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Stereoselective determination of unchanged and glucuroconjugated eliprodil, a new anti-ischaemic drug, in human plasma and urine by precolumn derivatization and column-switching high-performance liquid chromatography with fluorescence detection

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Abstract

An HPLC method was developed and validated for the determination in human plasma and urine of the enantiomers of eliprodil, (±)-α-(4-chlorophenyl-4[(4-fluorophenyl)methyl]piperidine-1-ethanol hydrochloride, a new anti-ischaemic agent administered as a racemate. Both enantiomers are present in human plasma in unchanged and glucuroconjugated form, whereas only the glucuroconjugated form is excreted into urine; as a consequence, such metabolites in human plasma and urine should be submitted to enzymatic deconjugation with β-glucuronidase (Escherichia coli) before being extracted. The general method involves a liquid–liquid extraction of eliprodil and internal standard from alkalinized plasma or urine with *n*-hexane, evaporation of the organic phase and derivatization with (*S*)-(+)-naphthylethyl isocyanate to give carbamate diastereoisomeric derivatives of (*S*)-(+)- and (*R*)-(−)-eliprodil and internal standard; after evaporation of the derivatizing mixture and dissolution of the residue in a small volume of phosphate buffer–acetonitrile (60:40, v/v), an aliquot is injected into a column-switching HPLC system. The derivatized sample extract is purified on a precolumn filled with C_8-bonded silica material, which is flushed with acetonitrile–water, then diastereoisomers of eliprodil and the internal standard are automatically transferred by the mobile phase to the analytical column. The analytical column is a C_8 type, specially deactivated for basic compounds, the mobile phase is 0.025 *M* phosphate buffer (pH 2.6)–methanol–acetonitrile (42:2:56) at a flow-rate of 1.2 ml min^{-1} and a fluorimetric detector operating at $\lambda_{ex} = 275$ nm and $\lambda_{em} = 336$ nm is used. The retention times, under these conditions, are about 16 and 17 min for (*S*)-(+)- and (*R*)-(−)-eliprodil diastereoisomers, respectively, and about 19 min for the first-eluted diastereoisomer of the internal standard. During the analysis time, the precolumn, reset in a different path from that of the analytical column, is back-flushed with different solvents, then re-equilibrated with acetonitrile–water before the next injection. Linearity in plasma, for unchanged eliprodil enantiomers, was assessed in the range 0.15–10 ng ml^{-1} and for total eliprodil enantiomers (unchanged + conjugated) in the range 0.75–500 ng ml^{-1}; the limit of quantitation (LOQ) is 0.15 ng ml^{-1} for each unchanged enantiomer and 0.75 ng ml^{-1} for each total enantiomer. Linearity was also assessed in urine for total

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(conjugated) eliprodil enantiomers in the range 50–25 000 ng ml^{-1}; the LOQ is 50 ng ml^{-1} for each enantiomer. The intra- and inter-day precision and accuracy of the method were investigated in plasma and urine and found to be satisfactory for pharmacokinetic studies. The method has been extensively used in pharmacokinetic studies in man treated with a 20-mg dose of eliprodil racemate and some results of this application are reported.

Keywords: Enantiomer separation; Derivatization, LC; Column switching; Eliprodil

1. Introduction

Eliprodil is a non-competitive receptor antagonist of N-methyl-D-aspartate (NMDA) on the polyamine modulator site, and it is being investigated as neuroprotector agent in irreversible focal cerebral ischaemia [1–4]. The compound, (±)-α-(4-chlorophenyl)-4[(4-fluorophenyl)methyl]piperidine-1-ethanol hydrochloride, contains in its structure (Fig. 1) a chiral carbon atom that gives rise to two optical isomers, (*S*)-(+)- and (*R*)-(−)-; both eliprodil enantiomers show comparable neuroprotective efficacy, thus justifying the clinical development of the racemate rather than one of its enantiomers [5]. The drug, in man, after repeated oral administration (5–15-mg dose range) shows a C_{max} in plasma ranging between 1.3 and 6.3 ng ml^{-1} on the first day of treatment, probably owing to an extensive first-pass effect. After 1 week of twice daily administration these values increased to 5.3 and 16.8 ng ml^{-1}, respectively. The mean overall ($n = 17$) biological half-life in plasma, calculated on the first day, was about 14 h, whereas on the seventh day it was about 48 h ($n = 24$), indicating the probable existence of a deep compartment from which the unchanged compound was slowly released. Nevertheless, the steady-state plasma levels showed linearity with the increased doses.

