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REVERSED-PHASE CHROMATOGRAPHY OF ZIMELIDINE AND SIMILAR DIBASIC AMINES

I. ANALYSIS IN BIOLOGICAL MATERIAL

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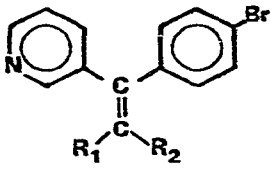
SUMMARY

Zimelidine, a new antidepressant, and its pharmacologically active metabolite, norzimelidine, have been determined quantitatively in biological material. The compounds are extracted from alkali-treated body fluid or tissue homogenate into diethyl ether–*n*-hexane (8:2, v/v), and re-extracted into a small volume of acidic aqueous phase (*ca.* 100 μl), which is injected onto the chromatographic column. The chromatographic support is Nucleosil C_{18} (5 μm) and the mobile phase is phosphate buffer (pH 2)–acetonitrile (91:9, v/v) with the addition of an aliphatic amine, N,N-dimethyl-N-octylamine ($4 \cdot 10^{-4}$ *M*), which improves the chromatographic performance. By using glass-lined stainless-steel tubes instead of the common precision-bore stainless-steel columns, peak symmetries were improved. Detection limits in plasma are in the range 2.3–4.7 nmol/l for extractions from 1 ml. It is demonstrated that the quality of the quantitations are improved by use of an internal standard, norzimelidine E-isomer. The precisions obtained for both compounds at the concentration levels of 300 and 1500 nmol/l are *ca.* 5–6% and 2% respectively for within-run variations, and 8–9% and 5% respectively for day-to-day-variations.

INTRODUCTION

Zimelidine, a new antidepressant and selective inhibitor of the uptake of serotonin[1], is demethylated in the body to norzimelidine, which has similar pharmacological properties and is the most important compound clinically since its steady state levels are about 3–8 times higher than those of zimelidine. Some other pharmacologically inactive metabolites have also been identified[2,3].

Several bioanalytical methods for determinations of the two compounds in plasma have been proposed: straight-phase ion-pair chromatography with chloride[4] or perchlorate[3,5] as counter ion; liquid chromatography on silica gel with an aqueous mobile phase[6]; or gas chromatography with electron capture detection[7] or a nitrogen-sensitive detector[8]. These methods suffer, however, from various drawbacks such as insufficient sensitivity for thorough pharmacokinetic studies[6–8] or high degree of sophistication[3–5].



Compound	R_1	R_2
Zimelidine	$-CH_2N(CH_3)_2$	H
Norzimelidine	$-CH_2NHCH_3$	H
ZIMELIDINE N-oxide	$-CH_2\overset{\overset{O}{\downarrow}}{N}(CH_3)_2$	H
Primary amine, metabolite	$-CH_2NH_2$	H
NorZIMELIDINE E-isomer	H	$-CH_2NHCH_3$

Fig. 1. Chemical structures.

The chromatographic system used in this paper is based on a silanized support, Nucleosil C_{18} (5 μm), and an acidic mixture of phosphate buffer and acetonitrile containing an aliphatic amine as the mobile phase. Its fundamental properties will be described elsewhere[9]; this study deals with bioanalytical applications.

EXPERIMENTAL

Apparatus

The equipment for liquid chromatography comprised a Milton Roy Minipump with a pulse dampener [Laboratory Data Control (LDC) Model 711-47], the UV-detectors with fixed wavelengths at 254 nm were LDC Model 1265 UV monitor and Waters Model 440. A Rheodyne Model 7120 equipped with home-made loops of different volumes (20–500 μl) was used for injections, the columns were precision-bore 316 stainless steel from Handy & Harman (100 nm × 1/4 in. O.D. × 4 mm I.D.) or glass-lined stainless-steel tubings (SGE, Scantec, Gothenburg, Sweden), 100 × 3 mm I.D., equipped with modified, zero dead volume, Swagelok end fittings and bed supports with 2-μm frits (Altex). Linear 285 (Linear Instruments Corp.) and Tekman TE 200 (Techtum Instruments) recorders were employed. The chromatography was performed at ambient temperature.

