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Short Communication

Determination of linopirdine and its N-oxide metabolites in rat plasma by liquid chromatography

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Introduction

Linopirdine [3,3-bis(4-pyridinylmethyl)-1-phenylindolin-2-one, Fig. 1] is a novel phenylindolinone derivative that enhances cognitive performance. It enhances the *in vitro* and *in vivo* release of acetylcholine, dopamine and serotonin in rat brain, and significantly enhanced the performance of rats and mice in behavioural test procedures [1, 2]. In man, linopirdine induced electroencephalographic changes indicative of an improvement in vigilance [3].

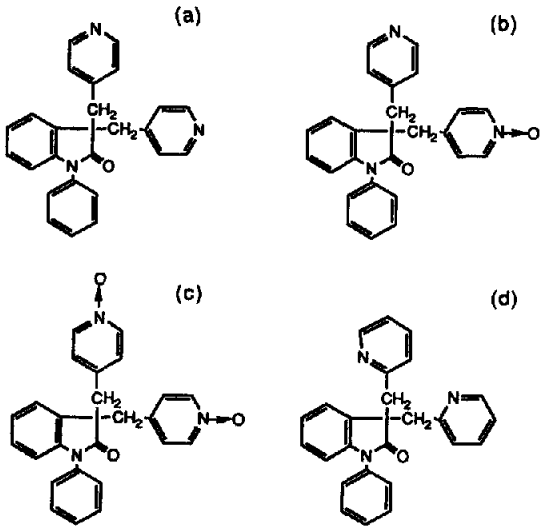

Figure 1
Chemical structures of linopirdine (a), linopirdine mono-N-oxide (b) and linopirdine bis-N-oxide (c) and internal standard (d).

Linopirdine is metabolized to its corresponding mono-N-oxide and bis-N-oxide in rats [4]. Oxidation of the pyridyl nitrogen is cytochrome P-450-mediated and is common to many pyridino-compounds. N-oxides of pyridine, nicotine, metyrapone, rosoxacin, and other pyridino-compounds have been isolated and characterized [5, 6]. In order to investigate the pharmacokinetics of linopirdine in animals, a high-performance liquid chromatographic method was developed. Since pyridino-N-oxides are readily reduced back to their pyridyl compounds, it is important to determine the kinetics of the reversible reaction both *in vitro* and *in vivo* [7]. This paper describes a HPLC method for the quantification of linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide in rat plasma.

Materials and Methods

Chemicals, reagents and control plasma

Linopirdine, linopirdine mono-N-oxide, linopirdine bis-N-oxide and the internal standard (3,3-bis(2-pyridinylmethyl)-1-phenylindolin-2-one) were synthesized by the Medicinal Chemistry Section of The DuPont Merck Pharmaceutical Company (Wilmington, DE). Each compound was reported to be >99% pure by the Analytical Research and Develop-

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ment Section of The DuPont Merck Pharmaceutical Company and was used without further purification. HPLC grade 1-octanesulphonic acid sodium salt was purchased from Eastman Kodak Company (Rochester, NY). All other chemicals and solvents were of the highest quality available. Control rat plasma was purchased from Buckshire Corporation (Perkasie, PA).

Chromatographic system

The liquid chromatograph consisted of a variable-wavelength detector (Model 783 Spectroflow Programmable Absorbance Detector, Kratos Analytical, Ramsey, NJ), a solvent pump (Model 590, Waters Associates, Milford, MA), an automatic injector (WISP 710B, Waters Associates) and a Model 4416 Data Acquisition System (Nelson Analytical, Cupertino, CA). The column was an APEX II RP 5 μm (250 mm × 4.6 mm i.d.) from Jones Chromatography (Littleton, CO).

Chromatography conditions

The mobile phase was an aqueous mixture of ammonium acetate (0.1 M)–1-octanesulphonic acid sodium salt (0.1 M)–acetic acid–triethylamine (740:5:4:1, v/v/v/v) and methanol (72.5:27.5, v/v). The flow rate was 1.5 ml min^{-1}, and detection was by UV at 254 nm. The separation of the analytes was conducted at ambient temperature.