The drug is mainly eliminated by metabolism, through the formation of glucuronides of its enantiomers. Such a conjugation pathway is stereoselective and glucuronides of eliprodil are present in both plasma and urine where they are mainly excreted [6]. Preliminary pharmacokinetic studies in man have been performed by using a non stereoselective HPLC method that utilized

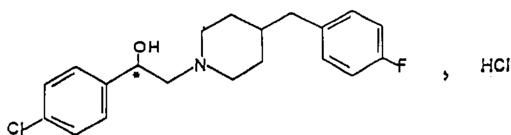
SL82.0715-10 (Eliprodil)

Structure

Molecular formula
$C_{20}H_{23}ClFNO.HCl$

Molecular weight
384.33

Ratio salt/base
1.1



SL83.0601-10

Structure

Molecular formula
$C_{20}H_{22}Cl_2FNO.HCl$

Molecular weight
418.77

Ratio salt/base
1.1

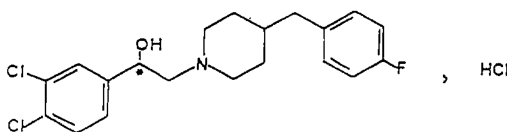


Fig. 1. Structures of eliprodil and the internal standard.

electrochemical detection (oxidation mode). The limit of quantitation (LOQ) of this method was about 0.2 ng ml^{-1} in plasma [7] for unchanged eliprodil. Further pharmacokinetic investigations in man required a highly sensitive stereoselective HPLC method. As a consequence, a method based on precolumn derivatization with (*S*)-(+)-naphthylethyl isocyanate (NEIC) and column-switching HPLC with fluorescence detection was investigated, developed and validated in both human plasma and urine, and is described in this paper.

2. Experimental

2.1. Chemicals, reagents and standards

Methanol, acetonitrile and tetrahydrofuran were of HPLC grade (Merck, Darmstadt, Germany), *n*-hexane was of Nanograde (Merck), sodium hydrogencarbonate powder was of analytical-reagent grade (J.T. Baker, Deventer, Netherlands), anhydrous sodium carbonate powder, anhydrous sodium dihydrogenphosphate, 85% orthophosphoric acid and sodium hydroxide and potassium hydroxide pellets were all of analytical-reagent grade (Merck), β-glucuronidase from *Escherichia coli* (200 I.U. ml^{-1}) was obtained from Sanofi-Pasteur (Paris, France) and (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate (NEIC) was of Fluka-type analytical-reagent grade (Aldrich, Milan, Italy). Pure water used for the reagent preparation and for the chromatographic eluent was of HPLC grade, prepared from deionized water by means of a reversed osmosis process (Milli-Ro Plus 60), then purified on a Milli-Q4 system and filtered through a 0.22-μm filter (Millipore, Bedford, MA, USA).

Sodium hydroxide solution (30%) was prepared from sodium hydroxide pellets, 1 *M* potassium hydroxide solution was prepared from potassium hydroxide pellets and 0.5 *M* carbonate buffer (pH 12) was prepared by dissolving 26.5 g of sodium carbonate and 21 g of sodium hydrogencarbonate in about 800 ml of pure water, adjusting the pH to 12 with about 30 ml of sodium hydroxide 30% solution, then diluting to 1 l with pure water. The derivatization mixture, 0.1% NEIC in acetonitrile, was prepared by sampling 25 μl of NEIC under a nitrogen flow, then diluting to 25 ml with acetonitrile; this solution can be stored under ambient conditions and is stable for 3 days. Phosphate buffer (0.025 *M*) (pH 2.6) used for the mobile phase was prepared by weighing 6.8 g of potassium dihydrogenphosphate, adding 3.4 ml of orthophosphoric acid, dissolving in and diluting with about 800 ml of pure water, then diluting to 1 l, giving a 0.1 *M* solution, and further diluting with water to give a 0.025 *M* solution. Phosphate buffer (0.02 *M*)(pH 6.5) used for the deconjugation reaction was prepared by weighing 136.08 g of potassium dihydrogenphosphate, dissolving in and diluting with 1 l of pure water, then diluting 20 ml of this solution with about 800 ml of pure water, adjusting the pH to 6.5 with 1 *M* potassium hydroxide solution and diluting to 1 l with water (0.2 *M* solution), finally diluting to give a 0.02 *M* solution. Phosphate solution (0.05 *M*) for the injection solvent was prepared by weighing 6.8 g of potassium dihydrogenphosphate, dissolving in about 800 ml of pure water and diluting to 1 l to give a 0.05 *M* solution.

The injection solvent was prepared by mixing 120 ml of 0.05 *M* potassium dihydrogenphosphate solution and 80 ml of acetonitrile. The mobile phase for chromatography was prepared mixing 420 ml of 0.025 *M* phosphate buffer (pH 2.6) solution, 20 ml of methanol and 560 ml acetonitrile to give phosphate–methanol–acetonitrile (42:2:56, v/v/v).

Eliprodil (SL 82.0715-10) and the internal standard, which is an analogue of eliprodil (SL 83.0601-10), both as racemates (Fig. 1), were of pharmaceutical grade from Synthélabo Recherche (Bagneux, France). (*S*)-(+)- and (*R*)-(−)- eliprodil enantiomers (SL 83.0936 and SL 83.0937, respectively), used for the chromatographic identification of each enantiomer in the racemate, were of pharmaceutical grade (Synthélabo Recherche).

Predose plasma utilized for preparing plasma standards and quality controls (QCs), containing sodium citrate as anticoagulant, was obtained from a blood bank (AVIS, Milan, Italy). Predose

urine, utilized for preparing urine standards and QCs, was obtained from pooled urine from healthy volunteers.

