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Simultaneous determination of lofepramine and desipramine by a high-performance liquid chromatographic method used for therapeutic drug monitoring

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Abstract

A simple reversed-phase HPLC method with ultraviolet detection for the simultaneous measurement of lofepramine and desipramine is described. Only a single alkaline extraction was used, with clomipramine as internal standard. The column used was a Supelco PCN column, and the mobile phase was acetonitrile–methanol–0.015 *M* phosphate buffer (120:35:100, v/v). The average recoveries were 78.8% for desipramine and 103.8% for lofepramine, and limits of quantitation were 25 and 5 nmol/l, respectively. The inter-assay C.V.s for lofepramine and desipramine were 6.0 and 7.6%, respectively. The method is specific and has excellent accuracy, and has been used for therapeutic drug monitoring of patients with depressions treated with lofepramine. Mean steady-state plasma concentrations found for lofepramine and desipramine were 8.5 ± 6.1 and 123.6 ± 120.6 nmol/l, respectively. It is concluded that lofepramine in itself has an antidepressive effect.

1. Introduction

Lofepramine is a tertiary amine, belonging to the tricyclic antidepressive drug group. It is metabolized in the liver by the P450 system to the active metabolite desipramine, which is a wellknown drug for treatment of affective disorders, and *p*-chlorobenzoic acid. Desipramine is further metabolized to 2-hydroxydesipramine.

Determination of lofepramine in biological samples is difficult, because the compound is very unstable in plasma and in aqueous standards, and is quickly converted to desipramine. Only two methods have been described for the determination of lofepramine [1,2], both of which involve reduction of lofepramine to the corresponding amino alcohol with sodium borohydride. In that way degradation of lofepramine during extraction is avoided.

The first method is a rather cumbersome gas chromatographic method with electron-capture detection of lofepramine and desipramine in two different runs [1]. Multiple extraction steps are used, followed by reduction of lofepramine to *p*-chlorobenzaldehyde. The *p*-chlorobenzaldehyde is distilled off and extracted with hexane. Desipramine is acylated with heptafluorobutyric anhydride and extracted twice with alkaline benzene. The limit of detection was estimated to 1 ng/ml for lofepramine and 2 ng/ml for desipramine.

Recently a reversed-phase HPLC method with

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electrochemical detection has been developed for determination of derivatised lofepramine [2]. Alkaline extraction at pH 9.3 is used, but it is necessary to repeat the extraction at pH 5.3, when the concentrations of lofepramine are low. The limit of detection for this method was 0.5 ng/ml for both lofepramine and desipramine.

The aim of the present study is to develop a simple HPLC method with UV detection for the simultaneous determination of derivatised lofepramine and desipramine. The method developed has been used for measurement of the plasma concentrations in depressed patients who were treated with lofepramine (Tymelyt®) under steady-state conditions. In two other studies the results of these determinations are correlated to the antidepressive effect [3] and various cardiovascular parameters [4].

2. Experimental

2.1. Chemicals

Lofepramine was kindly supplied by Leo Therapeutics (Helsingborg Sweden), clomipramine and desipramine by Ciba-Geigy (Basel, Switzerland), HPLC-grade methanol and hexane purchased from Mallinckrodt (Lexington, KY, USA). Acetonitrile, LiChrosolv-grade was from Merck (Darmstadt, Germany), sodium borohydride from Sigma (St. Louis, MO, USA). Water was purified by osmosis (Eurowater, Denmark) and further cleaned by a Milli-Q water system (Millipore, Milford, MA, USA). All other chemicals were of analytical grade.

The internal standard was made by dilution of 1 ml stock solution (3.5 mg clomipramine-HCl in 100 ml water) with 25 ml methanol. Phosphate buffer (0.015 *M*) was prepared by dissolving 1.035 g $NaH_2PO_4 \cdot 1H_2O$ in 500 ml of water. The buffer was filtered through a Millipore GS filter, 0.22 μm.