Stock solutions

Primary stock solutions containing 1 mg ml^{-1} of linopirdine, linopirdine mono-N-oxide, linopirdine bis-N-oxide and the internal standard were prepared by dissolving 50 mg of each compound in 50 ml of methanol. Linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide working stock solutions 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20 and 50 μg ml^{-1} were prepared by diluting the appropriate volumes of the primary stock solutions in methanol. An aliquot of the internal standard was diluted in methanol to prepare a working stock solution of 1.0 μg ml^{-1}.

Preparation of plasma standards and samples

Plasma standards for linopirdine and linopirdine mono-N-oxide were prepared by adding 100 μl of each linopirdine and linopirdine mono-N-oxide working stock solution and 100 μl of the internal standard working stock solution to 20 × 150 mm screw-cap test tubes. The mixtures were evaporated to dryness using a gentle stream of nitrogen at room temperature and 1 ml of control plasma was added and mixed with the compounds. The final concentrations of linopirdine and linopirdine mono-N-oxide were 0.01 or 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0 μg ml^{-1}. Plasma standards for linopirdine bis-N-oxide were prepared by adding 100 μl of each linopirdine bis-N-oxide working stock solution and 100 μl of the internal standard working stock solution to 12 × 75 mm culture tubes. The mixtures were evaporated to dryness using a gentle stream of nitrogen at room temperature and 1 ml of control plasma was added and mixed with the compounds. The final concentrations of linopirdine bis-N-oxide were 0.05, 0.1, 0.5, 1.0, 2.0 and 5.0 μg ml^{-1}. For plasma samples containing unknown concentrations of linopirdine and its N-oxide metabolites, only 100 μl of the internal standard solution was dried under nitrogen and mixed with 1 ml of the plasma samples.

Extraction procedures

Linopirdine and linopirdine mono-N-oxide. One millilitre of pH 9 buffer (pH 9.00 certified buffer, Fisher Scientific, Fair Lawn, NJ) and 5 ml of ethyl acetate was added to the test tubes containing either plasma standards or unknown samples of linopirdine and linopirdine mono-N-oxide. The tubes were capped and shaken for 10 min. After centrifugation (3000g), the upper organic layer was transferred to a clean 20 × 150 mm test tube and the aqueous layer was extracted again with 5 ml of ethyl acetate. The ethyl acetate extracts were combined and extracted with 1 ml of 0.1 N HCl. After centrifugation (3000g) and discarding the organic layer, the remaining acid was diluted with 1.25 ml of 0.1 N NaOH and extracted with 5 ml of ethyl acetate for 10 min. The ethyl acetate was evaporated to dryness using a gentle stream of nitrogen at room temperature. The residue was reconstituted in 200 μl of mobile phase and 100 μl was injected onto the HPLC column and chromatographed.

Linopirdine bis-N-oxide. Three millilitres spe®CN extraction columns (J.T. Baker Chemical Co., Phillipsburg, NJ) were preconditioned with 1 ml of methanol, 1 ml of water and 2 × 1 ml of 0.1 M Tris buffer (pH 10) using a vacuum manifold (Analytichem International, Harbor City, CA). The plasma and 1 ml of

0.1 M Tris buffer rinse of the culture tube were transferred to the column. The column was washed with 1 ml of Tris buffer. The retained linopirdine bis-N-oxide and internal standard were eluted off the column with 3 × 0.5 ml of methanol and collected in 13 × 100 mm screw-cap test tubes. The combined eluent was evaporated to dryness (using a gentle stream of nitrogen at room temperature). The residue was reconstituted in 200 μl of the mobile phase and 100 μl was injected onto the HPLC column and chromatographed.

Data analyses

Peak heights were measured by the Nelson Analytical Data System. The peak height ratios (PHR) of linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide to the internal standard were determined. Two linear regression equations were used to determine slopes, intercepts and correlation coefficients of each compound. The standards for the low standard curve ranged from 0.02 to 0.1, 0.02–0.1 and 0.05–0.5 μg ml^{-1} for linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide, respectively. The standards for the high standard curve ranged from 0.1 to 5.0, 0.1–5.0 and 0.5–5.0 μg ml^{-1} for linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide, respectively.

