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SEPARATION OF CYCLOBENZAPRINE FROM BIOLOGICAL SAMPLES AND ITS DETERMINATION BY THIN-LAYER CHROMATOGRAPHY FOLLOWED BY DENSITOMETRY

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SUMMARY

A sensitive method for the determination of cyclobenzaprine in biological samples is described. The extraction procedures used are different for urine and blood serum. The pH is a very important factor affecting the purity of the extract and the reproducibility of the recovery data, particularly for urine. By means of extraction, purification is achieved, while separation by thin-layer chromatography increases the specificity of the determination considerably. With a Vitatron TLD-100 densitometer, the substances separated can be measured as fluorescent spots from the thin-layer plates directly and quantitatively, with good reproducibility. The sensitivity is 1 ng and a linear relationship was obtained between 1 and 25 ng. Extensive recovery studies were made in view of the forthcoming clinical evaluation of cyclobenzaprine. A number of experiments showed that this method has so far produced better results than can be obtained with the most sensitive gas chromatographic detection methods.

INTRODUCTION

In the search for a specific assay to determine amitriptyline and nortriptyline when these drugs are combined with hypnotics and tranquillizers[1], a number of results were obtained which have led to the development of the assay procedure for cyclobenzaprine (MK-130) described here.

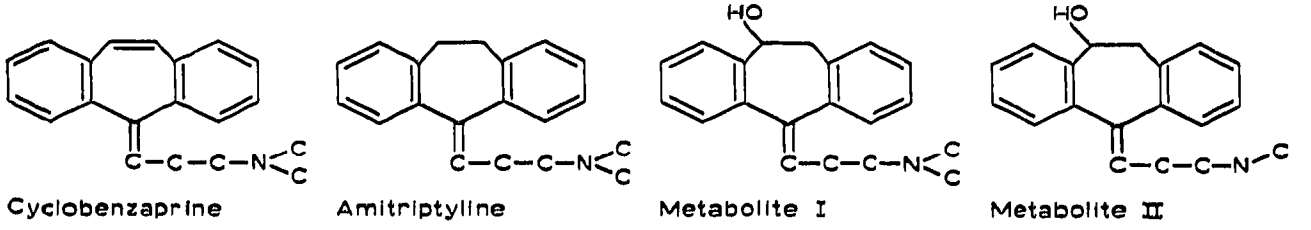
In 1958 and 1959, MK-130 was shown to have some activity in the treatment of depressive psychosis. It was, however, not as effective a tranquillizer as the standard phenothiazine, and not as effective an antidepressant as amitriptyline.

Within the past year, it has been demonstrated that MK-130 has a high consistent muscle relaxant activity in several animal models, exhibiting tonic skeletal muscle hyperactivity. At present, the compound is under extensive clinical testing, as it has been shown that MK-130 has the same consistent muscle relaxant activity in man.

Present clinical testing requires a study of the pharmacokinetics of the com-

pound. Therefore, an assay to determine MK-130 and/or its metabolites in human body fluids is required.

Cyclobenzaprine (MK-130, N,N-dimethyl-5 H-dibenzo[*a*,*d*]cycloheptenepropylamine hydrochloride) is very similar to amitriptyline in structure and physicochemical properties. It is therefore conceivable that the major metabolites are the 10-hydroxy compound (I) and the N-desmethyl-10-hydroxy compound (II).



Cyclobenzaprine Amitriptyline Metabolite I Metabolite II

During the development of an assay, a search was made for direct measurements on the thin-layer plate. Indirect methods, although often used for metabolic studies[2–6], are not sufficiently sensitive for determinations in blood. Based on results obtained earlier by densitometry, we compared the most sensitive gas–liquid chromatographic detection methods in a number of experiments (to be published).

PRELIMINARY STUDY OF INSTRUMENTAL ANALYSIS

Cyclobenzaprine can be separated from its metabolites by means of thin-layer chromatography. In this method, Merck Fertigplatten Kieselgel (Silica Gel G plates), size 20 × 20 cm, are used. The plates are heated in a drying oven for 30 min at 110° and samples are applied with a Hamilton dosing syringe, with absolute ethanol as the solvent. After application, the ethanol is evaporated off under a stream of nitrogen. Development takes place in a saturated tank, using an eluent of chloroform–methanol–glacial acetic acid (70:20:10). Before it was decided to use this eluent, more than 25 eluent systems were tested (see the section on the recovery of cyclobenzaprine from urine).

To convert cyclobenzaprine into a fluorescent compound, the TLC plate is immersed in 20% perchloric acid in ethanol–water (1:1), and subsequently heated at 110° in a drying oven for 5–7 min.

Immersion gives better results than spraying, with less background interference during densitometry. With standard solutions of cyclobenzaprine, under UV light of 350 nm, an orange-yellow fluorescence is obtained. Optimum excitation wavelength is achieved with a UVB filter (240–340 nm), and the exact emission wavelength of the fluorescent spots is 565 nm.

