Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 1831–1835
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Short communication

Method development and validation of repaglinide in human plasma by HPLC and its application in pharmacokinetic studies

Abu Bakar Ruzilawati [a,*], Mohd Suhaimi Abd. Wahab [a], Ahmad Imran [b], Zabidah Ismail [c], Siew Hua Gan [a]

[a] Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
[b] Department of Family Medicine, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia
[c] Faculty of Medicine, University College Sedaya International, Kuala Lumpur, Malaysia

Received 24 September 2006; received in revised form 21 November 2006; accepted 5 December 2006
Available online 16 December 2006

Abstract

In this study, the development and validation of a high-performance liquid chromatography (HPLC) assay for determination of repaglinide concentration in human plasma for pharmacokinetic studies is described. Plasma samples containing repaglinide and an internal standard, indomethacin were extracted with ethylacetate at pH 7.4. The recovery of repaglinide was 92% ± 55.31. Chromatographic separations were performed on Purospher® STAR C-18 analytical column (4.8 mm × 150 mm; 5 μm particle size). The mobile phase composed of acetonitrile–ammonium formate (pH 2.7; 0.01 M) (60:40, v/v). The flow rate was 1 ml/min. The retention time for repaglinide and indomethacin were approximately 6.2 and 5.3 min, respectively. Calibration curves of repaglinide were linear in the concentration range of 20–200 ng/ml in plasma. The limits of detection and quantification were 10 ng/ml and 20 ng/ml, respectively. The inter-day precision was from 5.21 to 11.84% and the intra-day precision ranged from 3.90 to 6.67%. The inter-day accuracy ranged 89.95 to 105.75% and intra-day accuracy ranged from 92.37 to 104.66%. This method was applied to determine repaglinide concentration in human plasma samples for a pharmacokinetic study.

Keywords: Repaglinide; HPLC; Method development; Validation

1. Introduction

Repaglinide is a new carbomoxylmethyl benzoic acid derivative, also known as 2-ethoxy-4-[2-[[3-methyl-1-[2-(1-piperidinyl)phenyl]butyl]amino]-2-oxoethyl] (Fig. 1a). It is a novel prandial glucose regulator for the treatment of type 2 diabetes mellitus [1,2]. It reduces the fasting glucose concentrations in patients with type 2 diabetes mellitus. It helps to control blood sugar by increasing the amount of insulin released by the pancreas. Repaglinide is rapidly absorbed from the gastrointestinal tract after oral administration. It differs from other antidiabetic agents in its structure, binding profile, duration of action and mode of excretion [3].

To date, several analytical methods are available for the determination of repaglinide in biological fluids, including liquid chromatography-tandem mass spectrometry (LC/MS/MS) [4,5]. However, these methods are costly and require the availability of expensive equipment. Furthermore, not many institutions have LC/MS/MS. To date, this is the only HPLC method available for repaglinide and no method for the determination of this drug in human plasma has been available. Gandhimathi et al. [6] and Berecka et al. [7] have reported an HPLC method for the determination of repaglinide in pharmaceutical dosage form.

In this paper, a newly developed and validated method using simple liquid-liquid extraction for the determination of repaglinide in human plasma using HPLC and diode-array detector is described. The developed method was applied to a pharmacokinetic study of repaglinide in healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Repaglinide standard was obtained from Novo Nordisk Pharma (Malaysia), batch no. 0004/1005858. The internal standard used was indomethacin (Fig. 1b) purchased from

* Corresponding author. Tel.: +609 7664187; fax: +609 7653370.
E-mail address: tieimran@yahoo.com (A.B. Ruzilawati).

