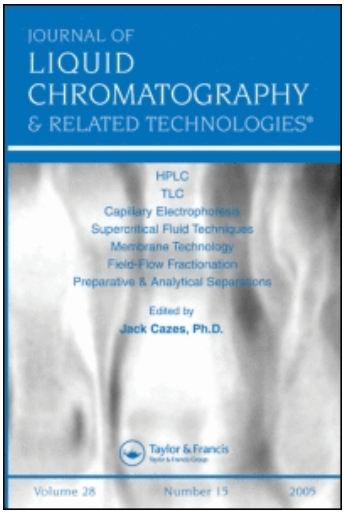

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DETERMINATION OF LINOPIRDINE AND ITS MONO-N-OXIDE METABOLITE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Sensitive and selective high-performance liquid chromatographic methods for the determination of linopirdine, a novel cognitive enhancer, and a major metabolite, linopirdine mono-N-oxide, in human plasma and urine are described. For plasma, alkalyzed samples were extracted with ethyl acetate. For urine, neutral samples were extracted with ethyl acetate and further treated by solid-phase extraction. The plasma residues were chromatographed on a Beckman CN HPLC column and the urine residues on a Jones Apex II CN HPLC column (both 4.6 x 25 cm). The mobile phase consisted of acetonitrile-ammonium acetate mixed with glacial acetic acid, 1-octane sulfonic acid and triethlyamine. The flow rate was 1.5 ml/min and the compounds were detected by UV at 254 nm. The lower limits of quantification for linopirdine and linopirdine mono-N-oxide were 2.5 ng/ml in plasma and 10.0 ng/ml and 100 ng/ml, respectively, in urine. The precision and accuracy, expressed as the percent coefficient of variation and percent difference, respectively, were <20 percent for the assays. The methods were used to study the pharmacokinetics of linopirdine and linopirdine mono-N-oxide in human subjects.

INTRODUCTION

Linopirdine, 3-3-bis(4-pyridinylmethyl)-1-phenylindolin-2-one, a phenylindolinone derivative, is being clinically evaluated as a potential cognitive enhancer for the treatment of Alzheimer's disease (1,2). Linopirdine is extensively metabolized in animals and man and linopirdine mono-N-oxide has been identified as a major metabolite (3). The mono-N-oxide metabolite is biologically inactive in an anti-amnestic screen in rats (data on file). However, quantification of linopirdine mono-N-oxide was included in the event that the metabolite might produce other pharmacological effects or assist in eludicating linopirdine disposition. To quantify both linopirdine and linopirdine mono-N-oxide in human plasma or urine, separate selective and sensitive high-performance liquid chromatographic methods were developed and validated. These procedures have been used to assay plasma or urine samples in a number of clinical trials.

MATERIALS

Linopirdine, linopirdine mono-N-oxide and the internal standard, E-4953, (3,3-bis(2-pyridinylmethyl)-1-phenyl-indolin-2-one hydrochloride) (Figure 1) were all obtained from The DuPont Merck Pharmaceutical Company (Wilmington, DE). HPLC grade acetonitrile, ethyl acetate, methanol, and reagent grade sodium hydroxide and hydrochloric acid were obtained from J. T. Baker (Phillipsburg, NJ). HPLC grade ammonium acetate, triethylamine, glacial acetic acid, certified reagent grade pH 9 buffer (0.1 M) and ACS certified reagent grade tris(hydoxymethyl)amino-methane (tris) were obtained from Fisher

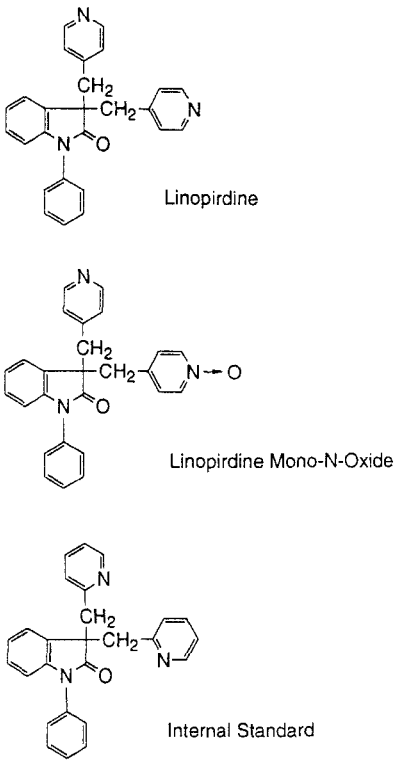

FIGURE 1. Chemical structures of linopirdine, linopirdine mono-N-oxide and internal standard.