2.2. Standard solutions and QCs

Standard methanolic solutions of eliprodil and internal standard were prepared from stock solutions (1 mg ml^{-1}) in order to obtain a wide range of concentrations suitable for calibrations (Table 1) in human plasma and urine. QCs in human plasma and urine were prepared at different concentrations (Table 1) by spiking predose plasma and urine with known amounts of eliprodil from standard methanolic solutions originating from an independent stock solution; 1-ml aliquots of the QC samples were transferred into screw-capped test-tubes and deep-frozen (−20°C) until analysis. Plasma and urine standards and QCs were run together with postdose samples.

2.3. Chromatographic system

Basic chromatographic system

The basic chromatographic system consisted of a Model 420 double-piston pump (flow-rate range 0.01–2 ml min^{-1}) (Kontron, Milan, Italy) set at a flow-rate of 1.2 ml min^{-1}, a Model 821-FP spectrofluorimetric detector provided with a xenon lamp and a 16-μl flow cell (Jasco, Tokyo, Japan), set at an excitation wavelength of

Table 1
Scheme for the preparation of the calibration standards and quality controls (QC) for unchanged eliprodil in human plasma, total eliprodil (unchanged + glucuroconjugated) in human plasma, and total eliprodil in human urine

Name of the substance	Standard solutions ($ng \cdot ml^{-1}$)[a]	Range of linearity ($ng \cdot ml^{-1}$)[a]	QC ($ng \cdot ml^{-1}$)[a]	Limit of quantitation ($ng \cdot ml^{-1}$)[a]
Unchanged eliprodil in human plasma				
SL 82.0715-10	1000–500		0.6	
Eliprodil[b]	100–50	0.3–20	3	0.3
	15		15	
SL 83.0601-10 Internal standard[c]	1250			
Total eliprodil in human plasma				
SL 82.0715-10	50000–10000		6	
Eliprodil[b]	2500–500	1.5–1000	60	1.5
	250–75		600	
SL 83.0601-10 Internal standard[c]	1250			
Total eliprodil in human urine				
SL 82.0715-10	2500–1000		0.4	
Eliprodil[b]	500–250	0.1–50.0	6.0	0.1
	50–25–5		30.0	
SL 83.0601-10 Internal standard[c]	20			

[a] Expressed as racemate.
[b] The concentrations of the two enantiomers *S*-(+) and *R*-(−) are half of those expressed for SL 82.0715.
[c] The concentrations of the two enantiomers are assumed to be 50% each.

275 nm and with emission at 336 nm, a Model 460 automatic sample injector (Kontron), an analytical column (150 × 4.6 mm I.D.) filled with 5-μm Hypersil C_8 BDS material (Shandon, Runcorn, UK) and a guard column (20 × 4.6 mm I.D.) filled with 40-μm Pelliguard LC_8 (Supelco, Bellefonte, PA, USA). The mobile phase was 0.025 *M* phosphate buffer (pH 2.6)–methanol–acetonitrile (42:2:56, v/v/v).

Extended chromatographic system

The extended chromatographic system (Fig. 3) was obtained from the basic system using a precolumn for on-line purification (20 × 4.6 mm I.D.) filled with 5-μm Supelguard LC_8 DB (Supelco), a Tracer Model MCS-670 switching apparatus (Kontron) provided with four high-pressure six-port valves and two six-port solvent selector valves. The whole apparatus was managed by an Anacomp 220 programmer (Kontron). An auxiliary Model 414 single-piston pump

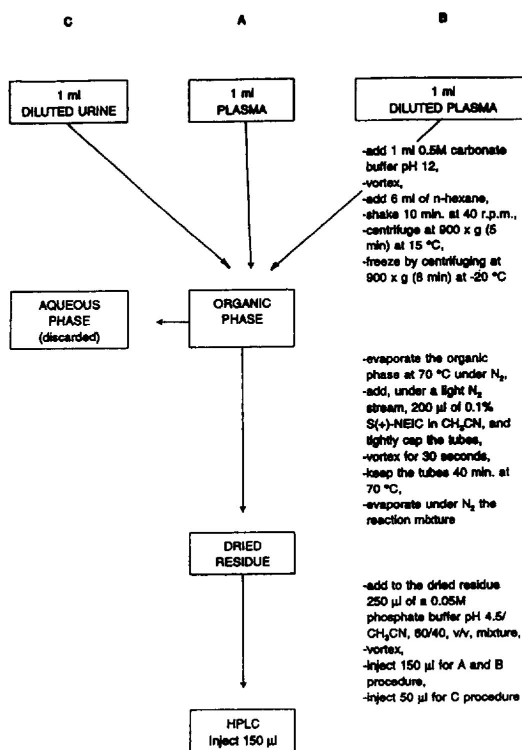


Fig. 2. Flow chart of sample preparation for eliprodil in human plasma and urine.

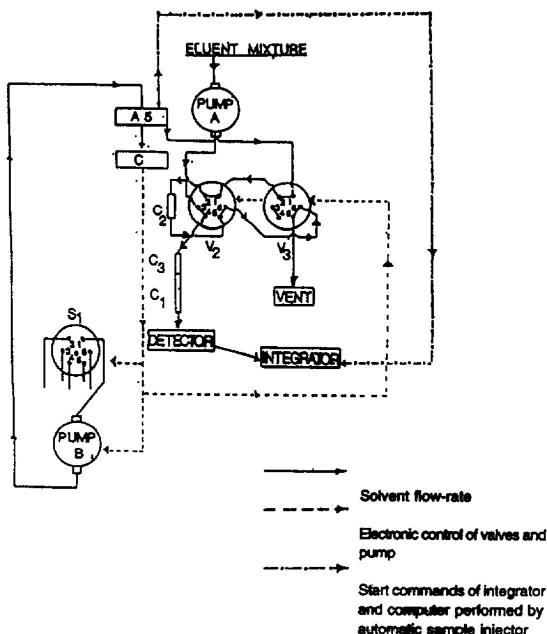


Fig. 3. Scheme of chromatographic apparatus used for automatic on-line clean-up of samples and HPLC with column switching.