Assay validation

The precision and accuracy of the method were evaluated by repetitive analysis of spiked rat plasma at different linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide concentrations. Three repeated analyses of spiked rat plasma samples were conducted on one day for intra-day variability and three sets of samples were analysed on three different days for inter-day variability. The extraction recovery of each compound was determined by comparing the peak height ratio from extracted samples to those of unextracted samples. The stability of each compound was assessed in rat plasma incubated at 37°C. In addition the stability of each compound was assessed in rat plasma frozen at −20°C containing 0.1–10.0 μg ml^{-1}. The samples for the stability evaluation were analysed in triplicate.

Rat pharmacokinetics

Male Sprague–Dawley rats (Charles River, Kingston, NY) weighing about 300 g were used for this study. A single bolus intravenous 2.5 mg kg^{-1} dose of linopirdine was injected into the tail vein of 44 rats. The dosing solution was prepared by dissolving linopirdine in 0.1 N HCl and adjusting the pH to 4. Blood samples (5 ml) were collected from four rats by cardiac puncture at prior to dosing, 0.03, 0.08, 0.17, 0.5, 1, 2, 4, 6, 8 and 12 h after dosing. The plasma was separated from the red blood cells by centrifugation at 3000*g* for 20 min and stored at −20°C pending analysis. The pharmacokinetic parameters were calculated using general noncompartmental procedures [8].

Results and Discussion

Extraction and chromatography

The extraction recovery of linopirdine and linopirdine mono-N-oxide were 102.3 ± 12.6 and 68.2 ± 3.8%, respectively, using the liquid–liquid extraction method. Linopirdine bis-N-oxide which could not be extracted by this technique was successfully extracted by a solid-phase method. The extraction recovery of linopirdine bis-N-oxide was 99.7 ± 1.6%. High recoveries of linopirdine and linopirdine mono-N-oxide were also achieved by the solid-phase method but their quantitation was hindered by co-elution of endogenous substances in the chromatography. Attempts to extract and chromatograph all three compounds simultaneously were unsuccessful. The two extraction methods, albeit lengthy procedures, were optimal for the compounds. The results of the extraction recovery at various concentrations are summarized in Table 1.

Preliminary chromatographic parameters were established on a reverse phase column using an ion-pairing solvent consisting of 0.1 M ammonium acetate–0.1 M 1-octanesulphonic acid solution salt–acetic acid–triethylamine (740:5:4:1, v/v/v/v) and an organic modifier at a ratio of 3:1, v/v. Table 2 shows the relative capacity factors for linopirdine, linopirdine mono-N-oxide, linopirdine bis-N-oxide and the internal standard using the three different organic modifiers; acetonitrile, tetrahydrofuran and methanol. The best separation of all compounds was obtained with the mobile phase containing methanol (Fig. 2). The composition of the mobile phase was further optimized to 27.5% methanol on the basis of peak shape and retention times. Under these conditions, linopirdine, linopirdine mono-N-oxide, linopirdine bis-N-oxide, and the internal

Table 1
Extraction recovery of linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide in rat plasma

Concentration added ($\mu g\ ml^{-1}$)	Extraction recovery (%)		
	Linopirdine*	Linopirdine mono-N-oxide*	Linopirdine bis-N-oxide†
0.05	116.9	72.6	98.0
0.50	94.5	65.9	100.0
5.00	95.6	66.0	101.1
Mean	102.3	68.2	99.7
(SD)	(12.6)	(3.8)	(1.6)

*Extracted by the liquid–liquid extraction method.
†Extracted by the solid-phase extraction method.

Table 2
Relative capacity factors (k') for linopirdine, linopirdine mono-N-oxide, linopirdine bis-N-oxide, and the internal standard on a Jones CN RP II HPLC column with a mobile phase consisting of an aqueous mixture* and an organic modifier (75:25, v/v)

Compounds	Organic modifier		
	Acetonitrile	Tetrahydrofuran	Methanol
Linopirdine	6.1	4.3	13.4
Linopirdine mono-N-oxide	2.3	2.9	6.2
Linopirdine bis-N-oxide	0.9	0.4	2.1
Internal standard	6.7	5.0	15.0

*Ammonium acetate (0.1 M)–1-octanesulphonic acid sodium salt (0.1 M)–acetic acid–triethylamine (740:5:4:1, v/v/v/v).

