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QUANTITATIVE THIN-LAYER CHROMATOGRAPHY FOR ROUTINE DETERMINATION OF NOMIFENSINE AND ITS METABOLITES IN HUMAN URINE

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SUMMARY

For pharmacokinetic studies with nomifensine, a thin-layer chromatographic (TLC) assay for human urine was introduced. Following acid cleavage of the N-glucuronides, nomifensine and its three main metabolites (M1, M2 and M3) were extracted at pH 10. An aliquot was transferred on to a silica gel plate. After chromatography, irradiation led to intense fluorescent yellow products, which were evaluated using a chromatogram spectrophotometer. Calibration graphs were defined by single parameters of non-linearity. The method is practicable, selective and accurate with detection limits of 0.2 μg/ml in urine for the four compounds of interest and can be used for assaying samples up to 24 h following dosage. Total nomifensine urine levels correlated well with those determined by a previous radioimmunoassay method. From cumulative excretions of nomifensine, complete relative bioavailability of a capsule formulation vs. oral solution was shown. Further, sex independence of urine excretion was demonstrated.

Pharmacokinetic data were computed using a two-compartment open model for nomifensine and its potent metabolite M1 or a one-compartment open model for M2 and M3.

INTRODUCTION

Nomifensine maleate (Alival®, Merital®) is a recently introduced antidepressive drug (Fig. 1). For pharmacokinetic purposes a practicable and selective analytical method is required for the determination of the parent drug and its metabolites M1, M2 and M3 in urine.

Previously published methods employed ^{14}C-labelled drug [1, 2], radioimmunoassay (RIA) [3], gas—liquid chromatography (GLC) [4—7] and high-performance liquid chromatography (HPLC) [8]. Using specific RIA, only the parent compound is determined in serum and urine. GLC for the

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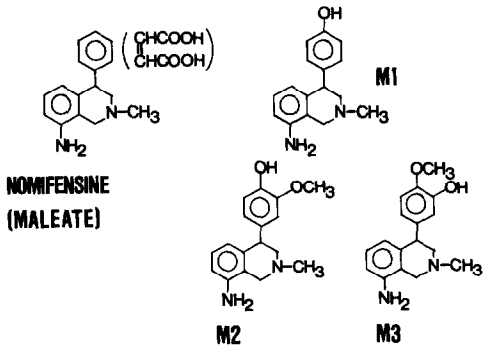

Fig. 1. Structures of nomifensine and metabolites.

determination of nomifensine in plasma requires derivatization steps. Using HPLC, nomifensine, the potent [9] metabolite M1 and the sum of M2 and M3 were determined only in serum. Total nomifensine plasma half-lives of approximately 2 h [3, 5, 7, 8] or approximately 4 h [4, 6] have been published. Total metabolite half-lives of approximately 1—2 h have been reported by Uihlein and Hajdu [8].

In urine, the glucuronides [2] of nomifensine and its metabolites are cleaved prior to analysis. On a TLC plate, fluorescent and intense yellow products of the separated compounds are formed on irradiation, which enhances the intensity of the spots and the sensitivity as well as the selectivity of the TLC method.

Analytical data have been calculated for the free base and denoted as total compound.

EXPERIMENTAL

Materials

Reagents. Analytical-reagent grade chemicals were used unless indicated otherwise. The reagents used were 0.1 mol/l hydrochloric acid, 0.1 mol/l carbonate buffer (pH 10), ethyl acetate, ethanol and concentrated ammonia solution (25%). Reference substances were supplied by Dr. K. Schmitt of Hoechst AG (nomifensine and M1 as maleates, M2 and M3 as free bases). The solvent system was ethyl acetate—ethanol—concentrated ammonia (8:2:0.2).