The Vitatron TLD-100 densitometer makes it possible to measure the intensity of the fluorescence of 0.01 μg of cyclobenzaprine with good reproducibility. This instrument (Fig. 1) is designed for the quantitative determination of irregularly shaped spots and spot patterns, where the distribution of molecules over the area of the spot is not necessarily homogeneous. The TLD-100 instrument scans the whole spot, signals are automatically summed, and the integrated value is automatically recorded[7,8].

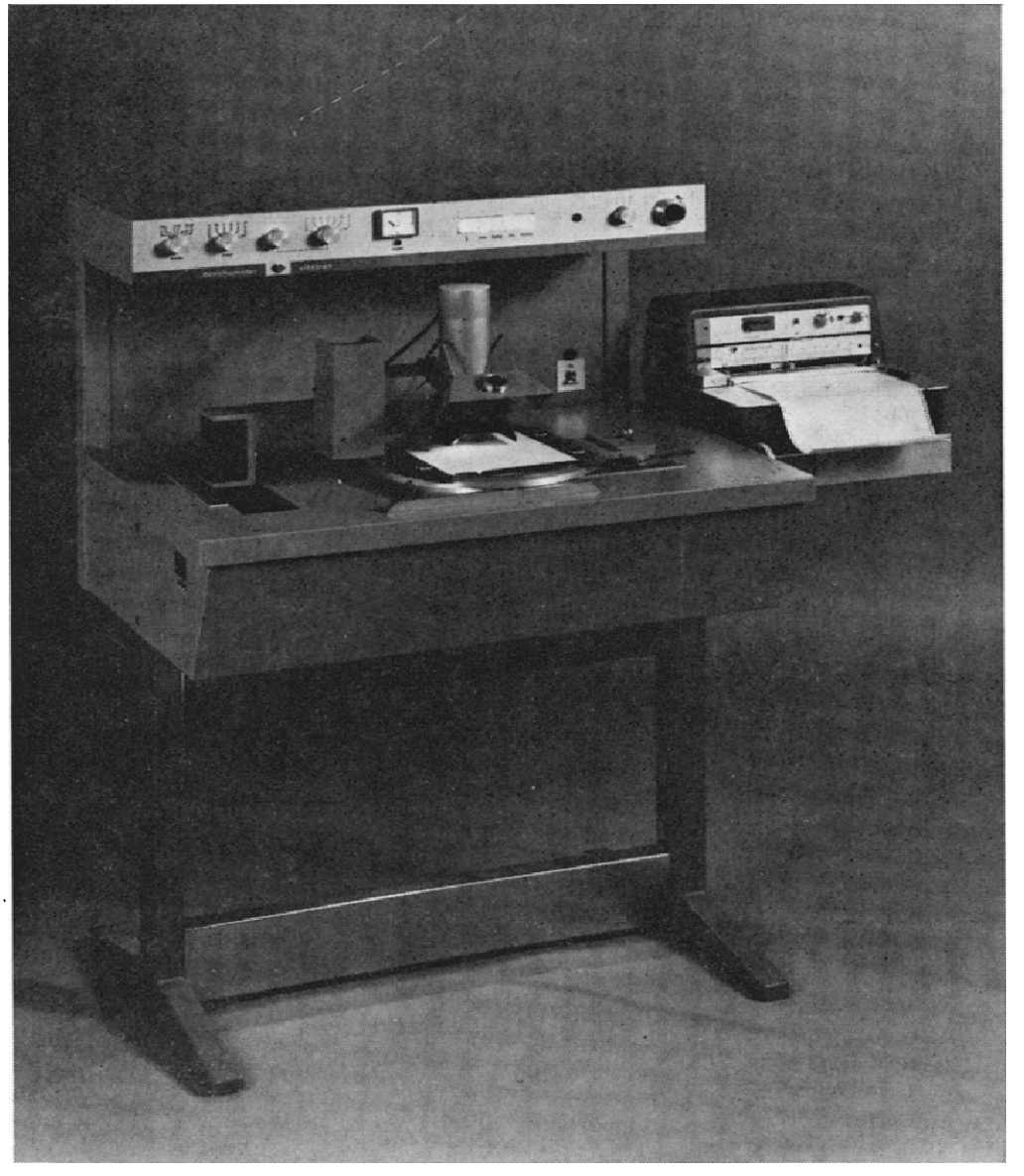


Fig. 1. Vitatron TLD-100 densitometer.

The operating conditions and settings of the densitometer are as follows:

Fluorescence	+ −
Light source	Mercury lamp
Filter (photomultiplier)	565 nm
Diaphragm	0.5
Level	f
Zero course	C6
Damping	2
Span	0.9
Filter (light source)	UVB (240–340 nm)
Scanning speed	1 cm/min
Swing	4–5 mm

Paper speed on recorder	1 cm/min
Sensitivity of integrator	6

During the preliminary study, ethanolic solutions of pure cyclobenzaprine hydrochloride were used in concentrations of 5, 10, 25, 50 and 75 ng per 10 μl. A 10-μl volume was always applied on to the TLC plate.

