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Development and validation of a bioanalytical method for the determination of the cholecystokinin type-1 (CCK_1) receptor antagonist dexloxiglumide in human plasma

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Abstract

A sensitive bioanalytical method for the measurement of dexloxiglumide, a new selective and potent cholecystokinin type-1 (CCK_1) receptor antagonist, in plasma, is reported. The method is based on reversed-phase liquid chromatography with ultraviolet absorption detection. Samples are extracted under acidic conditions into an organic solvent, and following evaporation, reconstitution and centrifugation stages, the supernatant is injected on to an ODS column with detection at 244 nm. The method has been validated over the concentration range 0.2–20 μg/ml, 0.2 μg/ml being the lower limit of quantification. The overall precision and accuracy (expressed as relative error) of the method was less than 6.1 and 2.3%, respectively. Dexloxigulmide was shown to be stable in plasma when stored at −20 °C for at least 200 days. The method is suitable for studying the pharmacokinetics of dexloxiglumide in man.

Keywords: Cholecystokinin type-1; Dexloxiglumide; Receptor antagonist

1. Introduction

The peptide hormone cholecystokinin (CCK) is a major regulator of gall bladder contraction and pancreatic enzyme secretion in response to food intake [1,2]. It also regulates motor and sensory functions in the gut [3], CCK receptors are present in the intestines and in the brain.

Dexloxiglumide, (*R*)-4-(3,4-dichlorobenzamido)-5-[*N*-(3-methoxypropyl)-*N*-pentylamino]-5-oxopentanoic acid, CR 2017 (Fig. 1a), is a selective and potent cholecystokinin type-1 (CCK_1) receptor antagonist being developed for the treatment of constipation-prone irritable bowel syndrome [4].

In order to investigate the pharmacokinetics of dexloxiglumide in man it was necessary to develop a simple sensitive and specific method for its measurement in human plasma. The only other previously published method for the measurement of dexloxiglumide in plasma [5] was based on the use of automated solid-phase extraction equipment, AASP (Varian, CA, USA), which is no longer commercially available. The study presented here describes a simpler bioanalytical method which has been developed and its validation.

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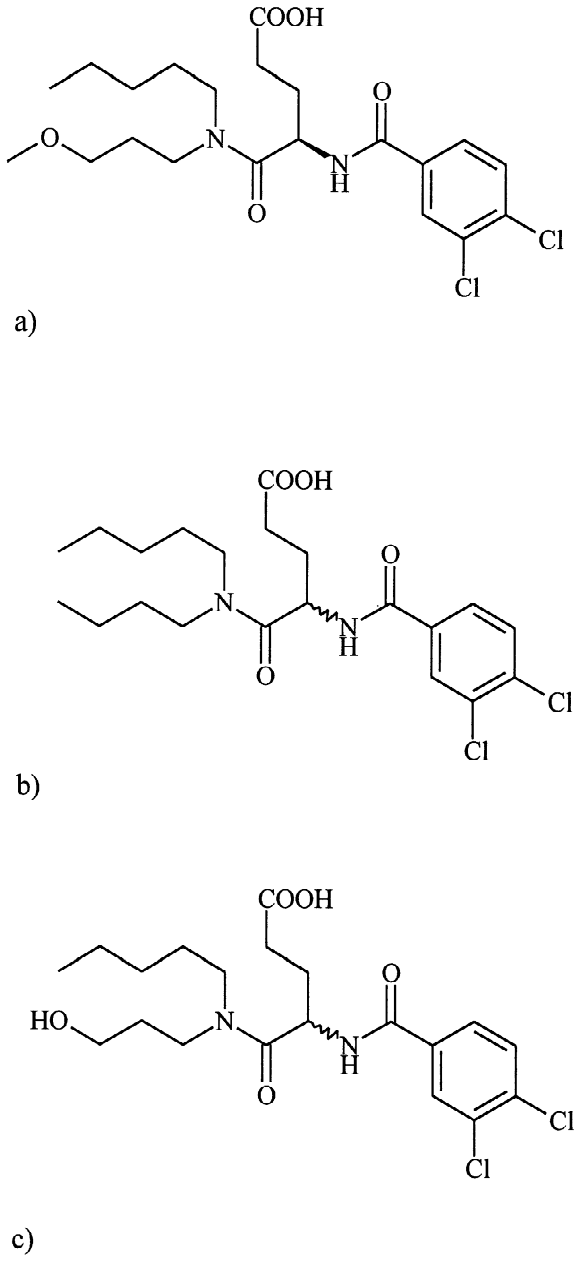

Fig. 1. (a) Dexloxiglumide (CR2017), (b) internal standard (CR 1409), (c) metabolite (CR 3529).