(Kontron) was used for the precolumn clean-up and restoring, set at a flow-rate of 2 ml min^{-1}. The auxiliary pump flushes the precolumn for 5 min with CH_3CN–H_2O (50:50, v/v), then the precolumn and column are connected in series for 2.5 min; after this time, the precolumn and column are disconnected and while the chromatography takes place on the analytical column, the precolumn is back-flushed with CH_3CN–H_2O (70:30, v/v), CH_3CN, CH_3OH and tetrahydrofuran, and finally it is re-equilibrated with CH_3CN–H_2O (50:50, v/v). The whole process takes about 30 min.

2.4. Sample preparation

In the sample preparation procedure, screw-capped tubes of borosilicate glass were utilized (Corning, New York, USA). Three different preliminary approaches were examined for the determination of unchanged and total (unchanged + glucuronated) eliprodil enantiomers in plasma and urine, as follows.

In procedure A (unchanged eliprodil in human plasma), 20 μl (25 ng of internal standard) were added to 1 ml of plasma that was then processed and derivatized according to the procedure shown schematically in Fig. 2.

In procedure B (total eliprodil = unchanged + glucuroconjugated in human plasma), to 0.2 ml of plasma, 0.2 ml of 0.02 *M* buffer (pH 6.5) and 20 μl (4 I.U.) of β-glucuronidase (*E. coli*) were added and mixed; after incubation for 24 h at 37°C, the sample was diluted to exactly 1 ml with water, 20 μl of internal standard solution (25 ng) were added to the diluted sample and the mixture was then processed according to Fig. 2.

In procedure C (total eliprodil in human urine), to 0.1 ml of urine, 0.1 ml of 0.02 *M* buffer (pH 6.5) and 50 μl (10 I.U.) of β-glucuronidase (*E. coli*) were added and mixed; after incubation for 24 h at 37°C, the sample was diluted to exactly 1 ml with water, 20 μl (400 ng) of internal standard solution were added to the diluted sample and the mixture was then processed according to Fig. 2.

The samples after extraction, concentration and derivatization with (*S*)-(+)-NEIC were evaporated under nitrogen, then reconstituted with a suitable solvent (the mobile phase is unsuitable as it causes peak broadening) and injected on to the precolumn.

2.5. Clean-up, column switching and analytical chromatography

The clean-up, flushing and restoring of the precolumn and the column switching are performed automatically by means of a programmable six-port solvent selector (S_1) (see Fig. 3) and two six-port high-pressure valves (V_2 and V_3, respectively), which can be managed through a computer (C) and a suitable computer program.

After automatic injection into the automatic sample injector (A5), the sample extract was further purified on the precolumn (C_2) as follows: for 5 min the precolumn was flushed with acetonitrile–water (50:50) by pump B; NEIC derivatives of (*S*)-(+)- and (*R*)-(−)-enantiomers of eliprodil and the internal standard were retained, while endogenous co-extracted substances, salts and unreacted NEIC were passed to waste. Next, the precolumn, guard column (C_3) and analytical column (C_1) were connected in series for 2.5 min; the analytes were transferred from the mobile phase, supplied by pump A, from the precolumn to the guard column and analytical column; in the meantime, the precolumn was back-flushed and restored with different solvents, then re-equilibrated with acetonitrile–water (50:50, v/v).

3. Results

3.1. Deconjugation of glucuroconjugates of eliprodil with β-glucuronidase

Diastereoisomeric glucuronides of eliprodil in plasma and urine should be submitted to hydrolytic deconjugation before extraction, which is then followed by derivatization with (*S*)-(+)-NEIC. The most reproducible hydrolytic conditions were achieved with β-glucuronidase (from *E. coli*) using 4 and 10 I.U. of enzyme for 0.2 ml of plasma and 0.1 ml of urine, respectively, and incubating at 37°C for 24 h.

3.2. Derivatization with (S)-(+)-NEIC

Chiral isocyanates have sometimes been used as chiral derivatizating agents for hydroxy compounds to give carbamates [8], and 1-(1-naphthyl)ethyl isocyanate (NEIC) was used successfully to produce diastereoisomeric carbamates of eliprodil and the internal standard. Optimum and reproducible reaction conditions with the highest derivatization yield were found with synthetic standards by using 200 μl of 0.1% (*S*)-(+)-NEIC in CH_3CN at 70°C for 40 min. The derivatization reaction, on plasma standards, gave a yield of about 80% over a wide range of concentrations (in comparison with the synthetic standards).