0731-7085/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2006.12.010

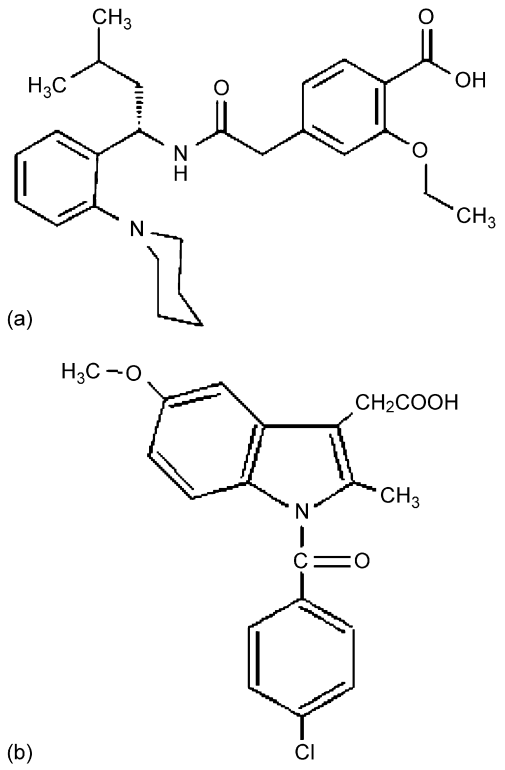

Fig. 1. Chemical structures of (a) repaglinide and (b) indomethacin (internal standard).

Sigma–Aldrich (St. Louis, MO, USA). All chemicals were of HPLC grade except for potassium dihydrogen orthophosphate (KH_2PO_4), ammonium formate, sodium hydroxide, isoamylalcohol, phosphoric acid (H_3PO_4) and formic acid 90% which were of analytical purity grade. Acetonitrile, dichloromethane, *n*-heptane, hexane, chloroform, ethylacetate, *tert*-methyl buthyl ethanol (MTBE), methanol and phosphoric acid (H_3PO_4) was from Merck® (Darmstadt, Germany). Potassium dihydrogen orthophosphate (KH_2PO_4), ammonium formate and sodium hydroxide were from Ajax Finechem (Australia). Isoamylalcohol and diethyl ether were from BDH Chemicals (England). Ethanol and formic acid 90% were from Fisher Scientific. The water used in the mobile phase was deionized and distilled using the Water Prodigy System (Labconco®, MO, USA).

2.2. Apparatus and chromatographic conditions

The HPLC system, Waters 2695 Separation Module consisted of a pump, a column heater, an autosampler, a degasser and Waters 2996 PDA Detector (Waters, Milford, MA, USA). Waters Empower software was used for data acquisition and processing, running on a Samsung PC. Chromatographic separations were performed on Purospher® STAR C-18 analytical column (4.8 mm × 150 mm; 5 μm particle size) (Merck, Darmstadt, Germany) attached to a guard column (LiChroCART Reverse Phase (RP-18) (5 μm: 150 mm × 4.6 mm i.d.) (Merck, Darmstadt, Germany). The mobile phase composed of acetonitrile–ammonium formate (pH 2.7; 0.01 M)(60:40, v/v). The formate buffer was prepared fresh daily with double-distilled deionized water. All solutions were filtered through a 0.45 μm membrane (Sartorius, Germany) prior to use. The flow rate was 1 ml/min and the column temperature was maintained at 30 °C. The channel on the diode-array detector was configured to acquire data at 244 nm. The volume of injection was 30 μl. The column was equilibrated for at least 20 min with the mobile phase flowing through the system before the injection of the drug standards. The run time was set at 10 min with the system operating at air-conditioned temperature (20 °C).

2.3. Preparation of stock solutions and standard working solution

Stock solutions of repaglinide (100 μg/ml) and indomethacin (100 μg/ml) were prepared by dissolving 1 mg of each drug in 10 ml ethanol. The standard solutions were stored at 4 °C in a clear glass volumetric flask and light-protected with aluminium foil. Repaglinide concentrations in the working solution chosen for the calibration curves were 20, 30, 60, 80, 100 and 200 ng/ml. Quality control (QC) samples (of low, medium and high concentration) at 40, 70 and 150 ng/ml were prepared in the same ways as the calibration standards. These working solutions were prepared fresh daily by making further dilutions of the stock solution in ethanol. Working standard solution of indomethacin was prepared by diluting the stock solution with ethanol at a concentration of 500 ng/ml. Fifty microlitres of indomethacin solution was used for every analysis.