Equipment. A Zeiss KM 3 chromatogram spectrophotometer with micro-optics and a Servogor (Metrawatt) recorder were used. Separation was performed on silica gel HPTLC plates (No. 5641; E. Merck, Darmstadt, G.F.R.) in a Camag twin-trough HPTLC chamber, 20 × 10 cm (No. 25254). For sample clean-up and spotting, a Vortex mixer, a centrifuge, glass-stoppered tubes (ca. 8 ml) and a Desaga Autospotter* were used.

Sample preparation

Hydrolysis of N-glucuronides. Nomifensine-N-glucuronide and N-glucuronides of metabolites M1, M2 and M3 were hydrolysed by incubation in

*Modified version: Tygon tubes of larger diameter [Technicon, flow-rated, code: 116-0549-09 (white)] and 60 cm long Hostaflon tubes were used.

TABLE I

AMOUNTS OF MATERIALS USED IN HYDROLYSIS REACTION

Dose of nomifensine maleate (mg)	Equivalent to nomifensine (mg)	Urine used (μl)	0.1 mol/l HCl added (μl)
200	134.5	100	10
100	67.2	200	20
50	33.6	500	50
25	16.8	1000	100

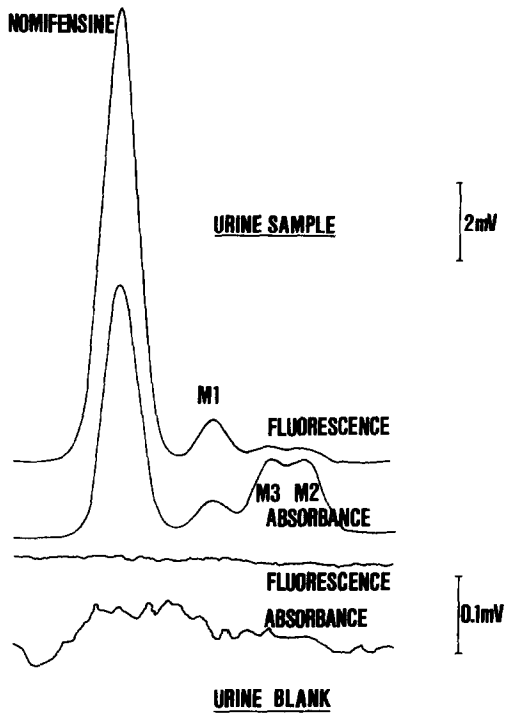

Fig. 2. Chromatography of urine extracts from a subject who had received 75 mg of nomifensine maleate orally. Urine sampled 0—5 h after application, containing 80 μl/ml of total nomifensine and 12 μg/ml of total M1, M2 and M3. A blank is shown for comparison.

0.1 mol/l hydrochloric acid. To cover the optimal range of the calibration graph, different aliquots of urine were used according to the scheme shown in Table I. The solution was incubated for 2 h in the dark at room temperature, then the pH was readjusted to pH 10 (Fig. 2) by adding 1 ml of 1 mol/l carbonate buffer (pH 10).

Extraction. The mixture was extracted with 5 ml of ethyl acetate for 30 sec on a Vortex mixer and centrifuged for 5 min. An aliquot of 100 μl (ca. 2%) of the organic phase was transferred for spotting.

Sample spotting. Using the Desaga Autospotter, 75 μl were applied on to the

HPTLC plate as a series of consecutive droplets of approximately 100 nl each. As each droplet was evaporated before the next one fell, a narrow spot was obtained suitable for HPTLC. Simultaneously, up to twelve extracts of samples, five standards and three controls were positioned on the plate (parameters: spotting speed, "2"; heating, "2"; ventilation,"2").

Chromatography

The twin-trough HPTLC developing chamber contained 10 ml of the solvent in one compartment. Chromatography was carried out at room temperature in the dark and without solvent saturation of the chamber. Within the developing time of 20 min, the solvent front moved 5 cm. Under UV light (254 nm), the HPTLC plate was heated on a heating plate for 2 h at 70°C. In this way, the parent compound and its metabolites were transformed into intense fluorescent yellow products [1].