3.3. Stability

Eliprodil enantiomers and the (*S*)-(+)-enantiomer of the internal standard were found to be stable in methanol (1 mg ml^{-1}) when maintained

at 0–5°C for at least 1 month. Eliprodil enantiomers, in addition, were demonstrated to be stable under different conditions and in different biological matrices: they were stable in human plasma and urine when maintained for 24 h at 37°C, in human plasma for 4 years at −20°C, in human urine for 1 month at −20°C (probably it is stable for much longer), in human plasma and urine submitted to two freezing–thawing cycles and in diluted human plasma and urine for 24 h under incubation conditions (with β-glucuronidase). Eliprodil enantiomers together with the internal standard [as the (S)-(+)-enantiomer] were stable under alkaline conditions (pH 12) for at least 4 h (pre-extraction conditions). Finally, eliprodil enantiomers and the internal standard, all as NEIC derivatives, were stable in the HPLC solvent for at least 24 h.

3.4. Selectivity

Several predose plasma and urine samples were tested for the absence of interfering compounds when performing or not the hydrolysis deconjugation reaction. In no case was any chromatographic interference found at the retention times of (S)-(+)- and (R)-(−)-eliprodil derivative or that of the (S)-(+)-enantiomer of the internal standard one. An example is reported in Fig. 4a.

3.5. Absolute recovery

The absolute recoveries of eliprodil enantiomers and the (S)-(+)-enantiomer of the internal standard (SL 83.0601) were evaluated from predose human plasma and urine samples spiked with the compounds of interest (in the unchanged form only, since the available synthetic glucuroconjugates of eliprodil were not pure enough to be used as reference standards); however, plasma and urine samples were also submitted to enzymatic hydrolysis (before extraction) to assess if such a process could affect the recovery.

The mean overall absolute recovery, from

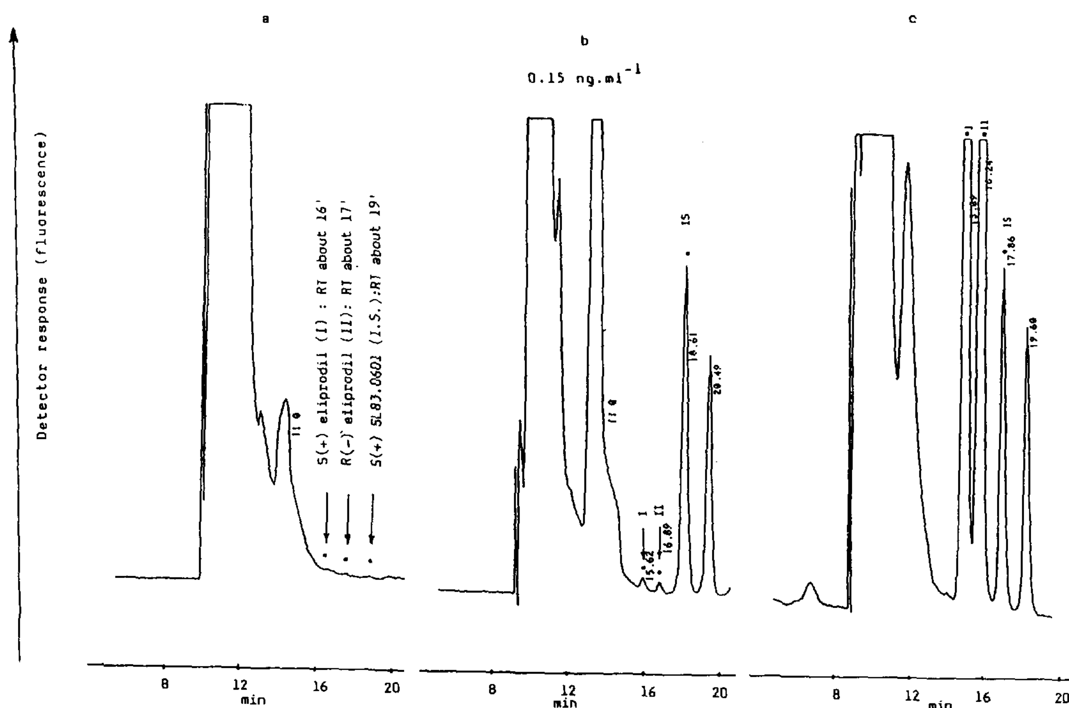


Fig. 4. (a) Chromatogram of predose plasma sample (method for unchanged eliprodil); (b) chromatogram of the LOQ in plasma for unchanged eliprodil; (c) chromatogram of plasma standard containing 500 ng ml^{-1} of each eliprodil enantiomer (total).

human plasma, of (S)-(+)- and (R)-(−)-eliprodil enantiomers, in the concentration range 0.5–5 ng ml^{-1}, was about 92% for samples not submitted to hydrolysis, and that from human plasma in the concentration range 3–300 ng ml^{-1} was about 100% for samples submitted to hydrolysis.

The mean overall absolute recovery, from human urine, of (S)-(+)- and (R)-(−)-eliprodil enantiomers, in the concentration range 200–1500 ng ml^{-1}, was about 80% for samples submitted to hydrolysis (the normal procedure since in dosed urine samples no unchanged eliprodil is present). The recovery from plasma is higher than that from urine, probably because the lipidic components of plasma enhance the quantitative extraction of eliprodil. The mean overall absolute recovery from human plasma and urine of the (S)-(+)-enantiomer of the internal standard was about 90% at the reported concentrations.