Scientific (Fair Lawn, NJ). HPLC grade 1-octane sulfonic acid was obtained from Eastman Kodak (Rochester, NY) and certified reagent grade pH 7 phosphate buffer was obtained from VWR Scientific (Bridgeport, NJ). Drug-free control human plasma was obtained from Biological Specialty Corp. (Lansdale, PA) and control human urine was obtained from in-house donors.

METHODS

Instrumentation

The chromatography systems consisted of a Spectra-Physics SP8810 isocratic pump (Palo Alto, CA), a Beckman 160 fixed wavelength UV detector (Fullerton, CA) and a Waters 712 autosampler (Milford, MA). The mobile phase consisted of a mixture of the following: acetonitrile: 0.05 M ammonium acetate:glacial acetic acid:0.1 M 1-octane sulfonic acid:triethylamine, 24:74.6:0.8:0.5:0.1 and 30:68.6:0.8:0.5:0.1 by volume for plasma and urine, respectively. With a flow rate of 1.5 ml/min, the retention times of linopirdine, linopirdine mono-N-oxide and internal standard in plasma and urine were 14.8, 7.3, and 17.7 minutes and 9.5, 5.0, and 11.0 minutes, respectively. Data acquisition and integration was achieved using a PE Nelson Turbochrom data acquisition system (PE Nelson, Cupertino, CA, USA) for plasma assays and a Chromperfect data acquisition system (Justice Innovations, Mountain View, CA) for urine assays.

Standard Preparation

Calibration standards were prepared from working stock solutions of linopirdine/linopirdine mono-N-oxide in methanol. A 200-µl aliquot of each stock solution was added to a glass culture tube. The working stock solution was evaporated to dryness under nitrogen. One ml of control plasma or urine was added and the tube was then vortexed to mix. Linopirdine/linopirdine mono-N-oxide standards were prepared at concentrations of 2.5 to 1500 ng/ml in plasma, 10 to 505 ng/ml for linopirdine in urine, and 100 to 10000 ng/ml for linopirdine mono-N-oxide in urine.

Plasma Extraction Procedure

Plasma samples (1.0 ml) were spiked with internal standard (0.2 ml of 1.0 µg/ml) and pH 9 buffer (1.0 ml). Ethyl acetate (5.0 ml) was added to the mixture and mixed. Following centrifugation, the ethyl acetate fraction was transferred to a clean tube. Extraction of the aqueous phase was repeated (an additional 5.0 ml of ethyl acetate) and the organic phases were combined. One ml of 0.1 N hydrochloric acid was added to the organic phase. It was then mixed and centrifuged. After discarding the organic phase, 0.1 N sodium hydroxide (1.25 ml) was added to the remaining aqueous portion and this mixture was extracted with ethyl acetate (5.0 ml). The ethyl acetate fraction was transferred to a clean tube, evaporated to dryness under nitrogen, and the residue was reconstituted in mobile phase (0.2 ml).

Urine Extraction Procedure

Urine samples (1.0 ml) were processed as described above for plasma, however, pH 7 phosphate buffer was substituted for pH 9 buffer. In addition, a solid phase extraction was employed to further isolate the compounds of interest. The final organic residue was reconstituted in methanol (0.2 ml) and 0.1 M pH 10 tris buffer (1.0 ml). To a conditioned Baker CN (500 mg) solid phase extraction column was added the sample mixture containing methanol and tris buffer layered between two aliquots of tris buffer (1.0 ml each). The buffer and sample mixture was passed through the column using vacuum. Following three water rinses (1.0 ml each), the column was eluted with three aliquots of methanol (0.5 ml each) and the eluent was evaporated to dryness under nitrogen. The residue was then reconstituted in mobile phase (0.2 ml).

