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Short Communication

Determination of the enantiomers of nisoldipine in human plasma using high-performance liquid chromatography on a chiral stationary phase and gas chromatography with mass-selective detection

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Abstract

A method is described that combines chiral HPLC and off-line GC with mass-selective detection for the quantitation of the enantiomers of nisoldipine [(±)-I] in human plasma. An isotope-labelled internal standard [nine-fold deuterated (±)-I] is used throughout the assay. The limit of quantification is 0.1 μg/l for each enantiomer. Data on the precision, accuracy and selectivity of the method are presented. Enantioselective analysis was performed in subjects receiving the racemic drug in tablet form. In healthy volunteers the maximum concentration and the area under the curve of the pharmacologically more active (+)-enantiomer were greater by 9-fold and 13-fold, respectively, compared to those of the (−)-enantiomer. In elderly hypertensive patients plasma concentrations of (+)-I were *ca.* five times as high as those of the (−)-enantiomer. Stereoselectivity was not affected by hepatic impairment. After intravenous administration of (±)-I there were no relevant differences between the plasma concentrations of the enantiomers.

1. Introduction

The calcium antagonist nisoldipine, (±)-3-isobutyl-5-methyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-pyridine-3,5-dicarboxylate ((±)-I, Fig. 1) is efficacious in the treatment of hypertension and coronary heart disease. Due to its asymmetrical ester functions at the dihydropyridine nucleus compound I is a chiral drug. It has been registered as racemate (baymycard®, syscor®).

The enantiomers differ with respect to their pharmacological activities and pharmacokinetic properties: The potency of (+)-I to inhibit K^+-stimulated contractions of isolated rabbit aortic

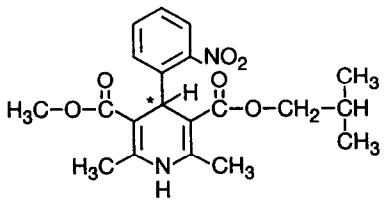

Fig. 1. Structure of nisoldipine (I).

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ring *in vitro* was 18.5-fold greater and its antihypertensive activity *in vivo* (rats) was 20-fold greater than the activities of the (−)-enantiomer [1]. In human volunteers receiving pseudoracemic I [^{13}C (+)-I and non-labelled (−)-I] the bioavailability of the (+)-enantiomer was 6.3-fold increased compared to that of the (−)-enantiomer, probably due to stereoselective differences in the intrinsic clearance [2].

While in this study stereoselective analysis had been achieved by GC–MS utilizing the isotope label of (+)-I, the objective of the present investigation was to establish an enantioselective assay for samples from clinical studies conducted with racemic I.

The low doses, high pharmacological activity, high volume of distribution (2.7 l/kg) and low bioavailability of 4–8% [3,4] lead to low plasma concentrations of the drug necessitating a sensitive analytical method.

Enantioselective methods for the separation of similar racemic dihydropyridine drugs have been previously described employing chiral HPLC with collection of fractions and off-line GC with mass-spectrometric [5,6] or electron-capture detection [7].

The light sensitivity of I [8] precludes on-line UV detection during the chiral separation by HPLC, and necessitates the use of stable isotope-labelled nisoldipine as internal standard to compensate for possible losses of analyte at this stage. Consequently, mass-spectrometric detection was chosen to quantify I in HPLC fractions. Thus the assay described here is similar to the enantioselective methods for nilvadipine [5] and nimodipine [6].

2. Experimental

HPLC-grade solvents were obtained from E. Merck (Darmstadt, Germany). Nanograde toluene was purchased from Promochem (Wesel, Germany). Nisoldipine [(±)-I] (Bayer, Leverkusen, Germany) and its enantiomers were used as certified reference compounds for quantitative analysis. The enantiomeric purity of I enantiomers exceeded 99.0% as determined by HPLC on Chiralcel OJ. The internal standard (I.S.), (±)-I deuterated nine-fold in the isobutyl moiety [(±)-D_9-I] contained less than 0.2% unlabelled (±)-I.

