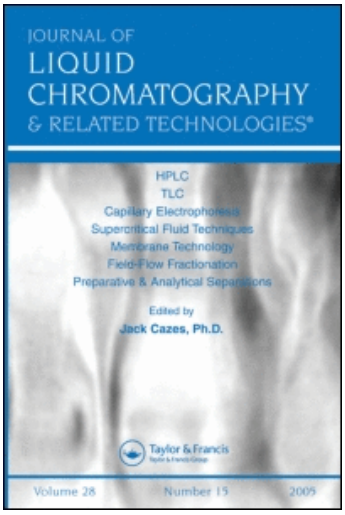

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STRAIGHT-PHASE ION-PAIR CHROMATOGRAPHY OF ZIMELIDINE AND SIMILAR DIVALENT AMINES
PART I BIOANALYSIS

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ABSTRACT

Methods are presented for the quantitative determination of ZIMELIDINE, a new antidepressant drug, and its active metabolite norZIMELIDINE in biological material (whole blood, plasma, urine and rat brain). The extraction is optimized regarding recoveries and blank chromatograms and the compounds are separated by high performance ion-pair liquid chromagraphy with perchlorate as counter ion in the stationary phase. Internal standards are chlorpheniramine and the geometrical isomer to norzimelidine. The precision for determinations in plasma ranges 2 - 7 % (CV) for the concentrations 100 - 5 ng/ml, and the detection limits are 150 pg/ml but can be lowered about five times by using larger sample volumes. The selectivity against metabolites is investigated and the use of the method in routine is discussed. The isolation and identification of the primary amine metabolite by collecting the peak for subsequent GC-MS-analysis is demonstrated.

INTRODUCTION

Zimelidine is a new antidepressant, which like its main metabolite, norZIMELIDINE is a rather selective inhibitor of the uptake of serotonin (1). Several current investigations have stressed the importance of monitoring the plasma levels of patients during treatment with such drugs since steady state levels, which are both too low and too high, seem to result in low efficacy, e.g. nortriptyline (2, 3, 4) amitriptyline (5, 6) protriptyline (7).

The determination of drugs and metabolites in biological material requires selective and in many cases very sensitive analytical methods. The selectivity is needed because frequently there is a close structural relationship between the drug and its metabolites and also because the presence of many endogenic substances may disturb the quantitation. High sensitivity is often needed, especially for pharmacokinetic investigations.

Such high demands on the analytical method are compatible only with rather sophisticated techniques. For example the tricyclic antidepressants have been determined in blood plasma by gas chromatography with flame ionisation (8), electron-capture (9) and nitrogen (10) detectors, as well as by mass fragmentography (11, 12), and by HPLC and UV-detection (13). The antihistaminic drug brompheniramine, structurally related to ZIMELIDINE, has been determined in biological fluids by gas chromatography using an electron capture detector (14).

The method presented in this paper is based on ion-pair partition chromatography, a technique in which medium-size supports are primarily used (15, 16), but where silica micro particles are equally suitable as supports for the stationary phase as demonstrated in studies on sulphonamides (17), aminophenols (18), tricyclic antidepressants (13), phenylacetic acid derivatives (19), glucuronides and sulfate-conjugates (20), and several amine-drugs and biogenic acids (21). Some studies on the chromatography of ZIMELIDINE have also been published: as chloride ion-pair on medium size cellulose (22), as perchlorate ion-pair on Partisil 10 (23), and with methanol/ammonium nitrate as mobile phase on Partisil 5 (24). A bioanalytical method based on gas chromatography and electron capture detection has also recently been submitted for publication (25).

The chromatographic system used in this study comprises an acidic perchlorate solution as stationary phase on irregular silica microparticles (5 μm) with a mixture of methylene chloride

and n-butanol as mobile phase. Its basic chromatographic properties will be described elsewhere (26). In this paper, application of this system to bioanalysis is described.

EXPERIMENTAL

Apparatus

The chromatographic equipment comprised a Milton-Roy Minipump with a pulse dampener (Laboratory Data Control (LDC) Model 711-47 solvent delivery system); a UV-detector with a fixed wavelength of 254 nm, (LDC Model 1265 UV monitor and a Waters Model 440, equipped with 8 and 10 µl flow cells respectively), and with variable wavelength (Cecil 212 with a 8 µl flow cell used at 262 nm); the injector was either Valco, 7000 or 6000 psi, equipped with a short teflon tube, 20 x 0.5 mm (1 x i.d.), at the loop inlet for direct injection with a syringe, or Rheodyne Model 7120.

The columns were of precision-bore 316 stainless steel (Altex or Handy & Harman), length 150 mm, ID 4 mm, OD 1/4 in., and were equipped with modified, zero dead volume, SVAGELOK end fittings and bed supports with 2 µm frits (Altex).

Parts of the liquid chromatograph were kept at a constant temperature in a cooling incubator, Termaks 2000 (Norway). An air-driven gas-amplifier pump, Haskel 7150-C, was used for the column packing.

Static photometric measurements were carried out with a Zeiss Spektralphotometer PMQ II and the pH measurements with an Orion Digital Ionalyzer model 801 A.

Ultrasonic disintegrations were performed with a Labsonic 1510 (B Braun Melsungen AG, West Germany), and ultrasonic homogenisations of the packing slurry with a Bransonic 220 (Branson, Heusenstamm, West Germany). Liquid scintillation measurements

were performed on a Packard Tricarb 2450. GC-MS studies were performed on a LKB 2091. Extractions were performed with a rotating device, Heto RK 20 VS (Denmark).

Chemicals and Reagents

ZIMELIDINE and norZIMELIDINE were used as the hydrochlorides, the norZIMELIDINE E-isomer (X) as the oxalate and chlorpheniramine as the maleate salt. The metabolites and other ZIMELIDINE-like compounds were used as bases, hydrochlorides or oxalates. The chemical structures of the compounds are given in Fig 3.

Dichloromethane, 70 % perchloric acid and sodium perchlorate were zur Analyse products from E Merck, (Darmstadt, GFH). The n-butylalcohol, "Pronalys", was from May & Baker (England). Diethyl ether, anhydrous, from May & Baker (England) and n-hexane, für die Spektroskopie, from E Merck (Darmstadt) were used for the extractions.

1,1,2,2-tetrabromoethane, purum, tetrachloroethylene, puriss, (E Merck, Darmstadt) and hexanes (certified; Fisher Scientific, US) were used for column packing.

All other chemicals used were of analytical grade or corresponding quality. INSTAGEL (Packard, USA) was used as scintillation cocktail.

Chromatographic Support

Partisil 5, irregular silica-microparticles (mean particle size 6 μm) from Whatman, England was used.

Column Packing and Coating

The columns were packed by a balanced density slurry technique. A slurry of 1.6 g of Partisil 5 and 16 ml of the balanced

density mixture (tetrabromoethane + tetrachloroethylene 6+4 w/w) is prepared by treatment with an ultrasonic bath for 1 min. The slurry is transferred to the reservoir, a stainless steel column 300 x 9 mm (1 x i.d.) to which the analytical column, 150 x 4 mm, filled with the balanced density mixture is attached. Another few ml of the balanced density mixture is put above the slurry and finally the column is completely filled with hexane and attached to a 5 m long stainless steel tube equipped with a manometer and a whitey-valve. With the valve in the closed position, the pressure is raised to about 400 bars by pumping hexane, the valve is then opened to push the slurry into the analytical column. When the balanced density mixture has passed, the column is washed with about 200 ml of hexane and 100 ml of dichloromethane and methanol before coating with the stationary phase.