In Fig. 2a, the integrated values are shown in a densitogram. The calibration curves for 5, 10, 25, 50 and 75 ng amounts were not linear (Fig. 2b). The calibration curve shows a plateau effect above 25 ng. However, between 5 and 25 ng, the correlation between concentration and number of pulses (integrated peak area)

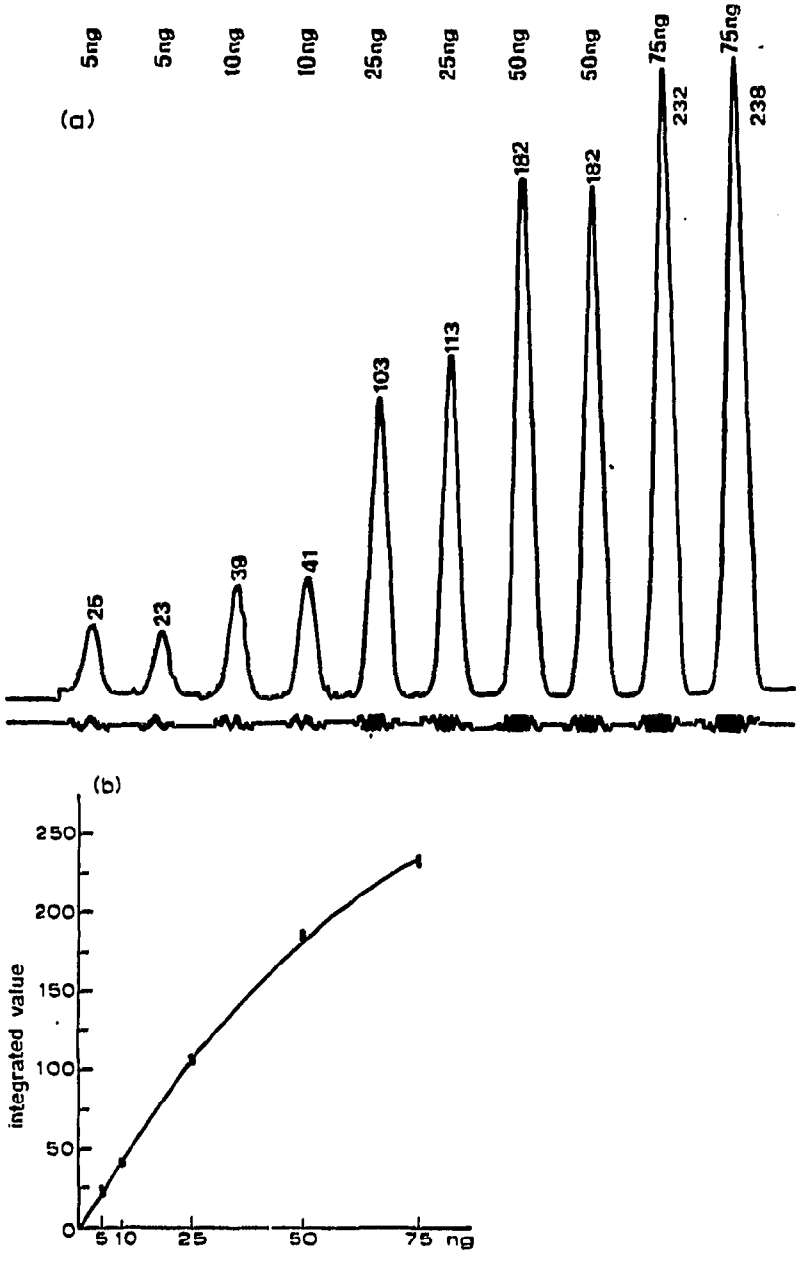


Fig. 2. (a) Densitogram of different amounts (5–75 ng) of pure cyclobenzaprine hydrochloride. (b) Calibration curve of cyclobenzaprine hydrochloride (5–75 ng).

warranted further investigation. In Fig. 3a the integrated values are shown in a densitogram. Further studies showed that the calibration curve is almost linear between 5 and 25 ng (Fig. 3b). The assay method appeared to be sensitive enough to differentiate the following concentrations: 1, 2, 5, 8 and 10 ng per 10 μl with a