2.2. Preliminary investigations

Initially we tried to avoid the derivatization of lofepramine with sodium borohydride, performing on-line extraction, injecting standards and samples directly on an extraction column dry packed with Lichroprep RP-18, 40–63 μm particles. This column replaced the Rheodyne loop, and in the load position it was washed with 1 ml of methanol and 2 ml of phosphate buffer. A 50-μl sample was injected onto the column, and the water-soluble substances were washed away with 1 ml of buffer (0.1 *M* phosphate buffer, pH 4.5). Then the Rheodyne was placed in the injection position, and the sample was transferred onto the analytical column.

2.3. Preparation of standards and sample handling

It proved to be necessary to derivatize lofepramine in standards and samples with sodium borohydride. Duplicates of each of the solutions mentioned below were made and used for preparation of half of the standards. A 6.00-mg amount of desipramine-HCl was added to 200 ml of water and 9.10 mg of lofepramine were added to 100 ml of methanol. The lofepramine solution could be stored at 4°C for 3 days and desipramine for several months in a deepfreezer at −20°C. From these standards 100 ml of human plasma were spiked with lofepramine and desipramine. The concentrations used were 50, 100, 200, 300 nmol/l for desipramine and 20, 50, 100, 200 nmol/l for lofepramine.

Desipramine was added first, then lofepramine and then the plasma was immediately stabilized with 200 mg of sodium borohydride. This solution was allowed to react for 15 min at room temperature, and the standards were then frozen in 5-ml aliquots at −20°C. Under these conditions the standards could be used for 12 months.

2.4. Extraction

The extraction procedure was performed as a single step alkaline extraction. All determinations were performed in duplicate. 2 ml standard or plasma was added to 10 ml glass tubes. The plasma was made alkaline with 200 μl 1.0 *M* sodium carbonate solution, pH 11. Then 50 μl of

internal standard was added, and thereafter 5 ml of 1% isoamyl alcohol in hexane. The tubes were shaken for 5 min, and then centrifuged for 10 min at 3000 *g*. The supernatant was transferred to clean tubes, and volatile components were evaporated at 60°C under an air stream. The residue was reconstituted in 100 μl mobile phase, and 70 μl was injected onto the HPLC system.

2.5. Equipment and chromatographic conditions

The HPLC system consisted of a Shimadzu pump LC-9A, a Shimadzu UV-spectrophotometer SPD-6A (Kyoto, Japan). The samples were injected with an autosampler (Shimadzu Sil-9A) or with a manual injection valve (Rheodyne 7125, Cotati, CA, USA). The chromatographic column was a Supelcosil LC-PCN, 15 cm × 4.6 mm I.D., 5 μm (Supelco cat. 5-8377, Struers, Denmark), protected by a 2-cm guard column, packed with the same material. The mobile phase was acetonitrile–methanol–0.015 *M* phosphate buffer (120:35:100, v/v). The flow-rate was 2.5 ml/min, and detection was performed at 254 nm with detection time constant of 1.5 s. The mobile phase was degassed in an ultrasound bath under vacuum before use.

2.6. Calculations

Data were collected with a Waters 745 integrator (Millipore, Milford, MA, USA). The regression equations were calculated as peak-height ratio between internal standard and different concentrations of standards, against the concentrations of standards. The values from the regression line were used for calculating the results for lofepramine and desipramine.

2.7. Patient material

Patient samples were from 25 depressed patients from various outpatient clinics. All had a score of more than 15 on the Hamilton depression scale-17. Blood samples were drawn prior to treatment with lofepramine, and then after 3 and 26 weeks of treatment, and 3 weeks after termination of treatment. The dose given ranged from 70–210 mg/day, most patients were given 140 mg/day.

Blood samples were taken before the patients had taken their medicine, and 45 min after administration of 70 mg of lofepramine. The samples were taken in heparinized Venoject tubes. The samples were centrifuged at 4°C, and as soon as possible after collection of the plasma, 3 ml of the plasma were at once transferred to tubes containing 5 mg of sodium borohydride. The samples were reacted for 15 min and then frozen at −20°C. The samples were sent to the laboratory in frozen condition. For more details about the patient material see Ref. [3].

