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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TRITHIOZINE AND ITS NEUTRAL METABOLITES IN HUMAN PLASMA AND URINE

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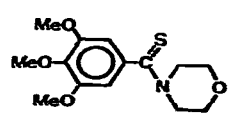
SUMMARY

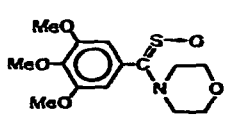
A high-performance liquid chromatographic method for the quantitation of the new antisecretory and antiulcer drug trithiozine in human plasma and urine is reported. The procedure is simple and precise; it allows the simultaneous determination of therapeutic doses of the drug and its three main metabolites, namely, 4-(3,4,5-trimethoxythiobenzoyl)tetrahydro-1,4-oxazine S-oxide, 4-(3,4,5-trimethoxybenzoyl)tetrahydro-1,4-oxazine, and 2-hydroxy-4-(3,4,5-trimethoxybenzoyl)tetrahydro-1,4-oxazine.

INTRODUCTION

Trithiozine (I) was selected in a systematic research program on new alkoxythiobenzamides [1, 2]. The compound displays considerable antisecretory and antiulcer activity and is devoid of anticholinergic, antihistaminic, ganglioplegic and cardiovascular activity [3, 4]. Its efficacy has been documented in several clinical trials [5—7].



I



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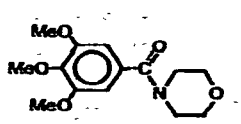

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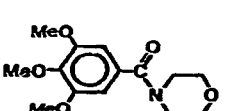

IV

A preliminary gas—liquid chromatographic (GLC) method for the determination of (I) has already been described for rat and dog plasma and urine [8]. Metabolic studies showed that the main neutral metabolites are the amide (III), the morpholinol (IV) and the sulphur-oxide (II). This last compound is converted by the GLC method [9] into the amide (III). In order to overcome this handicap, a high-performance liquid chromatographic (HPLC) method for the simultaneous quantitation of (I) and its main neutral metabolites was developed. The method was applied to the determination of plasma and urine levels of the four compounds in a volunteer after the oral administration of (I).

EXPERIMENTAL

Reagents and chemicals

The reference compounds [4-(3,4,5-trimethoxythiobenzoyl)tetrahydro-1,4-oxazine (I), 4-(3,4,5-trimethoxythiobenzoyl)tetrahydro-1,4-oxazine S-oxide (II), 4-(3,4,5-trimethoxybenzoyl)tetrahydro-1,4-oxazine (III), 4-(3,4,5-trimethoxybenzoyl)tetrahydro-2-hydroxy-1,4-oxazine (IV) and 4-(3,5-dimethoxythiobenzoyl)tetrahydro-1,4-oxazine (internal standard)] were synthesized in our laboratories as described elsewhere [1, 2, 8, 10, 11]. Their purity was tested by thin-layer chromatography: a single spot was detected by UV fluorescence quenching, except for (II) which cannot be completely purified from (I) and (III) even by several crystallizations or by liquid column chromatography [10—12].

Chloroform LiChrosolv and methanol LiChrosolv were obtained from Merck (Darmstadt, G.F.R.); double-distilled water and buffer solution (pH 10.0) were from Carlo Erba (Milan, Italy). Methanol and water were filtered before use through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). The siliconizing agent was Dri-Film SC 87 (Pierce, Rockford, IL, U.S.A.). Separating phase filters (Whatman, Maidstone, Great Britain) were used after extraction.

All the compounds were dissolved in methanol for the external calibration curves and in pH 10.0 buffer for the internal calibration curves.

Glassware

The test-tubes were cleaned with sulphochromic mixture, rinsed with water, dried in an oven and then silanized in a 10% toluene solution of Dri-Film SC 87. After 2 h, the glassware was air dried and used for analysis.

Chromatographic conditions

A Hewlett-Packard analytical liquid chromatograph (Model 1080) was combined with an LC-55 variable-wavelength ultraviolet detector (Perkin-Elmer) operating at 254 nm. A stainless-steel column (25 × 4.6 mm I.D.) packed with LiChrosorb RP-8 (10 μm) (Merck) and connected with a precolumn dry-packed with Perisorb RP-8 (30—40 μm) (Merck) was used. The mobile phase consisted of mixtures of methanol and double-distilled water with gradient elution: 30% methanol for 3 min, gradient to 50% methanol in the following 3 min, then 50% methanol for 5 min. The flow-rate was 2.5 ml/min at 30°C.

