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Enantioselective assay for the determination of nisoldipine in dog, rat and mouse plasma by chiral microbore high-performance liquid chromatography combined with gas chromatography–mass spectrometry

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ABSTRACT

A sensitive, selective and validated method for the enantioselective determination of (+)- and (−)-nisoldipine in rat, mouse and dog plasma following administration of nisoldipine racemate is described. The alkalized plasma samples containing [$^{13}C_4$]nisoldipine racemate as internal standard (ISTD) were extracted once with toluene. The enantiomers of nisoldipine were quantitatively separated by high-performance liquid chromatography on a 250 × 2 mm I.D. column containing tris(4-methylbenzoate)-modified cellulose on silica. The fractions containing either the (+) or (−)-enantiomer of the analyte and [$^{13}C_4$]ISTD were analysed by gas chromatography with mass-selective detection in the single-ion monitoring mode. The limits of determination and detection were 0.5 and 0.2 ng/ml, respectively, the total precision was better than 7% (R.S.D. at 5 and 50 ng/ml, $n = 35$) and the accuracy was better than 10% (0.5–100 ng/ml, $n = 23$). The sum of the concentrations of the enantiomers determined with this assay corresponds to the concentration of the racemate determined independently by capillary gas chromatography with electron-capture detection (accuracy better than 15%, 1–80 ng/ml). The method was used for the analysis of more than 500 plasma samples obtained from toxicokinetic studies.

INTRODUCTION

Nisoldipine (BAY k 5552) belongs to the group of dihydropyridine-type calcium antagonists and is a chiral drug with respect to the 4-position of the dihydropyridine moiety due to the non-identical ester functions (methyl, isobutyl) at the 3- and 5-positions (Fig. 1). Nisoldipine is marketed as a racemic (1:1) mixture of the (+)- and (−)-enantiomers (BAY r 1224 and BAY r 1223). Pharmacokinetic data for the enantiomers have been reported only after administration of the ^{13}C-labelled pseudo-racemate to humans followed by GC–MS analysis [1] and after separate administration of the enantiomers to rats [2]. In both instances a fivefold higher bioavailability of the pharmacologically more potent (+)-enantiomer was shown. HPLC on 4.6

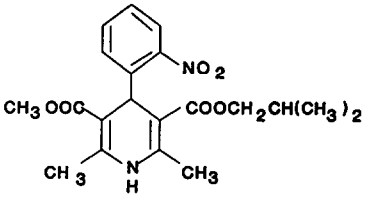

Fig. 1. Structure of nisoldipine, (±)-isobutylmethyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate.

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mm I.D. chiral columns followed by GC with electron-capture detection (ECD) or GC–MS analysis has been reported for other racemic calcium antagonists of the dihydropyridine type [3–7], but a quantitative enantioselective assay for nisoldipine was only briefly mentioned in the report by Breimer's group on the enantioselective determination of felodipine [5]. Instead of using GC–ECD [5], in this work GC–MS was used for the determination of the enantiomers following chiral HPLC separation on a microbore column. Mass-selective detection has the advantage over ECD that racemic $^{13}C_4$-labelled nisoldipine is used as internal standard (ISTD). Owing to the co-elution of the appropriate enantiomers of the analyte and the ^{13}C-labelled ISTD at the chiral stationary phase, high precision and accuracy are obtained with this method. Recently the enantiomeric separation of nisoldipine by coupling of two 250 × 4.6 mm I.D. Chiralcel OD columns was described [8]. The retention times were 45.5 and 52.0 min for the (−)- and (+)-enantiomer, respectively, and the resolution was 1.40 [9]. In contrast, we obtained a baseline separation with the method described here with retention times of 10.5 and 14.6 min for the (+)- and (−)-enantiomer, respectively, *i.e.*, with markedly shorter run times.

EXPERIMENTAL

Chemicals and materials

Nisoldipine (BAY k 5552, batch No. R-146-1), its (+)- and (−)-enantiomers (BAY r 1224 and BAY r 1223, batch Nos. 518905 and 518904) are products of Bayer (Wuppertal, Germany). Stock solutions of 10 mg per 50 ml in ethanol were diluted to final concentrations with ethanol. [$^{13}C_4$]Nisoldipine internal standard was kindly supplied by Dr. Pleiss (Pharma Research Centre, Bayer). Stock solutions 2 mg per 10 ml in ethanol were diluted with ethanol to a final concentration of 5 μg/ml.