3.6. Linearity

Linearity between the peak height-ratio of (S)-(+)- and (R)-(−)-eliprodil and the internal standard versus the concentration of (S)-(+)- and (R)-(−)-eliprodil was assessed by a weighted ($1/y$) regression equation for each assay just evaluating the individual residual values, for each calibration, not exceeding 15% of nominal concentration values. The individual residual (%) was calculated for each concentration according to the formula $(y'-y)/y \cdot 100$, where y is the nominal concentration and y' the calculated concentration. Linearity was checked in the range 0.15–10 ng ml^{-1} for each enantiomer in human plasma (without performing hydrolysis) at five levels, giving the equations $y=6.285x-0.047$ ($R^2=1$) and $y=7.142x-0.045$ ($R^2=1$) for the (S)-(+)- and (R)-(−)-enantiomer respectively, in the range 0.75–500 ng ml^{-1} for each enantiomer in human plasma (performing hydrolysis) at six levels, giving the equations $y=43.175x-0.092$ ($R^2=1$) and $y=47.503x-0.137$ ($R^2=1$) for (S)-(+)- and (R)-(−)-enantiomer, respectively, and in the range 0.05–25 $\mu g\ ml^{-1}$ for each enantiomer in human urine (performing hydrolysis) at seven levels, giving the equations $y=1.403x-0.011$ ($R^2=1$) and $y=1.540x-0.007$ ($R^2=1$) for the (S)-(+)- and (R)-(−)-enantiomer, respectively.

3.7. Limit of quantitation (LOQ)

The LOQ, taken as a chromatographic peak at least three times higher than the baseline noise, is 0.15 ng ml^{-1} for each unchanged eliprodil enantiomer in human plasma, 0.75 ng ml^{-1} for each total eliprodil enantiomer in human plasma and 50 ng ml^{-1} for each total eliprodil enantiomer in human urine. An example is reported in Fig. 4b. The LOQ is always the lowest point of the daily calibration graph.

3.8. Precision and accuracy

The intra- and inter-day precision and accuracy were evaluated using predose human plasma and urine samples spiked with different amounts of eliprodil (as racemate) and then processed according to the described methods. The results (Table 2) demonstrate acceptable precision and accuracy of the methods at the concentrations investigated. The precision was also evaluated (intra-day R.S.D.) at the LOQ ($n=4$) by means of predose human plasma and urine spiked with appropriate amounts of eliprodil (as racemate); in all the cases the R.S.D. was <10%. An example is reported in Fig. 4c.

3.9. Application to pharmacokinetics

The described method was applied to the determination of eliprodil enantiomers in plasma and urine from healthy subjects treated orally with 20 mg of eliprodil (as racemate). Typical chromatograms are reported in Fig. 5a–c. Fig. 6 reports the mean ($n=16$) plasma concentration vs. time plots of unchanged and total (unchanged + glucuronated) enantiomers and Fig. 7 shows the mean ($n=6$) cumulative urinary excretion of eliprodil enantiomers (as glucuronate) in the same subjects.

4. Discussion

The use of a pure optical isomer of NEIC was introduced for the first time for the formation of

Table 2
Precision and accuracy for eliprodil unchanged enantiomers in human plasma (A), total in human plasma (B) and in urine (C)

	Nominal concentration (ng · ml^{-1})		
	0.3	1.5	7.5
Unchanged eliprodil in human plasma			
Precision			
S-(+): Intra-day R.S.D. (%)	4.2 (*n* = 13)	5.1 (*n* = 15)	2.9 (*n* = 14)
R-(−): Intra-day R.S.D. (%)	4.5 (*n* = 13)	5.0 (*n* = 15)	3.0 (*n* = 14)
S-(+): Inter-day R.S.D. (%)	3.0 (*n* = 13)	1.0 (*n* = 15)	2.6 (*n* = 14)
R-(−): Inter-day R.S.D. (%)	1.9 (*n* = 13)	3.5 (*n* = 15)	2.3 (*n* = 14)
Accuracy			
S-(+): (Mean ± R.S.D.) (%)	90.4 ± 3.3 (*n* = 13)	91.4 ± 1.7 (*n* = 15)	89.6 ± 2.5 (*n* = 14)
R-(−): (Mean ± R.S.D.) (%)	87.1 ± 2.4 (*n* = 13)	90.0 ± 3.7 (*n* = 15)	90.3 ± 2.3 (*n* = 14)
	2.5	25.0	500.0
Total eliprodil in human plasma			
Precision			
S-(+): Intra-day R.S.D. (%)	3.0 (*n* = 3)	1.1 (*n* = 3)	2.5 (*n* = 3)
R-(−): Intra-day R.S.D. (%)	2.0 (*n* = 3)	1.1 (*n* = 3)	2.3 (*n* = 3)
S-(+): Inter-day R.S.D. (%)	4.0 (*n* = 16)	3.8 (*n* = 16)	0.6 (*n* = 16)
R-(−): Inter-day R.S.D. (%)	5.9 (*n* = 16)	4.1 (*n* = 16)	0.1 (*n* = 16)
Accuracy			
S-(+): (Mean ± R.S.D.) (%)	101.0 ± 4.8 (*n* = 6)	99.4 ± 5.3 (*n* = 6)	99.8 ± 0.6 (*n* = 6)
R-(−): (Mean ± R.S.D.) (%)	98.5 ± 6.1 (*n* = 6)	100.2 ± 5.5 (*n* = 6)	99.7 ± 0.4 (*n* = 6)
	0.25	10.0	25.0
Total eliprodil in human urine			
Precision			
S-(+): Intra-day R.S.D. (%)	5.4 (*n* = 3)	4.5 (*n* = 3)	5.2 (*n* = 3)
R-(−): Intra-day R.S.D. (%)	6.1 (*n* = 3)	4.4 (*n* = 3)	4.9 (*n* = 3)
S-(+): Inter-day R.S.D. (%)	4.0 (*n* = 5)	2.7 (*n* = 5)	1.1 (*n* = 5)
R-(−): Inter-day R.S.D. (%)	4.6 (*n* = 5)	2.8 (*n* = 5)	1.3 (*n* = 5)
Accuracy			
S-(+): (Mean ± R.S.D.) (%)	100.0 ± 3.8 (*n* = 4)	101.6 ± 2.4 (*n* = 4)	100.0 ± 1.3 (*n* = 4)
R-(−): (Mean ± R.S.D.) (%)	100.0 ± 3.3 (*n* = 4)	102.0 ± 2.3 (*n* = 4)	99.1 ± 1.1 (*n* = 4)