The R_F values were as follows: nomifensine, 0.55; M1, 0.50; M2, 0.40; and M3, 0.45.

Evaluation

Measurement of nomifensine and M1 was based on their fluorescence; an excitation wavelength of 313 nm (Hg) and a secondary filter cut-off wavelength of 460 nm (FL 46) were used. In contrast, M2 and M3 were quantitated by their absorbance in the visible range (410 nm) (Fig. 2).

Calibration

Calibration functions [10] were determined for each compound from the peak heights of the standards:

$$C = \frac{C_{max} \cdot E_{rel} \cdot K_m}{1 - E_{rel} + K_m}$$

where C_{max} = maximum calibration standard, E_{rel} = peak height/maximum peak height and K_m = parameter of non-linearity. K_m was obtained from a Hofstee plot following normalization of peak heights and concentrations (Fig. 3).

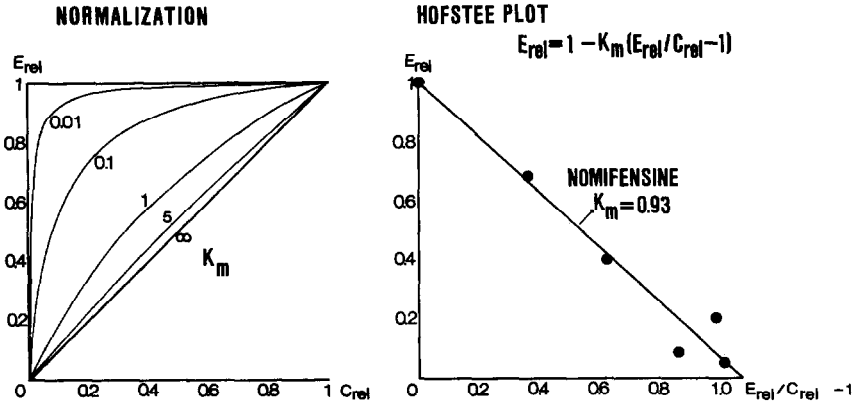

Fig. 3. Determination of K_m non-linearity parameter from a Hofstee plot.

RESULTS AND DISCUSSION

Physico-chemical properties of nomifensine and its metabolites

A knowledge of the physico-chemical properties of any compound is essential for obtaining optimal conditions for extraction from body fluids and for choosing the most suitable conditions of measurement.

Partition in octanol—water was studied in the pH range 3—12 (Fig. 4). At pH 8—10, maximal partition coefficients of 79 ± 9 were found for nomifensine (75 in diethyl ether—buffer [11]), 50 for M1 and 26 for M2.

In all instances pK_1 = 2.1 ± 0.1 (aminophenyl) was calculated from pH-dependent UV absorbances at 281 nm. For nomifensine, pK_2 = 7.0 ± 0.1 (amine) from aqueous titrations, equal to pK_2 = 7.1 ± 0.2 from the partition study. For M1, pK_2 = 7.4 ± 0.1 and pK_3 = 10.1 ± 0.1 (phenol) from titrations, equal to pK_2 = 7.5 and pK_3 = 10.1 from partition data. For M2, pK_2 = 7.5 and pK_3 = 10.2 were calculated from partition data.

Assay performance characteristics [12—14]

The compounds were mixed with blank urine at seven concentrations over the analytical ranges indicated in Table II. Each mixture was split into six portions of 0.5 ml, so that six equal series were formed. Each series was then analysed in turn so that a total of six independent analytical results were available for each concentration.