2.4. Extraction procedure

The internal standard, indomethacin and repaglinide solutions were added to blank plasma samples in round bottom glass tubes. One millilitre extraction buffer (0.1 mol/l potassium dihydrogen orthophosphate (KH_2PO_4, pH 5.9) was added to the tubes. After the mixture was vortexed, 5 ml ethylacetate and 50 μl isoamylalcohol were added and the pH adjusted to 7.4 with 2 M NaOH. The tubes were shaken on a rotater for 10 min. This was followed by centrifugation at 3000 rpm for 30 min. After centrifugation, the ethylacetate phase was transferred into V-tubes and evaporated to dryness under a stream of nitrogen at 45 °C. The dried extract was reconstituted with 70 μl of mobile phase, vortex-mixed and transferred to a clean autosampler vial. Thirty μl of this solution was injected into the HPLC system.

2.5. Method validation

Plasma calibration curves were prepared and assayed in triplicate on three different days to evaluate linearity, precision, accuracy, recovery, limit of quantitation (LOQ), limit of detection (LOD), selectivity and stability.

2.6. Application of method to healthy volunteers' sample

Fifty two healthy volunteers were given 4 mg dose of repaglinide. The study protocols were approved by our local Ethics Committee. All subjects were screened against the study's inclusion/exclusion criteria and gave written informed con-

sents. Blood samples were collected at 0, 30, 60, 120, 180 and 240 min after drug administration. Blood samples were centrifuged immediately at 3500 rpm for 10 min and the plasma obtained was stored at −20 °C until HPLC analysis.

3. Results and discussion

3.1. Optimization of chromatographic conditions

In preparing the mobile phase, several combinations of buffer and organic phase have been tested. It was found that the mixture of acetonitrile–ammonium formate (pH 2.7; 0.01 M) (60:40, v/v) produced good peak for repaglinide which was well separated from its internal standard, indomethacin and the solvent fronts. This method was an improved method when compared to the previous report by Venkatesh et al. [8] where ternary gradient have been used, increasing the cost of analysis. Fig. 2 shows the representative chromatograms of blank human plasma, plasma samples spiked with repaglinide at 30 ng/ml and plasma sample obtained from a healthy subject after 30 min following an oral

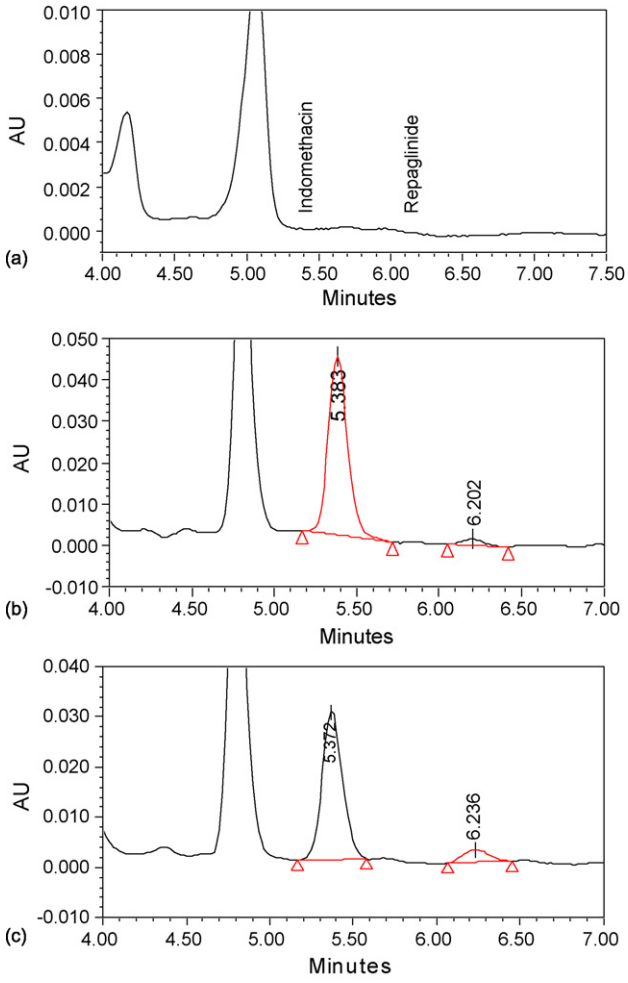

Fig. 2. Chromatograms showing: (a) blank human plasma, (b) plasma spiked with repaglinide (30 ng/ml) (6.202 min) and indomethacin (500 ng/ml) (5.383 min) and (c) plasma sample from a healthy subject after 30 min following a 4 mg oral dose of repaglinide, the plasma concentration was determined to be 71.63 ng/ml for repaglinide.