For coating about 100 ml of a mixture of the stationary phase and acetone, 75 + 25 v/v, is pumped through the column at a flow rate of about 1 ml/min. The column is transferred to the thermostated incubator, attached to the injection valve, and the detector and mobile phase, which has been carefully equilibrated with the stationary phase by shaking for at least 1 h, is pumped through the system at that flow rate which is to be used in the analytical determinations, normally about 0.8 ml/min.

The equilibration takes place overnight, but the system is then stable for months. The mobile phase in the reservoir, about 1/2 l, is covered by a layer of stationary phase, 75 - 100 ml, in order to maintain a careful equilibration of the phase.

The column, the detector and the supply of mobile phase where kept in an air thermostat at 23.0 $\pm$ 0.3°C.

The volume of the stationary phase (V_s) on the column was 0.79 ml as determined by eluting the column with anhydrous methanol and measuring the water content by a Karl Fischer titration. The void volume of mobile phase (V_m) was determined as 1.02 ml by injection of an unretained sample (toluene).

Determination of Distribution Data

Determination of extraction yields from plasma were performed with radioactive compounds; ^{14}C-ZIMELIDINE (specific activity = 7.41 x 10^7 becquerel/mmol, labelled in the aminomethyl group) and ^{3}H-norZIMELIDINE (specific activity = 2.53 x 10^7, becquerel/mmol unspecifically labelled). The compounds were incubated with plasma for = 30 min before the extraction that was performed in centrifuge tubes at room temperature. After centrifugation at 2000 - 3000 rpm for 5 - 10 minutes an aliquot of the organic phase was pipetted into a counting vial, 10 ml of INSTAGEL was added and the samples were measured by liquid scintillation. The quantitations were made in the external standard ratio mode and only chemical quenching was assumed.

Analytical Methods

Chromatographic system

Support:	Partisil 5
Stationary phase:	0.2 M $HClO_4$ + 0.8 M $NaClO_4$
Mobile phase:	dichloromethane + n-butanol (89 + 11 v/v) carefully equilibrated with the stationary phase
Flow rate:	0.7 - 0.8 ml/min

Extraction

A Plasma and whole blood

1 1.0 ml sample + 10 µl each of internal standard solutions CHLORPHENIRAMINE and norZIMELIDINE E-isomer + 1 ml 1 M NaOH are mixed and extracted for 20 min with 5.0 ml diethyl ether / n-hexane (80 + 20 v/v).

2 After centrifugation for 6 min at 2500 - 3000 rpm the organic phase is transferred with a pasteur pipette to a small

glass tube and evaporated at room temperature using a gentle stream of compressed air.

3 100 µl of the mobile phase is added to the tube and the residue is dissolved by treatment with a Whirlimixer for 10 - 15 s

4 The solution is injected on the separation column using an injection valve equipped with an 80 µl loop.
The quantitations are made from a standard curve relating the peak height ratios of the samples to the internal standards vs the concentrations, and obtained by analyzing known amounts of the compounds added to plasma.

B Urine
Urine normally contains such a high content of the compounds that the extraction can be performed on diluted samples. The same extractions as for plasma are applicable.

C Rat brain
The brain material has, like urine, a rather high content of the compounds to be analysed and determinations are normally performed on diluted samples.
The brains are homogenized by ultrasonic disintegration (output power: 350 W) after the addition of about 2 ml of deonized water. The homogenate is diluted to 25.00 ml with water, and 0.5 ml of the suspension is analyzed according to the same method given for plasma samples.

Identification of the ZIMELIDINE Primary Amine Metabolite

Five ml alkalinized urine was extracted with ten ml of the diethyl ether - hexane mixture and after the chromatographic separation the primary amine peak, containing 3 - 4 µg of the compound, was collected and the mobile phase was evaporated at room temperature under a gentle stream of air. The residue was dissolved in 100 µl of the mobile phase and rechromatographed for purification. After evaporation of the eluent from the collected peak,

equal volumes of an alkaline buffer and methylene chloride were added and the amine extracted from accompanying perchlorate ions. The organic phase was then evaporated and the residue was treated with 100 μl of trifluoroacetic anhydride at 50°C for 15 minutes. The solution was evaporated under nitrogen and the residue dissolved in 100 μl of ethyl acetate. The identification was performed by mass fragmentography by simultaneous recording of four typical fragments (m/e 384 (M^+), 271 (base peak), 386 and 273 (bromine isotopes)) and comparing with synthetic compound. (GC-conditions: 3 % OV-17 on Gas Chrom Q (100 - 120 mesh); 250°C).

RESULTS AND DISCUSSIONS

Extraction from Plasma

In extractions from biological materials it is important to avoid extraction systems that give very high distribution ratios since this increases the risk of coextraction of interfering endogenous compounds. The complex-binding by endogenous macromolecules (e.g. 27) can decrease the distribution ratio of compounds to be analysed, but an increase in extraction caused by the biological material has also been observed in some cases, e.g. for amines and a quaternary ammonium compound in serum (28). Quantitative extraction of the drug will of course increase the sensitivity of the analytical method and it is also beneficial for its reproducibility.

The amines can be extracted into an organic solvent as bases or as ion-pairs and both possibilities were tried. Hexane was chosen as the main solvent for the base extractions since it is non-polar and gives a low coextraction of endogenous compounds. Distribution data, $-\log k_d \times K'_{HA}$ is 6.52 for ZIMELIDINE and 8.4 for norZIMELIDINE, indicate that quantitative extraction should be achievable at pH $>$10.5 with equal phase volumes. The recoveries

from plasma at pH 11.5 were however, very low (Table 1), and since they increase with the concentration, losses by adsorption are indicated. Silanization of the equipment did not improve the results.

NorZIMELIDINE is a secondary amine that is solvated by hydrogen-accepting solvents. Diethyl ether, diisopropyl ether, ethyl acetate, butanol, toluene and the hydrogen-donating heptafluorobutanol were tried as extraction media. The best results were obtained with n-hexane-diethyl ether mixtures and a quantitative recovery is obtained with 80 % of diethyl ether (Table 2); The extract gave a good blank chromatogram (Fig 1) which has large peaks at the front but is clean after 3 minutes. The use of pure diethyl ether gives disturbances in the chromatogram necessitating further cleaning up extraction steps (c.f. 22,23). Different extraction times, 10 - 60 min, did not affect the recovery nor did a variation in pH between 10.5 and 11.5.

The compounds are divalent amines and can be extracted as

TABLE 1

Extraction of NorZIMELIDINE from Plasma with Hexane

Procedure: Plasma (pH 11.5) extracted with an equal volume of hexane after incubation for 60 min with norZIMELIDINE. The organic phase is evaporated and the residue is measured by liquid scintillation counting after addition of INSTAGEL.

ng/ml	Recovery (%)
20	2.0
50	12.5
100	28

Sample: ^{3}H-labeled norZIMELIDINE

TABLE 2

Extraction of NorZIMELIDINE from PLASMA with Hexane-Diethylether Mixtures

Sample: ^{3}H-labeled norZIMELIDINE, 250 ng/ml

Procedure: 2.0 ml plasma + 0.5 ml 5 M NaOH are extracted with 4.0 ml organic phase, followed by evaporation and measurements by liquid scintillation.

Extraction medium	% extracted
Diethyl ether	100
Diethyl ether + hexane (80 + 20 v/v)	99.7
Diethyl ether + hexane (50 + 50 v/v)	84.8

1 + 1 and/or 1 + 2 ion-pairs depending on pH and the hydrophobicity of the counter ion. With small ions the 1 + 1 ion-pair is usually easier to extract while large hydrophobic anions are expected to increase the extraction of the 1 + 2 ion-pair (28, 29). Screening experiments were made with 2-naphthalenesulfonate (BNS) and perchlorate at pH 3 using methylene chloride. It can be assumed that extraction of a 1 + 1 ion-pair dominates at this pH. The results, given in Table 3, show that rather low degrees of extraction are obtained for norZIMELIDINE both from aqueous solutions and plasma. An interesting fact is that addition of plasma gives a drastic decrease in the distribution ratio of the BNS ion-pairs, while a considerable increase is observed with perchlorate as the counter ion. The last observation is in accordance with the findings in ref (28), while the results with BNS might be due to strong protein-binding of this counter ion (c.f. 31).