The developed method comprises of a liquid–liquid extraction of acidified plasma (100 μl) containing a structurally related internal standard, lorglumide (CR 1409) (Fig. 1b). Following evaporation of the organic phase, reconstitution in acetonitrile, centrifugation, to remove particulates and after further evaporation, the extract is reconstituted in mobile phase and analysed by reversed-phase HPLC with UV detection at 244 nm. The method was shown to be specific for dexloxiglumide as a known dexloxiglumide metabolite, CR 3529 (Fig. 1c) formed by *O*-demethylation of the parent drug, is well resolved using these chromatographic conditions (Fig. 2). The method utilises 100 μl of human plasma and has been validated over the concentration range 0.2–20 μg/ml. This method was found to be suitable to analyse plasma samples from human volunteers during a study to determine the pharmacokinetics and metabolism of dexloxiglumide in male human subjects [6].

2. Experimental

2.1. Chemicals and reagents

Dexloxiglumide (CR 2017), internal standard (CR 1409), (*RS*)-4-[(3,4-dichlorobenzoyl)amino]-5-(dipentylamino)-5-oxopentanoic acid and metabolite (CR 3529), (*RS*)-4-[(3,4-dichlorobenzoyl)amino]-5-[(3-hydroxypropyl)pentylamino]-5-oxopentanoic acid were provided by Rotta Research Laboratorium, (Monza, Italy). Acetonitrile was far UV HPLC grade (Chromasolv), obtained from Reidel–de-Haen (Seelze, Germany). Ethyl acetate (Distol), potassium dihydrogen orthophosphate, di-potassium hydrogen orthophosphate trihydrate (analytical grade) were from Fisher Scientific (Loughborough, UK). Orthophosphoric acid (85%), (HiPerSolv) was obtained from VWR International (Poole, UK). Water was ultra high pure (Elga, High Wycombe, UK) and blank human plasma (Li heparin anticoagulant) was obtained from Charterhouse Clinical Research Unit (The Royal Masonic Hospital, London, UK). Stock solutions of analytical standards were prepared in acetonitrile–water (50:50, v/v) and stored at nominally +4 °C, under which conditions they were stable for at least 1 month.

2.2. Sample preparation procedure

Plasma samples were thawed and aliquots (100 μl) transferred into glass screw-capped extraction