Other chemicals used were toluene, nanograde (Promochem, Wesel, Germany), *n*-heptane, HPLC grade (Baker, Deventer, Netherlands), 2-propanol, pro analysi (Riedel-de Häen, Seelze, Germany), ethanol, absolute GR, and trifluoroacetic acid (TFA), Uvasol (Merck, Darmstadt, Germany).

HPLC on chiral stationary phase

A modular HPLC instrument (Spectra-Physics) consisting of an SP 8800 ternary pump, SP 8880 autosampler, SP 100 UV–Vis detector, SP 4290 integrator, Gilson Model 201 fraction collector with a water-cooled rack (5°C) and a column oven (Bayer workshops) was used.

The stationary phase was 10-μm aminopropylsilica coated with tris(4-methylbenzoate)-modified cellulose (Daicel, Japan). After washing, sieving and suspending in 2-propanol it was packed, using a descending slurry technique, in a 250 × 2 mm I.D. stainless-steel column (Grom, Herrenberg, Germany) and a precolumn (10 × 2 mm I.D.) packed with the same material with 2-propanol as the displacing medium at a pressure of 650 bar.

The operating conditions were as follows: mobile phase, *n*-heptane–0.2% TFA in 2-propanol (88:12, v/v); flow-rate, 0.2 ml/min; injection volume, 100 μl; and oven temperature, 40°C. Under these conditions, the retention times were 10.5 ± 0.2 min for the (+)- and 14.6 ± 0.3 min for the (−)-enantiomer. Fractions were collected between 9 and 13 min for the (+)- and between 13 and 20.5 min for the (−)-enantiomer. In addition to the high retention time stability, additional conformation was obtained from the UV signal of the co-eluting enantiomer of the $^{13}C_4$-labelled ISTD detected at 230 nm.

GC–MS system and operating conditions

A Hewlett-Packard (HP) Model 5890 II gas chromatograph directly interfaced to an HP Model 5971A mass-selective detector was used. A standard split–splitless injector operated in the splitless mode, with a 4-mm deactivated glass splitless sleeve (Restek, Bellefonte, PA, USA) packed with a plug of fused-silica wool (Restek), was used. The packed glass insert was silylated by typically five injections of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) into the hot injection port. A fused-silica capillary column (15 m × 0.25 mm I.D.) coated with 0.25-μm

DB-5 stationary phase (J & W Scientific, Rancho Cordova, CA, USA) was used. The chromatographic conditions were as follows: injection port temperature, 240°C; injection volume, 5 μl; split closed, 0–1 min; carrier gas, helium (4.6 grade) at 4 p.s.i. (27.6 kPa), resulting in a flow-rate of 0.95 ml/min through the column at 100°C. The oven temperature was held for 0.5 min at 100°C, then it was increased at 35°C/min, to 270°C, held there for 0.3 min, followed by a second ramp at 5°C/min to 285°C and a third ramp at 70°C/min to a final temperature of 320°C, which was held for 2 min.

The mass-selective detector was operated with electron impact (EI) ionization (70 eV) at an ion source temperature of 200°C. The capillary direct interface was set to 310°C. For quantification the ions at m/z 371 and 375 for the analyte and the ISTD, respectively, were detected in the single-ion monitoring (SIM) mode at a dwell time of 200 ms each. The electron multiplier voltage was set to 700 V above autotune.

The GC–MS system was controlled and data were processed by an HP DOS-Chemstation.

Sample preparation

A 1-ml volume of plasma (smaller volumes were adjusted to 1 ml with plasma from untreated animals) and 10 μl of internal standard solution were mixed in a glass centrifuge tube. After adding 0.1 ml of saturated NaCl solution, 0.1 ml of 1 *M* NaOH and 1.5 ml of toluene, the mixture was shaken at 300 rpm in a Mini-shaker (Adolf Kühner, Birsfelden, Switzerland) for 15 min. After centrifugation at 3000 rpm at 10°C for 10 min, the upper phase was removed and evaporated to dryness at 10 mbar for 25 min. The residue was reconstituted in 130 μl of the mobile phase and 100 μl were injected into the HPLC system.