3. Results

Fig. 1 shows the same plasma standard without derivatization of lofepramine, measured immediately after spiking and after 30 min at room temperature. The conversion of lofepramine to desipramine is obvious. We have tried various other ways to avoid the decomposition of lofepramine to desipramine, all with negative results.

The values of the standard curves for the method together with the limits of detection and quantitation are shown in Table 1. The difference between the limit of detection and the limit of quantitation of lofepramine is less than normally reported with a defined limit of quantitation at C.V. $<20\%$ as used here. The C.V. for the chosen value for the limit of quantitation was only 13.6% ($n = 6$). We think that this is due to the fact that the instrumental noise does not really interfere with the integration of the signal.

The absolute recoveries after extraction of the analytes are shown in Table 2. The average recovery was 78.8% for desipramine and 103.8% for lofepramine. We also investigated the recovery of the internal standard, clomipramine, and found it to be 88.0% on average.

The intra-day reproducibility and the inter-day reproducibility are shown in Table 3. The inter-day reproducibility was measured over the first 14 days of the assay. It can be seen that there is considerable variation in the C.V., which is

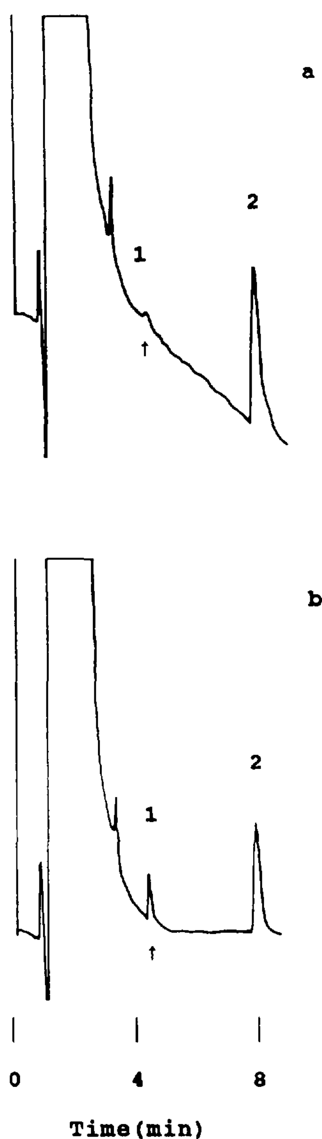

Fig. 1. Chromatograms of human plasma spiked with lofepramine (500 nmol/l). (a) Just after lofepramine was spiked. (b) The same sample after standing at room temperature for 30 min. Peaks: 1 = desipramine; 2 = lofepramine. HPLC conditions: 50 μl lofepramine extracted with on-line clean up; mobile phase: acetonitrile–methanol–0.02 *M* phosphate buffer (110:20:100, v/v), flow-rate 1.8 ml/min; other parameters as in Experimental.

relatively independent of the concentration level. Table 3 also shows that the method has a good accuracy.

Fig. 2a shows the chromatogram of a blank patient sample spiked with internal standard. No interference of endogenous substances is found. This was also true for all other samples from patients before treatment with lofepramine, and after termination of the treatment.

Fig. 2b shows the chromatogram of a standard with excellent separation of all analytes, and a total analysis time of 5 min.

Fig. 3a shows a typical chromatogram from a patient sample, and Fig. 3b shows the increase in lofepramine concentration 45 min after the intake of 70 mg lofepramine.

Table 4 shows the retention times of drugs tested for interference with the analysis. The only drug showing interference is haloperidol. Other tricyclic antidepressants are co-eluted with this method, but it is not common practice to co-administrate other antidepressive drugs with lofepramine. The commonly used benzodiazepines are eluted in the injection peak.

The method was used to determine the concentration of lofepramine and desipramine in 25 patients in steady state treated with 70–210 mg lofepramine per day. The measured means were 8.5 nmol/l lofepramine (range < 5–32.0 nmol/l) and 123.6 nmol/l desipramine (range < 25–504.0 nmol/l). We also measured the lofepramine concentration 45 min after intake of 70 mg lofepramine. The mean concentration was 33.0 nmol/l (range < 5–353.0 nmol/l).