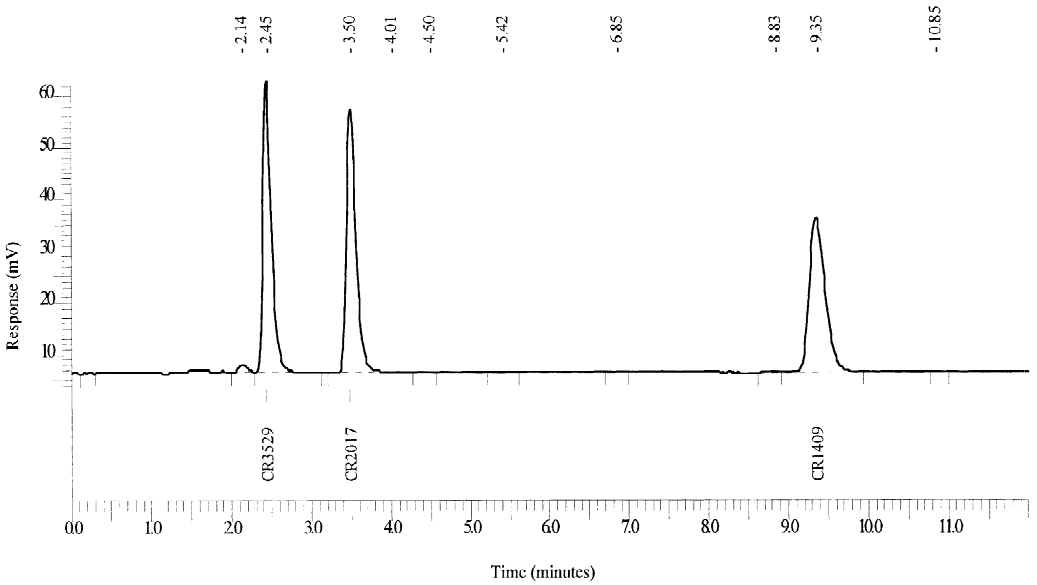

Fig. 2. Chromatogram of dexloxiglumide (CR 2017), internal standard (CR 1409) and metabolite (CR 3529) standards in mobile phase (50 μl injection of 5 μg/ml standard solution).

tubes. These samples were then subjected to the sample preparation procedure as outlined in Fig. 3.

2.3. Instrumentation

The HPLC system comprised of a Waters 2690 separation module coupled to a Waters 486 tunable absorbance detector (Waters, Watford, UK). Chromatographic data was captured and processed using a TurboChrom, Client/Server 6.1 data acquisition system (Perkin-Elmer, Watford, UK).

2.4. Data processing

Peak height ratios of dexloxiglumide to internal standard were calculated automatically by the computing integrator. Calibration lines were prepared with each analytical batch on the basis of peak height ratio versus concentration of dexloxiglumide in plasma. A $1/x$ (1/concentration) weighted, least-squares regression of the data (which improved the accuracy of measurement at low concentrations) was used to calculate slope, intercept and correlation coefficient and concentrations of dexloxiglumide in test and QC samples. Regression analysis and calculations were performed using in-house developed and validated software (Cal/Val, v2.0)

2.5. Chromatographic conditions

Sample extracts (50 μl) were injected on to a YMC-Pack ODS-AM-302 column 150×4.6 mm, mean particle size 5 μm; Capital HPLC, Broxburn, UK) which was maintained at nominally +35 °C. The analytical column was protected by a guard column (10×3.2 mm) containing the same packing material (Hichrom, Reading, UK). The mobile phase consisted of acetonitrile and phosphate buffer (0.05 *M*, pH 6) mixed in the ratio of 1:1, (v/v) at a flow-rate of 1 ml/min. The eluent was monitored at 244 nm and the analytical run time was 12 min. Under these conditions, dexloxiglumide (CR 2017), internal standard (CR 1409) and metabolite (CR 3529) were well resolved (Fig. 2).

2.6. Calibration standard solutions

Stock solutions of dexloxiglumide were prepared by weighing 10 mg of the free acid into a 10-ml volumetric flask, dissolving and making up to volume with acetonitrile–water (50:50, v/v). This solu-

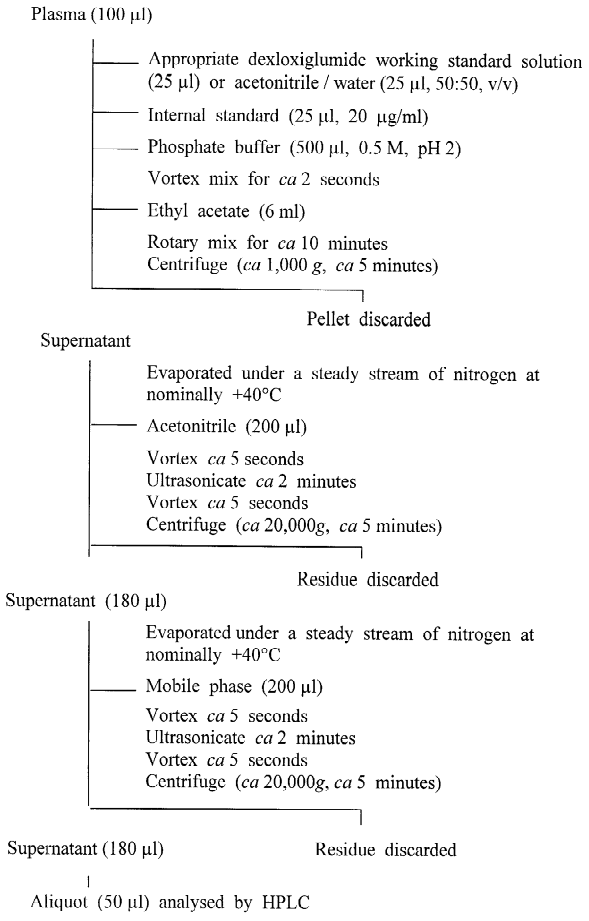

Fig. 3. Schematic representation of the sample extraction procedure.