Quality criteria of an analytical method are selectivity, precision, sensitivity

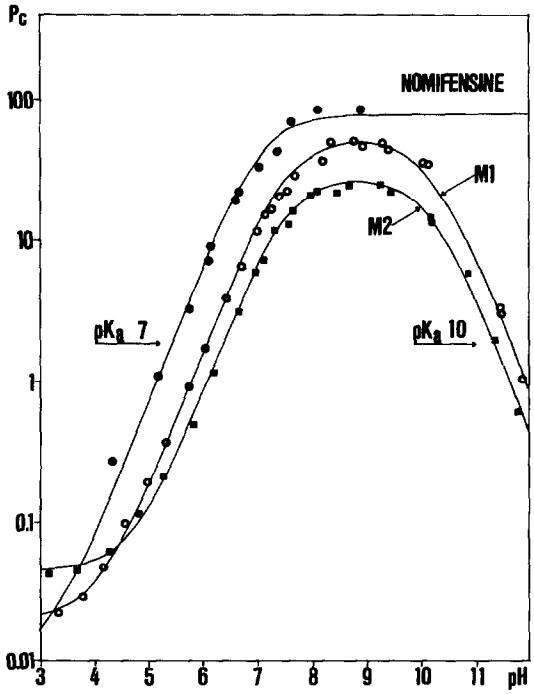

Fig. 4. Partition coefficients of nomifensine and metabolites in octanol—water.

TABLE II

NOMIFENSINE, M1, M2 AND M3 DETERMINATION BY TLC, RECOVERY AND INTRA-ASSAY PRECISION

$n = 6$; concentrations in μg/ml (mean ± S.D.).

Nomifensine		M1		M2		M3	
Added	Found	Added	Found	Added	Found	Added	Found
134.5	134.5 ± 1.6	34.3	34.2 ± 0.08	50.0	50.0 ± 0.57	50.0	50.0 ± 0.12
67.2	67.2 ± 1.4	13.7	14.5 ± 0.33	20.0	20.0 ± 0.39	20.0	20.6 ± 0.48
33.6	33.4 ± 0.64	6.9	6.7 ± 0.35	10.0	10.1 ± 0.20	10.0	9.9 ± 0.22
13.4	14.0 ± 0.31	3.4	3.4 ± 0.05	5.0	4.9 ± 0.10	5.0	4.7 ± 0.18
6.7	6.3 ± 0.18	1.4	1.3 ± 0.12	2.0	1.6 ± 0.21	2.0	1.6 ± 0.12
3.4	3.4 ± 0.19	0.7	0.7 ± 0.08	1.0	0.9 ± 0.15	1.0	0.9 ± 0.17
1.3	1.4 ± 0.10	0.3	0.3 ± 0.05	0.5	0.4 ± 0.12	0.5	0.5 ± 0.14
0	0	0	0	0	0	0	0
Accuracy (μg/ml):							
0.01 ± 0.31		0.06 ± 0.34		−0.09 ± 0.16		−0.04 ± 0.32	

TABLE III

NOMIFENSINE, M1, M2 AND M3 DETERMINATION BY TLC, INTER-ASSAY PRECISION

Control urines between August and November 1980; concentration in μg/ml (mean ± S.D.); C.V. = coefficient of variation.

Compound	Control 1	Control 2	Control 3
Nomifensine	65.9 ± 2.9 (C.V. 4%)	12.9 ± 1.3 (C.V. 10%)	3.4 ± 0.3 (C.V. 9%)
M1	6.8 ± 0.5 (C.V. 7%)	1.4 ± 0.2 (C.V. 14%)	0.34 ± 0.10 (C.V. 29%)
M2	9.5 ± 0.7 (C.V. 7%)	2.0 ± 0.3 (C.V. 15%)	0.49 ± 0.07 (C.V. 14%)
M3	9.7 ± 0.9 (C.V. 9%)	2.0 ± 0.3 (C.V. 15%)	0.47 ± 0.07 (C.V. 15%)

and accuracy. These parameters were derived from the analytical results given in Table II. As regards selectivity, the assay is free from interferences for all substances (Fig. 2).