4. Discussion

A simple method for the simultaneous determination of lofepramine and desipramine is described. We found it necessary, as did other authors [1,2], to derivatize the samples and standards with sodium borohydride to avoid disintegration of lofepramine. To obtain reliable results it was necessary to stabilize the material as soon as possible after preparation of the standards and blood samples. Our results indicate that decomposition of lofepramine begins immediately after dissolution of the drug.

The present method uses alkaline extraction at pH 11.0, and has a recovery for lofepramine of 100%. Virgili and Henry [2] mentioned a re-

Table 1
Standard curve values for the method

Drug	Regression equation of standards[a]	Correlation coefficient (r)	Linear range[b] (μmol/l)	Limit of detection[c] (nmol/l)	Limit of quantitation (nmol/l)
Desipramine	$y = 0.0140x - 0.129$	0.99	0–5	10	25
Lofepramine	$y = 0.0049x - 0.004$	0.99	0–1	3	5

[a] y = Peak high ratio drug/internal standard; x (nmol/l) = concentration.
[b] Standards determined in the mentioned concentration ranges.
[c] Signal-to-noise ratio 3:1.

Table 2
Recovery

Drug	Level (nmol/l)	Recovery[a] (%)	n[b]	Variation (%)
Desipramine	50	82.1	6	56.3–109.8
	100	75.9	10	59.4–100.4
	200	77.5	12	65.0–96.8
	300	79.8	12	66.4–90.8
Lofepramine	20	105.8	2	–
	50	102.5	2	–
	100	109.4	6	97.8–117.4
	200	97.6	6	87.8–105.1

[a] Extracted standards in % of the same amount derivatized and injected directly on the column.
[b] n = number of analyses with duplicate determinations.

Table 3
Reproducibility

Drug	Amount added (nmol/l)	Intra-day (n = 10)		Inter-day (n = 14)	
		Found (mean ± S.D.) (nmol/l)	C.V. (%)	Found (mean ± S.D.) (nmol/l)	C.V. (%)
Desipramine	50	51.6 ± 5.9	11.3		
	100	99.8 ± 6.0	6.0	99.8 ± 7.6	7.6
	200	204.3 ± 22.8	11.1		
	300	311.1 ± 32.4	10.4		
Lofepramine	20	18.4 ± 1.3	7.2		
	50	49.3 ± 1.9	3.8	50.0 ± 3.0	6.0
	100	95.1 ± 6.7	7.1		
	200	198.5 ± 9.2	4.6		

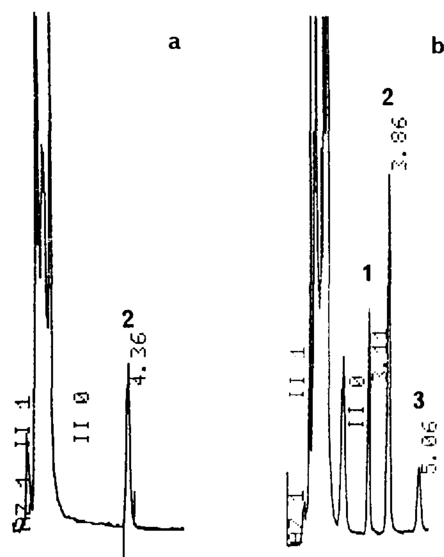

Fig. 2. Chromatograms (a) of extracted patient plasma before lofepramine intake, spiked with internal standard, (b) human plasma spiked with desipramine (100 nmol/l), and lofepramine (50 nmol/l). Peaks: 1 = desipramine; 2 = clomipramine (I.S.); 3 = lofepramine. HPLC conditions as described under Experimental.