4 mg dose of repaglinide. The inorganic buffer such as formate buffer has been found to be the most suitable buffer that does not absorb at low wavelength [9]. Ammonium formate buffer (0.01 M) was chosen because it was sufficient in concentration to avoid band tailing. Silica-based particles are unstable at low pH (pH < 2) [10], therefore in this study, pH 2.7 was chosen. Furthermore, the best area counts for both drugs with the least band tailing was seen at this pH. The run time was short requiring only 10 min (retention times for repaglinide and indomethacin were approximately 6.2 and 5.3 min, respectively). This short analytical time is considered good for plasma samples.

3.2. Liquid–liquid extraction

In this experiment, liquid–liquid extraction method was chosen. To develop a single step liquid–liquid extraction procedure with good recovery, a large range of extraction solvents such as dichloromethane, diethyl ether, ethylacetate, hexane, *n*-heptane, chloroform and methyl *t*-butyl ether (MTBE) were investigated. According to Nascimento et al. [11], extraction of drugs from biological fluids are usually the most difficult step in any analysis due to the presence of interferences. The absolute recoveries of repaglinide after single extraction from plasma using these organic solvents were less than 80% except when ethylacetate was used where the absolute recovery was high (more than 80% for both repaglinide and indomethacin). Ethylacetate was found to be a good extracting solvent most probably because of its high polarity, cheapness and volatility [10]. Ethylacetate was thus chosen to further investigate the effect of pH on extraction efficiencies. It was found that plasma pH of 7.4 gave the highest and best percentage recovery for repaglinide (92%) and indomethacin (87%). Thus, this pH was selected for our liquid–liquid extraction method.

3.3. Recovery

Recovery was quantified by finding the ratio of the slopes of the calibration curves for extracted to non-extracted samples. Recovery study for repaglinide in plasma by ratio of slopes found to be 109.09%.

3.4. Calibration curves, precision, accuracy and linearity

The calibration curve for repaglinide was linear in the concentration range of 20–200 ng/ml in human plasma. The inter- and intra-day calibration curves showed consistent linearity, as seen in consistency of intercept, slope and coefficient of correlation. A typical concentration curve for repaglinide had a slope of 0.0013, an intercept of 0.0085 and $r^2 = 0.9930$. The precision and accuracy of the assay were determined from the low (40 ng/ml), medium (70 ng/nl) and high (150 ng/ml) QC plasma samples. Inter-day assay was determined by analyzing QC samples in triplicates and was analyzed on three different days. Intra-day was determined for each QC sample in plasma, each in triplicate on one day. Intra- and inter-day precision in this study was expressed as percent of coefficient of variation (CV). Accuracy was expressed as the mean percentage of ana-

Table 1
Precision and accuracy of the method for the determination of repaglinide in human plasma

Concentration (ng/ml)	Mean (ng/ml)	S.D.	Precision (CV)	Accuracy (%)	Percentage difference
Repaglinide intraday (within batch) ($n=3$)					
40	36.95	2.46	6.67	92.37	−7.63
70	73.26	2.86	3.90	104.66	4.66
150	150.51	7.80	5.19	100.34	0.34
Repaglinide interday (between batch) ($n=9$)					
40	36.85	3.06	8.32	92.11	−7.89
70	74.02	3.86	5.21	105.75	5.75
150	134.93	15.97	11.84	89.95	−10.05

lyte recovered in the assay [12]. According to Center for Drug Evaluation Research (CDER)[13], the precision determined at each concentration should not exceed 15% of CV except for LOQ where it should not exceed 20% of the CV. The results of the precision and accuracy determined of the method intra- and inter-days are shown in Table 1.

3.5. Specificity and selectivity

In this developed HPLC method, there were no co-eluting peaks occuring with other drugs including antidiabetics and antihypertensives drugs. For the selectivity study, paracetamol, loratadine, metformin, gliclazide, glibenclamide, acarbose, captopril, metoprolol, amlodipine, enalapril, prazosin, pravastatin, gliceryl trinitrate and frusemide were tested. Fig. 3 shows the chromatogram of one of the tested drugs (gliclazide) and the peaks of interests. Therefore, we conclude that this method is selective and suitable for quantification of repaglinide in samples of subject taking other medications.