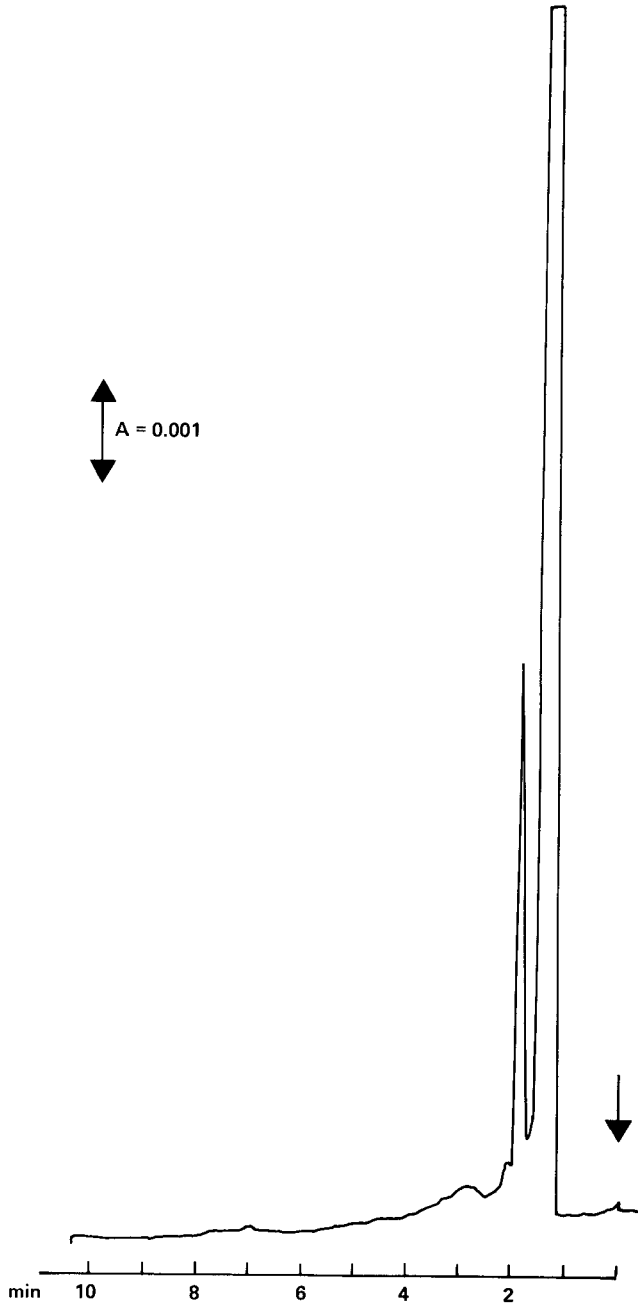


Fig 1 Blank Chromatogram from Plasma

Procedure: According to "Analytical Method"

TABLE 3

Ion-Pair Extraction of ZIMELIDINE and NorZIMELIDINE from Plasma

Sample: ^{14}C-labeled ZIMELIDINE and ^{3}H-labeled norZIMELIDINE

Procedure: Aqueous or plasma solutions of the sample are mixed with counter ion solution (final pH = 3) and extracted with an equal volume of methylene chloride. The organic phase is evaporated and measured by liquid scintillation counting.

Counter ion solutions: 2-naphthalenesulfonate (BNS) 0.2 M
perchlorate 1 M

Compound	ng/ml	Counter ion	Milieu	% extracted
ZIMELIDINE	20	BNS	water	44.2
"	"	"	plasma	19.9
"	100	"	water	53.0
"	"	"	plasma	8.9
"	20	ClO_4	water	48.0
"	"	"	plasma	93.3
"	100	"	water	47.7
"	"	"	plasma	93.2
NorZIMELIDINE	20	BNS	water	13.4
"	"	"	plasma	4.2
"	100	"	water	11.5
"	"	"	plasma	2.8
"	20	ClO_4	water	8.4
"	"	"	plasma	27.7
"	100	"	water	5.9
"		"	plasma	21.8

Internal Standard

It is well-known that amines often have the tendency of getting adsorbed to glass boundaries in extraction and evaporation vessels (32) giving rise to losses of the compounds during an analytical procedure. The ionic strength, the technique of transferring the organic phase and silanization of the equipment may reduce the losses (28) as well as the addition of adsorption inhibitors such as alcohols (e.g. 28), or amines (33), and by using reextraction instead of evaporation (34).

Preliminary extraction studies showed that severe adsorption losses frequently occurred with norZIMELIDINE and several of the above mentioned possibilities of reducing the losses were tried, but none increased the extraction to an acceptable level.

Compensation for adsorption losses by use of an internal standard puts severe restrictions on the structure of the intended compound. The geometrical isomer of norZIMELIDINE (X in Fig 3) was tried as internal standard for both norZIMELIDINE and ZIMELIDINE and the resulting standard curves are shown in Fig 2. This works well with norZIMELIDINE, but for ZIMELIDINE the ratio increases almost exponentially with concentration illustrating the higher adsorption losses of the standard compared to the tertiary amine.

The adsorption losses of ZIMELIDINE are in most cases negligible, and it is possible to use external standards for this compound. The application of an internal standard has however the important advantage of largely simplifying the routine work: it is not necessary to work with exactly known volumes in the different transfer procedures in the analytical method. Furthermore small variations in the chromatographic system (e.g. the flow rate) that affect the peak shapes can also be compensated by the internal standard technique, improving the precision and reproducibility. Chlorpheniramine was found to give a suitable retention time and good standard curves and was chosen as the internal standard for ZIMELIDINE.