The fractions obtained by HPLC were immediately evaporated at room temperature under a gentle stream of nitrogen. The residues were stored in a refrigerator below −20°C. For GC–MS analysis the residues were reconstituted in 0.1 ml of toluene and 5 μl were injected.

All operations must be done under light protection in a darkened room with yellow light (Osram L 62 W 40).

Calibration and quality control samples

Calibration and quality control samples were processed as outlined above. Quality control samples (QCs) were prepared by spiking control plasma samples (1 ml) with racemic nisoldipine at two concentrations (low and medium) on the day of blood sampling. The QCs were stored together with the unknown samples at temperatures below −20°C. Six QCs ($n = 3$ at both concentrations) were run in each sample sequence.

Calibration samples were prepared by spiking control plasma samples (1 ml) with racemic nisoldipine at six concentrations levels ranging from 1 to 210 ng/ml (two samples at each level) and racemic $^{13}C_4$-labelled nisoldipine (ISTD) at 50 ng/ml on the day of sample work-up. The ratios of the peak areas of the appropriate enantiomer of the analyte and ISTD in each fraction were calculated. Calibration graphs were constructed by linear regression.

Determination of racemic nisoldipine by GC–ECD

An aliquot of each unknown plasma sample and each QC sample was sent to a contract laboratory (Institute of Clinical Pharmacology–Prof. Dr. Lücker, Grünstadt, Germany) where the concentration of racemic nisoldipine was determined by non-enantioselective GC–ECD. The concentration of racemic nisoldipine was compared with the sum of the concentrations of the enantiomers determined by the enantioselective assay described here. This was an additional confirmation for the validity of the method.

Method specifications

The analytical working range of the method was 0.5–200 ng/ml. The limit of determination was 0.5 ng/ml, which was established during prestudy method validation and which was checked each day. The concentration of 0.5 ng/ml was included as the lowest calibration level for every calibration graph. The limit of detection was about 0.2 ng/ml. During evaporation of the fractions obtained by HPLC, 9 and 11% of the (+)- and (−)-enantiomer ($n = 12$ each), respectively, were lost. The extraction recovery was 91% at 0.5 ng/ml ($n = 7$), 98% at 10 ng/ml

($n = 9$) and 98% at 50 ng/ml ($n = 9$). The total precision derived from QCs at 5 and 50 ng/ml ($n = 35$) was better than 7% and at the limit of determination was better than 15%. The accuracy for spiked plasma samples (validation and QC samples) was better than 10% through the whole working range of the assay and at the limit of quantification was better than 15%. The deviation between the results obtained with this assay compared with those obtained independently in the contract laboratory by GC–ECD was less than 15%. During prestudy method validation no significant differences were found for the method specifications between plasma samples obtained from rat, mouse or dog. No sex dependence was observed.

The positive-ion EI mass spectra of nisoldipine and the ISTD [$^{13}C_4$]nisoldipine are shown in Fig. 2. The molecular peaks are at m/z 388 and 392. For quantitative determinations the ions at m/z 371 and 375 (base peaks, $[M - OH]^+$) for nisoldipine and the ISTD, respectively, were measured in the SIM mode. Typical chromatograms for a calibration plasma sample (1 ml) spiked with 1 ng of racemic nisoldipine and the ISTD are shown in Fig. 3. This represents the limit of determination, corresponding to 0.5 ng/ml for each enantiomer. There is no interference of plasma matrix components at m/z 371 and 375 at the retention time of 7.18 ± 0.01 min for the analyte and ISTD.