Validation of the Assays

For calibration, plasma or urine standards were spiked with known amounts of internal standard and with linopirdine and linopirdine mono-N-oxide at concentrations ranging between 2.5 to 1500 ng/ml and 10.0 to 10,000 ng/ml for plasma and urine, respectively. Calibration curves were obtained by power curve fit of the peak height ratio of linopirdine or linopirdine mono-N-oxide to the internal standard against the corresponding known concentration. The intra- and interday precision of the assays for linopirdine and linopirdine mono-N-oxide were estimated by measuring plasma/urine quality control samples (standards were used instead for linopirdine in urine) at day 1 (n=5 or 6 per concentration) and on two subsequent assay days (n=3 per concentration). The accuracy of the assays was evaluated by assaying quality control samples containing known amounts of each compound. The extraction efficiency was determined by comparing the peak heights of extracted standards to the corresponding unextracted standards. Selectivity and specificity was determined by injection of standard solutions of 42 various drugs which were selected based on concomitant medication information from linopirdine clinical trials. Any peak with a relative retention time ± 0.5 minutes of linopirdine, linopirdine mono-N-oxide or the internal standard was considered a potential interference. If a potential interference was observed, that drug was spiked into drug-free control human plasma, extracted, and chromatographed. The stability of linopirdine and linopirdine mono-N-oxide in human plasma and urine stored at -20°C was determined. Plasma samples were spiked at concentrations of 166 or 1660 ng/ml of linopirdine and 50 or 1000 ng/ml of or linopirdine mono-N-oxide. Urine samples were spiked

at concentrations of 40 or 400 ng/ml of linopirdine and 320 or 840 ng/ml of linopirdine mono-N-oxide. Samples were assayed immediately and at various times after storage.

Application to Clinical Trials

The assays were used in a clinical study to assess the safety and pharmacokinetics of linopirdine after a single 40 mg oral dose in healthy elderly volunteers. Urine and venous blood samples were collected at various time points. Aliquoted plasma and urine samples remained frozen (-20°C) until assayed.

RESULTS AND DISCUSSION

The methods described in this paper were developed to quantitatively assay linopirdine and a major metabolite, linopirdine mono-N-oxide in human plasma and urine. The procedures consisted of a series of extractions and, for urine only, an additional isolation step employing a solid phase extraction column. Separation of the analytes and internal standard was achieved using a cyano HPLC column followed by ultraviolet detection at 254 nm. Typical chromatograms of blank and 'spiked' human plasma and urine are shown in Figure 2. Also shown in Figure 2 are chromatograms of a plasma sample extract from an elderly patient taken 3 hours after receiving a 30-mg oral dose of linopirdine (c) and a urine sample extract from an elderly volunteer taken at a 0-6 hour collection interval after receiving a 40-mg oral dose of linopirdine (f). The lower limits of quantification (i.e., the lowest concentration that could be determined with precision and accuracy estimates of less than or equal to 20%) for linopirdine and linopirdine