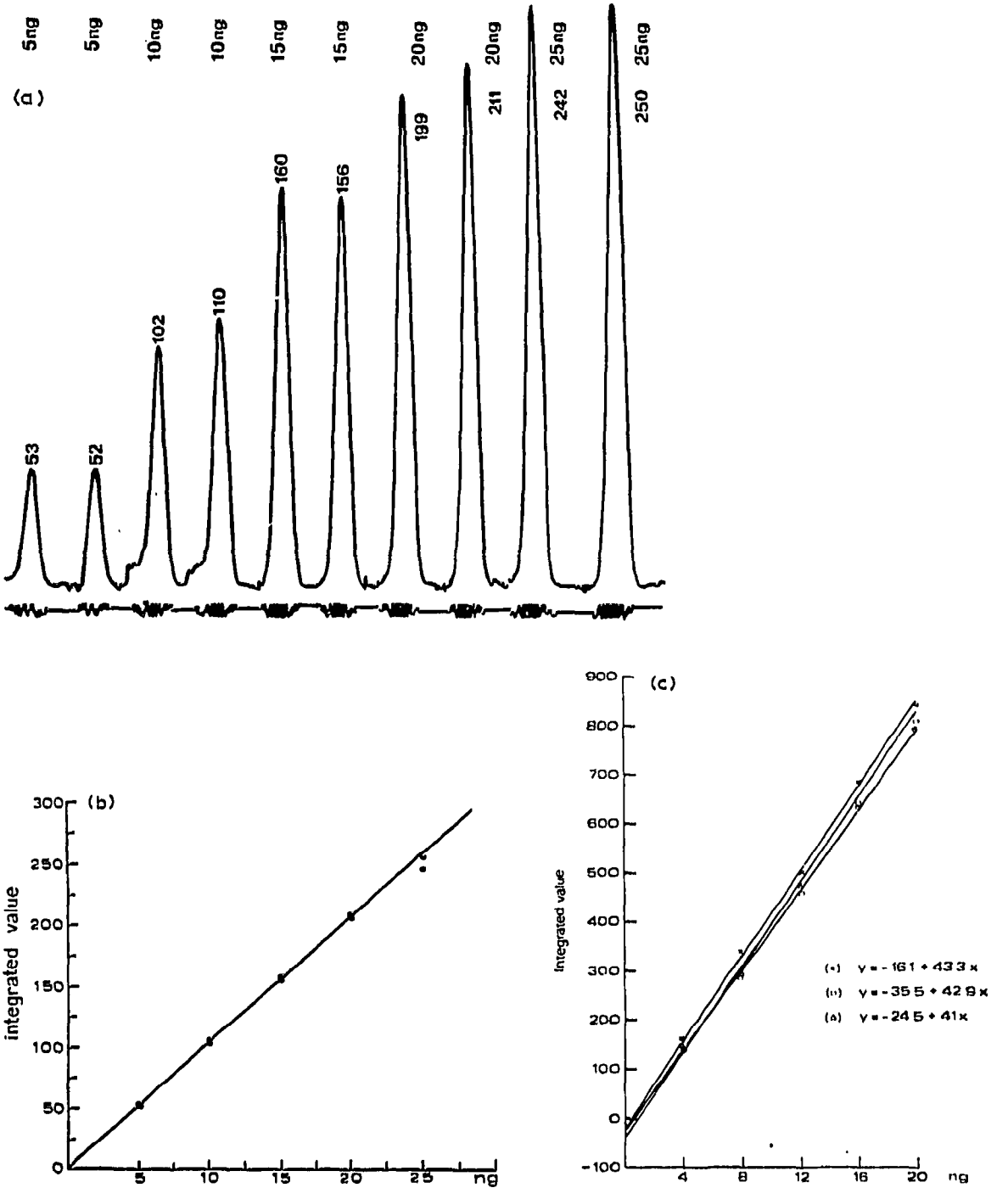


Fig. 3. (a) Densitogram of different amounts (5–25 ng) of pure cyclobenzaprine hydrochloride. (b) Calibration curve of cyclobenzaprine hydrochloride (5–25 ng). (c) Calibration curves of cyclobenzaprine hydrochloride obtained from different TLC plates.

TABLE I

STATISTICAL ANALYSIS OF RESULTS FROM THE DETERMINATION OF THE DEVIATION FROM LINEARITY

Series No.	Variables[a]						Values
1	g	−2	−1	0	1	2	$\Sigma g(y_1 + y_2) = 1024$
	y_1	51	110	165	201	250	
	y_2	44	117	166	216	262	$b = \frac{1024}{100} = 10.24$
	$g(y_1 + y_2)$	$-2(95) = -190$	$-1(227) = -227$	$0(331) = 0$	$1(417) = 417$	$2(512) = 1024$	$a = 4.6$ $F_{\text{lin.}} = 2.65$ $p > 0.05$
2	g	−2	−1	0	1	2	$\Sigma g(y_1 + y_2) = 852$
	y_1	45	81	133	176	214	
	y_2	45	89	134	190	204	$b = \frac{852}{100} = 8.52$
	$g(y_1 + y_2)$	$-2(90) = -180$	$-1(170) = -170$	$0(267) = 0$	$1(366) = 366$	$2(418) = 836$	$a = 1.3$ $F_{\text{lin.}} = 2.93$ $p > 0.05$
3	g	−2	−1	0	1	2	$\Sigma g(y_1 + y_2) = 953$
	y_1	54	96	144	192	251	
	y_2	56	110	158	211	237	$b = \frac{953}{100} = 9.53$
	$g(y_1 + y_2)$	$-2(110) = -220$	$-1(206) = -206$	$0(302) = 0$	$1(403) = 403$	$2(488) = 976$	$a = 7.9$ $F_{\text{lin.}} = 0.12$ $p \gg 0.05$
4	g	−2	−1	0	1	2	$\Sigma g(y_1 + y_2) = 716$
	y_1	43	84	132	164	182	
	y_2	46	86	145	168	184	$b = \frac{716}{100} = 7.16$
	$g(y_1 + y_2)$	$-2(89) = -178$	$-1(170) = -170$	$0(277) = 0$	$1(332) = 332$	$2(366) = 732$	$a = 16$ $F_{\text{lin.}} = 15.79$ $p < 0.01$
5	g	−2	−1	0	1	2	$\Sigma g(y_1 + y_2) = 819$
	y_1	57	86	134	174	216	
	y_2	58	89	152	182	218	$b = \frac{819}{100} = 8.19$
	$g(y_1 + y_2)$	$-2(115) = -230$	$-1(175) = -175$	$0(286) = 0$	$1(356) = 356$	$2(434) = 868$	$a = 13.7$ $F_{\text{lin.}} = 1.17$ $p > 0.05$

linear curve. The number of pulses is linearly related to the concentration in the concentration range 1–25 ng per 10 μl.