Chromatographic technique

The columns were packed by the upwards slurry packing technique described by Bristow *et al.*[10], with methyl isobutyl ketone as the slurry medium and dichloromethane as the packing solvent. All column parts were cleaned by treatment in an

ultrasonic bath for 5 min with the solvents dichloromethane, acetone, nitric acid (5 *M*), acetone and dichloromethane, and then dried at *ca.* 150°. The slurry concentration was *ca.* 3% (w/v) and the applied pressure 350–400 bar. After packing, the support was purified by pumping 100 ml each of dichloromethane and methanol at a flow-rate of 2–3 ml/min through the column. Column performance was tested according to Bristow and Knox[11].

Mobile phases were degassed in an ultrasonic bath and allowed to stand overnight at ambient temperature before use. Column equilibrium is achieved within 30 min at a flow-rate of *ca.* 1 ml/min. The void volumes of mobile phase were determined either by composition disturbance or by the injection of potassium nitrate. All reported chromatographic data are the means of duplicate or triplicate determinations.

Analytical method

Extraction. A 1.0-ml volume of plasma + 10 μl internal standard solution (norzimelidine E-isomer, 330 nmole/l) + 1 ml of 1 *M* NaOH are extracted for 20 min with 4.0 ml diethyl ether–*n*-hexane (8:2, v/v) in 10-ml tubes*. After centrifugation for 6 min at 1600–1900 *g*, as much as possible of the organic phase is transferred to a silanized glass tube (4 ml), whereupon *ca.* 105 μl phosphate buffer, pH 2, are added and the contents mixed for *ca.* 15 sec on a Whirlimixer. After another centrifugation for 6 min, 100 μl of the aqueous phase are injected onto the column.

Chromatography. Support: Nucleosil C_{18} (5 μm) in a stainless-steel column (100 × 4 mm) or a glass-lined stainless-steel column (100 × 3 mm). Mobile phase: phosphate buffer (pH 2)–acetonitrile (91:9, v/v), $4 \cdot 10^{-4}$ *M* N,N-dimethyl-N-octylamine (DMOA); flow-rate, 1 ml/min. UV-detection at 254 nm.

Quantitations are performed from a standard curve of the ratio of the peak heights of the sample and the internal standard *vs.* concentration, obtained by analyzing known amounts of the compounds added to plasma.

RESULTS AND DISCUSSION

Extraction

The optimization of the extraction from biological material was studied in ref. 3, and it was found that diethyl ether–*n*-hexane (8:2, v/v) gave quantitative extractions of zimelidine and norzimelidine together with minimal blank disturbances in the chromatogram. This mixture of organic solvents was also used in the present method, but followed by reextraction with a small volume of acidic buffer solution instead of evaporating as in ref. 3. The ratio of the volumes of organic and aqueous phases is *ca.* 40, and it can be calculated from distribution data (log $k_d \times K'_{HA} = -7.57$ for norzimelidine) that even a ratio of *ca.* 400 should give a quantitative reextraction. As shown below, however, the chromatographic system can accept quite large sample volumes without its performance being impaired so it is not necessary to minimize the volume of the aqueous phase in this step.

* It is essential that these tubes are absolutely clean and this is achieved by treatment with dichromate and sulphuric acid for 15 min after the ordinary washing procedure, followed by thorough rinsing with deionized water.

The elimination of the evaporation step has the advantage compared to the method in ref. 3 that problems of adsorption of the secondary amine are avoided, and the use of internal standards is not necessary. Norzimelidine E-isomer is however added as internal standard in order to simplify the technical procedure by eliminating the need to transfer exact volumes and to increase the precision in quantitative determinations[12], see below.

Chromatography

The best available support for reversed-phase chromatography of zimelidine and similar compounds (see Fig. 1 for chemical structures) was chosen after systematic studies of chromatographic parameters as reported in ref. 9. Nucleosil C_{18} (5 μm) was found to give the best performance; a low pH of the mobile phase gives the best chromatographic efficiency, and in all experiments reported below the pH was 2. Acetonitrile was chosen as the organic modifier and a concentration of 9% (v/v) was found to be suitable[9] for the studied compounds and was used throughout this work.

