ng/ml ($n=18$) (Tables 1 and 2). The mean relative error of measurement across the range 0.1–20.0 ng/ml was ±7% or less.

3.4. Extraction recovery

Although liquid–liquid extraction was carried out in the presence of sodium ions, sodium adducts were not evident in the spectra obtained (limit <1% of analyte response). Sodium adduct formation is less of a concern when heated nebuliser-based methods are used than with those involving ionspray.

Table 2
Analysis of replicate quality control samples on three occasions: inter-batch precision[a] and accuracy

Concentration (ng/ml)	Mean concentration measured (ng/ml)	C.V. (%)	RE (%)	n
0.1	0.09	11	−7	18
0.3	0.31	9	3	17
10.0	9.83	9	−2	18
20.0	19.77	8	−1	18

SD, standard deviation; C.V., coefficient of variation; RE, relative error of mean. Mean, SD, C.V. and RE are all calculated using non-rounded data.

[a] Inter-day precision was calculated using a one-way analysis of variance (ANOVA).

The mean extraction recovery of YF476 from human plasma was 99±10% at 0.3 ng/ml ($n=6$), 97±2% at 10.0 ng/ml ($n=6$) and 92±5% at 20.0 ng/ml ($n=6$). The mean recovery of CH-3058A from human plasma was 90±2% at 5 ng/ml ($n=6$), the internal standard concentration used in the bioanalytical method.

3.5. Specificity

Although the extraction procedure in the bioanalytical method was relatively simple, high specificity was achieved using tandem mass spectrometry by monitoring the structurally specific ion fragmentations, m/z 499→351 (corresponding to the analyte) and m/z 527→351 (corresponding to the internal standard) as shown in Figs. 2 and 3. No matrix effects occurred during the analysis of control human plasma from six different subjects as the responses from YF476 and internal standard were similar in each case: the coefficients of variation of measurement of YF476 fortified in these six control plasma samples at 0.1 and 20 ng/ml were ±12% and ±3%, respectively and of the internal standard fortified alone was ±4% (area). No constituents of control human plasma from six individually sourced samples were found to interfere with the method nor were possible metabolites of YF476 considered to have compromised the robustness of the method as judged by the consistency of the pharmacokinetic data obtained during analysis of clinical samples and the constant response obtained for the internal standard. Interfering metabolites would be expected to either affect the peak shape for YF476 or to be separated from YF476 during LC. Expected metabolites of YF476 (formed by oxidation and/or side-chain cleavage) would have different masses from YF476 and the internal standard, and consequently interference from metabolites is most unlikely. The probability of a metabolite having the same extraction, chromatographic behaviour and MRM transition as the parent drug is remote particularly since the closely structurally related YF476 and the internal standard were easily separated during chromatography (see Fig. 3). The possibility of ''cross-talk''

was excluded by the LC–MS–MS instrument equipped with Q2 settling software.

3.6. Storage stability

YF476 was shown to be stable in human plasma at ambient temperature (ca. 22 °C) for up to 2 h, or when frozen at ca. −20 °C for at least 3 months or following three freeze–thaw cycles (Table 3).

3.7. Effect of dilution

The concentration of YF476 found in control samples ($n=6$) following a 10- or a 100-fold dilution with blank matrix were in good agreement (RE 5% and 4%, respectively) with the theoretical spiked concentrations after correction for the appropriate dilution factor. These data effectively confirm the increase of the analytical range of the method such that plasma samples with YF476 concentrations up to 2500 ng/ml can be quantified.

3.8. Injection carry-over

No injection carry-over of YF476 or CH-3058A was observed to an extent likely to interfere with the results obtained using the method.

3.9. Cross-matrix and cross-species validation

Although the cross-validation results are not included in this publication, the bioanalytical method has been adapted and validated for use with human urine, as well as rat and dog plasma. The performance specifications of these cross-validated methods are similar to those described for the human plasma.