3.6. Stability

Stability studies were carried out according to CDER guideline [13]. Two concentrations of repaglinide in plasma were prepared in triplicates at low (60 ng/ml) and high concentrations (200 ng/ml) and kept frozen at −20 °C until analysis. For short-term stability tests, five hours thaw, freeze thaw cycles and stability in autosampler carousel were studied. Five-hour stability was examined by leaving replicates of the low and high plasma quality control samples at room temperature on the bench five hours before injection. Freeze–thaw stability of the samples was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h and freezing for 12–24 h for each cycle respectively. Autosampler stability of repaglinide was tested by analyzing processed and reconstituted low and high plasma quality control samples which have been left in the autosampler tray for 24 h. For long-term stability tests, 1 week, 1 month and 3 months storage were studied. Sufficient samples were prepared and stored in the freezer for thawing and analysis for the whole of duration.

The concentration of repaglinide after each storage period was compared with the initial concentration that was determined for samples that were freshly prepared and immediately processed. In all of these stability studies, both repaglinide and indomethacin did not show any significant degradation (Table 2). When stored in plasma at −20 °C, the peaks of repaglinide and indomethacin did not have any interference with the matrix components during storage. These results confirmed that repaglinide was stable in plasma under the storage conditions and during injections.

3.7. Clinical samples

The method has been successfully applied to the analysis of samples from a pharmacokinetic study consisting of 52 healthy volunteers. A pharmacokinetic profile of repaglinide in human plasma after administration of 4 mg of repaglinide to a healthy

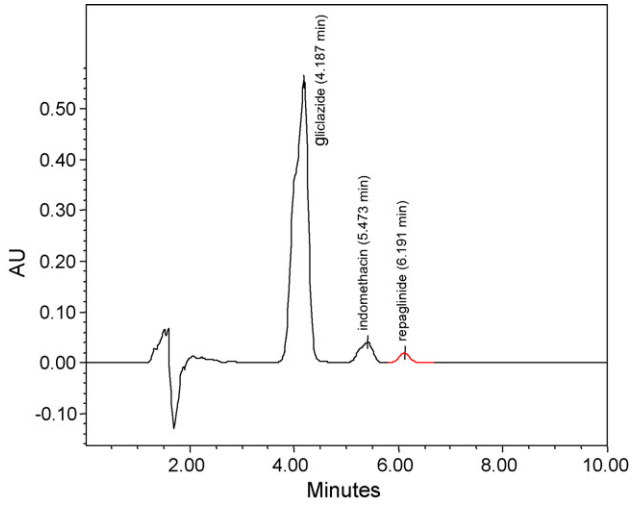

Fig. 3. Chromatogram of gliclazide (8 mg/ml) (4.187 min), indomethacin (500 ng/ml) (5.473 min) and repaglinide (200 ng/ml) (6.191 min).

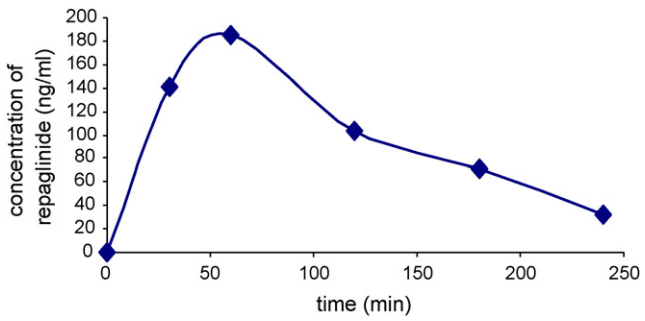

Fig. 4. A pharmacokinetic profile of repaglinide after administration of 4 mg of repaglinide to a healthy volunteer.