Duplicate determinations were carried out on solutions containing 5, 10, 15, 20 and 25 ng. It appeared that the values of the original and duplicate determinations were almost always the same. The regression coefficients and F and p values are given for the deviation from linearity (Table I). Series number 4 is a failure. From the others, the deviation from linearity is not statistically significant at a threshold value of 5 %.

Eventually, we chose a more sensitive adjustment of the TLD-100 densitometer. This resulted in a linearity in the complete range from 4 to 20 ng. The degree of fluorescence may vary, but this does not affect the linearity (Fig. 3c). Fig. 3c illustrates the linearity of three single determinations, also on different thin-layer plates, of solutions containing 4, 8, 12, 16 and 20 ng.

To provide confirmation, a trained analyst obtained the results on three unknown solutions given in Table II.

TABLE II

MANUAL ASSAY OF THREE UNKNOWN SOLUTIONS

Solution	*Concentration in sample (ng per 10 µl)*	*Concentration determined by assay (ng per 10 µl)*
I	40	43
II	125	124
III	10	10

RECOVERY OF CYCLOBENZAPRINE FROM URINE

Instrumental analysis with the densitometer enables cyclobenzaprine to be determined directly on the thin-layer chromatogram provided that it is measured in the linear area of the concentration fluorescence curve. This linear area occurs below 25 ng of cyclobenzaprine. The blood levels and urinary concentrations to be anticipated are favourable as regards this range, so that extraction from blood and urine provides concentrations within this linear area. Higher concentrations in the extract can be brought within the area desired by dilution. It is necessary to carry out a recovery study for the extraction, as the extraction does not necessarily give a yield of 100%, however accurately it may be carried out. It is less important to obtain an almost 100% yield than to have a reliable and reproducible recovery, so that later one specific recovery factor can be used in the determinations.

In searching for an extraction method that is both reproducible and produces as high a yield as possible, we took into account the well known similarities in chemical and physical behaviour of cyclobenzaprine to amitriptyline. Unfortunately, little uniformity is to be found in the literature with respect to the extraction of amitriptyline[9–15], and recovery studies are hardly, if ever, mentioned. Consequently, it was necessary to carry out preliminary studies to obtain more insight into factors such as pH, extraction agents, desiccating agents, purification, and testing conditions of thin-layer chromatography.

Preliminary study

The standard solutions were prepared by adding 100 μl of a solution of cyclobenzaprine in ethanol (50 μg/ml) to 10 ml of urine. The extraction of 5 μg of cyclobenzaprine from 10 ml of urine was carried out with initial purification by extraction with chloroform at pH 1–2, followed by extraction with three 20-ml volumes of chloroform at pH 9. The chloroform was shaken with magnesium sulphate and activated carbon, and evaporated on a water-bath after filtration; the last portion was removed in a tube attached to a rotating film evaporator. The residue was dissolved in 1 ml of ethanol and 10 μl of this solution were applied to the thin-layer plate. On the chromatogram, there was no spot at the R_F value of cyclobenzaprine, and tests with higher concentrations of cyclobenzaprine did not produce any results with the above method. A search for the cause of this failure gave the following results.

Activated carbon must be used to purify the cyclobenzaprine after extraction because cyclobenzaprine is markedly adsorbed by activated carbon. The extraction of cyclobenzaprine from water with the 20-ml volumes of chloroform at pH 9 resulted in a low recovery (*ca.* 30%). To increase the recovery, 5 μg of cyclobenzaprine were added to 10 ml of urine and extracted with five 20-ml volumes of chloroform at pH $\geqslant$ 10. The chloroform was dried over magnesium sulphate. By using thin-layer chromatography, a good separation was obtained without contaminations at the R_F value of cyclobenzaprine. The recovery was about 60–70%. The yield could be increased: extraction with six 25-ml volumes of chloroform resulted in a recovery of 80–90%, while recovery from water produced a yield of 95%. This effect is probably due to the partial elimination of the cyclobenzaprine spot on the thin layer owing to varying extents of contamination in the extract, so that no reproducible values could be obtained. The recoveries varied daily from 60 to 95%. Initial purification by extraction at pH 1 with chloroform, followed by the usual procedure, did not give any result. Cyclobenzaprine also appeared to be extractable as the hydrochloride salt, but the recoveries were low (60–70%). Although cyclobenzaprine has a pK_b value of 8.4 and at high pH values ($>$10.4) it is completely in the form of the free base (Fig. 4, titration curve), a number of extractions were carried out at pH 6.8[16]. Better results were obtained, so that it was decided to continue working at pH 6.8.