tion was further diluted to produce a stock solution at a concentration of 400 μg/ml. Working solutions of dexloxiglumide were prepared by dilution of this stock solution to produce concentrations at 0.8, 2, 4, 8, 20, 40 and 80 μg/ml. Aliquots (25 μl) of these working solutions were added to blank human plasma (100 μl) to give plasma calibration standards at concentrations of 0.2, 0.5, 1, 2, 5, 10 and 20 μg/ml.

Stock solutions of internal standard were prepared by weighing 5 mg of CR 1409 into a 10-ml volumetric flask, dissolving and making up to volume with acetonitrile/water (50:50, v/v). Working internal standard solution was prepared by diluting this solution to produce working stock solution at a concentration of 20 μg/ml. An aliquot (25 μl) of this solution was added to each plasma sample.

2.7. Quality control samples

Quality control (QC) samples to assess the precision and accuracy of the analytical method, the stability of dexloxiglumide in plasma and the recovery of dexloxiglumide from plasma were prepared independently using standard solutions prepared separately from those used to calibrate the method. Four levels of QC samples were prepared in bulk (10 ml) at concentrations equivalent to the lower limit of quantification (LLOQ) of the method and at low, middle and high concentrations over the calibration range, by the addition of appropriate standard solutions of dexloxiglumide to volumetric flasks and made up to volume with blank plasma to give dexloxiglumide concentrations of 0.2, 0.4, 8 and 18 μg/ml, respectively. These samples were divided into suitable aliquots and stored in polypropylene tubes. Replicate ($n=6$) samples at each of the four concentrations were analysed immediately, the remainder were stored at nominally −20 °C until taken for analysis. A dilution QC was prepared in plasma at a concentration ten-fold greater than the high QC concentration, equivalent to 180 μg/ml. This sample was subjected to a one in ten dilution with blank human plasma, in replicate ($n=6$) prior to analysis.

2.8. Studies in human subjects

Plasma samples for analysis were generated during a study to determine the pharmacokinetics and metabolism of dexloxiglumide in four male human subjects following oral and intravenous infusion doses of 200 mg ^{14}C-dexloxiglumide, details of the clinical study are reported separately [6].

3. Method validation

The method was fully validated [7] to determine the following parameters.

3.1. Linearity

The linearity of the bioanalytical method was determined by plotting detector response (peak

height ratio of dexloxiglumide to internal standard) against the theoretical concentration of dexloxiglumide present. Calibration measurements were subjected to least squares regression analysis by computer program, to provide information on the slope, *y*-intercept, correlation coefficient (*r*) and the back-calculated calibration standard concentrations.

3.2. Precision

The precision of the method was evaluated for within- (intra-) and overall- (inter-) batch analyses by determining the coefficients of variation (C.V.) of the mean concentrations of dexloxiglumide measured in replicate samples. Four levels of QC samples, at the LLOQ, low, middle and high concentrations over the calibration range were analysed in replicate (nominally $n=6$) on three separate occasions.

3.3. Accuracy

The accuracy of the bioanalytical method was determined by measuring the concentrations of dexloxiglumide in replicate QC samples and comparing them with the theoretical concentrations of dexloxiglumide in these samples. The accuracy was calculated by dividing the mean concentration found by the theoretical concentration and was expressed in terms of relative error (RE) of measurement (i.e. inaccuracy):

$$\text{RE}\,(\%) = \frac{(\text{Mean calculated concentration} - \text{theoretical concentration}) \times 100}{\text{Theoretical concentration}}$$

3.4. Sensitivity

The sensitivity of the method, in terms of LLOQ was considered to be the lowest calibration standard at which acceptable precision (C.V. $\pm 20\%$) and accuracy ($100 \pm 20\%$) was observed.