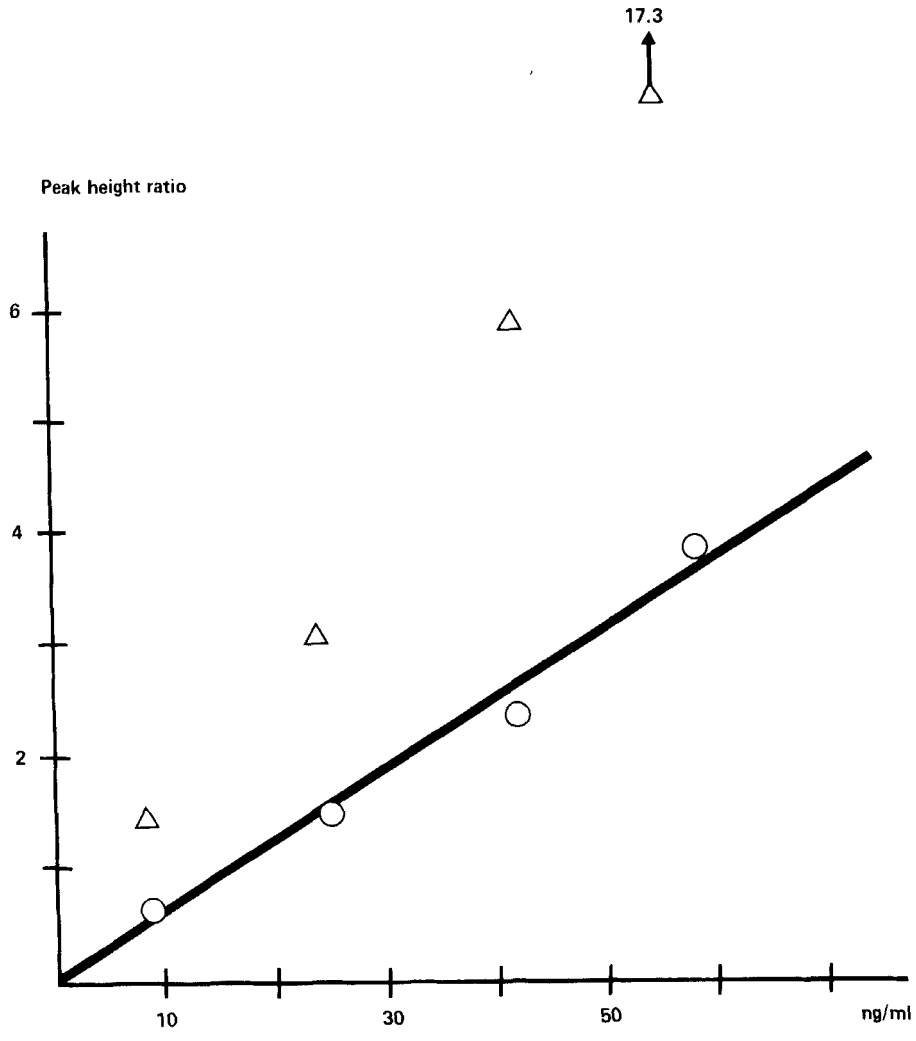


Fig 2 Standard Curves of ZIMELIDINE and NorZIMELIDINE
Internal Standard: Geometrical Isomer to NorZIMELIDINE

△ ZIMELIDINE

○ norZIMELIDINE

Content of internal standard: 100 ng/ml

Selectivity

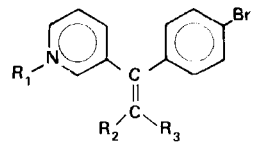
The metabolites of ZIMELIDINE (35) isolated from rat urine after oral and intravenous administration are summarized in Fig 3. The main metabolites are norZIMELIDINE, the amine oxide (I) and the acids (VI and VII). These compounds as well as the primary amine (XI) have also been found in human urine.

Capacity ratios and selectivity factors of the most important metabolites relative to ZIMELIDINE, norZIMELIDINE, the E-isomer of norZIMELIDINE (X) and chlorpheniramine are given in Table 4. It is shown that a complete chromatographic separation is easily acheived. It is not likely that the amine oxides (I, II, IV, VIII, and IX) and the acids (VI and VII) are extracted in the "Analytical method for biological samples". The aldehyde (V) is probably more hydrophobic than the acids and will consequently be eluted near the front giving very high selectivity factors.

Other agents that may be used as well as ZIMELIDINE in the clinical studies are bensodiazepines, which are eluted very early in the chromatogram (diazepam $k'_f = 0$, nitrazepam $k'_f = 0.47$, and oxazepam $k'_f = 0.20$) and consequently do not interfere in the determinations. The antidepressants amitrypline, imipramine, chloroimipramine, nortriptyline, desipramine and protriptyline being hydrophobic monovalent amines are also eluted with the front, while opipramole, a divalent amine, gives $k'_f = 1.78$, i.e. a selectivity factor against ZIMELIDINE of 1.33, but which is eluted together with the internal standard chlorpheniramine.

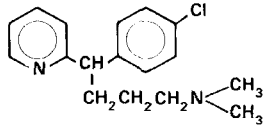
Determination in plasma

A chromatogram from a representative sample of human plasma is shown in Fig 4. The peaks are eluted on a clean baseline within 8 minutes and the nice appearance is due to the rather specific extraction step where only hydrophobic basic and neut-



Compound	R_1	R_2	R_3
Zimelidine	–	$-CH_2N(CH_3)_2$	H
Norzimelidine	–	$-CH_2NHCH_3$	H
I	–	$-CH_2\overset{\uparrow}{N}(CH_3)_2$ O	H
II	O	»	H
III	–	$-CH_2NHCOCH_3$	H
IV	O	»	H
V	–	–CHO	H
VI	–	–COOH	H
VII	–	H	–COOH
VIII	O	–COOH	H
IX	O	H	–COOH
X[a)]	–	H	$-CH_2NHCH_3$
XI	–	$-CH_2NH_2$	H

Chlorpheniramine[a)]



Compounds I – IX were identified as metabolites by J. Lundström [34]

[a)] Internal standard

Fig 3 Chemical Structures of ZIMELIDINE and Metabolites

TABLE 4

Selectivity between Analytical Objects and Metabolites

Compound	log k'_f	log α vs Chlorphenirамine	ZIMELIDINE	NorZIMELIDINE	NorZIMELIDINE E-isomer
XI (prim. amine)	0.821	0.571	0.449	0.330	0.243
I (amine oxide)	0.137	0.114	0.236	0.354	0.441
VI (acid)	0.523	0.773	0.896	1.014	1.100
III (acetylated prim. amine)	<-0.52	>0.77	>0.90	>1.01	>1.10

ral compounds are extracted, and furthermore that ion-pairs of monovalent amines have much higher distribution ratios than those of divalent amines of similar size in the chromatographic system.

Plasma from rats, dogs and humans have been analyzed and no disturbing peaks have yet been observed.

Some data on recovery and precision (CV) are given in Table 5. Concentrations down to 1 ng/ml can be determined with acceptable precision provided that 1 ml plasma is taken for analysis, and the detection limits, defined as 3 times the noise of the baseline (detector - Waters model 440) are about 150 pg/ml under these conditions, but they can be improved further by taking larger plasma samples as demonstrated in the table. An illustration of the appearance of the chromatogram from the determination of 100 pg/ml is also given (Fig 5). Such a high sensitivity is of vital importance in pharmacokinetic studies.

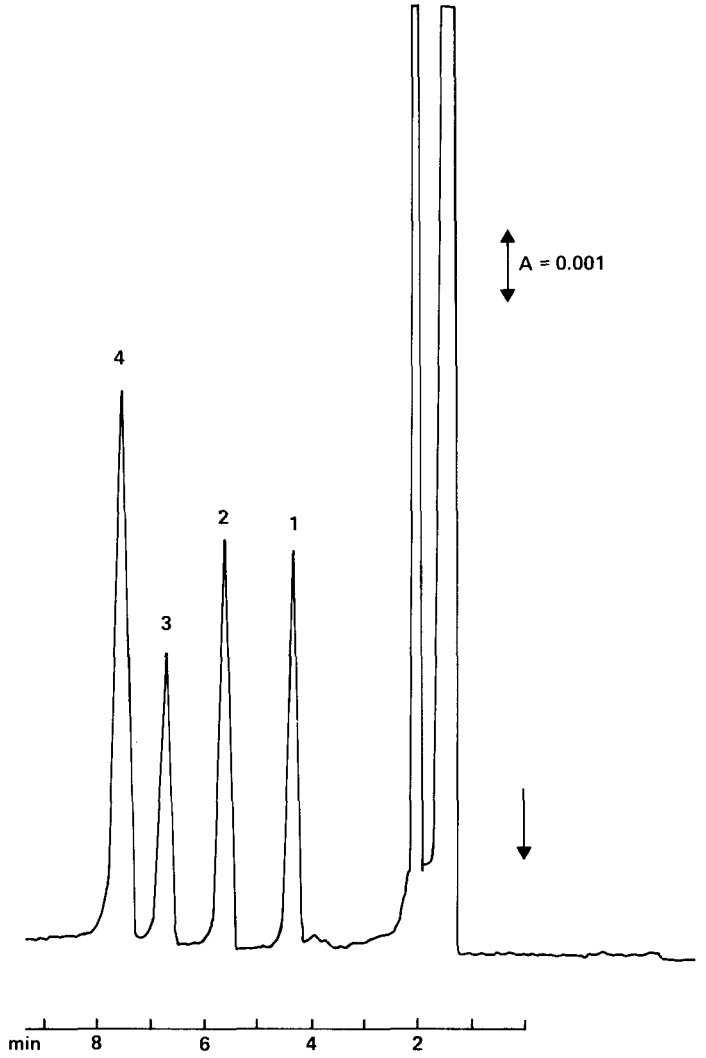


Fig 4 Chromatogram from Plasma Sample
Analysis according to "Analytical Methods"