2.1. Sample preparation

In a stoppered test tube 10 μl of the I.S. working solution (0.5 μg/ml (±)-D_9-I in toluene) and 250 μl of 5 *M* NaOH (Dilut-It; Baker, Groß-Gerau, Germany) were added to 1.0 ml of plasma. The mixture was extracted with 2.0 ml of toluene (60 min) and the organic phase was separated by centrifugation for 20 min at 2000 *g* (−5°C). Toluene was evaporated at 40°C (Vortex evaporator, Haake-Buchler, Saddle Brook, USA). The residue was reconstituted in 130 μl of the mobile phase, 100 μl of which were injected onto the HPLC. Prior to each sequence *ca.* 50 ng (±)-I were repeatedly injected to ascertain stable retention times and define the fractions of interest (UV detection). During the sequence the UV detector was switched off. The fractions of (+)-I and (−)-I were collected separately [1.0 ml (+)-I, 1.6 ml (−)-I, 0.2 ml between fractions were discarded] and evaporated under nitrogen at 37°C. The residue was reconstituted in 30 μl of toluene, 10 μl of which was injected onto the GC.

Sample preparation and instrumental analysis were carried out under yellow light (L 40/62, Osram).

2.2. Instrumentation and operating conditions

The modular instrument (Spectra Physics, Darmstadt, Germany) consisted of a SP 8800 ternary pump, SP 8880 autosampler, SP 4290 integrator and SP 100 UV-Vis detector (230 nm). The fraction collector was a Model 201 (Gilson, Villiers Le Bel, France) with water-cooled rack (5°C). The column oven (40°C) was custom-built. For separation of the enantiomers tris(4-methyl-benzoate)cellulose on silica, 8 μm (Chiralcel OJ; Baker, Groß-Gerau, Germany), 10 × 2 mm I.D. (guard column) and 250 × 2 mm I.D. (analytical column) was used. The mobile phase was *n*-heptane–isopropanol/trifluoroacetic acid 0.2% (88:12, v/v). The flow-rate was 0.2 ml/

min. The hold-up time (t_0) was determined by injection of (1,3,5)tris(*tert.*-butyl)benzene.

For quantification a gas chromatograph HP 5890 and mass selective detector HP MSD 5970 (Hewlett-Packard, Waldbronn, Germany) were used, equipped with a split/splitless injector (temperature: 280°C, split closed from 0.0–1.0 min). The injection port employed a glass insert, silylated with bis(trimethylsilyl)acetamide (E. Merck). The detector was operated in the single-ion monitoring mode with electron-impact ionization. The signals at m/z 371 (I) and 380 (D_9-I) were used for quantification. A fused-silica 100% dimethylpolysiloxane capillary column (film thickness 0.33 μm, 12 m × 0.2 mm I.D.) (Ultra-1, Hewlett-Packard) was used with helium (0.7 bar) as carrier gas.

The specified temperature program was: Initial temperature: 100°C, initial time: 1 min; rate A: 30°C/min, temperature A: 200°C, hold time A: 0 min; rate B: 4°C/min, temperature B: 240°C, hold time B: 0 min; rate C: 20°C/min, temperature C: 320°C, hold time C: 1 min; transfer line temperature: 280°C, total run time: 19 min.

2.3. Calibration and quality control

Calibration samples were prepared by spiking aliquots of working solutions of (±)-I (in acetonitrile) into blank plasma to yield eight concentrations (0.1–5.0 μg/l per enantiomer). Quality control (QC) samples at 0.4, 0.8 and 1.5 μg/l were obtained by spiking (+)-I and (−)-I, respectively, into blank plasma. QC samples were stored together with unknown samples at −20°C. Calibration, QC and unknown samples were processed further as described above.

The (+)- and (−)- fraction of each sample was quantified by GC–MS. Calibration curves were obtained for each enantiomer by plotting concentration *vs.* peak-height ratio (I/D_9-I) and fitting the equation $y = a + bx^c$ to the data points.

2.4. Assay validation

The stability of I enantiomers in the mobile phase was investigated by repeated GC analysis of spiked solutions. Longterm stability was examined in study samples containing (±)-I after 4 and 8 months storage at −20°C.