3.5. Recovery

The recoveries of dexloxiglumide and internal standard through the extraction procedure were determined by comparing the responses of the analytes extracted from replicate QC samples (nominally $n=6$) with the responses of analytes in non-extracted standard solutions at equivalent concentrations. Recoveries were determined at low, middle and high concentrations over the calibration range for dexloxiglumide and at a single concentration for the internal standard.

3.6. Specificity

The specificity of the method was assessed qualitatively for the presence of interfering peaks and changes in retention times by comparison of chromatograms of extracts of blank human plasma, plasma containing internal standard and plasma containing internal standard together with low and high concentrations of dexloxiglumide. Six independent sources of blank human plasma were checked for the presence of interfering endogenous peaks. The chromatographic system was also checked for injection carry-over, by injection of mobile phase immediately after a high concentration sample extract. A known metabolite of dexloxiglumide, CR 3529 was analysed to further assess the specificity of the method.

3.7. Stability

The stability of dexloxiglumide was assessed in plasma by analysing replicate QC samples (nominally $n=6$) at low and high concentrations over the calibration range. Samples were analysed immediately after preparation, after ≈ 2 h at ambient room temperature (nominally $+22\,^{\circ}\text{C}$), after storage, frozen (nominally $-20\,^{\circ}\text{C}$) for 7, 33, 56 and 203 days and after three and five freeze–thaw cycles. The samples subjected to three and five freeze–thaw cycles were stored at nominally $-20\,^{\circ}\text{C}$ and on up to five separate days thawed to ambient room temperature for ≈ 2 h. In addition, the stability of dexloxiglumide after extraction and reconstitution was assessed at $+22\,^{\circ}\text{C}$ for ≈ 24 h. The concentrations of dexloxiglumide measured in the stability samples were compared to time zero concentrations.

3.8. Sample dilution

The effect of dilution was assessed at a concentration ten-fold greater than the highest QC sample. This sample was diluted in replicate ($n=6$) 1 in 10 with blank human plasma and the concen-

tration of dexloxiglumide measured in the diluted samples was compared to the theoretical concentration.

4. Results and discussion

4.1. Linearity

The relationship between peak height ratio of dexloxiglumide to concentration of dexloxiglumide in plasma was linear (mean $r=0.9998$, $n=9$) over the calibration range 0.2–20 μg/ml. The back-calculated concentration values for each calibration standard expressed as mean relative error were between −1.4 and 1.8% over the calibration range (Table 1).

4.2. Intra-batch precision and accuracy

Within-batch precision and accuracy measurements as indicated by the C.V. and relative error, respectively, of the measured concentrations of replicate ($n=6$) QC samples in three batches were well within ±10% (Table 2).

4.3. Inter-batch precision and accuracy

Between-batch precision and accuracy measurements were also well within ±10% (Table 3).

4.4. Sensitivity

The sensitivity of the method in terms of LLOQ was 0.2 μg/ml based on a plasma sample volume of 100 μl. At this concentration the within-batch precision ranged from 3.3 to 6.3% and the accuracy of measurement ranged from 97.1 to 107.6% and the analyte response was on average at least five times the response compared to a blank sample.

4.5. Recovery

The mean recovery (extraction efficiency) of dexloxiglumide from plasma was 79.8% (±4.4 SD, $n=6$) at 0.4 μg/ml, 81.4% (±3.9 SD, $n=6$) at 8 μg/ml and 83.0% (±3.6 SD, $n=6$) at 18 μg/ml. The mean recovery of internal standard was 61.3% (±3.0 SD, $n=6$) at 5 μg/ml.

4.6. Selectivity

In extracts of five of six separate batches of blank human plasma there were no interfering peaks present in chromatograms corresponding to the retention times of dexloxiglumide or internal standard which gave a detector response greater than ≈20% of the lowest calibration standard. In one batch, there was an endogenous peak present in its chromatogram corresponding to the retention time of dexloxi-