The chromatography was further optimized by the additions of an aliphatic tertiary amine, DMOA, which has a substantial effect on chromatographic efficiency, peak symmetry and capacity ratios (*cf.*, ref. 13). Regarding chromatographic efficiency the optimal concentration range of DMOA seems to be *ca.* $4–8 \cdot 10^{-4}$ *M*. The retention of zimelidine and related compounds decreases with increasing DMOA concentration. In blank chromatograms from plasma, a large peak of an endogenous substance has similar retention times to the compounds of interest. The capacity ratio of this unknown compound also decreases with increasing DMOA concentration as illustrated in Fig. 2, but not to the same extent, which means that an adequate chromatographic selectivity is obtained at [DMOA] $\geqslant 4 \cdot 10^{-4}$ *M*. With [DMOA] >

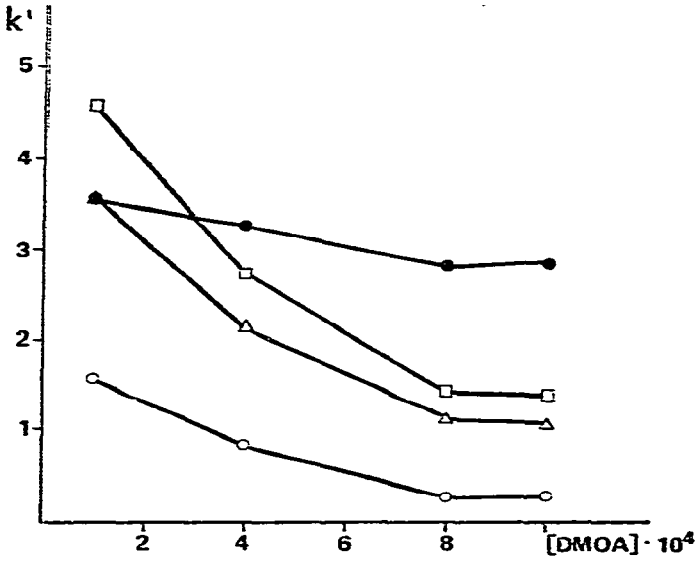


Fig. 2. Influence of DMOA concentrations on capacity ratios. Column: stainless steel. Support: Nucleosil C_{18} (5 μm). Mobile phase: phosphate buffer (pH 2)–acetonitrile (91:9, v/v) + DMOA. ○, norzimelidine E-isomer; △, norzimelidine; □, zimelidine; ●, endogenous compound.

$4 \cdot 10^{-4}$ *M* the internal standard, norzimelidine E-isomer, will be disturbed by early eluting endogenous substances. Representative blank and sample chromatograms with the optimum DMOA concentration ($4 \cdot 10^{-4}$ *M*) are shown in Fig. 3, and typical chromatographic data with this mobile phase are given in Table I.

Glass-lined stainless-steel tubes were preferable because of the superior peak symmetries obtained compared to conventional precision-bore stainless-steel tubes[9] (*cf.*, ref. 14).