Mean concentrations measured were linearly correlated with the standard deviations (S.D.s) and in this way the intra-assay precision was defined. Sensitivity was expressed as the detection limit (D.L.) and was taken as the intercept × 2. For nomifensine, precision = 1.9% of the result + 0.07 μg/ml, D.L. = 0.2 μg/ml; for M1, precision = 2.1% of the results + 0.07 μg/ml, D.L. = 0.2 μg/ml; for M2, precision = 1.2% of the result + 0.12 μg/ml, D.L. = 0.2 μg/ml; and for M3, precision = 1.6% of the result + 0.11 μg/ml, D.L. = 0.2 μg/ml.

The inter-assay precision was tested on three spiked control urines over a 4-month period. The results are presented in Table III.

Accuracy was considered to be the deviation (bias) at the mean value of the concentrations found from the concentrations mixed with blank urine. In all instances the average accuracy was < 0.1 μg/ml. Regression coefficients were greater than 0.9996.

Accuracy was further tested by comparison with radioimmunoassay. Urine samples collected after oral administration of 50 mg of nomifensine maleate

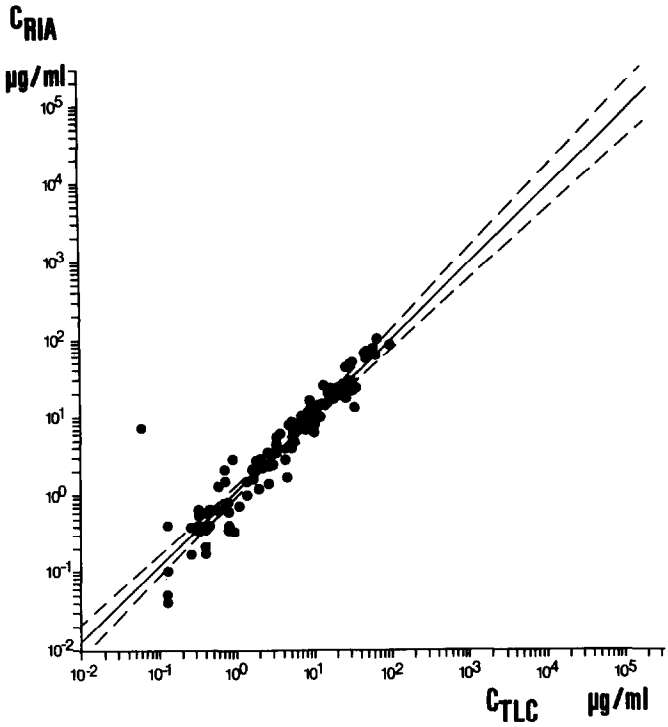

Fig. 5. Parallel determination of total nomifensine in urine by TLC and RIA (n = 131).

(solution) to healthy subjects [15] were measured by both TLC and RIA [3]. Results ranging over four decades were correlated by unweighted log-log linear regression (Fig. 5). Identity requires an intercept of zero and a slope of one. From 131 determinations, an intercept of 0.05 with a 95% probability range of —0.01 to 0.10 and a slope of 0.97 with a 95% probability range of 0.91 to 1.03 were calculated, thus including zero and one, respectively. Therefore, identity of the results of the two methods was verified.

The capacity is such that 100 samples can be processed per person per week.

Pharmacokinetics

In a cross-over study with different oral dosage forms, i.e., oral solution vs. capsule, 50 mg of nomifensine maleate were administered to 23 male volunteers. Details of the human experiment and sample collection were described previously [15].

By TLC, urine levels of the compounds were found in ranges 0.2—150 µg/ml of total nomifensine and 0.2—20 µg/ml of total metabolite(s).

By analogy with plasma levels, urinary excretion rates were used in order to obtain pharmacokinetic data (Fig. 6). For nomifensine and its potent metabolite M1, a bi-exponential decrease was found with identical terminal half-lives of 4 h. In contrast, M2 and M3 were excreted mono-exponentially with identical half-lives of 2 h.