1 CHLORPHENIRAMINE 100 ng/ml

2 ZIMELIDINE 20 ng/ml

3 norZIMELIDINE 20 ng/ml

4 norZIMELIDINE E-isomer (X) 100 ng/ml

Detector: Waters model 440 at 254 nm

TABLE 5

Quantitative Determinations of ZIMELIDINE and NorZIMELIDINE in plasma

The determinations were made according to the "Analytical method" but performed with different amounts of plasma:

A 1 ml of plasma; the standard curve comprising 10 standards in the range 1 - 150 ng/ml

B 5 ml of plasma; the standard curve comprising 6 standards in the range 0.1 - 1.5 ng/ml

Compound	Sample volume (ml)	Added (ng/ml)	Found (ng/ml)	CV (%)	n
ZIMELIDINE	1	1.01	1.14	13.4	6
		5.27	5.30	4.6	11
		101.1	102.6	1.9	11
	5	0.202	0.202	14.4	6
		1.01	0.95	7.8	6
NorZIMELIDINE	1	1.00	1.00	17.5	6
		10.01	9.93	6.9	11
		100.9	103.6	3.2	11
	5	0.200	0.213	16.0	6
		1.00	1.04	4.1	6

In determinations of unknown samples with added standards, the detection limit as defined above may however not be valid since then the confidence limits of the standard curve must also be con-

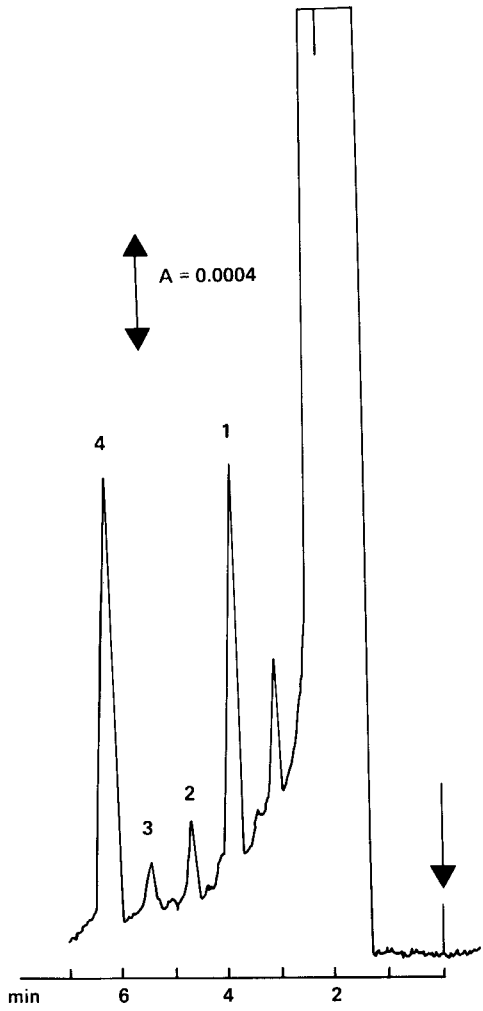


Fig 5 Determination of Picogram Amounts in Plasma
Procedure: 5 ml plasma treated according to "Analytical Method"

1 CHLORPHENIRAMINE 20 ng/ml

2 ZIMELIDINE 100 pg/ml

3 norZIMELIDINE 100 pg/ml

4 norZIMELIDINE E-isomer (X) 10 ng/ml

Detector: Waters model 440 at 254 nm

sidered. This problem has been dealt with by Hubaux and Vox (36) and their definitions are used in the following discussion. A universal computer program for linear regression (Fortran IV REGSTA) developed at Astra was utilized and examples of its performance are shown in Fig 6 by two standard curves for ZIMELIDINE, one for the low concentration range, 1 - 10 ng/ml, and the other for the range 5 - 202 ng/ml. The detection limits (X_D) ranges 0.8 - 1.1 ng/ml and 12 - 17 ng/ml at confidence limits 90 - 99 % for the both cases respectively, and as discussed by Hubaux and Vox (36) this value of course depends on the precision of the method; that is the degree of fit to the straight line, as well as the range of contents of the standards, the modes of repartition of the standards and on the replication of the unknown. As a consequence the detection limits may differ from day to day since the performance of the standards varies.

Some limited studies on the stability of ZIMELIDINE and norZIMELIDINE have shown that they remain unchanged in plasma stored on a laboratory bench at room temperature for a fairly long time, ZIMELIDINE for 7 days and norZIMELIDINE for 5 days: the compounds also remain unchanged after storage in a freezer at -20^{o}C for 2 months. Further studies on this subject are in progress.

Determination in Whole Blood

The concentration of a drug at the site of action, the biophase, is traditionally supposed to be related to its plasma level. Many drugs are however distributed to the erythrocytes; for ZIMELIDINE and norZIMELIDINE the concentration ratios blood cells/plasma are 1.18 and 1.44 respectively (37) and if this equilibrium is subjected to an individual variation it might be more relevant to correlate the biological response with drug levels in whole blood rather than in plasma.