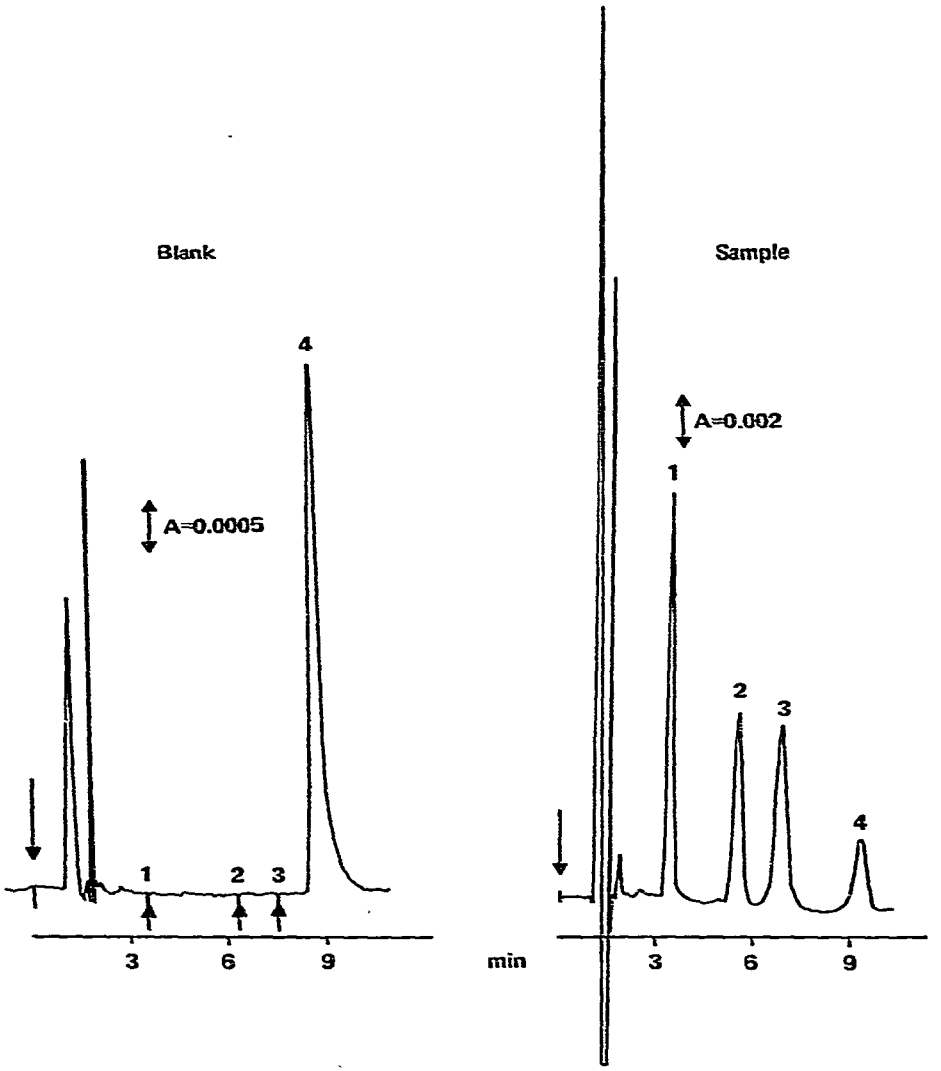


Fig. 3. Sample and blank chromatograms from plasma. Mobile phase: phosphate buffer (pH 2)–acetonitrile (91:9, v/v) + $4 \cdot 10^{-4}$ *M* DMOA; flow-rate, 1 ml/min. Other conditions as in Fig. 2. Peaks: 1 = norzimelidine E-isomer, 100 ng/ml; 2 = norzimelidine, 35 ng/ml; 3 = zimelidine, 35 ng/ml; 4 = endogenous compound.

TABLE I

CHROMATOGRAPHIC DATA

Support: Nucleosil C_{18} (5 μm) in a glass-lined stainless-steel column [100 × 3 mm I.D.]. Mobile phase: phosphate buffer (pH 2)–acetonitrile (91:9, v/v) + $4 \cdot 10^{-4}$ *M* DMOA; flow-rate, 0.83 ml/min. k' = Capacity ratio, with potassium nitrate as the unretained compound; α = selectivity factor.

Compound	k'	α	*HETP (μm)*	*Asymmetry factor**
Norzimelidine E-isomer	1.02		32	1.00
		2.00		
Zimelidine primary amine metabolite	2.04		29	1.13
		1.25		
Norzimelidine	2.55		25	1.05
		1.22		
Zimelidine	3.11		26	1.02
		1.87		
Zimelidine N-oxide	5.82		25	0.93

* At 10% of peak height.

Zimelidine is extensively metabolized[2,3], and the selectivity of the extraction procedure with respect to the metabolites was discussed in ref. 3. The primary amine, zimelidine N-oxide and the acetylated primary amine are compounds that might be co-extracted to a certain extent, and chromatographic data for the first two compounds are included in Table I. The acetylated primary amine is not available in adequately pure form for chromatographic studies, but owing to the reduced polarity and different protolytic properties compared to the primary amine it will probably be eluted later than the N-oxide. The selectivity factors are sufficiently large to permit baseline separation of all these compounds with almost completely symmetric peaks using a glass-lined stainless-steel column as demonstrated in Fig. 4.

The selectivity with respect to established antidepressants (amitriptyline, imipramine, chloroimipramine, nortriptyline, desipramine, protriptyline and opipramole) and to benzodiazepines (diazepam, nitrazepam and oxazepam), that sometimes are used in combination with antidepressants, is very high since only opipramole, with $k' = 100$, is eluted within a reasonable time (<3 h at a flow-rate of 0.8 ml/min) in the chromatographic system.

If samples are dissolved in the mobile phase and large volumes are injected onto the column, the chromatographic efficiency will decrease as has been shown in several papers (*e.g.*, refs. 15 and 16), but by utilizing a trace enrichment effect[17,18], which is achieved when samples are dissolved in aqueous solutions resulting in high initial capacity ratios, the efficiency can be maintained up to the injection of several millilitres. In the present system up to 500 μl of sample dissolved in buffer, pH 2, were injected without any effect on the chromatographic parameters.