To be sure the best results were being obtained, some other experiments analogous to the well known extraction methods for amitriptyline were carried out. The extraction method for amitriptyline described by ESCHENHOF AND RIEDER[2], using benzene at pH 9 after initial purification with benzene at pH $<$ 3, gave even lower recoveries (*ca.* 10%). Following the work of HERMANN AND PULVER[17] we extracted cyclobenzaprine with 1,2-dichloroethane in urine buffered with 1 *N* sodium carbonate. It was found that one extraction with 150 ml of chloroform using a shaker for 20 min instead of the procedure of six extractions with 25 ml of chloroform resulted in recoveries of 39 and 31%. The same procedure carried out with 1,2-dichloroethane instead of chloroform was not successful and gave lower recoveries. On the chromatogram, the cyclobenzaprine spot was disturbed by a large fluorescent spot with tailing, so that cyclobenzaprine could hardly be distinguished. A further extraction with petroleum ether[18] was carried out. Apart from a markedly contaminated chromatogram, the extraction method was inadequate because a very low recovery of cyclobenzaprine was obtained.

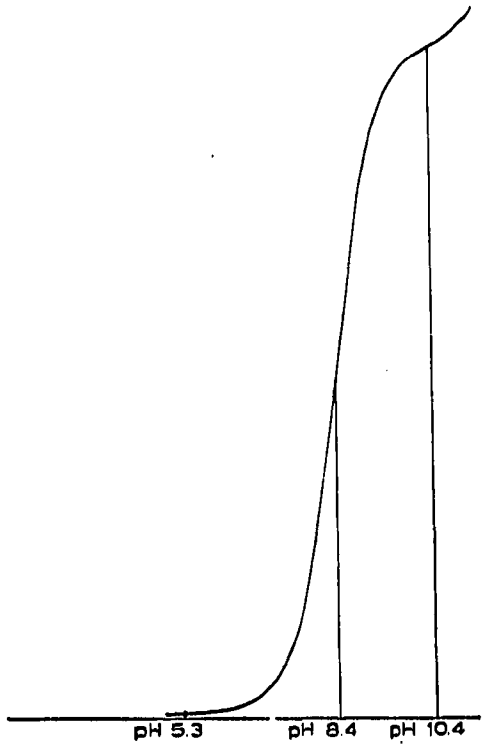


Fig. 4. Titration curve of cyclobenzaprine.

Thus the extraction of cyclobenzaprine from urine was difficult. However, it is now possible to determine cyclobenzaprine quantitatively in human urine. It must be remembered, however, that pH is a very important factor affecting the purity of the extract and the reproducibility of the recovery data as follows:

(1) Above pH 10, extraction gives a high recovery of *ca.* 95%. Cyclobenzaprine is then present as the free base. Impurities interfere to such an extent that the reproducibility is minimal with a variation of *ca.* 35 %.

(2) At pH 1.0 the recovery is minimal and the variation considerable.

(3) It was found that the best results were obtained by carrying out the extraction in urine buffered to pH 6.8. The recovery is then lower than in (1), but the results vary little. Moreover, the impurities that remain are well separated from cyclobenzaprine on the chromatogram.

Extraction procedure in urine

A 10-ml volume of urine is adjusted to pH 6.8 with 0.1 *N* NaOH–0.1 *N* HCl, using a magnetic stirrer, and 5 ml of Sorensen buffer of pH 6.8 are added. (In our recovery studies, 2 μg of cyclobenzaprine were then added to the buffered urine.) The urine is extracted with six 25-ml volumes of chloroform, subsequently dried over magnesium sulphate and filtered, and the chloroform is distilled off in a water-bath at 60°.

The concentrate (with residual amounts of chloroform) is placed in a centrifuge tube with a ground-glass stopper and evaporated in a rotating film evaporator (Rotavapor). The residue is dissolved in 1.0 ml of absolute ethanol, and 10 μl of this solution are applied to the TLC plate and treated as discussed under INSTRUMENTAL ANALYSIS.

Results

Table III summarizes the recovery results from 25 determinations on 8 days according to the above procedure carried out on human urine containing 2 μg of cyclobenzaprine per 10 ml.

Thus, the extraction of cyclobenzaprine from urine containing an unknown concentration of cyclobenzaprine as well as the subsequent densitometry have to be carried out in duplicate.

In order to study the effect of the cyclobenzaprine concentration on recovery, a number of experiments were performed in which various amounts of cyclobenzaprine were added to the urine. The results obtained proved that there was hardly any difference in recovery at the various concentrations. Fig. 5 shows a densito-

TABLE III

RECOVERY OF CYCLOBENZAPRINE FROM URINE ON CONSECUTIVE DAYS

Day	*Recovery* (%)[a]
1	80, 80, 90 (83.3)
2	77, 77, 74 (76)
3	83, 87, 84 (84, 67)
4	77, 77, 73 (75, 67)
5	84, 84, 88 (85, 3)
6	82, 84, 86 (84)
7	79, 76, 78 (77, 67)
8	80, 86, 83 (83)

[a] Standard deviation of daily means = 4.0%. Average recovery (in parentheses) = 81.2 ± 4.0%.