Six spiked samples of each enantiomer at concentrations 0.1, 0.4 and 1.5 μg/l were analysed within one day to yield the intra-day variability. This procedure was repeated on three consecutive days to calculate the inter-day variability.

Absence of matrix interferences was confirmed by analysis of blank plasma. Carry-over between fractions (HPLC) was investigated by determination of I in the (−)-fraction of samples spiked with (+)-I (and *vice versa*).

Quality control during routine analysis: Accuracy, precision and specificity were calculated based on results of QC samples.

2.5. Determination of racemic nisoldipine in plasma

Prior to enantioselective analysis the concentrations of (±)-I in plasma were determined by GC with electron-capture detection [3] using (±)-nitrendipine (certified reference compound, Bayer AG) as internal standard. The limit of quantification (LOQ) was 0.1 μg/l.

2.6. Application

I enantiomers were determined in plasma samples obtained in clinical pharmacological studies:

Study A: Single dose of 40 mg (±)-I controlled release (CR) formulation, healthy male volunteers (aged 22–38 years).

Study B: Steady-state profile of 20 mg (±)-I-CR dose in hypertensive patients (male and female, aged 66–77 years).

Study C: Steady-state profile of 10 mg (±)-I dose (immediate release tablet) in patients with liver cirrhosis (male and female, aged 40–64 years) and profile following intravenous (i.v.) infusion of 0.5 mg (±)-I.

All subjects gave written informed consent of participation in each study performed in accordance with the declaration of Helsinki and Good Clinical Practice Rules.

2.7. Data evaluation

$AUC(0–t_n)_{norm}$ was obtained by dividing $AUC(0–t_n)$ (area under the curve up to the time of the last concentration above LOQ) by dose per body weight. AUC was calculated by the lin–log trapezoidal rule. Descriptive statistics were performed by calculation of arithmetic means and standard deviations.

3. Results and discussion

3.1. Validation

The separation of I enantiomers was performed on a Chiralcel OJ column (Fig. 2) using a modification of the mobile phase described by Soons *et al.* [7]. The variation in retention times for (+)- and (−)-I was low (C.V. = 1.9–2.0%, $n = 6$). Previously, baseline chiral separation of (±)-I had been described by Ohkubo *et al.* [9] who used an alternative stationary phase [Chiralcel OD, tris(3,5-dimethylphenylcarbamate)-cellulose on silica].

There was no degradation of I in the mobile phase up to 29 h (conditions of storage in fraction collector). Upon storage at −20°C I was stable in plasma for at least eight months.

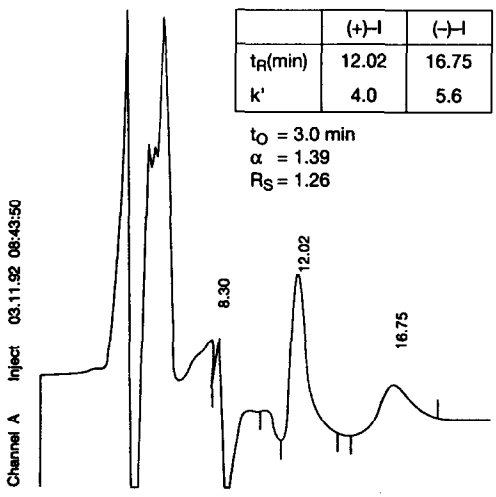

Fig. 2. Separation of nisoldipine (I) enantiomers on a chiral stationary phase [Tris(4-methylbenzoate)cellulose on silica, Chiralcel OJ]; UV detection at 230 nm.

Toluene extraction of I from plasma yielded quantitative recovery as reported previously [3]. No interferences coeluting with I or the I.S. were present in blank plasma when the HPLC fractions were quantified by GC with single-ion monitoring MS. Analyte and I.S. were detected at a t_R of *ca.* 14.5 min and readily separated due to the nine-fold deuterium label of the I.S.