On average, 58 ± 13% of an oral solution was found in urine, 41.0 ± 9.4%

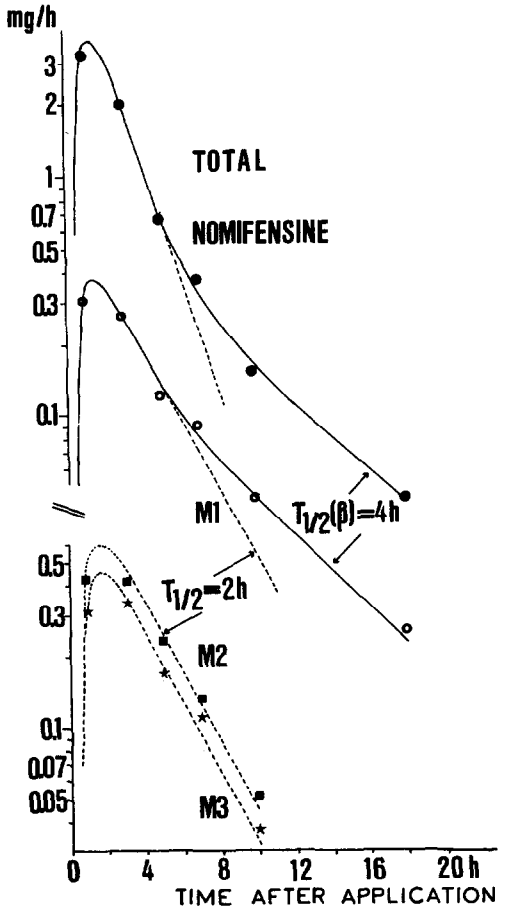

Fig. 6. Half-lives of total nomifensine and total metabolites from mean renal excretion rates (23 volunteers). ——, Two-compartment model; — — —, one-compartment model.

was excreted as total nomifensine, 5.4 ± 1.4% as total M1, 6.2 ± 2.0% as total M2 and 5.0 ± 1.5% as total M3 (Fig. 7) [1].

In capsule form, the same amount was administered to the same subjects. With a delay of just 20 min (Fig. 8), the following amounts were excreted renally: 60 ± 10% the dose was found in urine, 41.6 ± 8.0% was excreted as total nomifensine, 5.7 ± 1.4% as total M1, 7.0 ± 1.7% as total M2 and 5.7 ± 1.5% as total M3.

Excretion ratios were identical following both oral dosage forms. Therefore, nomifensine in capsules is 100% bioavailable compared with that in aqueous solution.

In a further study*, the drug was given to male and female volunteers. The details of the human experiment were similar to those of the bioavailability study (see above). Following an oral dose of 1.3 mg of nomifensine maleate

*This study was performed by Drs. W. Rupp and M.J. Badian of Hoechst AG.