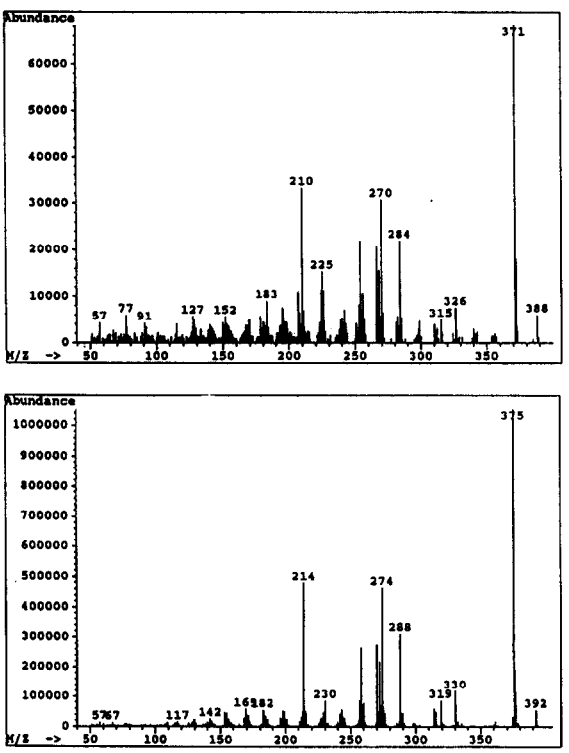

Fig. 2. Positive-ion EI mass spectra of nisoldipine (above) and the ISTD [$^{13}C_4$]nisoldipine (below).

The calibration graphs obtained from 1-ml plasma samples spiked with 1–210 ng of racemic nisoldipine range from 0.5 to 105 ng/ml for the enantiomers and are identically shaped. The curve was linear and passed through origin; the linear fit was described by the equation $y = (1.04 \pm 0.02)x$ (where y is the detector response in arbitrary units and x is concentration in ng/ml) for both enantiomers throughout all runs. That means that the sensitivity of the assay was identical for both enantiomers and during all runs. On each day a calibration graph covering the whole analytical working range was run in duplicate with the unknown samples.

The accuracy is the percentage deviation from the conventional true value. For spiked plasma or QC samples the true value is the spiked amount. For comparison of the enantioselectively determined sum of the enantiomers with the GC–ECD results, the GC–ECD values were considered to be true.

The precision was calculated as the relative standard deviation: R.S.D. = (S.D./mean) × 100%. If not stated otherwise, the precision is given in terms of total precision, which is the sum of the intra- and inter-day precision.

The limit of determination was reached when the accuracy exceeds 10% and/or the precision exceeds 20%. The limit of detection was estimated at a signal-to-noise ratio of 3 following injection of blank sample matrix.

RESULTS

The assay was used in toxicokinetic studies. The plasma concentrations of the (+)- and (−)-enantiomers were determined following subchronic (13-week) oral administration of racemic nisoldipine mixed with food at 0 (control) and 3 doses to male and female rats and mice. Racemic