3.10. Pharmacokinetics/pharmacodynamics

Some pharmacokinetic data obtained in humans using this method have been reported: after a single oral dose of 25 mg of YF476, a peak mean drug concentration of 89.5 ng/ml was reached at 0.5 h. Concentrations declined with a mean half-life of 4.3 [13]. YF476 exerted an antisecretory effect for at least 13 h postdose [13].

4. Conclusion

A highly sensitive and specific LC–MS–MS method for the determination of YF476 in human plasma has been developed and validated, to achieve a lower limit of quantification of 0.1 ng/ml. Validation results show that the bioanalytical method has

Table 3
Stability measurements of YF476 in human plasma

Time of storage (temperature)	Concentration (ng/ml)	Mean concentration measured (ng/ml)	SD	C.V. (%)	Recovery (%)
Day 0	0.3	0.34	0.03	8	112
	20.0	19.83	1.11	6	99
2 h	0.3	0.32	0.01	4	107
(ca. +22 °C)	20.0	19.96	1.07	5	100
3×FT	0.3	0.30	0.03	10	100
	20.0	19.25	0.81	4	96
1 week	0.3	0.32	0.01	5	107
(ca. −20 °C)	20.0	21.83	0.58	2	109
1 month	0.3	0.33	0.01	3	110
(ca. −20 °C)	20.0	21.20	0.40	3	106
3 months	0.3	0.33	0.01	3	112
(ca. −20 °C)	20.0	19.41	0.34	2	97

Number of replicates=6. SD, standard deviation; C.V., coefficient of variation; FT, freeze–thaw cycles. Mean, SD, C.V. and recovery of mean concentrations measured were calculated using non-rounded data.

good precision and accuracy over a wide concentration range (0.1–25.0 ng/ml).

This relatively simple, rapid and robust bioanalytical method has been used for processing large batches of clinical samples on a daily basis, and permits the higher throughput required to quantify YF476 in large numbers of biological samples from clinical trials.

References

[1] S.R. Bloom, R.G. Long, J.M. Polak, in: D.J. Weatherall, J.G.G. Ledingham, D.A. Warrell (Eds.), Oxford Textbook of Medicine, 2nd edition, Oxford Medical Publications, Oxford, UK, 1987, Section 12.52.
[2] S.A. Wank, J.R. Pisegna, A. de Weerth, Ann. N.Y. Acad. Sci. 713 (1994) 49.
[3] J. Calam, Acta Gastroenterol Belg. 56 (1993) 251.
[4] G. Semple, H. Ryder, D.P. Rooker, A.R. Batt, D.A. Kendrick, M. Szelke, M. Ohta, M. Satoh, A. Nishida, S. Akuzawa, K. Miyata, J. Med. Chem. 40 (1997) 331.
[5] Unpublished data. Yamanouchi Assay no. 95-1006 dated 21 August 1995.
[6] D. Thomas, S.D. Clarke, H.M. Hill, T.A.G. Noctor, J.N. Robson, in: E. Reid, H.M. Hill, I.D. Wilson (Eds.), Biofluid Assay For Peptide-related and Other Drugs, Royal Society of Chemistry, London, 1996, p. 230.
[7] K.N. Cheng, L.F. Chasseaud, Xenobiotic Metab. Dispos. 13 (1998) 382.
[8] J.F. Van Bocxlaer, K.M. Clauwaert, W.E. Lambert, D.L. Deforce, E.G. VandenEeckhout, A.P. DeLeenheer, Mass Spectrom. Rev. 19 (2000) 165.
[9] O.H. Drummer, J. Chromatogr. B 713 (1998) 201.
[10] K. Heinig, J. Henion, J. Chromatogr. B 732 (1999) 445.
[11] J. Zweigenbaum, K. Heinig, S. Steinborner, T. Wachs, J. Henion, Anal. Chem. 71 (1999) 2294.
[12] H. Yuan, Z. Mester, H. Lord, J. Pawliszyn, J. Anal. Toxicol. 24 (2000) 718.
[13] M. Boyce, S. Warrington, A. Johnston, A. Harris, Br. J. Clin. Pharmacol. 49 (2000) 493P.