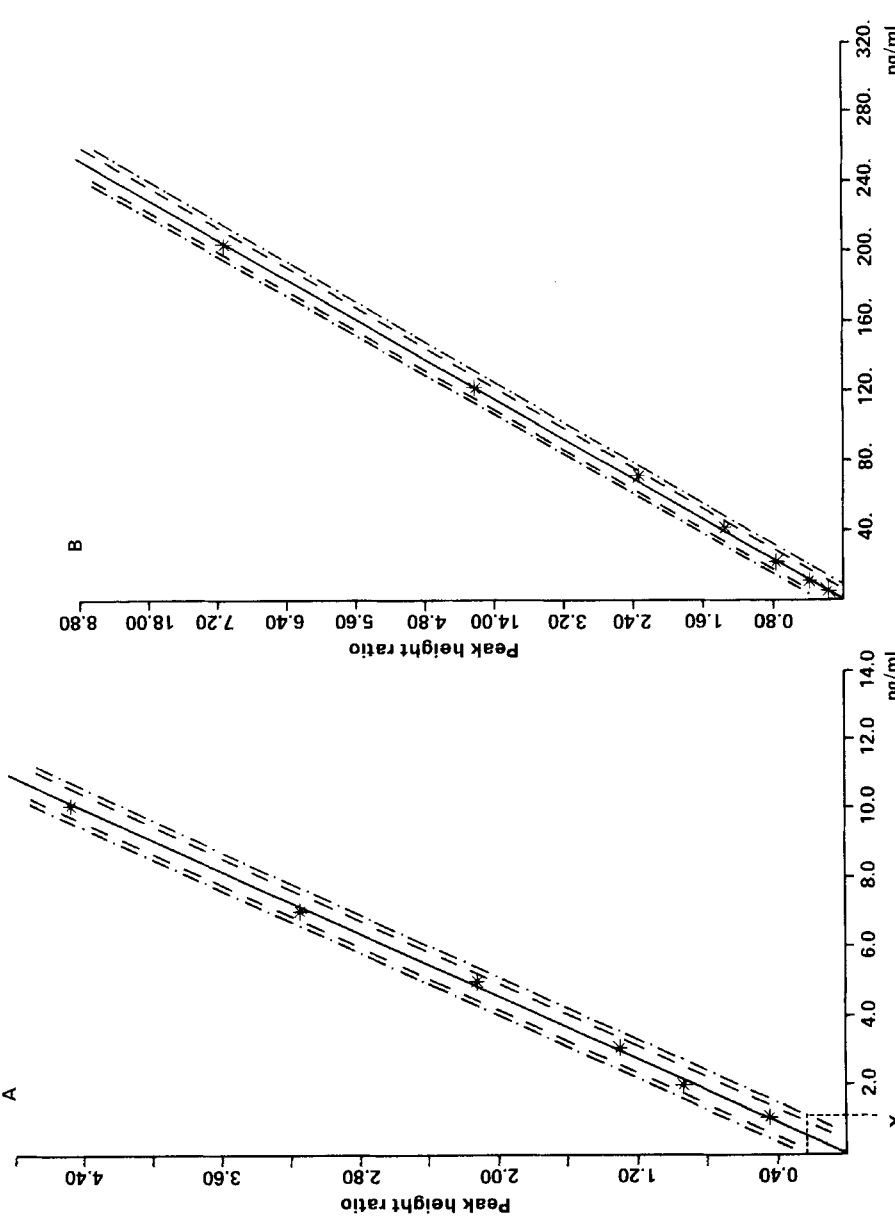


Fig. 6. Standard Curves from Plasma Extractions. -- Regression line 90% confidence level; -.- Regression line 99% confidence level; X_D = detection limit; A Determination of small

It was found that whole blood could be analyzed by the same procedure as plasma; blank and sample chromatograms are shown in Fig 7. The recoveries and precision (see Table 6) are rather similar to those obtained from plasma.

Determination in Urine

Urine contains more polar compounds than blood and interfering peaks appear in the chromatogram if the same extraction procedure as above is applied to 1 ml of urine, Fig 8. The magnitude of the disturbances can furthermore be expected to vary from day to day and even hourly since the degree of dilution of urine varies. The concentration of drugs and metabolites in urine is, however, often much higher than in plasma and diluted samples can be used. An example of such an analysis is shown in Fig 9 where the extraction procedure is applied to 100 μl of urine. The sample contained the primary amine metabolite to ZIMELIDINE (peak 5).

Determination in Rat Brain

The accuracy in the determination of drugs in the brain, as well as other tissues, is difficult to determine since it is impossible to simulate the penetration of a compound to the site of action in the preparation of standards. The brains of rats which had received 10 mg/kg of ZIMELIDINE were analyzed after homogenisation and dilution 50 times, and after this procedure standards were added. It was confirmed that a variation of the equilibration time between 5 and 60 minutes in the extraction step did not affect the relative recovery. A representative chromatogram is shown in Fig 10, where peak No 5 probably is the primary amine metabolite of ZIMELIDINE.

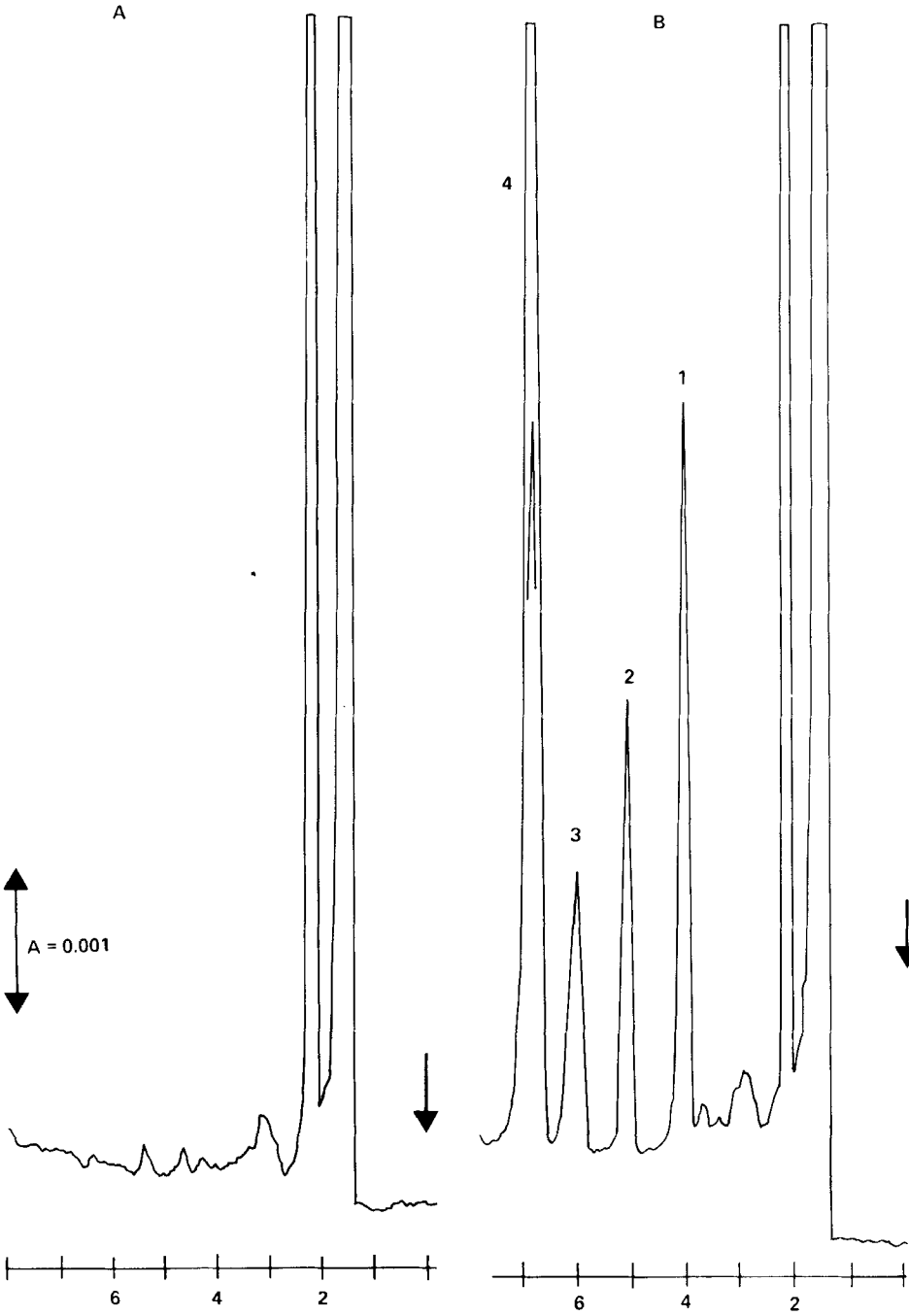


Fig. 7. Chromatograms from Human Blood. Procedure: According to "Analytical Methods". A Blank. B Sample. 1 Chlorpheniramine 100 ng/ml; 2 Zimelidine 10 ng/ml; 3 norZimelidine 10 ng/ml; 4 norZimelidine E-isomer (X) 200 ng/ml. Detector: Waters model 440 at 254 nm.

TABLE 6

Quantitative Determinations of ZIMELIDINE and NorZIMELIDINE in Whole Blood

The determinations were made according to the "Analytical method" and the standard curve comprised 8 standards in the range 5 - 200 ng/ml.