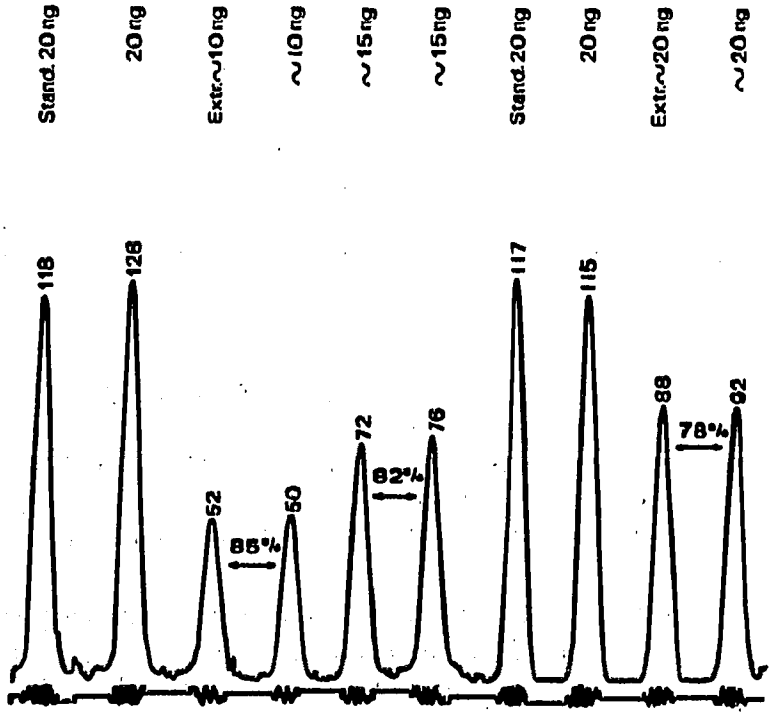


Fig. 5. Densitogram of different amounts (10–20 ng) of pure cyclobenzaprine hydrochloride and cyclobenzaprine hydrochloride isolated from urine.

gram representing the results of one of the recovery experiments in which 1.0, 1.5 and 2.0 μg of cyclobenzaprine were added to 10 ml of urine.

RECOVERY OF CYCLOBENZAPRINE FROM SERUM

In blood, cyclobenzaprine can be protein-bound, which will therefore have to be taken into account in the determinations. Several methods can be used for breaking the protein bond, such as ultrasonic vibrations and denaturation by (fractionated) salt precipitation. Preference has, however, been given to complete denaturation with absolute ethanol. The ethanol has a dual function: the denaturation of the proteins, and the extraction of cyclobenzaprine. In our recovery studies, pure cyclobenzaprine was added to 1 ml of serum. A mixture of 1 ml of serum and 4 ml of absolute ethanol is shaken on the vibromixer for 2 min, and then heated in a water-bath for 30 min at 60°. This results in denaturation and precipitation of serum proteins. Ethanol is also used as the solvent. After cooling, the ethanol layer is separated by centrifuging for 15 min at 3200 r.p.m. Extraction of the residue is repeated once.

Subsequently, both of the ethanol extracts are concentrated by evaporation in a rotary evaporator. The residue is dissolved in 1.0 ml of absolute ethanol, and from this solution aliquots can be applied directly on to the TLC plate. Further purifications are unnecessary because cyclobenzaprine is separated from impurities with chloroform–methanol–glacial acetic acid (70:20:10) as eluent.

Table IV summarizes the results of a series of recovery experiments performed

TABLE IV

RECOVERY (%) OF CYCLOBENZAPRINE FROM SERUM ON CONSECUTIVE DAYS

Day	*Cyclobenzaprine concentration (ng per 10 µl)*			
	5	*10*	*15*	*20*
1	—	—	—	99 100 85
2	—	—	—	90 100 94
3	—	88 96	— —	100 90
4	—	92	—	94
5	—	90 100	— —	96 93
6	100 100	95 94	—	—
7	90 98	93 95	— —	— —
8	100	96	95	98
9	98	94	100	88

Over-all recovery (n = 32) 95.0 ± 4.3%. Range = 85–100%.

with concentrations varying from 0.5 to 2 μg per ml of serum. In Fig. 6 the result of a recovery experiment is shown in a densitogram.

The anticipated concentration of cyclobenzaprine in blood from patients undergoing treatment is most likely to be lower than that mentioned above. Therefore, a study was undertaken to demonstrate that lower concentrations can also be measured. To 1.0 ml of horse serum was added 1 μg of cyclobenzaprine. After extraction, the residue was dissolved in absolute ethanol, and the recoveries are given in Table V.

These results do suggest that anticipated blood levels (probably between 10 and 100 ng/ml) can be measured. The residue can be dissolved, not in 1.0 ml, but in 0.1 ml of absolute ethanol, and/or by using more than 1 ml of serum and/or spotting more than 10 μl on the TLC plate.