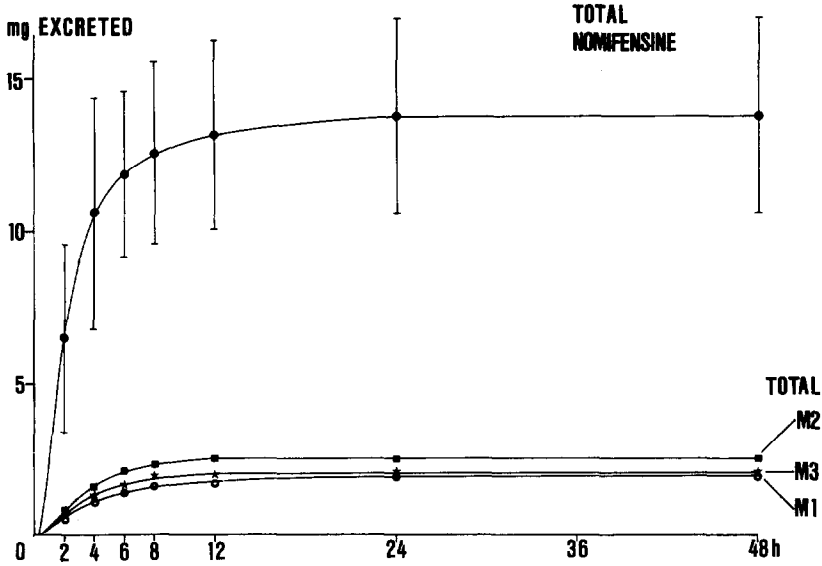

Fig. 7. Mean cumulative renal excretion of total nomifensine, M1, M2 and M3 by 23 male volunteers after administration of an oral solution of 50 mg of nomifensine maleate (= 33.6 mg of nomifensine).

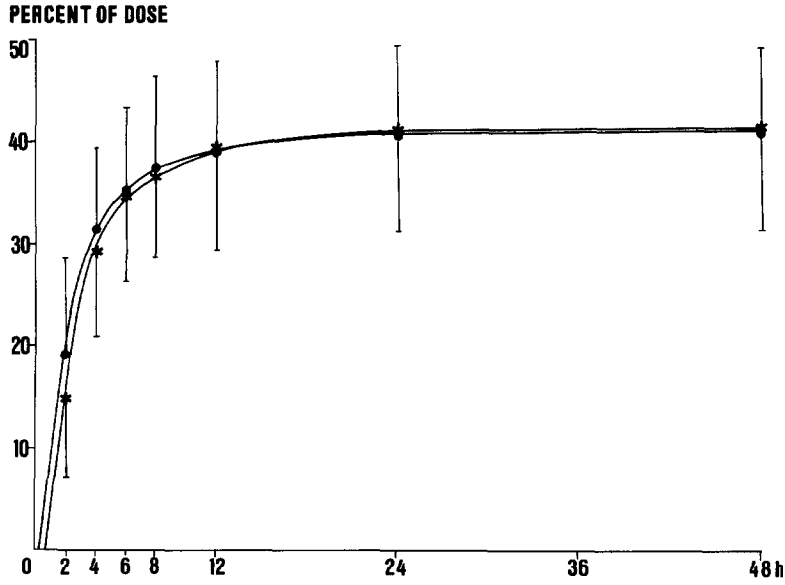

Fig. 8. Relative bioavailability: cross-over study of 23 male volunteers, capsule (⋆) vs. oral solution (•) of 50 mg of nomifensine maleate. Total nomifensine excretion as percentage of dose.

per kilogram body weight, approximately 60% of the dose was accounted for in urine (Table IV).

Sex-independent renal excretion is therefore verified.

TABLE IV

CUMULATED URINE EXCRETIONS AS PERCENTAGE OF ORAL DOSE

Compound	8 males, 69 ± 5 kg: mean ± S.D. (%)	8 females, 56 ± 4 kg: mean ± S.D. (%)
Total nomifensine	36.6 ± 6.6	35.4 ± 6.6
Total M1	6.4 ± 0.9	6.8 ± 2.2
Total M2	8.7 ± 0.8	8.2 ± 1.7
Total M3	7.6 ± 0.8	7.2 ± 1.8
Sum	59 ± 7	58 ± 11

CONCLUSION

TLC has been demonstrated to be suitable for the assay of nomifensine in urine. In our laboratory, this technique has proved to be reliable, selective, sensitive and efficient method suitable for routine assay, and may be employed in most instances whenever radioimmunoassay is not available or all metabolites have to be determined separately.

Further improvements are expected in the future. New spotting devices, for instance the Fenimore Contact Spotter or the Camag Automatic TLC Sampler, should allow more efficient use of the TLC layer. Automation of measurements by Ebel's system [16] will certainly alleviate operator requirements. With a minimum of instrumental expense, new and selective reagents for derivatization of compounds will help to increase sensitivity and selectivity. In our experience, this is of utmost importance whenever interferences from unknown or undeclared biological materials or co-medications occur.

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