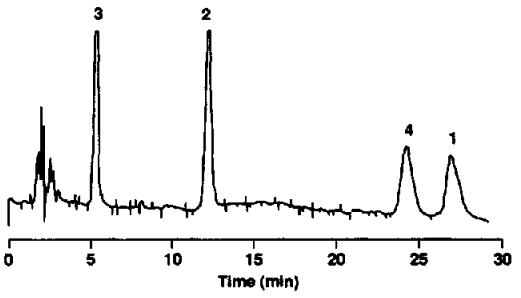

Figure 2
Chromatogram of linopirdine, linopirdine mono-N-oxide, linopirdine bis-N-oxide, and internal standard obtained using a mobile phase consisted of methanol–(0.1 M) ammonium acetate–(0.1 M) 1-octanesulphonic acid sodium salt–acetic acid–triethylamine (250:740:5:4:1, v/v/v/v/v). 1 = linopirdine, 2 = linopirdine mono-N-oxide, 3 = linopirdine bis-N-oxide and 4 = internal standard.

standard had k' values of 14.0, 4.8, 1.6 and 10.5, respectively.

Representative chromatograms of rat plasma extracted by the liquid–liquid and solid-phase methods are shown in Figs 3 and 4. Linopirdine, linopirdine mono-N-oxide and the internal standard were well separated without any interference from endogenous substances after the liquid–liquid extraction method. The retention times for linopirdine, linopirdine mono-N-oxide and the internal standard were approximately 23.5, 11.1 and 20.6 min, respectively. The chromatograms for the solid-phase extraction were also well separated for linopirdine bis-N-oxide and the internal standard; retention times were 5.3 and 20.6 min, respectively. Correlation coefficients for the standard curves usually exceeded 0.99.

Assay validation and stability

Results for the precision and accuracy of the assays are shown in Table 3. The intra-day precision results (RSD), ranged from 1.03 to 8.0%, 2.66–9.09% and 3.71–19.95% and the inter-day precision results ranged from 0.06 to 8.70%, 0.06–16.67% and 0.60–5.88% for linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide, respectively. The accuracy of the assays for all three compounds ranged from 91.0 to 130.0%. When incubated in rat plasma at 37°C for 2 h, linopirdine and both N-oxides were stable. The stability at −20°C was at least 62, 60 and 30 days for linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide, respectively. The minimum quantifiable limits using 1 ml of rat plasma for extraction were 0.02, 0.01 and 0.05 $\mu g\ ml^{-1}$ for linopirdine, linopirdine

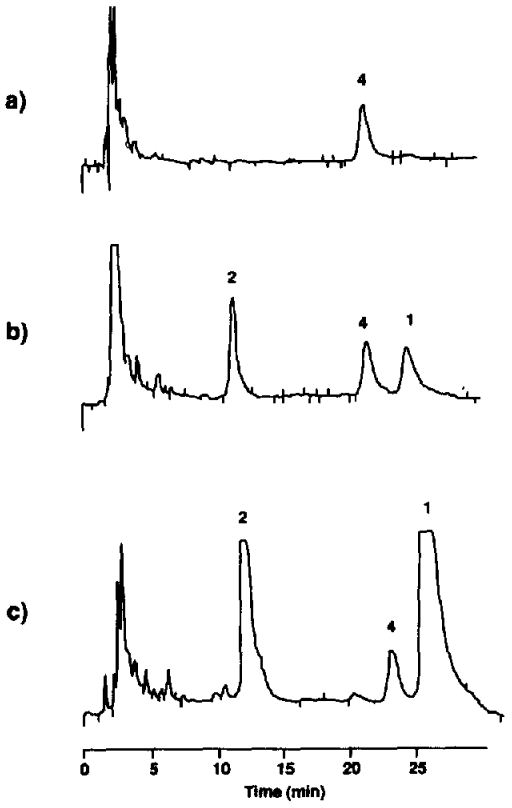

Figure 3
Typical chromatograms of rat plasma obtained using liquid–liquid extraction using a mobile phase consisted of: methanol–0.1 M ammonium acetate–0.1 M 1-octanesulphonic acid sodium salt–acetic acid–triethylamine (275:715:5:4:1, v/v/v/v/v): (a) control plasma containing the internal standard, (b) control plasma spiked with 0.1 μg ml^{-1} of linopirdine and linopirdine mono-N-oxide, (c) rat plasma 0.5 h after a single bolus 2.5 mg kg^{-1} intravenous dose of linopirdine. 1 = linopirdine, 2 = linopirdine mono-N-oxide and 4 = internal standard.