Quantitative determinations

Detection limits in plasma, defined as the concentrations which give a signal equal to twice the baseline noise, correspond to 4.7 and 2.3 nmol/l for zimelidine and norzimelidine respectively. These values can however be improved by performing the extractions from larger volumes, as demonstrated in Fig. 5 by a representative blank chromatogram obtained after an extraction from 5 ml plasma.

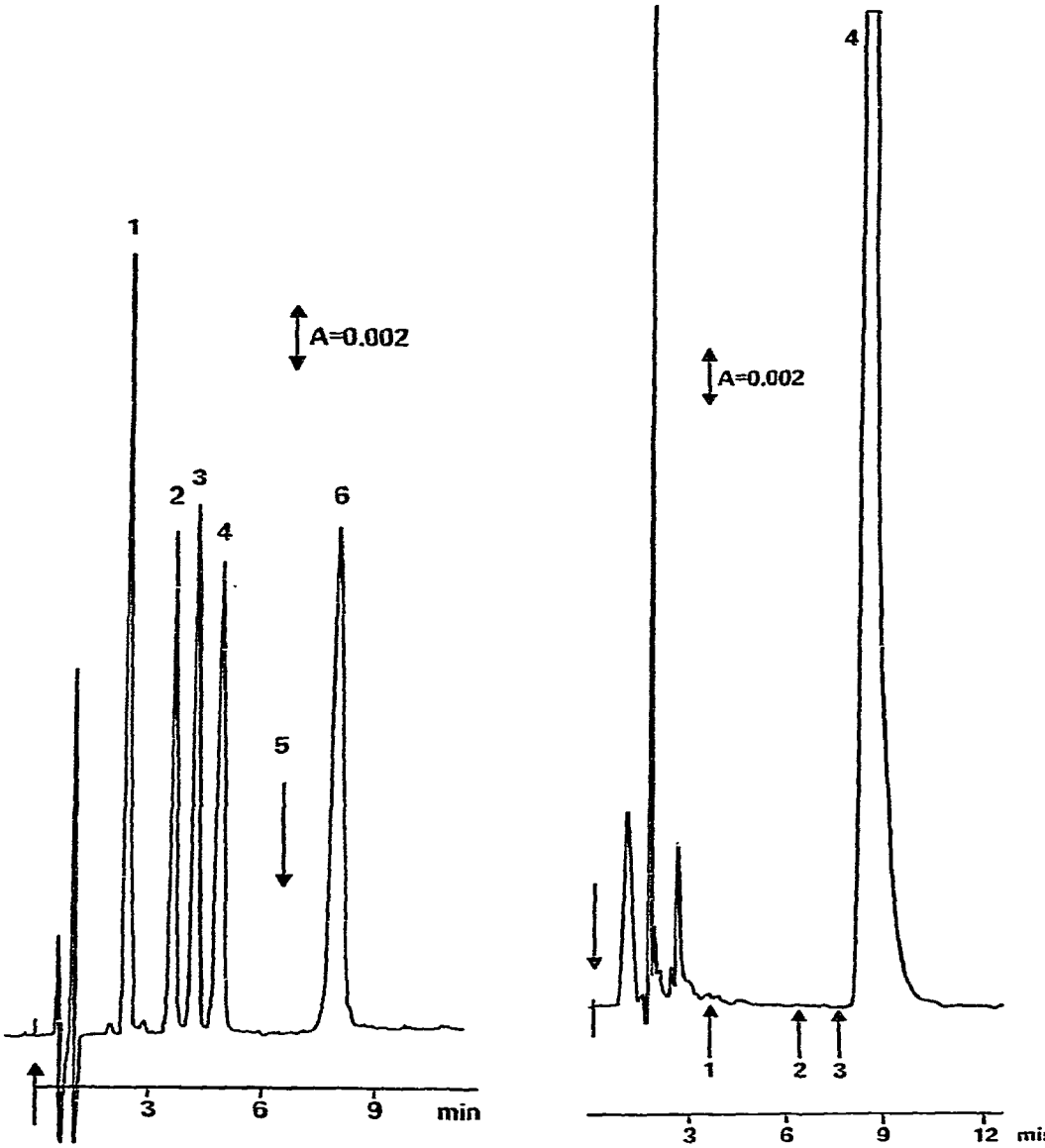


Fig. 4. Chromatogram of zimelidine and metabolites. Support: Nucleosil C_{18} (5 μm) in a glass-lined stainless-steel column (100 × 3 mm I.D.). Mobile phase: phosphate buffer (pH 2)–acetonitrile (91:9, v/v) + $4 \cdot 10^{-4}$ *M* DMOA; flow-rate, 0.9 