(±)-D_9-I was added as I.S. prior to work-up thus compensating for possible losses of analyte during extraction or collection of fractions. Calibration curves were obtained by plotting concentration *vs.* peak-height ratio (I/D_9-I). Due to the slight concavity of the resulting curve a non linear equation ($y = a + bx^c$) was fitted whereby relative residuals were usually $\leq 10\%$.

Inter- and intra-day precision of the assay was $\leq 10\%$ and $\leq 20\%$ at a limit of quantification (LOQ) of 0.1 μg/l, respectively, for the (+)- and (−)-enantiomer (Table 1). This concentration was also reported as LOQ by Fischer *et al.* in a similar assay for another racemic dihydropyridine, nimodipine [6]. The limit of detection was estimated to be 0.01–0.03 μg/l. The amount of (−)-I detected in blank plasma spiked with (+)-I was $\leq 0.5\%$ and, conversely, $\leq 0.9\%$ of (+)-I was detectable in plasma spiked with (−)-I (Table 1). Taking into account the enantiomeric purity of (+)- and (−)-I reference compounds ($\geq 99.0\%$), it can be concluded that the assay was specific for each enantiomer and no carry-over between fractions occurred.

Assay performance during routine analysis was checked by quality control (QC) samples containing pure I enantiomers: Precision was 5.6–12.3% [(+)-I] and 12.2–19.0% [(−)-I], respectively. Accuracy [92–100% and 92–93% for (+)- and (−)-I, respectively] was also satisfactory. As on-line detection for the chiral separation could not be applied, QC samples were the only means of ensuring that fractions were collected appropriately. In QC samples spiked with (+)-I, 0.5–2.8% I was detectable in the (−)-fraction. Likewise, QC samples of (−)-I contained 2.7–3.6% of I in the (+)-fraction,

Table 1
Intra- and inter-day precision, accuracy and selectivity of determination of nisoldipine (I) enantiomers in human plasma

Day	0.10 μg/l[a]			0.40 μg/l[a]			1.50 μg/l[a]		
	Mean (μg/l)	Precision (%)	Accuracy (%)	Mean (μg/l)	Precision (%)	Accuracy (%)	Mean (μg/l)	Precision (%)	Accuracy (%)
(+)-I									
1	0.10	20.0	101	0.36	5.3	90	1.31	6.5	87
2	0.09	17.7	90	0.34	6.4	85	1.24	10.3	82
3	0.09	7.9	87	0.31	9.4	78	n.s.[d]	n.c.[e]	n.c.[e]
Days 1-3 (inter-day)	0.09	17.1	93	0.34	8.8	84	1.27[b]	8.4[b]	85[b]
I in (−) fraction (ng/l)	< LD[f]			< LD			7 ± 15[c]		
(-)-I									
1	0.10	14.1	98	0.35	4.3	87	1.36	7.9	91
2	0.10	4.8	99	0.35	2.1	87	1.37	5.3	91
3	0.11	7.3	106	0.33	6.7	81	1.39	6.4	93
Day 1-3 (inter-day)	0.10	9.7	101	0.34	5.6	85	1.37	6.3	91
I in (+) fraction (ng/l)	< LD			1 ± 4[c]			13 ± 17[c]		

[a] Nominal concentration.
[b] Mean of days 1 and 2.
[c] Mean ± S.D.; the given numbers are estimates as they are generally below the limit of quantification.
[d] n.s. = no sample.
[e] n.c. = not calculated.
[f] < LD = below limit of detection.

indicating that also during routine analysis the assay was sufficiently selective for each isomer.

There was good agreement between the results of this assay [as sum of the (+)- and (−)-enantiomers] and the concentrations of (±)-I determined previously by a non-stereoselective method (Fig. 3).

3.2. Application

The plasma concentration *vs.* time profile of I enantiomers is shown in Fig. 3. Additionally the parameters C_{max} and AUC including three further subjects are given (Study A).