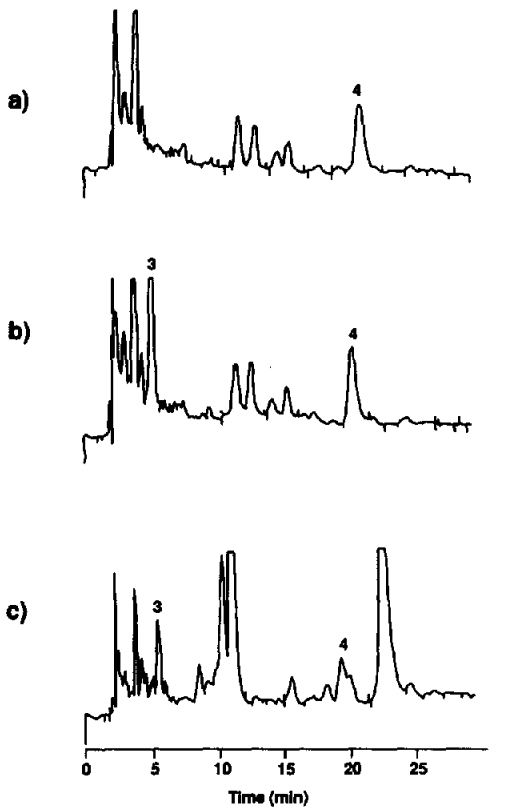

Figure 4
Typical chromatograms of rat plasma obtained using solid phase extraction using a mobile phase consisted of: methanol–0.1 M ammonium acetate–0.1 M 1-octanesulphonic acid sodium salt–acetic acid–triethylamine (275:715:5:4:1, v/v/v/v/v): (a) control plasma containing the internal standard, (b) control plasma spiked with 0.1 μg ml^{-1} of linopirdine bis-N-oxide, (c) rat plasma 0.5 h after a single bolus 2.5 mg kg^{-1} intravenous dose of linopirdine (depicting minor interference with the internal standard from endogenous materials). 3 = linopirdine bis-N-oxide and 4 = internal standard.

mono-N-oxide and linopirdine bis-N-oxide, respectively.

Rat pharmacokinetics

The method was used to determine plasma concentrations of linopirdine and its N-oxide metabolites after a single intravenous dose of linopirdine. Figure 5 depicts the plasma concentrations of each compound in rats as a function of time after a single 2.5 mg kg^{-1} dose of linopirdine. Immediately (2 min) after linopirdine administration, linopirdine plasma concentrations of 2.77 ± 0.08 μg ml^{-1} were observed in the rat. The plasma concentrations declined rapidly and were below the quantifiable limit of the assay (<0.02 μg ml^{-1}) by 4 h. The terminal half-life was 0.6 h. The apparent systemic clearance and volume of distribution were 37.3 ml h^{-1} kg^{-1} and 2.0 l kg^{-1}, respectively. As early as 5 min after dosing linopirdine mono-N-oxide was detected in the plasma. Its concentration continued to increase with time reaching peak concentrations of 0.27 ± 0.18 μg ml^{-1} at 0.5 h. After the peak the concentrations of linopirdine mono-N-oxide declined at approximately the same rate as that observed for linopirdine. Linopirdine bis-N-oxide was only detected in the plasma at 30 min after dosing and its concentration was 0.07 μg ml^{-1}. The results demonstrate that the method is suitable to evaluate the pharmacokinetics of each compound. The method presented has also been used to investigate the intravenous and oral disposition, *in vivo* formation and reversible metabolism of linopirdine. The results of these studies will be reported elsewhere.