ml/min. Detectors: Waters Model 440 at 254 nm. Peaks: 1 = norzimelidine E-isomer; 2 = zimelidine primary amine metabolite; 3 = norzimelidine; 4 = zimelidine; 5 = endogenous compound; 6 = zimelidine N-oxide.

Fig. 5. Blank chromatogram from 5 ml plasma. Chromatographic conditions as in Fig. 4. Peaks: 1 = norzimelidine E-isomer; 2 = norzimelidine; 3 = zimelidine; 4 = endogenous compound.

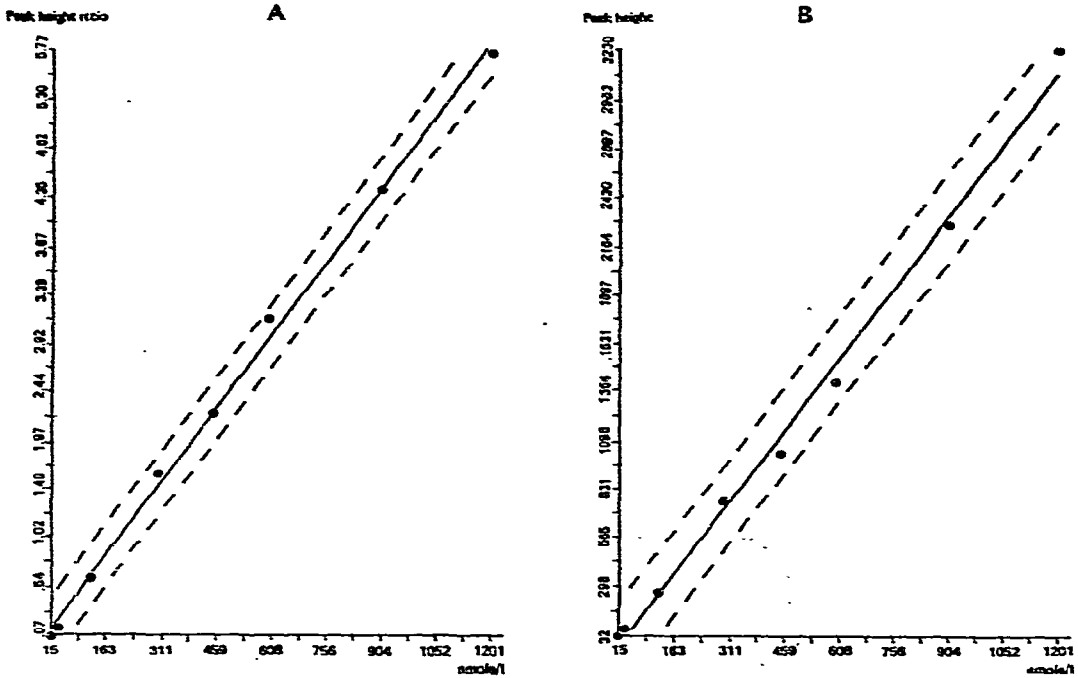


Fig. 6. Zimelidine standard curves in plasma, with and without internal standard. Analysis performed according to *Analytical method*. Column: glass-lined stainless steel. – – –, 95% confidence limits. A, With internal standard, norzimelidine E-isomer (659.6 nmol/l); B, without internal standard.