diastereoisomeric ureas from primary and secondary amines, such as β-adrenergic antagonists [9], and then extended to the formation of carbamates from hydroxy compounds [8]. For eliprodil, derivatization with (*S*)-(+)-NEIC seems to work well if it is carried out in completely anhydrous conditions and, most important, if a large (*S*)-(+)-NEIC excess is guaranteed. Unfortunately, the weak points of such an approach are the derivatization residues and by-products that give rise, during chromatography, to several long-retained peaks which lengthen the chromatographic run to 45–50 min.

The method described here takes advantage of the extremely lipophilic properties of NEIC carbamate derivatives of eliprodil that, after formation, can be submitted to an extensive clean-up (solid-phase extraction), being retained on a 5-μm C_8 precolumn by flushing the precolumn for 5 min with water–acetonitrile (1:1) at 2 ml min^{-1}. After this automatically performed step, the derivatized and purified extract containing the analytes of interest is switched to the analytical column for chromatography of the diastereoisomeric carbamates of eliprodil and the internal standard. The clean-up step allows the

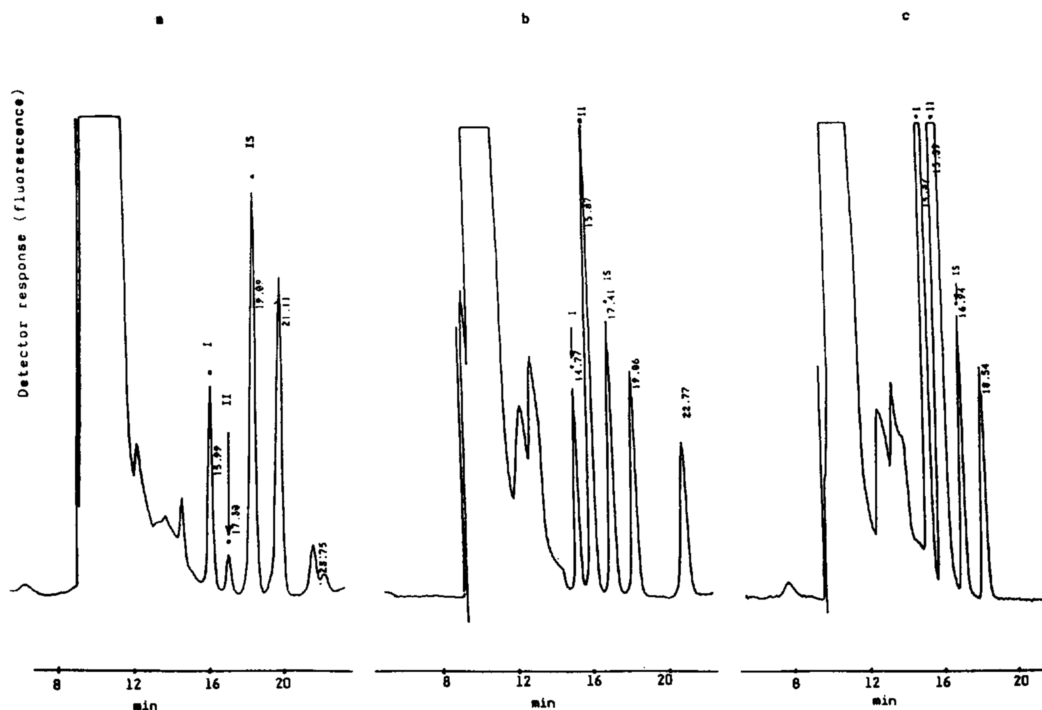


Fig. 5. (a) Chromatogram of plasma sample from a healthy volunteer treated with 20 mg of eliprodil; sample collected 8 h after drug intake; concentrations found for unchanged enantiomers: 4 and 0.4 ng ml^{-1} for the (*S*)-(+)- and (*R*)-(−)-enantiomers, respectively. (b) Chromatogram of plasma sample (after deconjugation) from a healthy volunteer treated with 20 mg of eliprodil; sample collected 3 h after drug intake; concentrations found for total enantiomers: 29.7 and 77.6 ng ml^{-1} for the (*S*)-(+)- and (*R*)-(−)-enantiomers, respectively. (c) Chromatogram of urine sample (after deconjugation) from a healthy volunteer treated with 20 ng of eliprodil; 0–2 h urinary fraction; concentrations found for total (glucuronated) enantiomers: 10.1 and 32.6 μg ml^{-1} for the (*S*)-(+)- and (*R*)-(−)-enantiomers, respectively.