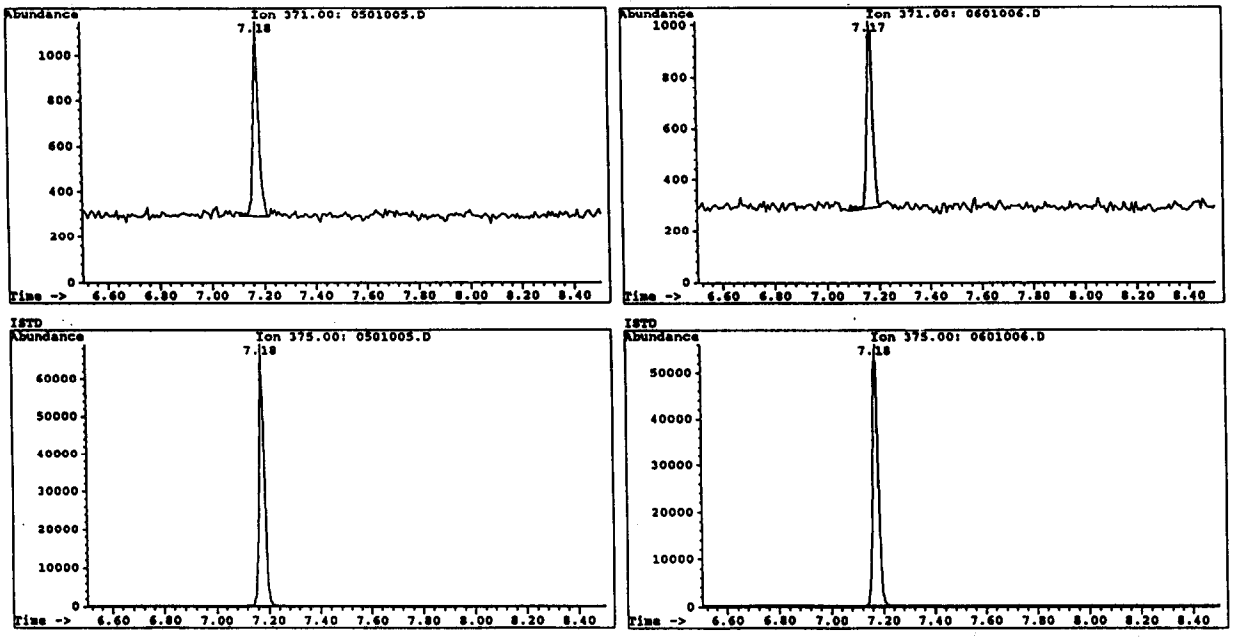

Fig. 3. 0.5 ng/ml of the (+)- (left) and (−)-enantiomer (right) and the corresponding ISTD peaks following chiral HPLC of a calibration plasma sample (1 ml) spiked with 1 ng of racemic nisoldipine and the ISTD: SIM of *m/z* 371 (nisoldipine) and 375 (ISTD).

nisoldipine (BAY k 5552) was administered to five animals per dose and sex. Blood samples were taken on the 10th and 85th days of the study. Examples are shown in Table I for male rats and in Table II for male mice. In the last column of each table the geometric mean and standard deviation of the racemate concentration determined independently by GC–ECD are given. Note that the pooled samples from five animals per dose and sex used for enantioselective assay were not obtained from equal amounts of plasma for each individual animal. Neverthe-

TABLE I

PLASMA CONCENTRATION OF THE INDIVIDUAL (+)- AND (−)-ENANTIOMERS OF BAY k 5552 AFTER SUBCHRONIC ORAL ADMINISTRATION TO MALE RATS

Dose (ppm)	Day	(+)-Enantiomer (μg/l)	(−)-Enantiomer (μg/l)	Ratio, (+)/(−)	Sum, (+) + (−) (μg/l)	BAY k 5552 (μg/l)[a] (mean ×/: S.D.)
Control	10	n.d.[b]	n.d.			<0.2
	85	n.d.	n.d.			<0.2
50	10	n.d.	n.d.			<0.2
	85	n.d.	n.d.			<0.2
300	10	1.78	0.74	2.4	2.52	1.76 ×/: 1.91
	85	0.63	(0.3)[c]	2.0	0.93	0.84 ×/: 1.44
1800	10	10.57	2.56	4.13	13.13	12.65 ×/: 2.02
	85	2.71	1.19	2.3	3.90	4.06 ×/: 1.52

[a] These values were obtained independently by GC–ECD.
[b] n.d. = Not determined, because the racemate concentration was below the limit of detection.
[c] Value in parentheses is between the limits of determination (0.5 μg/l) and detection (0.2 μg/l).

TABLE II

PLASMA CONCENTRATIONS OF THE INDIVIDUAL (+)- AND (−)-ENANTIOMERS OF BAY k 5552 AFTER SUBCHRONIC ORAL ADMINISTRATION TO MALE MICE

Dose (ppm)	Day	(+)-Enantiomer (μg/l)	(−)-Enantiomer (μg/l)	Ratio, (+)/(−)	Sum, (+) + (−) (μg/l)	BAY k 5552 (μg/l)[a] (mean ×/: S.D.)
Control	10	n.d.[b]	n.d.			<0.2
	85	n.d.	n.d.			<0.2
100	10	1.87	1.19	1.6	3.06	1.76 ×/: 2.32
	85	2.77	1.69	1.6	4.46	4.09 ×/: 1.62
300	10	4.66	2.37	2.0	7.03	6.76 ×/: 2.28
	85	7.69	3.62	2.1	11.31	11.79 ×/: 1.58
900	10	27.25	11.95	2.3	39.20	40.35 ×/: 1.87
	85	20.71	9.79	2.1	30.50	31.25 ×/: 2.24

[a] These values were obtained independently by GC–ECD.
[b] n.d. = Not determined, because the racemate concentration was below the limit of detection.

less, there is good agreement between the enantioselectively determined sum of the plasma concentrations of the enantiomers and the GC–ECD results for the racemate concentration. The accuracy is 14.2% for the rat data ($n = 10$) and 5.5% for the mouse data ($n = 12$). The ratio of the (+)- to the (−)-enantiomer is 2.6 ×/: 1.37 in male and 0.6 ×/: 1.28 in female rats (geometric mean and standard deviation). In mice the ratio of the (+)- to the (−)-enantiomer is significantly lower in male (1.9 ×/: 1.16) than in female (2.5 ×/: 1.27) animals ($\alpha < 0.05$).