Table 1
Calibration measurements of dexloxiglumide in human plasma

Calibration number	Back-calculated concentrations (μg/ml)							Slope	Intercept	r
	0.2	0.5	1	2	5	10	20			
1	0.21	0.52	0.94	1.96	5.01	9.82	20.25	0.4714	−0.0003	0.9998
2	0.21	0.51	0.97	1.93	4.96	10.21	19.92	0.4388	0.0037	0.9999
3	0.20	0.51	0.97	1.99	5.04	10.08	19.90	0.4486	0.0102	1.0000
4	0.20	0.51	0.97	2.01	5.03	9.77	20.21	0.4667	0.0074	0.9999
5	0.19	0.49	1.01	2.01	5.15	10.39	19.46	0.4873	0.0149	0.9995
6	0.22	0.48	1.00	1.97	4.85	10.17	20.03	0.4547	0.0029	0.9998
7	0.21	0.50	1.00	1.95	4.91	9.93	20.20	0.4330	−0.0032	0.9999
8	0.19	0.50	1.01	2.04	5.12	10.01	19.83	0.4799	0.0218	0.9999
9	0.20	0.50	1.00	1.98	4.94	10.10	19.98	0.4562	0.0136	1.0000
Mean	0.20	0.50	0.99	1.98	5.00	10.05	19.97	0.4596	0.0079	0.9998
SD	0.01	0.01	0.02	0.03	0.10	0.20	0.25	0.0183	–	–
C.V. (%)	3.8	2.5	2.4	1.7	2.0	1.9	1.2	4.0	–	–
RE (%)	1.8	0.2	−1.4	−0.9	−0.0	0.5	−0.1	–	–	–

C.V. = coefficient of variation; SD = standard deviation; r = correlation coefficient; RE = mean relative error.

Table 2
Intra-batch precision and accuracy measurements for batches 1–3

Batch number		Theoretical concentration (μg/ml)			
		0.20	0.40	8.00	18.00
1	Mean back-calculated concentration (μg/ml)	0.22	0.42	8.06	18.23
	SD	0.01	0.01	0.19	0.49
	C.V. (%)	3.3	2.3	2.4	2.7
	RE (%)	7.6	4.2	0.8	1.3
2	Mean back-calculated concentration (μg/ml)	0.20	0.41	8.26	18.47
	SD	0.01	0.01	0.14	0.35
	C.V. (%)	3.8	1.6	1.7	1.9
	RE (%)	2.2	2.4	3.3	2.6
3	Mean back-calculated concentration (μg/ml)	0.19	0.39	7.97	18.09
	SD	0.01	0.01	0.25	1.08
	C.V. (%)	6.3	1.7	3.2	5.9
	RE (%)	−2.9	−3.2	−0.4	0.5

glumide which gave a response of ≈25% of the lowest calibration standard. A known metabolite of dexloxiglumide, CR 3529 was resolved and did not interfere with the measurement of dexloxiglumide (Fig. 1). Typical chromatograms of plasma extracts after oral and intravenous doses of dexloxiglumide to human volunteers are illustrated in Fig. 4.

4.7. Stability

Dexloxiglumide was shown to be stable in plasma at ambient room temperature (nominally +22 °C) for ≈2 h, stored frozen at nominally −20 °C for up to 203 days and following freeze–thaw cycles. Dexloxiglumide was also stable in extracted samples for ≈24 h at nominally +22 °C (Table 4).

Table 3
Inter-batch precision and accuracy measurements

	Theoretical concentration (μg/ml)			
	0.20	0.40	8.00	18.00
Mean back-calculated concentration (μg/ml)	0.20	0.40	8.10	18.27
SD	0.01	0.01	0.23	0.65
C.V. (%)	6.1	3.6	2.8	3.6
RE (%)	2.3	1.1	1.2	1.5
n	18	18	18	17

n = number of replicates.

4.8. Dilution

The concentration of dexloxiglumide found in QC samples following a ten-fold dilution in blank plasma were in good agreement with the theoretical concentration (RE, 2.5%, C.V., 11.4%).

4.9. Test sample analysis

Plasma concentrations following administration of 200 mg dexloxiglumide as a single intravenous infusion dose (over 20 min) or following oral capsule administration are shown in Fig. 5. A C_{max} of 19.5 (±2.9 SD) μg/ml was reached after the end of the infusion period. After oral dosing, a mean C_{max} of 22.0 (±0.3 SD) μg/ml was reached after 1.44 h (±0.52 SD) after dosing. The mean availability of dexloxiglumide after oral dosing was 48% (±10 SD) [6].