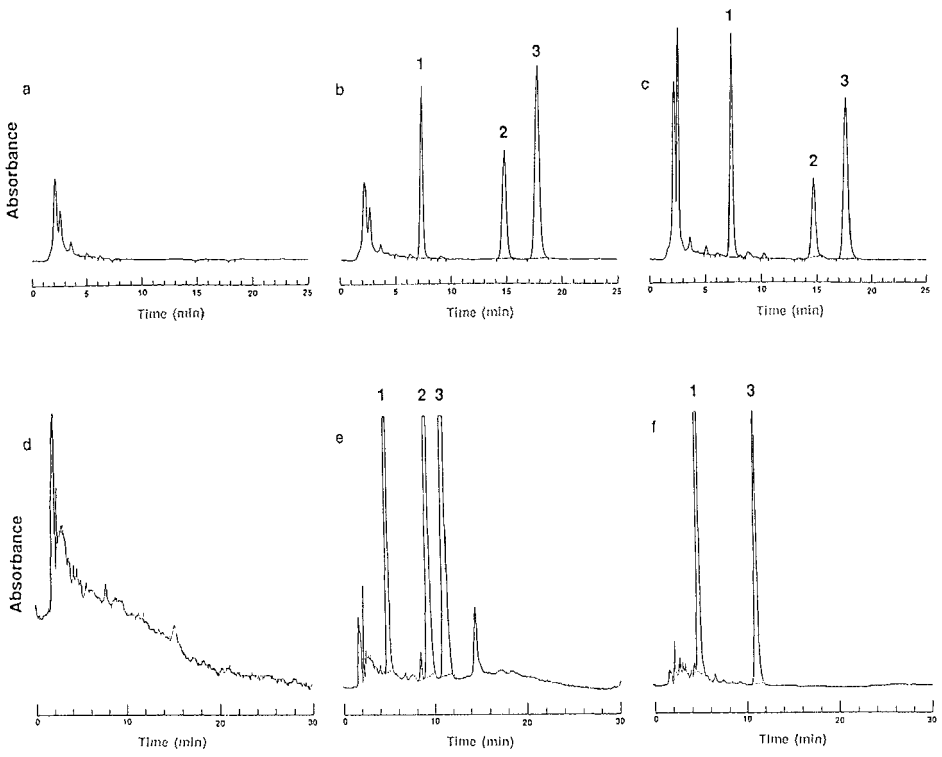

FIGURE 2. Chromatograms of (a) an extract of drug-free human plasma, (b) a spiked human plasma sample containing 100 ng/ml each of linopirdine and linopirdine mono-N-oxide, (c) a patient's plasma sample containing 85 ng/ml linopirdine and 160 ng/ml linopirdine mono-N-oxide, (d) an extract of drug-free human urine, (e) a spiked human urine sample containing 1500 ng/ml each of linopirdine and linopirdine mono-N-oxide, and (f) a subject's urine sample containing 4200 ng/ml linopirdine mono-N-oxide. Linopirdine mono-N-oxide, linopirdine, and the internal standard (E4953) are denoted 1, 2, and 3, respectively.

mono-N-oxide were 2.5 ng/ml in plasma and 10.0 ng/ml and 100 ng/ml, respectively, for urine. The intraday precision and accuracy of the assays for the determination of linopirdine and linopirdine mono-N-oxide in human plasma and urine are shown in Tables 1 and 2, respectively. The %CV ranged from 1.2% to 12.5% and 3.0% to 10.6% for linopirdine in

TABLE 1

Intraday Precision and Accuracy of Linopirdine and Linopirdine Mono-N-oxide in Human Plasma

Concentration Added (ng/ml)	Difference (%)		Mean Concentration Found ± SD (ng/ml)	Mean Difference (%)	CV (%)
Linopirdine					
2.5	-8.0	-12.0	2.4 ± 0.3	-4.0	12.5
	-16.0	16.0			
	4.0				
10.0	5.0	-1.0	10.2 ± 0.3	2.0	2.9
	6.0	1.0			
	1.0				
100.3	1.2	0.9	100.1 ± 1.2	-0.2	1.2
	-1.3	-1.3			
	-0.7				
1003.0	1.5	0.3	1021.2 ± 13.0	1.8	1.3
	1.3	2.2			
	3.8				
1504.5	3.6	-0.9	1542.2 ± 56.9	2.5	3.7
	-1.8	4.3			
	7.3				
Linopirdine Mono-N-oxide					
2.5	4.0	-4.0	2.7 ± 0.4	8.0	14.8
	0.0	32.0			
	8.0				
10.0	4.0	-3.0	10.1 ± 0.5	1.0	5.0
	8.0	-1.0			
	-1.0				
100.4	1.0	-3.3	95.4 ± 3.8	-5.0	4.0
	-8.1	-7.3			
	-7.2				
1004.5	-2.1	-3.9	985.7 ± 14.8	-1.9	1.5
	-2.0	0.3			
	-1.7				
1506.8	-1.1	-7.1	1463.2 ± 51.7	-2.9	3.5
	-5.6	-2.0			
	1.3				

TABLE 2

Intraday Precision and Accuracy of Linopirdine and Linopirdine Mono-N-oxide in Human Urine