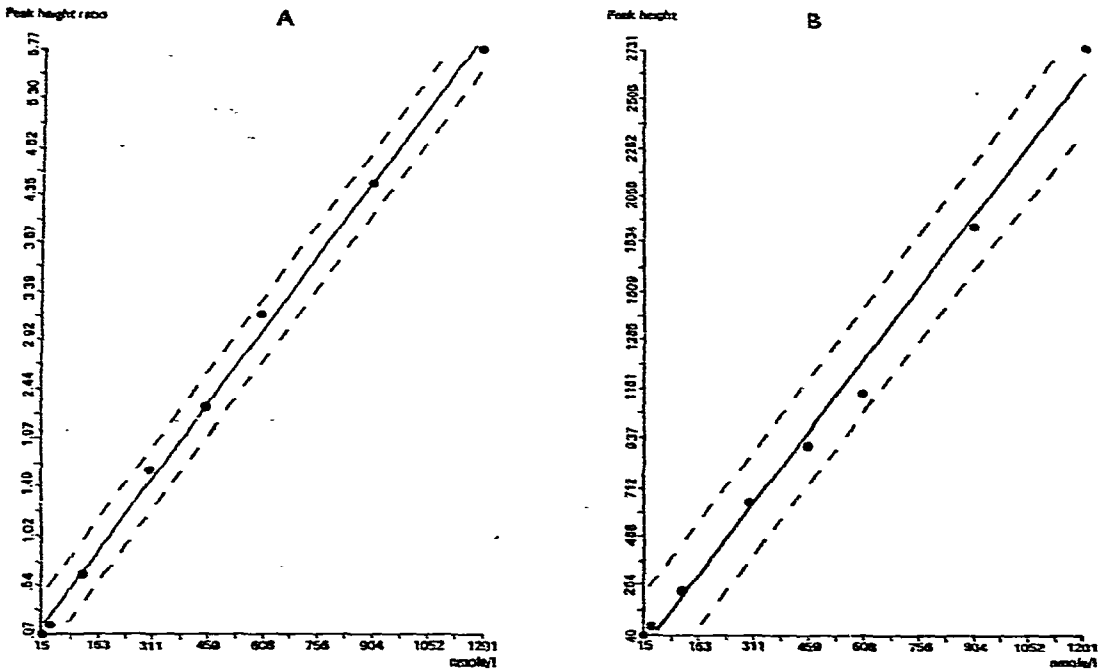


Fig. 7. Norzimelidine standard curves in plasma, with and without internal standard. Details as in Fig. 6.

TABLE II

LINEAR REGRESSION ANALYSIS OF STANDARD CURVES

Data obtained by inverted predictions on the standard curves shown in Figs. 6 and 7. IS = With internal standard; NIS = without internal standard.

Amount (nmol/l)		*Accuracy (%)*	*Limits at P = 0.95*	*Mode*
Added	*Found**			
Zimelidine				
120.1	114.5	−4.7	52.8– 174.7	IS
120.1	123.0	+2.4	33.8– 209.4	NIS
450.5	448.2	−0.51	389.2– 507.1	IS
450.5	413.3	−8.3	328.1– 498.2	NIS
1201	1178	−1.9	1112 –1247	IS
1201	1245	+3.7	1148 –1348	NIS
Norzimelidine				
120.1	116.8	−2.7	78.9– 154.2	IS
120.1	124.7	+3.8	15.3– 229.8	NIS
450.2	468.1	+4.0	431.6– 504.5	IS
450.2	429.6	−4.6	325.5– 533.4	NIS
1201	1190	−0.92	1148 –1233	IS
1201	1256	+4.6	1138 –1384	NIS

* Obtained from the regression equation of the standard curve.

TABLE III

STUDIES ON QUANTITATIVE DETERMINATIONS

Analysis performed according to *Analytical method.*
Column: glass-lined stainless steel, 100 × 3 mm I.D. Standard curves: for concentrations ≲75 nmol/l, seven points in the range 8–160 nmol/l; for higher concentrations, seven points in the range 150–1800 nmole/l. Internal standard: Norzimelidine E-isomer, 98.9 and 659.6 nmol/l for low and high concentrations respectively.

Compound	*Amount*		s_{rel}%	*n*
	Added (nmol/l)	*Found (%)*		
Within-run variations				
Zimelidine	15.0	98.4	8.19	9
Norzimelidine	15.0	103.0	8.20	9
Zimelidine	31.5*	97.2	5.40	6
Norzimelidine	33.0*	102.4	6.00	6
Zimelidine	75.1	102.0	5.34	7
Norzimelidine	75.0	105.0	4.12	7
Zimelidine	300.3	94.9	5.17	6
Norzimelidine	300.1	104.0	6.24	6
Zimelidine	1502	98.8	2.30	6
Norzimelidine	1501	94.0	1.80	6
*Day-to-day variations***				
Zimelidine	300.3	99.2	9.70	15
Norzimelidine	300.1	99.8	7.60	17
Zimelidine	1502	99.7	4.89	18
Norzimelidine	1501	96.0	5.16	18

* Column: precision-bore stainless steel, 100 × 4 mm (1 × I.D.).
** Analyses were performed on four different days on samples from a common pool.