Compound	Added	Found	CV (%)	n
ZIMELIDINE	10.09	10.31	4.5	6
	100.9	105.3	1.9	6
NorZIMELIDINE	10.00	8.73	5.2	5
	100.0	103.3	2.1	6

Isolation and Identification of the Primary Amine Metabolite of ZIMELIDINE in Human Urine and Dog Plasma

As mentioned above, a peak with the same retention time as the primary amine (XI) was observed in chromatograms from human urine, rat brain and dog plasma, it is also frequently observed in human plasma samples. The compounds isolated from the body fluids by liquid chromatography were identified by GC-MS after derivatization with trifluoroacetic anhydride as described in the experimental part.

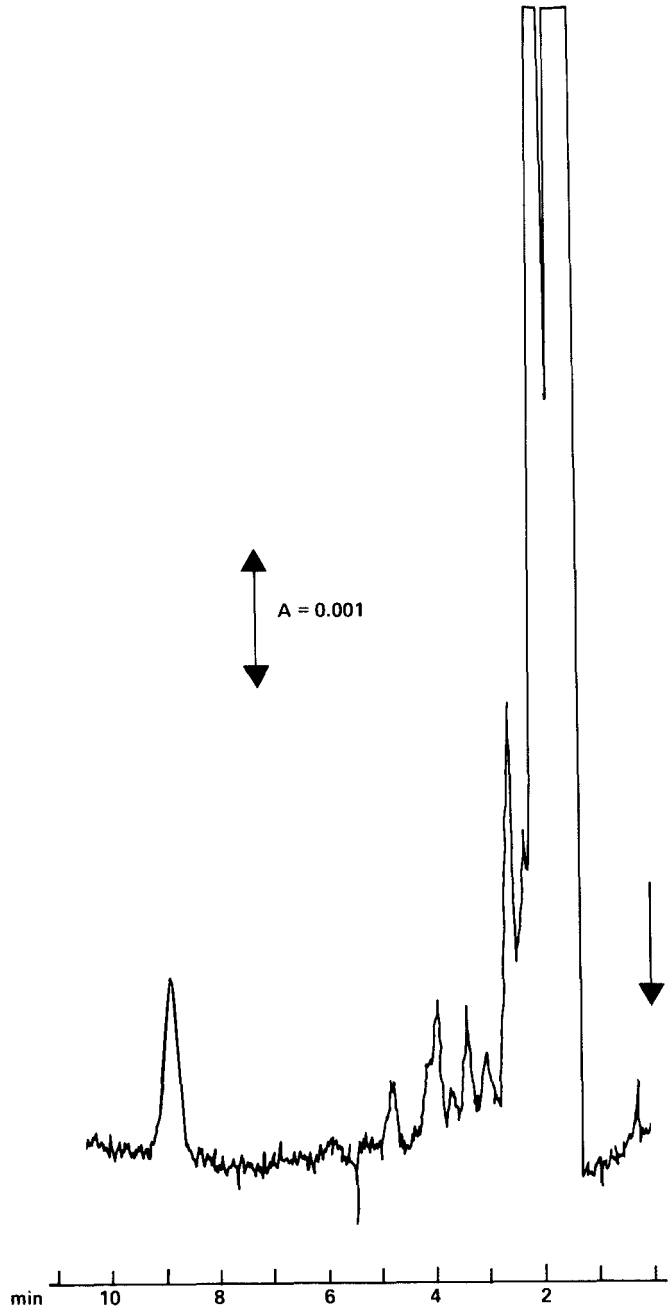


Fig 8 Blank Chromatogram from Urine
Procedure: According to "Analytical Method"
Urine volume: 1 ml

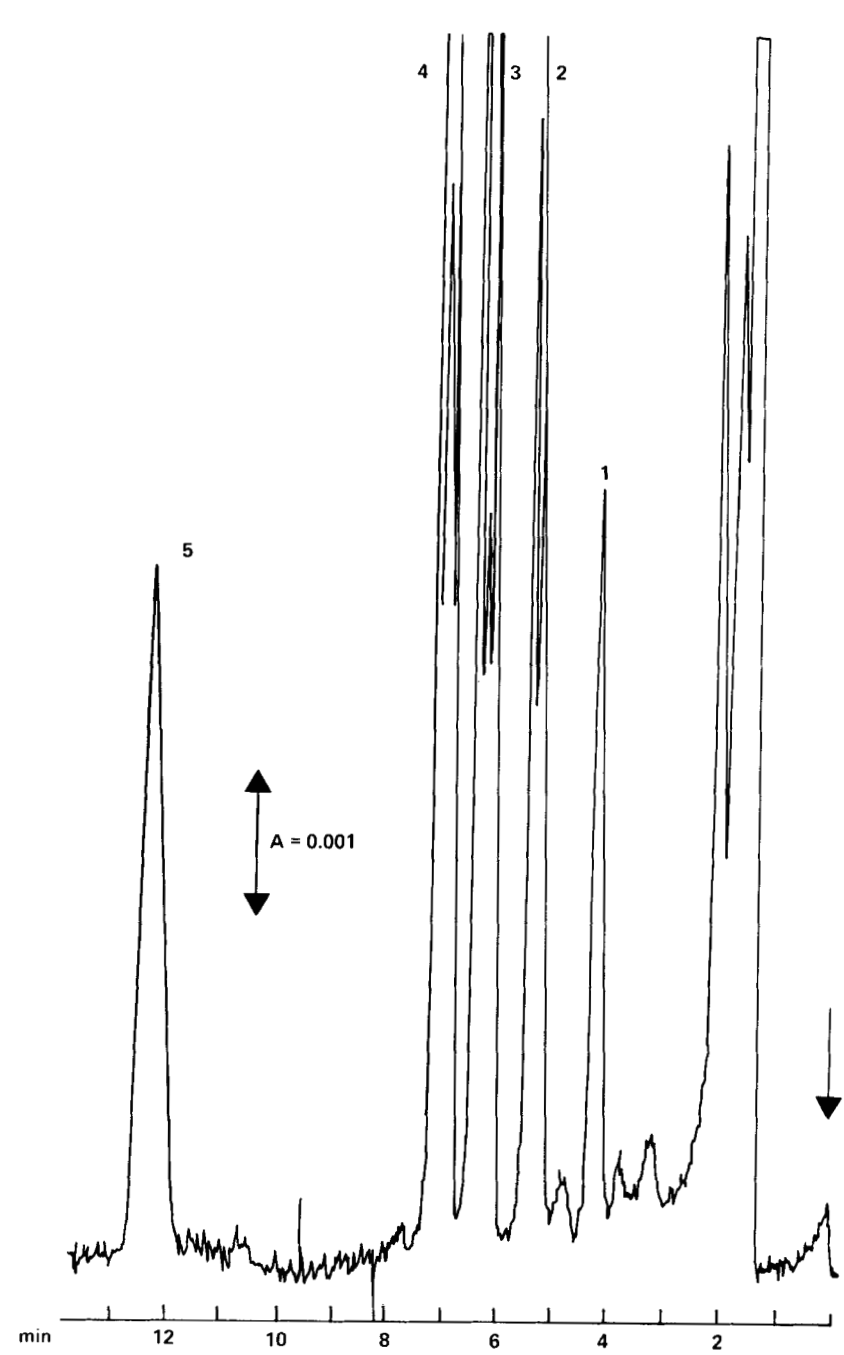


Fig. 9. Sample Chromatogram from Human Urine. Analysis according to "Analytical method". Urine volume: 100 µl (diluted to 1 ml with water). 1 Chlorpheniramine 1000 ng/ml; 2 Zimelidine 400 ng/ml; 3 norZimelidine 1350 ng/ml; 4 norZimelidine E-isomer (X) 2000 ng/ml; 5 Primary amine (XI) 900 ng/ml. Detector: LDC UV-monitor 1285 at 254 nm.