While the concentrations of (−)-I were hardly above the LOQ (0.1 μg/l), the profile of the (+)-enantiomer was almost identical to that of racemic I, determined independently by a non-stereoselective assay. The stereoselectivity ratio (SR) of the concentrations [(+)-I/(−)-I, given as mean ± S.D., $n = 4$) was greater during the initial peak of the profile (16.2 ± 0.4 *vs.* 15.4 ± 5.9 at 2.5 and 4.0 h, respectively) and remained almost constant between 7.0 and 14.0 h (6.8 ± 1.0–9.2 ± 1.8). Compared to (−)-I the C_{max} and AUC_{norm} of the (+)-enantiomer were greater by 9.2-fold and 12.8-fold, respectively. These results are confirmed by the results from a previous study with pseudoracemic I [2], where the C_{max} and AUC_{norm} of (+)-I had been increased by 8.5-fold and 6.3-fold, respectively.

Stereoselectivity was found to be slightly less pronounced in the target population of the drug, namely elderly hypertensive patients (study B, SR = 4.3 ± 1.4–5.5 ± 1.2, given as mean ± S.D.; $n = 8$). Yet there was a clear preponderance of the pharmacologically more active (+)-I. While an influence of age on the pharmacokinetics of (±)-I had been shown previously [10], these results suggest that with age also the ratio of stereoisomers in plasma may change.

Likewise, it was shown that liver cirrhosis affects the pharmacokinetics of (±)-I since the drug is hepatically metabolized [11]. Theoret-

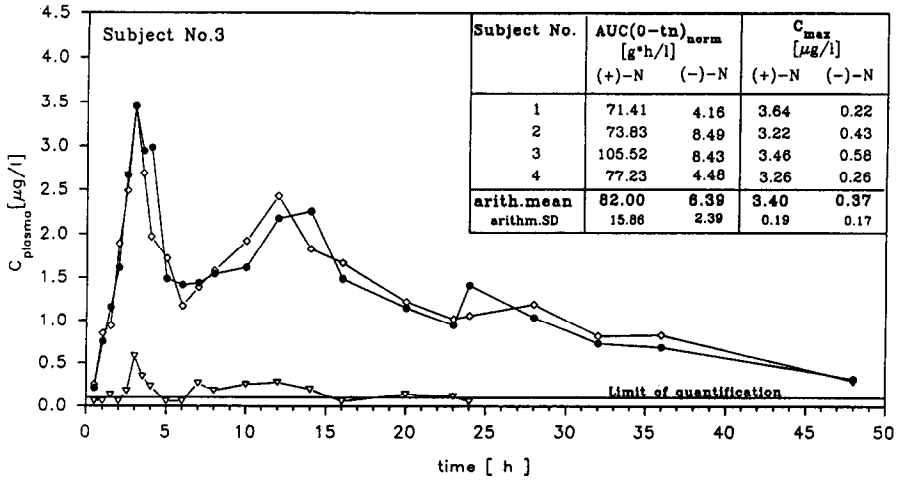

Fig. 3. Plasma concentrations of nisoldipine (I) racemate and enantiomers in a healthy volunteer receiving 40 mg (±)-I as controlled release tablet and derived AUC and C_{max} values including three other subjects. (▽) (−)-I, (●) (+)-I, (◇) (±)-I determined by GC with electron-capture detection.

ically, liver impairment might also alter the stereoselectivity of the drug metabolism. However, after oral administration, patients with liver cirrhosis (study C) showed a degree of stereoselectivity (SR = 7.9 ± 4.8–12.1 ± 9.9, given as mean ± S.D.; $n = 6$) that was comparable to that in healthy volunteers.

There were no relevant stereoselective differences after i.v. administration (study C, SR = 1.3 ± 0.2–1.5 ± 0.4, given as mean ± S.D.; $n = 6$), which confirms the previously reported absence of significant stereoselectivity after i.v. administration of pseudoracemic drug [2]. Thus the degree of stereoselectivity also depends on the route of administration and is different for oral and intravenous dosing.

4. Conclusions

Determination of nisoldipine enantiomers after administration of the racemic drug is of particular importance as there are differences in their pharmacological properties, the (+)-enantiomer being more potent in terms of antihypertensive activity. After oral administration the concentrations of the racemic drug largely represent the pharmacologically more active (+)-enantiomer. These results are in good agreement with a previous study employing the pseudoracemate technique to investigate the pharmacokinetics of I enantiomers.

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