In two further studies, the (+)- and (−)-enantiomers (BAY r 1224 and BAY r 1223) were administered as an intravenous bolus at doses of 0 (control) and 0.02, 0.06 and 0.2 mg/kg and racemic nisoldipine (BAY k 5552) at 0.2 mg/kg body mass to male and female Beagle dogs. On the first, seventh and fourteenth days of the study the plasma concentrations of the parent compound were determined by GC–ECD directly before and 5 min, 15 min and 0.5, 1, 2 and 3 h after administration. All samples following administration of BAY k 5552 racemate and the samples obtained 15 min after administration of the enantiomers were additionally analysed by the enantioselective assay described here. Examples shown here are from the seventh day of the study. Again, the good agreement between the enantioselectively determined sun of the enantiomer concentration and the GC–ECD results is worth mentioning. Following administration of one of the enantiomers, no chiral inversion into the opposite enantiomer was observed (Table III). The administered enantiomer was contaminated by about 1.4% by its opposite enantiomer. This contamination was also found in plasma samples in the 0.06 and 0.2 mg/kg dose groups. Following intravenous administration of racemic nisoldipine (BAY k 5552), a 1:1 ratio of the concentrations of the enantiomers could be demonstrated (Fig. 4).

Typical plasma concentration *vs.* time courses following i.v. administration of 0.2 mg/kg body mass of racemic nisoldipine to a male dog are shown for illustration in Fig. 4. The concentration ratio of the enantiomers is 1:1, and the sum of the concentration of the enantiomers is the same as the racemate concentration determined independently by GC–ECD.

DISCUSSION

A sensitive and specific enantioselective assay for the determination of the (+)- and (−)-enantiomers BAY r 1224 and BAY r 1223 of nisoldipine when the racemic nisoldipine BAY k 5552 is administered to rats, mice or Beagle dogs has been described. No species difference was found, considering method specifications. The limit of determination was 0.5 ng/ml and the limit of detection about 0.2 ng/ml throughout all runs.

TABLE III

PLASMA CONCENTRATIONS OF BAY r 1224 (+) AND BAY r 1223 (−) DETERMINED BY ENANTIOSELECTIVE ASSAY OF PLASMA SAMPLES TAKEN 15 min AFTER INTRAVENOUS ADMINISTRATION OF BAY r 1224 TO BEAGLE DOGS ON THE SEVENTH DAY OF TREATMENT

Dose (mg/kg)	Dog No. (sex)	Concentration (μg/l)		
		GC–ECD[a]	BAY r 1224 (+)	BAY r 1223 (−)[b]
Control	291 (m)	<0.4	<0.2	<0.2
	299 (m)	<0.4	<0.2	<0.2
	278 (f)	<0.4	<0.2	<0.2
	292 (f)	<0.4	<0.2	(0.2)
0.02	279 (m)	3.62	4.62	(0.2)
	281 (m)	4.28	4.67	<0.2
	282 (f)	3.97	4.31	(0.2)
	284 (f)	3.88	4.39	(0.2)
0.06	285 (m)	9.51	8.92	(0.3)
	295 (m)	11.67	11.28	0.52
	288 (f)	13.37	13.71	(0.4)
	298 (f)	11.82	10.43	(0.2)
0.2	287 (m)	71.00	87.55	1.16
	297 (m)	46.99	54.08	0.63
	286 (f)	28.34	41.41	0.78
	294 (f)	55.06	75.99	0.99

[a] Values in this column were determined independently (non-enantioselectively) by GC–ECD and represent the sum of both the (+)- and the (−)-enantiomers.

[b] Values in parentheses are between the limit of detection (0.2 μg/l) and determination (0.5 μg/l).