Analytical procedure

External calibration curves. To aliquots of (I), (III) and (IV) ranging from 1 to

30 μg, 1 ml of internal standard (10 μg/ml) was added and the mixture was evaporated to dryness under a stream of nitrogen at 30°C. Methanol (100 μl) was added and 20 μl of this solution were injected. The external calibration curve for (II) was done separately because (I) and (III) are always present as impurities. The exact amount of (II) in the synthetic sample was calculated by subtracting the quantities of (I) and (III), calculated from their calibration curves, from the weighed amount.

Determination of (IV) as methyl ether. An aliquot of (IV) in methanolic solution (from 5 to 50 μg/ml) was evaporated to dryness under nitrogen in screw-capped tubes and then treated with 2 ml of anhydrous 3 *N* HCl—methanol in an oil bath at 60°C for 20 min. To the mixture was added 1 ml of internal standard; the solution was then evaporated, and the residue was dissolved in 100 μl of methanol; 20 μl were injected.

Internal calibration curves: urine. To aliquots of 4 ml of urine, 1 ml of internal standard (10 μg/ml) and 1 ml of a solution of (I) at concentrations ranging from 2 to 80 μg/ml were added. The mixture was brought to a volume of 7 ml with buffer (pH 10.0). Extraction was performed with 10 ml of chloroform by mechanical shaking for 20 min. After centrifugation at 2900 *g* for 5 min, the organic phase was filtered through a phase-separation filter and then evaporated to dryness at 30°C under nitrogen. The residue was redissolved with 100 μl of methanol and 20 μl were injected.

The same procedure was followed for (II), (III) and (IV); in the last case, the chloroform phase, after being reduced to dryness, was treated with 2 ml of anhydrous 3 *N* HCl—methanol as previously described.

Internal calibration curves: plasma. To 2-ml aliquots of plasma, 1 ml of internal standard (10 μg/ml) and 1 ml of a solution of (I) at concentrations ranging from 1 to 40 μg/ml were added. The volume of the mixture was brought to 4 ml with buffer and extracted with 9 ml of chloroform. Subsequent handling was as described for urine. Metabolites II, III and IV were treated in the same way.

Peak area ratios of compounds to internal standard were measured and plotted against their concentrations.

Application

A healthy male volunteer (R.R., 70 kg, 30 years' old) was administered a single oral dose (400-mg capsules of Tresanil[R])*. Blood samples, collected at 0, 1, 2, 4, 6, 8, 10 and 24 h after administration, were mixed with heparin and centrifuged. The separated plasma was immediately analyzed in order to avoid decomposition of (II) [12].

Urine was collected every 8 h for 48 h. An aliquot of 4 ml was taken at each time for the quantitation of (I), (II) and (III), and a second 4-ml aliquot taken for quantitation of (IV).

RESULTS AND DISCUSSION

The separation of trithiozine from its three main metabolites was optimized by using gradient elution (Fig. 1). Under such conditions there are no inter-

*Tresanil[R], I.S.F. — Icar Leo.

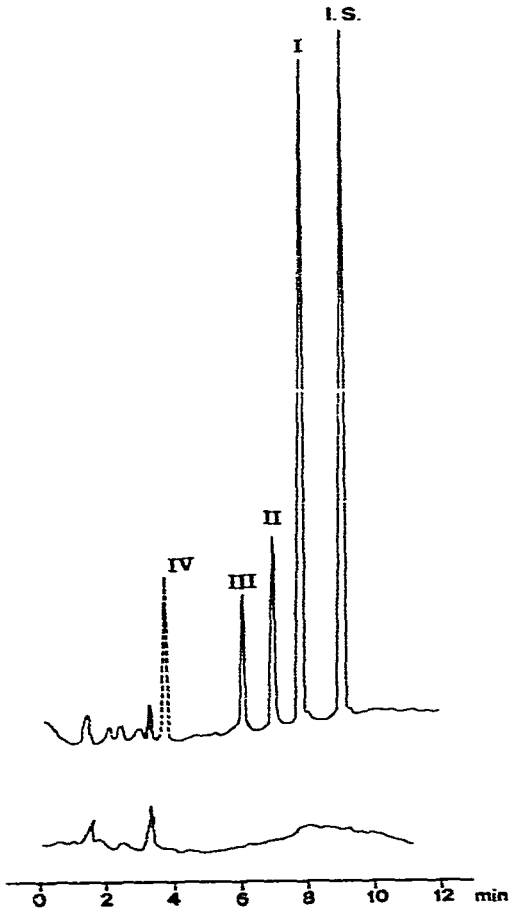

Fig. 1. HPLC profile of a neutral plasma extract (man, 400 mg of trithiozine, single oral dose). The dotted line has been used to show the separation of (IV) though it is only present in human plasma in amounts below the limit of sensitivity of the method. I.S. = internal standard.

ferences with plasma blank. The retention times are 3.90, 5.91, 6.72 and 7.70 min for (IV), (III), (II) and (I), respectively.