Table 2
Stability study

Quality control sample	Repaglinide 60 ng/ml (low concentration)	Repaglinide 200 ng/ml (high concentration)
Short term stability study		
(a) Five hours thaw		
Replicate 1	52.87	153.38
Replicate 2	59.25	154.53
Replicate 3	70.08	124.99
Mean	60.74	167.29
S.D.	8.70	7.72
CV	14.32	4.87
(b) Freeze–thaw cycle		
Replicate 1	57.99	215.45
Replicate 2	47.91	240.86
Replicate 3	65.84	220.36
Mean	57.25	225.56
S.D.	7.98	13.48
CV	14.13	5.98
(c) Autosampler		
Replicate 1	54.53	225.53
Replicate 2	69.23	237.82
Replicate 3	54.96	193.80
Mean	59.58	219.05
S.D.	8.37	22.72
CV	14.04	10.37
Long term stability study		
(a) One week stability		
Replicate 1	61.17	218.25
Replicate 2	56.25	233.19
Replicate 3	59.84	237.97
Mean	59.07	229.80
S.D.	2.55	10.29
CV	4.31	4.48
(b) One month stability		
Replicate 1	69.55	216.32
Replicate 2	68.93	230.26
Replicate 3	64.80	217.91
Mean	67.76	221.50
S.D.	2.58	7.63
CV	3.81	3.45
(c) Three months stability		
Replicate 1	39.96	278.72
Replicate 2	41.37	251.37
Replicate 3	36.97	256.33
Mean	39.43	262.14
S.D.	2.25	14.57
CV	5.70	5.56

volunteer is shown in Fig. 4. The maximum repaglinide plasma concentration (C_{max}) was 185.75 ng/ml, time to C_{max} (t_{max}) was 1.1 h and half-life ($t_{1/2}$) was 1.0 h. The parameters are in good agreement with those reported previously [2].

4. Conclusion

The developed and validated HPLC method coupled with liquid–liquid extraction for sample preparation is simple and convenient for the quantitation of repaglinide in human plasma samples. The method has good linearity, accuracy, precision, selectivity and stability over the relevant therapeutic concentration range. In conclusion, the method described is suitable for clinical monitoring of plasma level of repaglinide in patients and in pharmacokinetic studies.

Acknowledgements

This study was financially supported by Universiti Sains Malaysia IRPA grant (grant no. 305/PPSP/6112222). The author was supported by Universiti Sains Malaysia Academic Staff Training Scheme. We are grateful to Mrs Maria Yoharnis (HUSM) for her help in clinical studies.

References

[1] T.M. Marbury, J.L. Ruckle, V. Hatorp, M.P. Andersen, K.K. Nielsen, W.C. Huang, P. Strange, Clin. Pharmacol. Ther. 67 (2000) 7–15.
[2] V. Hatorp, H. Won-Chin, P. Strange, Clin. Ther. 21 (1999) 702–710.
[3] J.R. Culy, B. Jarvis, Drugs 61 (2001) 1625–1660.
[4] M. Niemi, J.T. Backman, M. Neuvonen, Clin. Pharmacol. Ther. 68 (2000) 495–500.
[5] A. Grenschel, K. Beschke, H. Rapp, W. Roth, J. Chromatogr. 568 (1991) 246–252.
[6] M. Gandhimathi, T.K. Ravi, S.K. Renu, Anal. Sci. 19 (2003) 1675–1677.
[7] A. Berecka, A. Gumieniczek, H. Hapkala, J. AOAC Int. 89 (2006) 319–325.
[8] P. Venkatesh, T. Harisudhan, H. Choudhury, R. Mullangi, N.R. Srinivas, Biomed. Chromatogr. 20 (2006) 1043–1048.
[9] J.W. Dolan, L.R. Snyder, in: J.W. Dolan, L.R. Snyder (Eds.), Troubleshooting LC Systems, Humana Press, New Jersey, 1989, pp. 292.
[10] J. Chamberlain, in: J. Chamberlain (Eds.), Analysis of Drugs in Biological Fluids, CRC Press, Boca Raton, Florida, 1985, pp. 192.
[11] T.G. Nascimento, E.J. Oliveira, R.O. Macedo, J. Pharm. Biomed. Anal. 37 (2005) 777–783.
[12] S.H. Gan, R. Ismail, J. Chromatogr. B 759 (2001) 325–335.
[13] Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services, Food and Drug Administration. Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001.