5. Conclusion

A selective, sensitive, precise and accurate method for the measurement dexloxiglumide has been developed and used in clinical pharmacokinetic investigations with dexloxiglumide. The method was fully validated to current regulatory guidelines in-

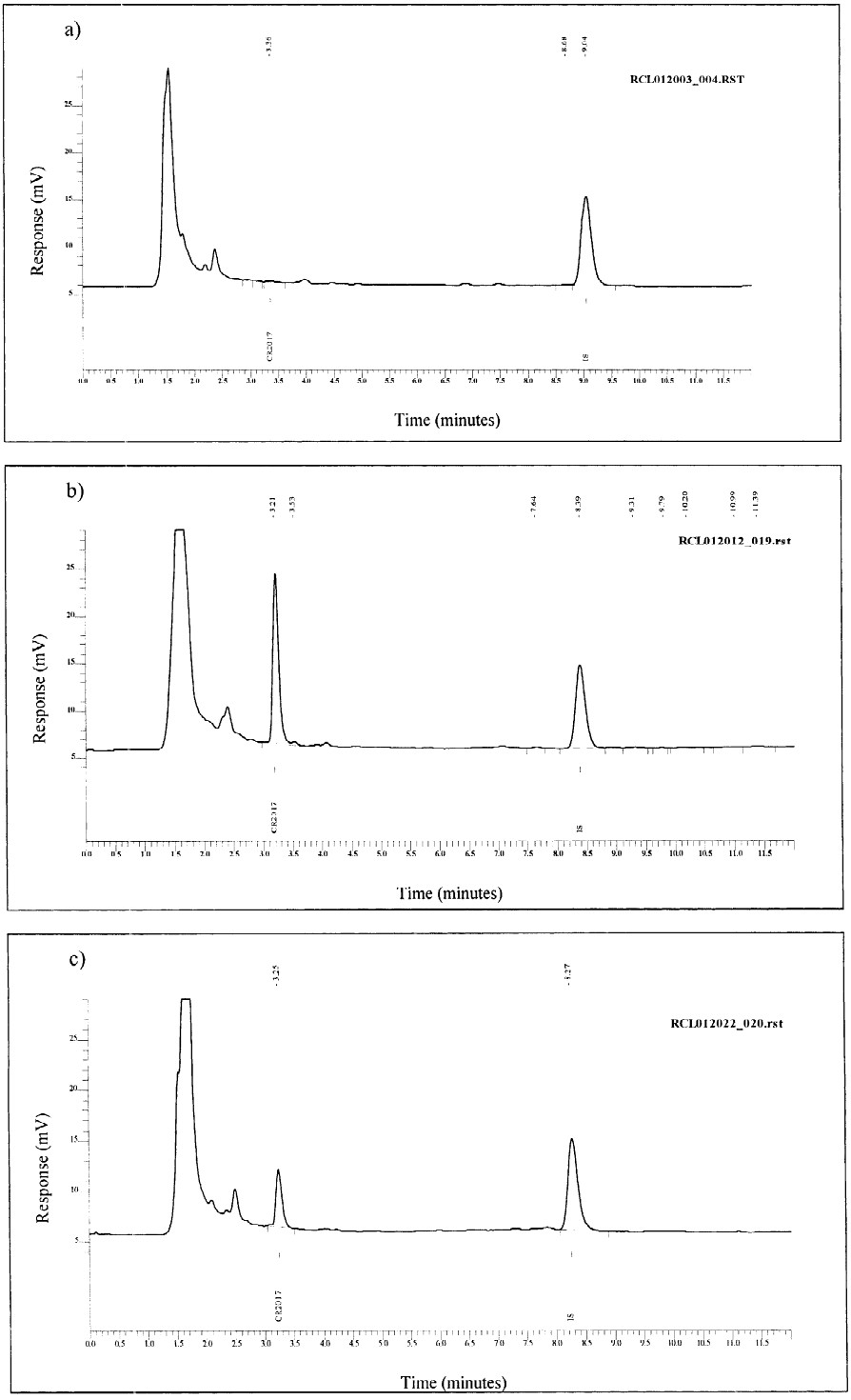

Fig. 4. Chromatograms of human plasma extracts: (a) predose plasma, (b) 30 min after intravenous dosing (containing 4.55 μg/ml), (c) 2 h after oral dosing (containing 1.44 μg/ml). CR 2017 = dexloxiglumine; I.S. = internal standard.