The internal calibration curves for the quantitation of the four compounds in plasma are reported in Fig. 2. Each point is the mean of three values. Good linearity was found in plasma with concentrations ranging from 0.5 to 20.0 μg/ml. The detection limits were empirically estimated to be 0.1 μg/ml for all the compounds. The recoveries, over the whole concentration range, and the reproducibility of the method calculated with the pooled standard deviation are reported in Table I.

Because of the presence of interfering substances in the urine, which vary between individuals and with time of collection, it was often impossible to separate compound (IV) as such from the urine blank. Therefore (IV) was derivatized to its methyl ether, as described in the Experimental section. Although this compound has a retention time too close to that of (II) (7.42 min), its

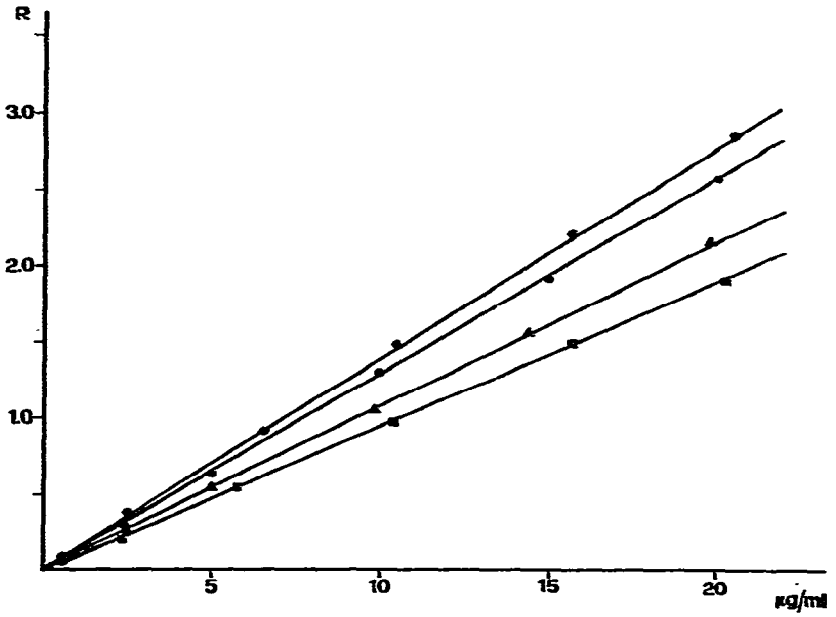

Fig. 2. Internal calibration curves of trithiozine (*) and metabolites II (▲), III (●) and IV (■) in human plasma.

TABLE I

RECOVERIES AND REPRODUCIBILITY IN THE DETERMINATION OF TRITHIOZINE AND ITS NEUTRAL METABOLITES

Compound	Recovery (%)		Pooled standard deviation*	
	Plasma	Urine	Plasma	Urine
I	75.0	98.0	2.8	1.7
II	75.0	86.6	1.7	1.5
III	98.0	96.0	2.1	1.7
IV	73.0	78.5**	2.3	1.7

*Pooled standard deviation = $\frac{\Sigma(s_i/x_i)}{n} \times 100$, where s_i = standard deviation, x_i = mean value at point i, n = number of points on the calibration curve.

**Detected as methyl ether.

quantitation was possible given the complete degradation of (II) under the derivatization conditions [9]. A typical chromatographic profile of a urine extract before and after derivatization is shown in Fig. 3. The internal calibration curves for the quantitation in urine are reported in Fig. 4. Each point is the mean of three values. The detection limits were estimated to be 0.1 μg/ml for all the compounds. The recoveries, over the whole concentration range, and the reproducibility of the method are listed in Table I.

Plasma concentration—time curves of (I), (II) and (III) for a single subject following oral administration of 400 mg of trithiozine, and the cumulative

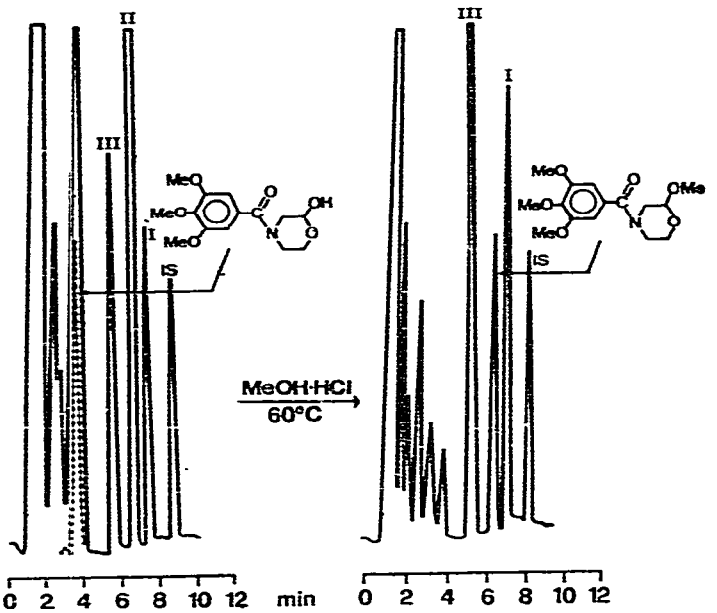

Fig. 3. HPLC profile of a urine extract before (left) and after (right) derivatization.