Table 3
Precision and accuracy results of linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide for rat plasma by HPLC assays

	Intra-day			Inter-day		
Added conc. ($\mu g\ ml^{-1}$)	Found conc. ($\mu g\ ml^{-1}$)	RSD (%)	Accuracy (%)	Found conc. ($\mu g\ ml^{-1}$)	RSD (%)	Accuracy (%)
Linopirdine						
0.020	0.025 ± 0.002	8.00	125.0	0.023 ± 0.002	8.70	116.3
0.050	0.047 ± 0.002	4.26	94.0	0.048 ± 0.002	4.17	96.0
0.100	0.097 ± 0.001	1.03	97.0	0.098 ± 0.001	1.02	98.0
0.500	0.535 ± 0.041	7.66	107.0	0.525 ± 0.017	3.24	105.0
1.000	0.939 ± 0.023	2.45	93.9	0.954 ± 0.025	2.62	95.4
5.000	5.007 ± 0.259	5.17	100.1	5.005 ± 0.003	0.06	100.1
Linopirdine mono-N-oxide						
0.010	0.011 ± 0.001	9.09	110.0	0.012 ± 0.002	16.67	120.0
0.020	0.026 ± 0.001	3.85	130.0	0.023 ± 0.003	13.04	115.0
0.050	0.050 ± 0.003	6.00	100.0	0.051 ± 0.001	1.96	102.0
0.100	0.091 ± 0.004	4.40	91.0	0.094 ± 0.002	2.13	94.0
0.500	0.517 ± 0.044	8.51	103.4	0.512 ± 0.006	1.17	102.4
1.000	0.939 ± 0.025	2.66	93.9	0.963 ± 0.021	2.18	96.3
5.000	5.007 ± 0.273	5.45	100.1	5.004 ± 0.003	0.06	100.1
Linopirdine bis-N-oxide						
0.050	0.053 ± 0.002	3.77	106.0	0.051 ± 0.003	5.88	102.0
0.100	0.103 ± 0.002	1.94	103.0	0.101 ± 0.003	2.97	101.0
0.500	0.537 ± 0.105	19.95	113.8	0.528 ± 0.023	4.36	105.6
1.000	0.979 ± 0.058	5.92	97.9	1.005 ± 0.033	3.28	100.5
2.000	1.838 ± 0.243	13.32	91.9	1.892 ± 0.092	4.86	94.6
5.000	5.056 ± 0.126	2.49	101.1	5.035 ± 0.030	0.60	100.7

Mean ± SD, $n = 3$.

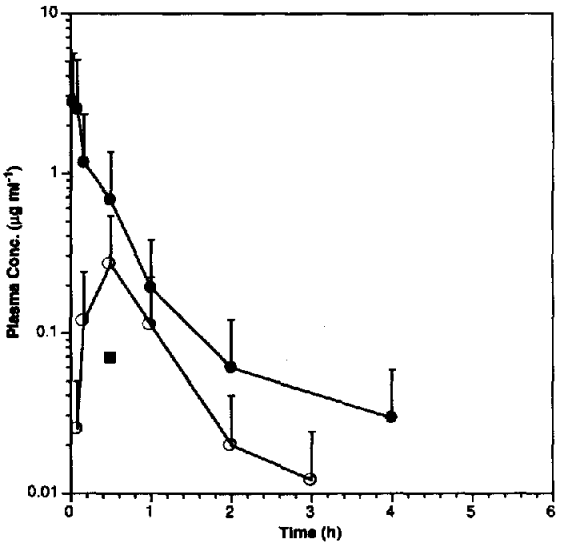

Figure 5
Mean linopirdine (●), linopirdine mono-N-oxide (○) and linopirdine bis N-oxide (■) plasma concentrations in rats (mean ± SD, $n = 4$) following a single bolus 2.5 mg kg^{-1} intravenous dose of linopirdine.

Conclusions

An LC method is described for the determination of linopirdine and its N-oxide metabolites in rat plasma. Linopirdine and linopirdine mono-N-oxide were extracted simultaneously from alkaline plasma into ethyl acetate while linopirdine bis-N-oxide was extracted from a separate sample of plasma by solid-phase extraction. The extracts containing linopirdine and linopirdine mono-N-oxide and the extracts containing linopirdine bis-N-oxide were assayed separately on one LC method which consisted of a reversed-phase CN column and an ion-pairing mobile phase. Detection was by UV at 254 nm. The minimum quantifiable limits of the assay using 1 ml of plasma for extraction were 0.02, 0.01 and 0.05 $\mu g\ ml^{-1}$ for linopirdine, linopirdine mono-N-oxide and linopirdine bis-N-oxide, respectively. The method was suitable in studying the pharmacokinetics of linopirdine in rats after a single intravenous dose.

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