Table 4
Stability measurements of dexloxiglumide in human plasma

Storage period (nominal temperature)	Theoretical concentration (μg/ml)	Mean concentration (μg/ml)	C.V. (%)	*n*	Relationship to time zero (%)
0 h	0.4	0.42	2.5	6	–
2 h (+22 °C)		0.42	1.2	6	100.0
24 h (+22 °C)[a]		0.39	1.4	5	92.9
3 freeze–thaws[b]		0.38	3.7	6	90.5
5 freeze–thaws[b]		0.38	8.7	6	90.5
7 days (−20 °C)		0.39	2.1	6	92.9
33 days (−20 °C)		0.39	1.9	6	92.9
56 days (−20 °C)		0.39	3.2	6	92.9
203 days (−20 °C)		0.42	8.8	5	100.0
0 h	18	18.23	2.7	6	–
2 h (+22 °C)		17.06	2.6	5	93.6
24 h (+22 °C)[a]		18.34	1.6	5	100.6
3 freeze–thaws[b]		18.41	4.7	6	101.0
5 freeze–thaws[b]		17.13	0.6	6	94.0
7 days (−20 °C)		18.09	6.0	5	99.2
33 days (−20 °C)		17.28	1.3	6	94.8
56 days (−20 °C)		16.40	1.8	6	90.0
203 days (−20 °C)		19.78	0.9	6	108.5

SD = standard deviation; C.V. = coefficient of variation; *N* = number of replicates.
[a] After sample extraction.
[b] Cycles of freezing (nominally −20 °C) and thawing (nominally +22 °C, ≈2 h).

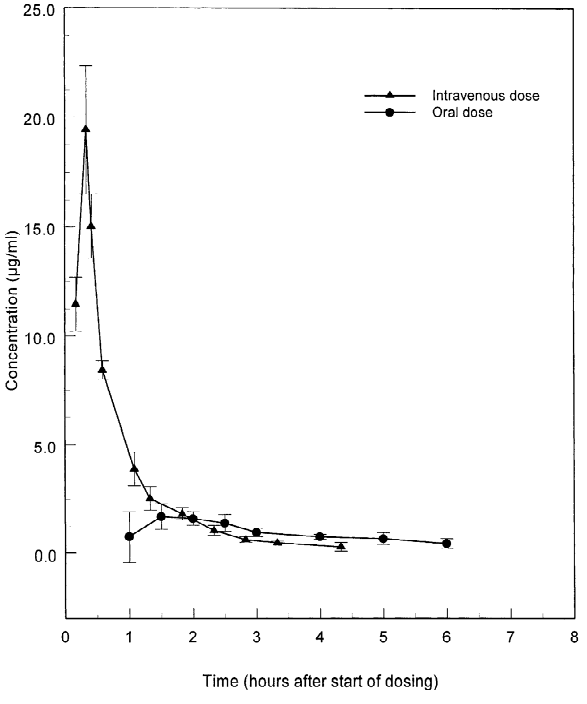

Fig. 5. Mean dexloxiglumide plasma concentrations (±SD) in four healthy male volunteers following administration of a single oral and an intravenous infusion of 200 mg.

cluding extensive stability experimentation to show that dexloxiglumide was stable in frozen plasma (nominally −20 °C) for at least 200 days and after several freeze–thaw cycles. Other experiments have indicated that no chiral inversion of dexloxiglumide into its antipole occurs in man (unpublished results). Therefore, further development of a chiral method for determination of dexloxiglumide enantiomers is not foreseen. In addition, the limit of quantification (0.2 μg/ml) of this method was sufficient to measure dexloxiglumide in plasma for up to 6 h after both oral and intravenous dosing. The specificity of the method with respect to one of the main dexloxiglumide's metabolites, CR 3529 and endogenous compounds was satisfactory. In conclusion, the described bioanalytical method for the determination of dexloxiglumide in human plasma has proved suitable for the investigation of drug plasma pharmacokinetics in man.

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