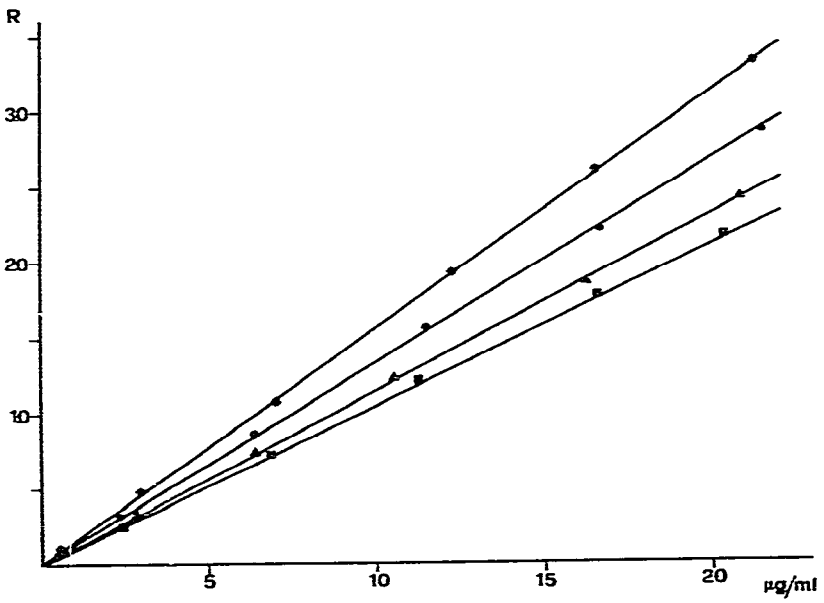

Fig. 4. Internal calibration curves of trithiozine (*) and metabolites II (▲), III (●) and IV (■) in human urine.

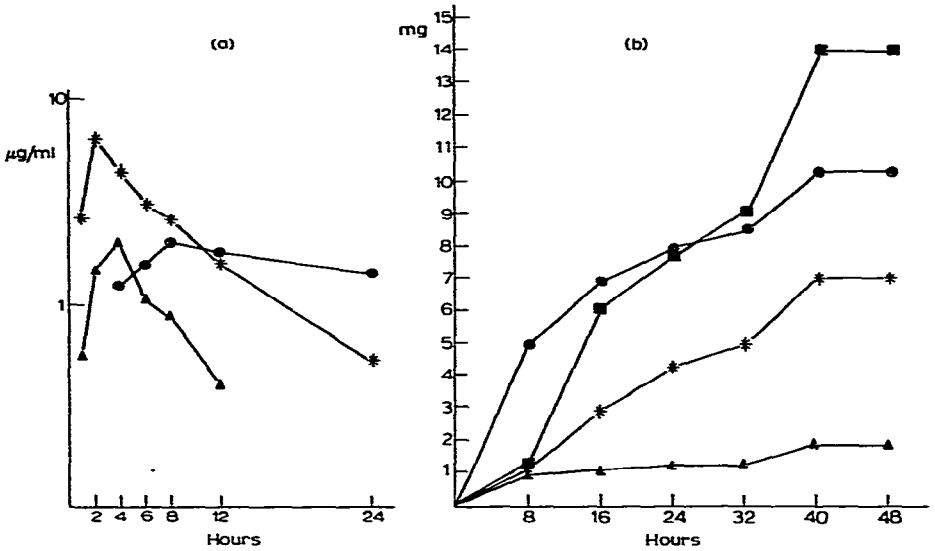

Fig. 5. Human plasma levels (a) and urinary cumulative excretion (b) of trithiozine (*) and its metabolites II (▲), III (●) and IV (■).

urinary excretion of (I), (II), (III) and (IV) were determined as shown in Fig. 5. In this case (IV) in plasma was found to be below the limit of sensitivity of the method. In contrast to the GLC procedure described elsewhere [8], the HPLC method permits the quantitation of the unchanged drug and its main neutral metabolites without formation of artifacts and is simple, accurate and precise enough for routine monitoring of clinical samples.

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