Concentration Added (ng/ml)	Difference (%)		Mean Concentration Found ± SD (ng/ml)	Mean Difference (%)	CV (%)
Linopirdine					
10.1	-6.9	5.9	10.4 ± 1.1	3.0	10.6
	1.0	18.8			
	-10.9	9.9			
25.2	7.1	5.2	26.0 ± 1.6	3.1	6.2
	4.4	-9.5			
	7.9	3.6			
101	-4.7	-6.3	98.7 ± 3.0	-2.3	3.0
	0.0	-2.5			
	2.0	-2.2			
505	10.9	2.6	518 ± 23.2	2.6	4.5
	0.2	4.0			
	-2.2	0.0			
Linopirdine Mono-N-oxide					
100	-9.0	-6.0	91.5 ± 2.9	-8.5	3.2
	-8.0	-5.0			
	-13.0	-10.0			
300	-0.7	-0.3	304 ± 5.6	1.3	1.8
	2.7	0.0			
	3.0	3.3			
750	-2.3	1.7	747 ± 11.9	-0.4	1.6
	0.8	-0.9			
	0.4	-1.9			
3000	2.9	0.5	3104 ± 54.1	3.5	1.7
	4.1	3.9			
	3.2	6.0			
10000	-1.6	-0.7	9825 ± 498	-1.8	5.1
	-2.7	-5.2			
	7.1	-7.4			

plasma and urine, respectively. The corresponding values for linopirdine mono-N-oxide were 1.5% to 14.8% and 1.6% to 5.1%. The mean percent difference for linopirdine ranged from -4.0% to 2.5% and -2.3% to

3.1% in plasma and urine, respectively. The corresponding values for linopirdine mono-N-oxide were -5.0% to 8.0% and -8.5% to 3.5%. The interday precision of the assays is shown in Tables 3 and 4. The %CV ranged from 0.1% to 8.5% and 2.0% to 8.0% for linopirdine in plasma and urine, respectively. The corresponding values for linopirdine mono-N-oxide were 0.0% to 6.2% and 0.5% to 3.9% The overall

TABLE 3

Interday Precision of Linopirdine and Linopirdine Mono-N-oxide in Human Plasma

Concentration Added (ng/ml)	Mean Concentration Found[a] (ng/ml)	CV (%)
Linopirdine		
2.5	2.3	4.3
5.0	4.7	8.5
10.0	10.0	4.0
15.0	14.8	5.4
25.1	24.8	2.0
50.2	50.4	0.2
100.3	100.0	1.9
501.5	504.0	2.2
1003.0	1017.7	0.1
1504.5	1549.3	1.3
Linopirdine Mono-N-oxide		
2.5	2.6	0.0
5.0	4.9	2.0
10.0	9.7	6.2
15.1	14.5	2.8
25.1	24.0	2.5
50.2	49.6	4.6
100.4	97.9	4.9
502.2	489.5	3.2
1004.5	1016.2	3.1
1506.8	1521.0	1.7

[a] n=3.

TABLE 4

Interday Precision of Linopirdine and Linopirdine Mono-N-oxide in Human Urine

Concentration Added (ng/ml)	Mean Concentration Found[a] (ng/ml)	CV (%)
Linopirdine		
10.1	10.1	8.0
25.2	24.8	3.9
101	102	3.1
505	507	2.0
Linopirdine Mono-N-oxide		
100	95.1	3.9
200	205	1.0
400	390	1.0
1500	1489	0.7
5000	5012	0.8
7000	6973	1.3
10000	10101	0.5

[a] n=3.

extraction recovery averaged 60.7% and 74.3% for linopirdine and 43.3% and 50.9% for linopirdine mono-N-oxide in plasma and urine, respectively. Of the 42 drugs tested for potential interference, only bupropion and nifedipine yielded a relative retention time within ± 0.5 minutes of linopirdine, linopirdine mono-N-oxide, or internal standard. However, no chromatographic interferences were present following extraction of bupropion and nifedipine in control human plasma. A list of the drugs tested is shown in Table 5. The stability of linopirdine and linopirdine mono-N-oxide in frozen plasma and urine was studied. In plasma, no degradation of linopirdine or linopirdine mono-N-oxide was observed following storage at -20°C for at least 13

TABLE 5

Relative Retention Times for Potentially Interferring Drugs in Plasma Assay[a,b,c]