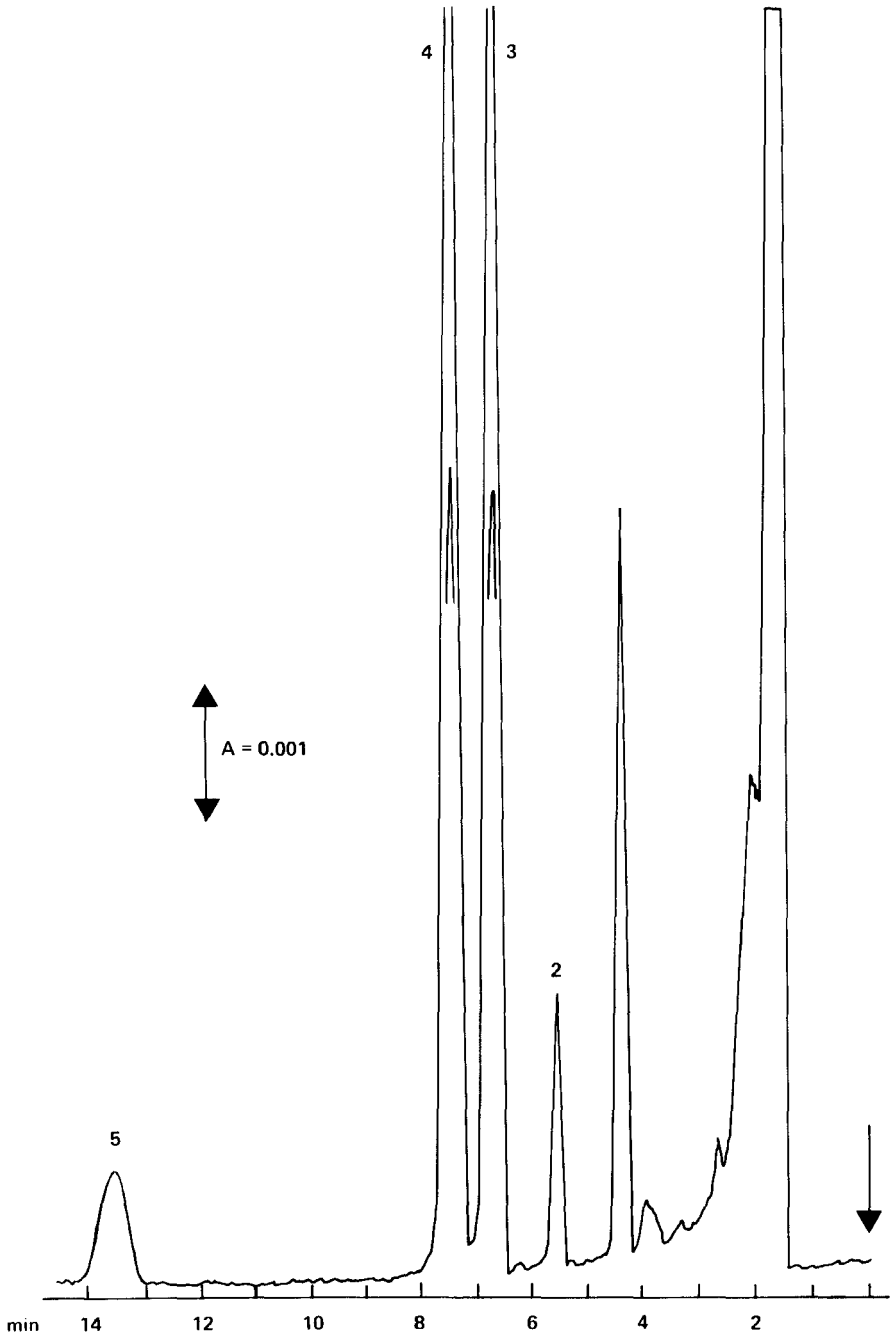


Fig. 10. Sample Chromatogram from Rat Brain. Analysis according to "Analytical Method". Rat brain diluted to 1/50. 1 Chlorpheniramine 100 ng/ml; 2 Zimelidine 6 ng/ml; 3 norZimelidine 81 ng/ml; 4 norZimelidine E-isomer (X) 200 ng/ml; 5 Primary amine (XI); Detector: Waters model 440 at 254 nm.

Routine Use of the Method

The analytical method has been used routinely for about 18 months and the columns have shown an excellent stability, having taken at least 40 samples a day for several months without any change of the chromatographic parameters. When signs of bad resolution appear, the chromatographic performance can be restored by the injection of stationary phase, normally 30 - 40 µl once a week is sufficient. A column can last for 12 - 18 months when treated in this way. A highly contaminated column can be recoated after washing off the stationary phase by elution with 100 - 150 ml of acetone or methanol.

The mobile phase (a reservoir of 1/2 l) is recycled continously for 5 days during the routine determinations. The content of impurities in the reservoir will of course increase, but no disturbances have been noted in the chromatograms; the rise in the baseline is easily corrected electronically. After 5 days the mobile phase is replaced by a freshly prepared one; equilibration time about 30 minutes.

The amines are injected as bases and will remove perchlorate from the stationary phase at the top of the column. No disturbances have, however, been found since the sample concentration is very low and the sample capacity of the ion-pair system on the column is high because of high counter ion concentration and low extraction constants (cf ref 16). The removed ions are furthermore continously replaced by ions from the mobile phase which, as shown in ref 26, contain a rather high concentration, 2×10^{-3}M, of perchlorate.

Problems with the stability will only arise if the temperature control is not adequate; a decrease in temperature causes aqueous microdrops to appear in the mobile phase making the baseline unstable.

TABLE 7

Influence of Blood Collection Tubes on Measured Plasma Levels

Blood samples from healthy volunteers participating in a pharmacokinetic study were collected in both kinds of tubes simultaneously and the samples were analyzed according to "Analytical method".

Vacu = VACUTAINER tubes
Veno = VENOJECT tubes
R = yield by Vacu / yield by Veno

The contents are given in ng/ml plasma

Patient nr	Time after dose (h)	ZIMELIDINE Veno	Vacu	R (%)	NorZIMELIDINE Veno	Vacu	R (%)
1	2	66	52	78.8	34	28	82.4
1	4	90	78	86.7	96	85	88.5
2	4	20	19	95.0	11	10	90.0
3	4	71	62	87.3	95	66	69.5
4	2	19	19	100.0	8	8	100.0
5	4	16	16	100.0	18	16	88.9
6	4	49	26	53.1	106	68	64.2
8	4	19	15	78.9	17	14	82.4
9	4	55	53	94.6	74	61	82.4

Collection of Blood Samples

A study on the effects of the collection tubes on the plasma levels of ZIMELIDINE and norZIMELIDINE was undertaken as a consequence of a paper by Cotham et al (38), who found that blood sam-

ples collected in VACUTAINER tubes gave spuriously lower concentrations of propranolol than samples collected in all glass tubes or in another commercial brand of collection tubes, VENOJECT. This is due to the presence of a substance in the stopper of the VACUTAINER tubes, identified as tris butoxyethyl phosphate by Piafsky and Borgå (39). The substance is dissolved in the sample and competes with propranolol for the binding sites in the plasma protein. The ratio between free and bound drug increases and results in a redistribution of propranolol in the blood involving the distribution of a larger amount of the drug to the erythocytes.

A comparison of VACUTAINER and VENOJECT tubes for collection of ZIMELIDINE samples is given in Table 7. The yields from the VACUTAINER tubes varies between 53 - 100 % and 64 - 100 % for ZIMELIDINE and norZIMELIDINE respectively. These compounds are protein bound in the range 70 - 90 % and they are also as mentioned above distributed between the blood cells and the plasma (37) and it is consequently highly probable that the mechanism related above for propranolol is operative also for these compounds.

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