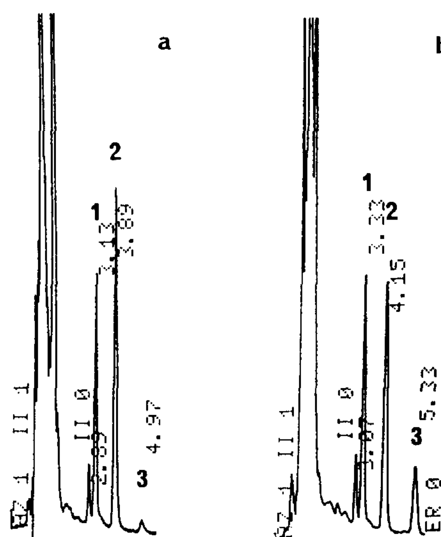

Fig. 3. (a) Chromatogram of patient in steady state on 140 mg lofepramine per day. Peaks: 1 = desipramine (114.0 nmol/l); 2 = I.S.; 3 = lofepramine (10.2 nmol/l). (b) Chromatogram of a patient 45 min after oral intake of 70 mg lofepramine. Peaks: 1 = desipramine (137.1 nmol/l); 2 = I.S.; 3 = lofepramine (66.9 nmol/l). HPLC conditions as described under Experimental.

covery of 90% at pH 5.3. However, at pH 5.3 desipramine is extracted with a much lower recovery. We find a recovery for desipramine of ca. 79.0%, comparable with the results obtained by others [1,5,6]. However, some problems occurred for the absolute recovery of lofepramine, as the derivatized lofepramine shows a smaller UV response than the underivatized substance, and because even minimal amounts of sodium borohydride destroyed the chromatographic column.

Virgili and Henry [2] found an inter-assay C.V. $<4.5\%$ for lofepramine determinations with extraction at pH 5.3, but they do not have results for extraction at pH 9.3. The intra-assay C.V. for desipramine found in the present study is larger than that obtained by others [5,6], even for low concentrations of desipramine [7,8]. This cannot be explained as being due to the derivatization of lofepramine, as desipramine is not changed by that [1]. One explanation could be that on ageing the CN columns, as indicated by our own experience and that of others, are changed in such a way that desipramine is reversibly bound to the column material [6].

UV detection was selected because it is easy to work with, although it is less sensitive than the electron capture and electrochemical detection

Table 4
Compounds tested for interference

Drug	Retention time (min)
2-Hydroxydesipramine	2.20
Haloperidol	2.69
Desipramine	2.80
Nortriptyline	2.85
Zuclopentixol	2.99
Imipramine	3.01
Perphenazine	3.05
cis-Flupentixol	3.05
Amitriptyline	3.06
Desmethylclomipramine	3.19
Clomipramine	3.46
Lofepramine	4.40

Measured as derivatised plasma standards at high therapeutic levels.

systems used in previous studies [1,2]. In these studies no values for the quantitation limits of lofepramine and desipramine are given, but with the method presented here it is possible to measure lofepramine and desipramine in plasma 12 h after intake of 70 mg lofepramine, in patients in steady-state conditions.

Only few clinical investigations on steady-state plasma concentrations of lofepramine and desipramine have been published. Our findings in depressed patients show very low concentrations of desipramine and lofepramine, compared to those of other tricyclic antidepressants. Others have also found the same low values for lofepramine and desipramine [9,10]. Our results show, as do others [9], a large variation in the range of concentrations, even at the same lofepramine dose. We have found concentrations of lofepramine up to 300 nmol/l 45 min after lofepramine intake, with great variation between patients.

The recommended therapeutic window for desipramine is 405–592 nmol/l [11], and it is therefore highly unlikely that it is only desipramine that is responsible for the antidepressive effect in our patients. It has been found that desipramine is three times more potent as an inhibitor of norepinephrine reuptake than lofepramine [9,10]; since the lofepramine concentration is too low to cause the observed effect, the in-vivo inhibition must come from desipramine.

Treatment with tricyclic antidepressants has a lag-time before the antidepressive effect can be measured, and it is therefore likely that the effect is caused by some adaptations in the central catecholamine neurons (for review see Ref. [12]). In-vitro investigations show [13,14] that lofepramine binds more easily to desipramine binding sites than desipramine, and gives greater inhibition of c-AMP in cortical β-receptors, although the desensitization of the β-receptors is less. The clinical profile of lofepramine is also different from that of imipramine/desipramine, both with regard to anticholinergic adverse effects and cardiovascular toxicity [4,14]. Because of this it is very likely that lofepramine itself shows anti-depressive activity, or acts synergistically with desipramine.

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