Drug	Retention Time Relative to Linopirdine Mono-N-oxide (min)	Retention Time Relative to Linopirdine (min)	Drug	Retention Time Relative to Linopirdine Mono-N-oxide (min)	Retention Time Relative to Linopirdine (min)
Acebutolol	-3.4	-11.1	Levothyroxin	17.2	9.3
Acetominophen	-4.3	-11.5	Lidocaine	-3.1	-10.9
Allopurinol	-5.3	-13.0	Lorazepam	2.8	-4.9
Alprazolam	5.6	-2.1	Metoprolol	-3.2	-11.0
Atenolol	-4.6	-11.9	Metronidazole	-4.3	-11.8
Brompheniramine	2.8	-4.9	Nadolol	-4.3	-11.8
Bupropion	-0.5	-8.2	Nifedipine	11.0	3.6
Chlorpheniramine	1.8	-5.7	Nortriptyline	17.7	9.8
Colchicine	-2.6	-10.0	Promethazine	12.5	4.6
Dextromethorphan	2.6	-5.3	Ranitidine	-2.7	-10.4
Diltiazem	8.7	0.8	Salbutamol	-4.6	-12.1
Dipyridamole	2.3	-5.1	Sulfacetamide	-4.2	-12.1
Doxylamine	-1.5	-9.0	Tetracycline	4.0	-3.3
Ephedrine	-4.4	-12.2	Theophylline	-4.7	-11.9
Famotidine	-4.7	-12.2	Timolol	-3.0	-10.3
Haloperidol	19.2	11.7	Triamterene	-3.1	-10.3
Hydralazine	-4.5	-11.7	Trimethoprim	-3.7	-11.2
Labetalol	2.0	-5.5	Verapamil	26.4	18.7

[a] Relative retention times were calculated as the difference between the retention time of test drug to the retention time of either linopirdine or linopirdine mono-N-oxide.

[b] Test solutions contained 50 μg/ml except lorazepam (1000 μg/ml), alprazolam (250 μg/ml), and bupropion (25 μg/ml).

[c] Meclizine, terfenadine, pilocarpine, thioridazine, phenylpropranolamine, and doxycycline were also tested and no chromatographic peak was observed within 60 minutes following injection.

months and 5 months, respectively. In urine, the corresponding stability values were 4 months and 18 months.

The assays were used to quantitate plasma and urine concentrations in volunteers after oral administration of linopirdine. Figure 3 shows a semi-logarithmic plot of mean plasma concentrations of linopirdine and linopirdine mono-N-oxide in elderly volunteers (aged 67-79 years) following a single oral dose of 40-mg linopirdine. In six subjects, the mean Cmax linopirdine plasma concentration was 775 ng/ml. The mean Cmax linopirdine mono-N-oxide plasma concentration was similar,

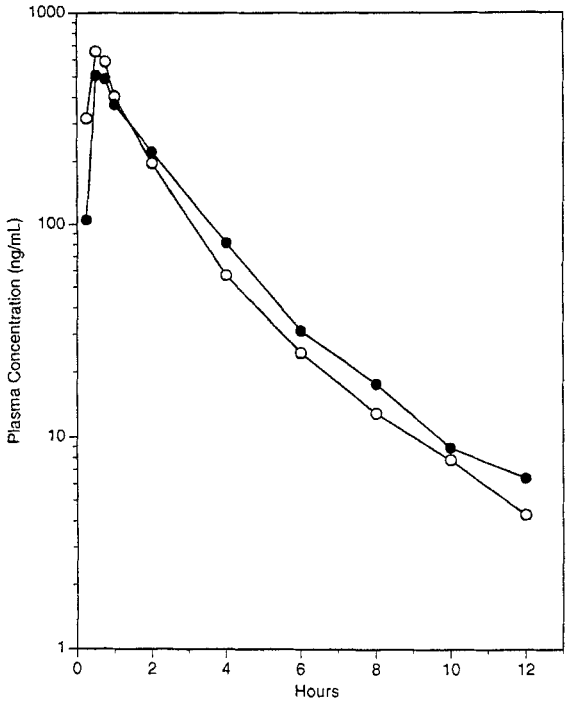

FIGURE 3. Mean plasma concentration versus time plot in healthy elderly subjects (n=6) following a single oral dose of 40-mg linopirdine. (O, linopirdine; ●, linopirdine mono-N-oxide.)

653 ng/ml. The urinary excretion of linopirdine was negligible as can be seen in Figure 2(f). The urinary excretion of linopirdine mono-N-oxide over 24 hours ranged from 2.2% to 8.6% of the linopirdine dose.

In summary, separate sensitive and selective high-performance liquid chromatographic methods for the determination of linopirdine and a major metabolite, linopirdine mono-N-oxide, in human plasma and urine were developed. They were successfully used to study the pharmacokinetics of linopirdine in humans.

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