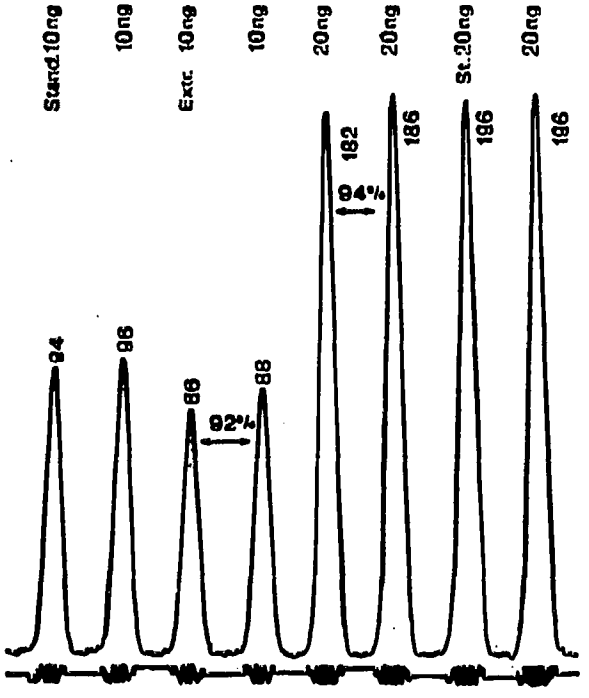


Fig. 6. Densitogram of different amounts (10–20 ng) of pure cyclobenzaprine hydrochloride and cyclobenzaprine hydrochloride isolated from serum.

TABLE V

RECOVERY OF CYCLOBENZAPRINE FROM HORSE SERUM

Volume of ethanol used to dissolve residue (ml)	*Volume applied on TLC plate (μl)*	*Recovery (%)*
1.0	10	100
0.5	5	86
0.5	5	96
0.1	1	88

METABOLISM OF CYCLOBENZAPRINE

A study of the two metabolites was performed on a number of samples, while amitriptyline and nortriptyline were superficially compared with cyclobenzaprine. The separation of these five compounds on the thin-layer chromatogram was good, as can be seen from the R_F values in Table VI.

TABLE VI

R_F VALUES OF CYCLOBENZAPRINE; AMITRIPTYLINE, NORTRIPTYLINE AND THEIR METABOLITES

Substance	*R_F value*
Cyclobenzaprine	0.51
Metabolite I	0.34
Metabolite II	0.10
Amitriptyline	0.57
Nortriptyline	0.24

The eluent system used was ethyl acetate–*n*-hexane–concentrated ammonia (90:10:12). It should be noted that the fluorescence with cyclobenzaprine was stronger than that with the other compounds, which is probably caused by the double bond between C_{10} and C_{11}. However, the two possible major metabolites may be transformed into cyclobenzaprine and desmethylcyclobenzaprine by boiling an acidic solution of the residue for 30 min, rendering them equally sensitive to determination.

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REFERENCES

1 D. B. FABER, to be published.
2 E. ESCHENHOF AND J. RIEDER, *Arzneim.-Forsch.*, 19 (1969) 957.
3 R. E. MCMAHON, F. J. MARSHALL, H. W. CULP AND W. M. MILLER, *Biochem. Pharmacol.*, 12 (1963) 1207.
4 H. B. HUCKER, *Pharmacologist*, 4 (1962) 171.
5 G. B. CASSANO, S. E. SJÖRSTRAND AND E. HANSSON, *Psychopharmacologica (Berlin)*, 8 (1965) 1.
6 M. E. AMUNDSON AND J. A. MANTHEY, *J. Pharm. Sci.*, 55 (1966) 277.
7 H. J. KOOPMANS, *Quantitative in situ Analysis of Coloured or Artificially Coloured Compounds on Thin-Layer Adsorbent Material by means of the Vitatron Densitometer TLD-100 in Transmission Measurements*, Application Laboratory of Vitatron Scientific Instruments N.V., Dieren, The Netherlands, 1969.
8 J. GOLDMAN AND R. R. GOODALL, *J. Chromatogr.*, 47 (1970) 386.
9 E. C. MUNKSGAARD, *Acta Pharmacol. Toxicol.*, 27 (1969) 129.
10 G. L. CORONA AND R. MAFFEI FACINO, *Biochem. Pharmacol.*, 17 (1968) 2045.

11 R. MAFFEI FACINO AND G. L. CORONA, *Farmaco*, 23 (1968) 366.
12 M. AASBERG, B. CRONHOLM, F. SJÖQVIST AND D. TUCK, *Br. Med. J.*, 4 (1970) 18.
13 F. SJÖQVIST, F. BERGLUND, O. BORGÅ, W. HAMMER, S. ANDERSSON AND C. THORSTRAND, *Clin. Pharmacol. Ther.*, 10 (1969) 826.
14 J. E. WALLACE AND E. V. DAHL, *J. Forensic Sci.*, 12 (1967) 484.
15 J. COCHIN AND J. W. DALY, *J. Pharmacol. Exp. Ther.*, 139 (1963) 160.
16 R. H. DROST AND J. F. REITH, *Pharm. Weekbl.*, 105 (1970) 1129.
17 B. HERMANN AND R. PULVER, *Arch. Int. Pharmacodyn. Ther.*, 126 (1960) 454.
18 R. A. BRAITHWAITE AND J. A. WHATLEY, *J. Chromatogr.*, 49 (1970) 303.