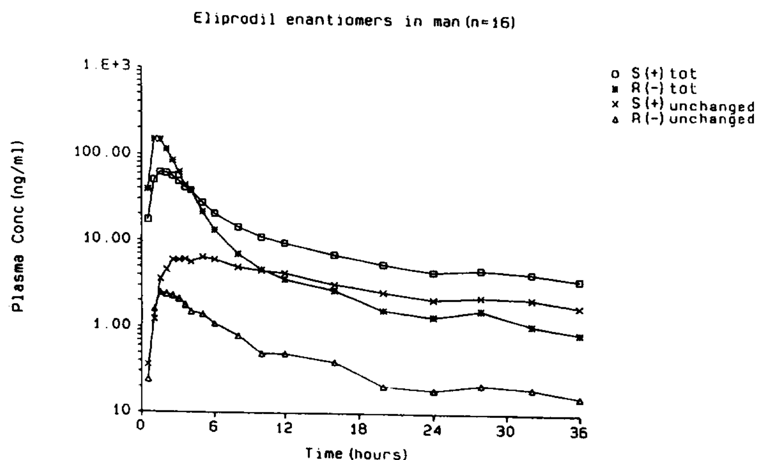


Fig. 6. Mean plasma concentration–time course of unchanged and total eliprodil enantiomers in sixteen healthy volunteers treated with 20 mg of eliprodil (as racemate).

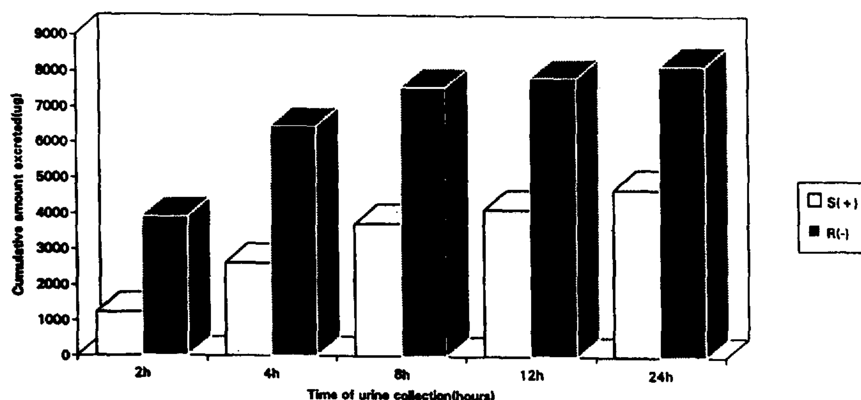


Fig. 7. Mean cumulative urinary excretion of eliprodil enantiomers (as glucuronate) in healthy volunteers ($n = 16$) after oral administration of 20 mg of eliprodil (as racemate).

analytical run time to be reduced to about 30 min but, more importantly, it avoids overloading the analytical column with secondary products which, if more polar than the analytes, are flushed from the precolumn with acetonitrile–water (1:1) mixture, or, if less polar than the analytes, are trapped from the precolumn and then back-flushed with different selective solvents. Finally, the selected switching conditions allow the extraction procedure to be simplified to a single step, without problems of interferences, thus obtaining high recoveries for the analytes.

Concerning the enzymatic hydrolysis of eliprodil as the glucuroconjugated metabolite in plasma and urine, it was found, working on pooled plasma and urine samples obtained from subjects treated orally with 20 mg of eliprodil racemate, that β-glucuronidase from *E. coli* was able to give rise to complete hydrolytic cleavage of glucuronides of both (*S*)-(+)- and (*R*)-(−)-enantiomers while β-glucuronidase from *H. pomatia* allowed complete hydrolysis of only the (*S*)-(−)-enantiomer, the (*R*)-(+)-enantiomer being partially recovered even when using a higher enzyme content and/or longer incubation times. The different behaviours of the two eliprodil glucuroconjugated diastereoisomers may be explained on the basis of different affinities of the catalytic sites of the two types of β-glucuronidase for the (*R*)-(−)-eliprodil glucuronide diastereoisomer.

The formation of (*S*)-(+)-NEIC diastereoisomeric derivatives, which exhibit strong fluorescence properties, is easy and reproducible, allowing very high sensitivity to be achieved. The method has been tested on over 1000 samples from in vivo studies, demonstrating its feasibility, its robustness and its high reproducibility (assessed by the small R.S.D. of the slope of the calibration graph and QCs). The method was applied to pharmacokinetic studies in man, giving results which were in close agreement with those obtained by analysing the drug as the racemate.

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