The use of internal standards in quantitative determinations has been discussed[12,19]. Representative standard curves, with 95% confidence limits outlined, obtained for zimelidine and norzimelidine on the same samples and calculated using both peak heights and peak height ratios (Figs. 6 and 7) demonstrate the advantage of incorporating an internal standard in this method. Data on confidence limits at various concentration levels (Table II) further support this statement. In all cases the limits are broader for calculations without an internal standard and the accuracies, calculated as the difference between the nominal value and the value obtained from the regression equation of the standard curve, are with only one exception better when using the internal standard. The differences obtained are especially large for the secondary amine, probably because of its close chemical relationship to the internal standard. Some data obtained by use of an internal standard on within-run and on day-to-day variations (Table III) illustrate the reliability of the method.

Some blank chromatograms obtained from some other kinds of biological material (Fig. 8) illustrate the possibilities of carrying out determinations in whole blood, urine and rat hypothalamus. Analysis in whole blood does not seem to present any new problems compared to plasma, while in urine there is a peak that interferes with the tail of the internal standard. However, it is probable that a slight change in the composition of the mobile phase, for example an increase of its DMOA content (see Fig. 2), would improve the separation or alternatively, as discussed above, it may be possible to perform the analysis without any internal

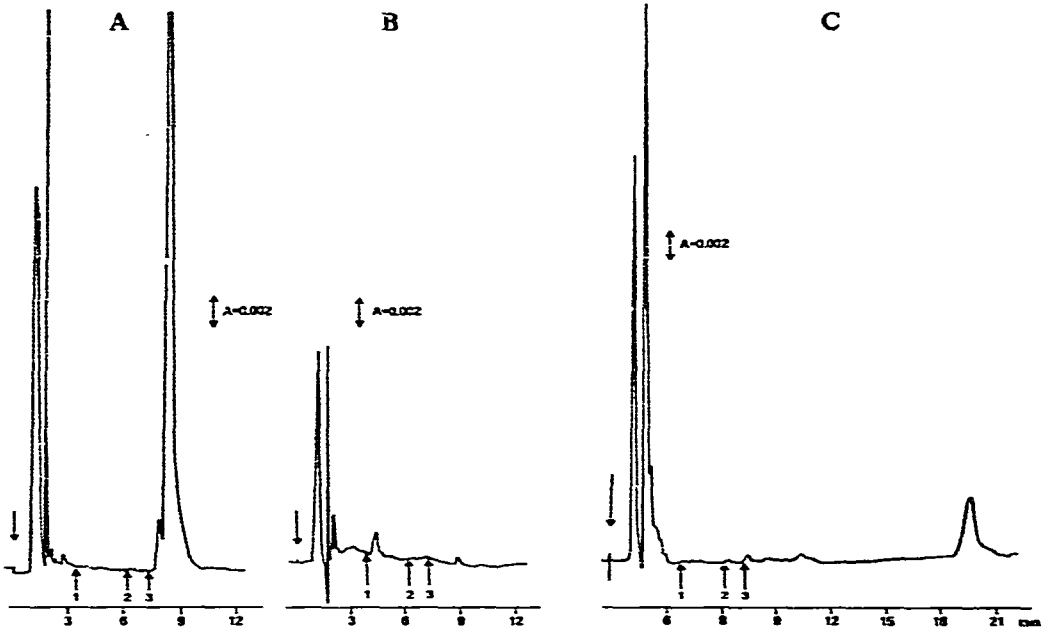


Fig. 8. Blank chromatograms from human whole blood, human urine and rat hypothalamus. Extraction and chromatographic conditions according to *Analytical method*, after adjustment of reagent volumes. Column: stainless steel. Peaks: 1 = norzimelidine E-isomer, 2 = norzimelidine; and 3 = zimelidine. A, Human whole blood, extraction of 2.5 ml; B, human urine, extraction from 100 μl; C, rat hypothalamus, homogenized together with 2 ml deionized water before the extraction.

standard. The blank chromatogram obtained from rat hypothalamus is quite clean in the region where the compounds of interest are eluted; the only problem seems to be a peak which is eluted late (20 min) and which increases the time of analysis.

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