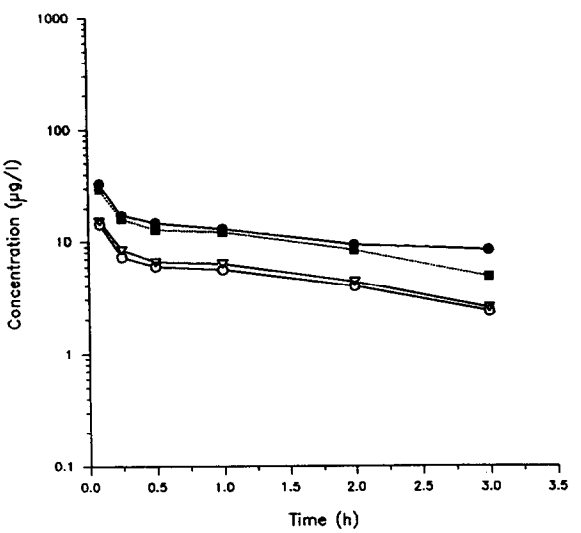

Fig. 4. Plasma concentrations of individual enantiomers (HPLC followed by GC–MS), sum and independently determined (GC–ECD) racemate concentrations following intravenous administration of racemic nisoldipine (BAY k 5552) at 0.2 mg/kg to Beagle dogs. Dog No. 283, male, seventh day. ● = BAY k 5552 (GC–ECD); ▽ = (+)-enantiomer; ○ = (−)-enantiomer; ■ = sum of (+)- and (−)-enantiomers.

With the exception of the paper by Ohkubo *et al.* [8] and a brief mention in a paper by Breimer's group on felodipine [4], there is no method in the literature for the determination of nisoldipine enantiomers following administration of the racemate. Ohkubo *et al.* stated that Chiralcel OF and OJ columns (250 × 4.6 mm I.D.) are inefficient for the enantiomeric separation of dihydropyridine derivatives including nisoldipine using *n*-hexane–2-propanol as the mobile phase. They combined two 250 × 4.6 I.D. mm Chiralcel OD columns [tris(3,5-dimethylphenylcarbamate)-modified cellulose on silica] to obtain a baseline separation ($R = 1.40$) of the enantiomers of nisoldipine and nilvadipine using a mobile phase composition of *n*-hexane–2-propanol–ethanol (95:4:1). This resulted in long run times of more than 1 h (retention times 45.5 and 52.0 min for the (−)- and (+)-enantiomers of nisoldipine, respectively). We found that a 250 × 2 mm I.D. microbore column packed with tris(4-methylbenzoate)-modified cellulose on amino-

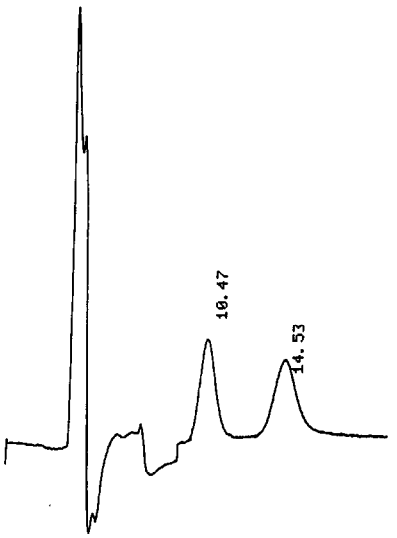

Fig. 5. HPLC showing the separation of racemic nisoldipine on a microbore column (250 × 2 mm I.D.) packed with tris(4-methylbenzoate)-modified cellulose on aminopropylsilica as stationary phase: (+)-enantiomer at 10.47 min and (−)-enantiomer at 14.53 min.

propylsilica as the stationary phase is useful for the separation of racemic nisoldipine (Fig. 5). Using *n*-heptane–2-propanol–trifluoroacetic acid (88:12:0.2) as the mobile phase the retention times are 10.5 and 14.6 min for the (+)- and (−)-enantiomer ($k_1' = 2.50$, $k_2' = 3.87$), respectively, resulting in a sample throughput of 3/h. The enantiomers are baseline separated, the resolution is $R_s = 2.40$ and the selectivity is 1.55. The dead time (t_0) was estimated with 1,3,5-tri-*tert.*-butylbenzene. The plate numbers are 700 and 590 for the (+)- and the (−)-enantiomer, respectively.

This type of column is also useful for the separation of racemic nimodipine (BAY e 9736) into its enantiomers, resulting retention times of 25.0 and 21.0 min for the (+)- and (−)-enantiomer, respectively [2].

The main advantage of using mass-selective detection over ECD is the possibility of using a stable isotope-labelled ISTD, which is in every case the best choice for an ISTD. All sample losses due to extraction, evaporation, chiral separation and injection are compensated for by this type of ISTD, resulting in high accuracy and precision of the whole assay.

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